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CADMIUM CHRONIC EXPOSURE. MORPHOLOGICAL AND BIOCHEMICAL CHANGES ON THE SPORES FROM *ATHYRIUM FILIX-FEMINA* (L.) ROTH, *DRYOPTERIS FILIX-MAS* (L.) SCHOTT AND *DRYOPTERIS AFFINIS* (LOWE) FRASER-JENKINS

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Abstract
In this study we evaluate the influence of cadmium (Cd) on the spores from Athyrium filix-femina (Linnaeus) Roth (1799), *Dryopteris filix-mas* (Linnaeus) Schott (1834) *and D. affinis* (Lowe) Fraser-Jenkins (1979) *starting with the gametophyte and sporophyte differentiation and continued with the biochemical changes. For spores cultivation we used soil treated with Knop solution in which different amounts of Cd acetate were dissolved and we obtained the folowing variants: $V_1Cd = 0{,}01g\ Cd^{2+}kg^{-1}$; $V_2Cd = 0{,}02\ g\ Cd^{2+}kg^{-1}$; $V_3Cd = 0{,}05\ g\ Cd^{2+}kg^{-1}$; $V_4Cd = 0{,}1\ g\ Cd^{2+}kg^{-1}$. For 4 months we monitored the gametophyte and sporophyte differentiation and after that we determinate the amount of photosynthetic pigments and the content of polyphenols. Over time, the differences between the variants regarding the differentiation of gametophyte were reduced, and the sporophyte appeared in all variants. The smallest concentration of Cd stimulate the amount of assimilatory pigments and the content of polyphenols increased at the highest concentrations of Cd.*

Keywords: cadmium, pigments, polyphenols, spores.

1. INTRODUCTION

One of the heavy metals that has a high impact on environment is cadmium (Ibrahim et al., 2017); due to its mobility and toxicity it can affect plant growth (Manquián-Cerda et al., 2016), by disrupting the physiological and biochemical processes in plants (Atabayeva et al., 2020). Exposure to heavy metals in plants leads to an increase in reactive oxygen species (ROS) which are harmful (Okem et al., 2015). In higher plants, the biosynthesis of the photosynthetic pigments is inhibit by heavy metal stress; the decrease of the pigments is particularly pronounced during differentiation of seedlings (Myśliwa-Kurdziel and Strzałka, 2002) and because of that the amounts of chlorophyll and carotenoids are used as important indicators of metal toxicity (Loría et al., 2019). Wahid et al. (2008) reported that the content of chlorophyll and its biosynthesis were highly damaged by increased cadmium levels.

To mediate Cd toxicity plants have developed a number of strategies that involves both instantaneous as well as long-lasting responses (Khanna et al., 2019). Many studies reported production of phenolics in plants during stress conditions (Đogić et al., 2017). Phenolic compounds

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(PCs) are important plant constituents (Elguera et al., 2013) and they include a complex group varying from simple phenols to highly polymerized compounds (Cetin et al., 2014). Phenols have a major influence on the dynamic process of Cd in the soil-plant interface (Li et al., 2016). Ferns (Monilophyte and Lycophyte) are estimated at about 4% of Earth's plants – about 15.000 species (Chapman, 2009; Ballesteros and Pence, 2018), the abundance of species increase from Pole`s to the Equator (Moran, 2004). Due to their extensive biogeographical distribution and to their unique habitats, pteridophytes have the possibility to adapt to stressors action so they can be used in research (Catalá et al., 2011).
In this study we evaluate the influence of Cd on the spores from *Athyrium filix-femina*, *Dryopteris filix-mas* and *D. affinis*: starting with the gametophyte and sporophyte differentiation and continued with the biochemical changes (the content of photosynthetic pigments and phenols).

2. MATERIALS AND METHODS

The biological material was represented by spores of *Athyrium filix-femina* (L.) Roth *(Aff)*, *Dryopteris affinis* (Lowe) Fraser-Jenk. *(Da)* and *Dryopteris filix-mas* (L.) Schott *(Dfm)* colected from several individuals from the Vâlsan Valley.
The soil was taken from the forest, sterilized at 105°C in an oven for 2 and a half hours. The soil was treated with Knop solution in which different amounts of Cd acetate were dissolved to obtain progressive concentration: $V_1Cd=0{,}01g\ Cd^{2+}kg^{-1}$; $V_2Cd=0{,}02\ g\ Cd^{2+}kg^{-1}$; $V_3Cd=0{,}05\ g\ Cd^{2+}kg^{-1}$; $V_4Cd=0{,}1\ g\ Cd^{2+}kg^{-1}$; the soil was homogenous distributed in Petri dishes (50 mg/box) and after that the spores where added. After 4 months in which the Petri dishes were maintained in the growth chamber we use the biological material (gametophyte and young sporophyte) to determinate the content of pigments and polyphenols.
The determination of photosynthetic pigments (mg g^{-1} fresh weight) (chlorophyll *a* and *b*) and that of carotenoid pigments were performed spectrophotometrically using the Holm (Holm, 1954) formulae.
The total polyphenol content (% gallic acid equivalents - GAE/dry weight) was determined through the spectrophotometric method, using Folin-Ciocâlteu reagent (Merck), by measuring absorbance at 765 nm (Orțan et al. 2015).
The statistical interpretation was performed using SPSS (version 16 for Windows). We calculated the mean, the standard deviation and used the Duncan test for comparisons between the means.

3. RESULTS AND DISCUSSIONS

After 1 month of exposure at *Athyrium filix-femina* in the Control was observed the most advanced stage of the gametophyte - chordate prothalia with antheridia and archegonia (Fig. 1-2.). In the variants $V_{1\text{-}3}Cd$, in *Athyrium filix-femina*, the gametophyte is in chordate stage but only with male gametangia and viable antherozoids (Fig. 3). Also chordate prothalia stage was observed in Control, V_1Cd (Fig. 6.79) and V_3Cd (Fig. 4) in the case of *Dryopteris affinis* species. For *Dryopteris filix-mas* young chordate prothalia without gametangia and prothallium blade were predominant (Fig. 6) (Tab. 1).
At the second monitoring (after 2 months of exposure), along with the gametophyte the sporophyte (Fig. 8) was identified in all variants, except for Control (Fig. 7) and V_4Cd (Tab. 2) in *Dryopteris affinis*. Over time, these differences were reduced, and after 4 months the sporophyte appeared in all variants (Fig. 9-13).

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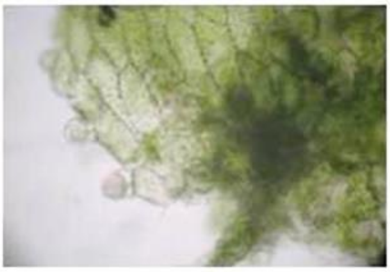

Fig. 1. *Aff* C 1 month (x100)

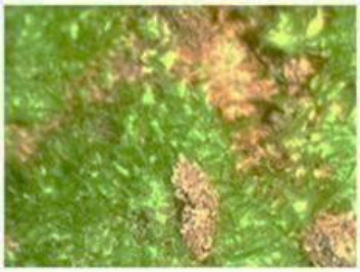

Fig. 2. *Aff* C 1 month (x10)

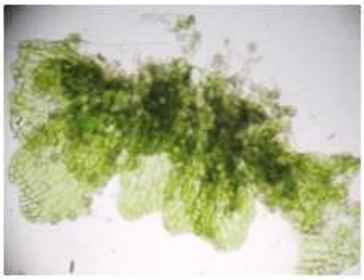

Fig. 3. *Aff* V2Cd 1 month (x40)

Fig. 4. *Da* V_3Cd 1 month (x40)

Fig. 5. *Da*V_1Cd1 month (x10)

Fig. 6. *Dfm* V_4Cd 1month (x40)

Fig. 7. *Da* C 2 months (x10)

Fig. 8. *Aff* C 2 months (x10)

Fig. 9. *Aff* V_2Cd 4 months

Fig. 10. *Dfm* V_1Cd 4 months

Fig. 11. *Dfm* V_2Cd 4 months

Fig. 12. *Dfm* V4Cd 4 months

Fig. 13. *Da*V2Cd 4 months

Tab.1 The gametophyte differentiation after 1 month of Cd exposure

VARIANTS	*Athyrium filix-femina (Aff)*	*Dryopteris filix-mas (Dfm)*	*Dryopteris affinis (Da)*
Control / C	chordate prothalia, antheridia, archegonia	young chordate prothalia	chordate prothalia
V_1Cd	chordate prothalia, antheridia, viable antherozoids	young chordate prothalia without gametangia	chordate prothalia
V_2Cd	chordate prothalia, antheridia, viable antherozoids	prothallium blade and young prothalia	prothallium blade
V_3Cd	chordate prothalia, antheridia	young chordate prothalia	chordate prothalia
V_4Cd	chordate prothalia	young chordate prothalia without gametangia	prothallium blade

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Tab.2 The gametophyte differentiation after 2 months of Cd exposure

VARIANTS	*Athyrium filix-femina (Aff)*	*Dryopteris filix-mas (Dfm)*	*Dryopteris affinis (Da)*
Control / C	$G_{n+}S_{2n}$	$G_{n+}S_{2n}$	chordate prothalia
V_1Cd	$G_{n+}S_{2n}$	$G_{n+}S_{2n}$	$G_{n+}S_{2n}$
V_2Cd	$G_{n+}S_{2n}$	$G_{n+}S_{2n}$	$G_{n+}S_{2n}$
V_3Cd	$G_{n+}S_{2n}$	$G_{n+}S_{2n}$	$G_{n+}S_{2n}$
V_4Cd	$G_{n+}S_{2n}$	$G_{n+}S_{2n}$	chordate prothalia

Legend: sporophyte S_{2n}, gametophyte: G_n

In *Athyrium filix-femina,* after 4 months from the beginning of the experiment, an increase in the content of chlorophyll *a* was found in all variants with Cd compared to Control (Fig. 14); the highest increase (62%) was determinated at V_1Cd, and for the others variants the increases were between 0,5% şi 14%. The growth trend was maintained at V_1Cd for both chlorophyll *b* (71%) and carotenoids (65%). In the case of chlorophyll *b* there were no significant differences between Control and the other variants with Cd (V_{2-4}). Instead in carotenoids the highest value were recorded in V_4.
The content of assimilatory pigments in V_1Cd also increased in the 2 species of *Dryopteris* (Fig. 15 and Fig. 16). In *Dryopteris filix-mas* the increase in Cd concentration led to a significant descrease in the amount of chlorophyll *a* at the variants V_{2-4}. A similary situation was observed for carotenoids. For *Dryopteris affinis* (Fig. 16) Cd exposure stimulated the production of the pigments.
Some authors consider that heavy metals in low concentrations stimulate de content of pigments (Singh şi Pandey, 2011; Doganlar şi colab., 2012; Shah şi colab., 2017)

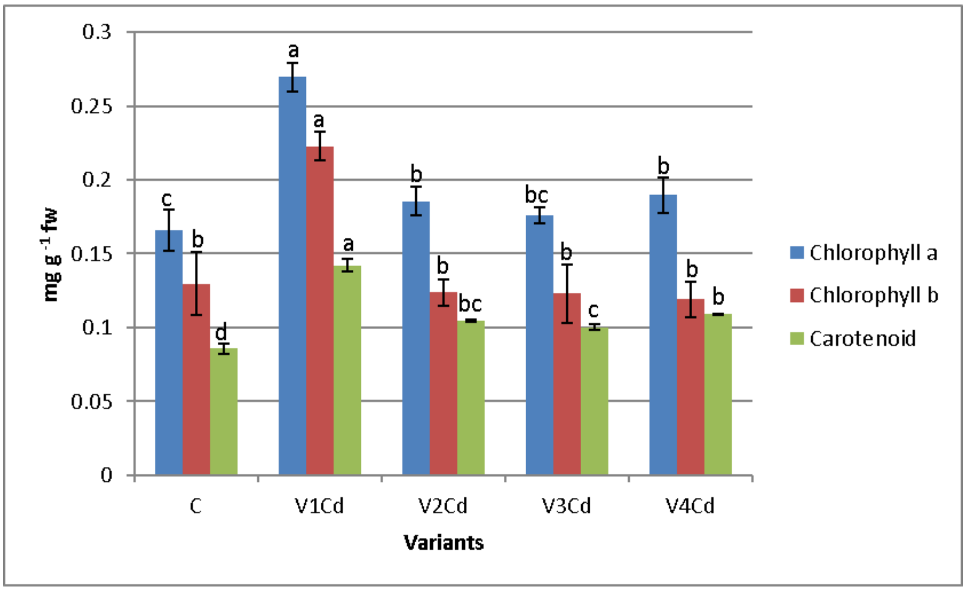

Fig. 14. ***Athyrium filix-femina*** **pigments content after 4 months of exposure**

Jain et al. (2006) found that the concentration of 0.01 mM $CdCl_2$ stimulated the formation of chlorophyll in corn, while at the variant with 0.5 mM $CdCl_2$ inhibited it. At high concentration of Cd (300 and 400 mg kg^{-1} Cd), the amount of chlorophyll increase greatly in *Lycopersicon esculentum* (Shekar et al. 2011).

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Research on *Gossypium hirsutum* showed that after 10 days of treatment the amount of chlorophyll descreased, and because of that the differences between the variants with Cd and Control were significant: at the bigest concentration (100 μmol L^{-1} Cd) the decrease was 50,1% and 56,7% for the two varieties NDM9 şi GXM3 – compared to the Control (Liu et al., 2016).

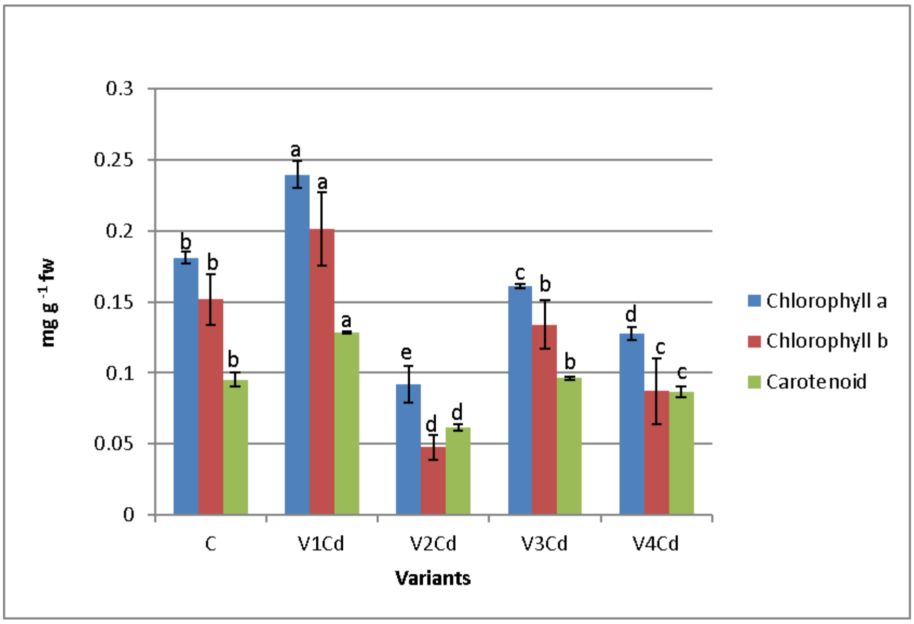

Fig. 15. ***Dryopteris filix-mas*** **pigments content after 4 months of exposure**

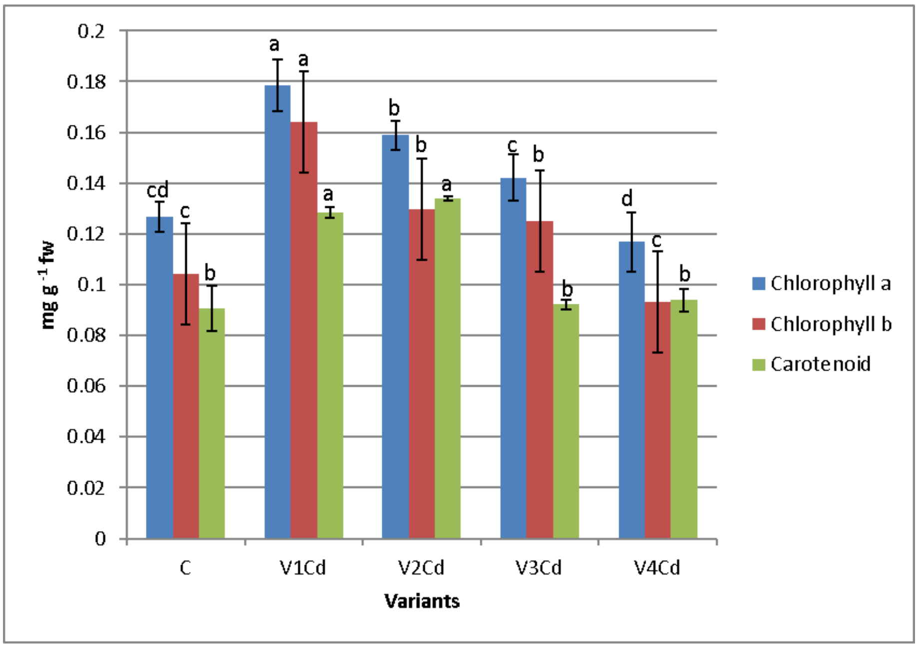

Fig. 16. ***Dryopteris affinis*** **pigments content after 4 months of exposure**

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In *Cajanus cajan* in the variant with 20 mg L^{-1} Cd the decrease in the amount of chlorophyll was 68,96% compared to control (Aruna şi Mohanty, 2014), and the amount of carotenoids in the leaves of *Eichhornia crassipes* decreased with the increasing of Cd concentration. (Borker et al., 2013). It`s considered that the decrease in the amount of chlorophyll is caused by the inhibition of important enzymes associated with chlorophyll biosynthesis (Gowthami şi Vasantha, 2015). The reducing of pigments cand be seen as a plant specific response to metal stress which leads to chlorophyll degradation and inhibition of photosynthesis (Devi Chinmayee et al., 2014).
In *Tagetes erecta* the amount of chlorophyll increased continuously up to the variant with the concentration of 18 mg kg^{-1} Cd and then decreased continuously to high concentration (Shah et al., 2017). In *Triticum aestivum* the reduction in the amount of chlorophyll was 20% in the variant with to 50 mg kg^{-1} Cd and by about 50% in the variant with100 mg kg^{-1} Cd, compared to the control (Bheemareddy, 2013).
The application of Cd in different concentration (2.6-10.5 M) in the nutrient solution in *Phaseolus coccineus* led to an increase in chlorophyll accumulation in the early stages, and to a significant decrease in chlorophyll amount at the end of the growth of the primary leaf (Skórzyńska et al., 1995).
In *Lonicera japonica* the amount of pigments increased in the variants with low concentration of Cd (5-50 µmol L^{-1}): the increase was by 17,18%; 8,76%; 65% compared to the control. At high concentration of Cd (100 µmol L^{-1}) the content of pigments decreased by 58,56%; 27,01% and 55%. These results indicate that at low concentration Cd may be eneficial to plants (Jia et al., 2012).
Certain concentrations of Cd can stimulate biomass production and chlorophyll synthesis (Tang et al., 2009), this is associated with the phytochelatins formation which have a role in detoxification (Prasad, 2004).
Sabeen et al. (2013) studied the effect of different concentration of Cd (0, 50, 100, 250, 750, 1000 µg) on pigments in *Arundo donax* L. In plants grown under hydroponic conditions, the highest amount of chlorophyll *a* and *b* was observed in the variant with 250 µg L^{-1}, and for the variants with soil at 1000 µg g^{-1}. In the variants grown on soil, chlorophyll *b* tended to increase, reaching a maximum at 250 µg g^{-1}; after that it decreased with the increase of Cd concentration. For both variants the maximum for carotenoids was obtained at the 100 µg.
According to Wang et al. (2013) chlorophyll *b* had a higher sensitivity to stress caused by Cd exposure compared to chlorophyll *a*, and the carotenoid pigments are less affected than chlorophyll (Stoeva et al., 2005).
Uraguchi et al. (2006) observed the increase of the amount of chlorophyll and carotenoids in *Crotalaria juncea* after the exposure to concentration of 1 mg L^{-1} and 5 mg L^{-1} Cd for a period of 4 weeks, and in *Avena strigosa* no significant reduction of pigments was observed.
Lemna trisulca was able to tolerate high concentrations of Cd (up to 10 mM) without significant changes in pigments (Prasad et al., 2001).
Carotenoids which usually grow in stress conditions (Munne-Bosch şi Alegre, 2000) don`t have a certain tendency in plants exposed to the action of Cd (Parmar et al., 2013).
Thus there are 3 possibilities:
- to increase - in *Cucumis sativus* (Burzynski et al., 2007), *Zea mays* (at 100 µM) (Chaneva et al.,2010) and in *Thalassia hemprichii* (at 10 and 100µmol L^{-1}) (Lei et al., 2012);
- to decrease - in *Pfaffia glomerata* (at 20, 60, 80 µM) (Skrebsky et al., 2008), *Vigna radiata* (at 3, 6, 9 and 12 mg kg^{-1}) (Wahid et al., 2008), in *Pisum sativum* (at 7 mg kg^{-1}) (Hattab et al., 2009), in *Hordeum vulgare* (at 20 and 30 mM) (Gubrelay et al., 2013);

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– not to be significantly different - in *Pteris vittata* (at 60 and 100µM) (Balestri et al., 2014).

The highest value (52% GAE) obtained at the variants exposed to Cd in the case of polyphenols content was at the variant V_4 for *D. filix-mas*; and for *A. filix-femina* and *D. affinis* at the variant V_3Cd. (Fig. 17).

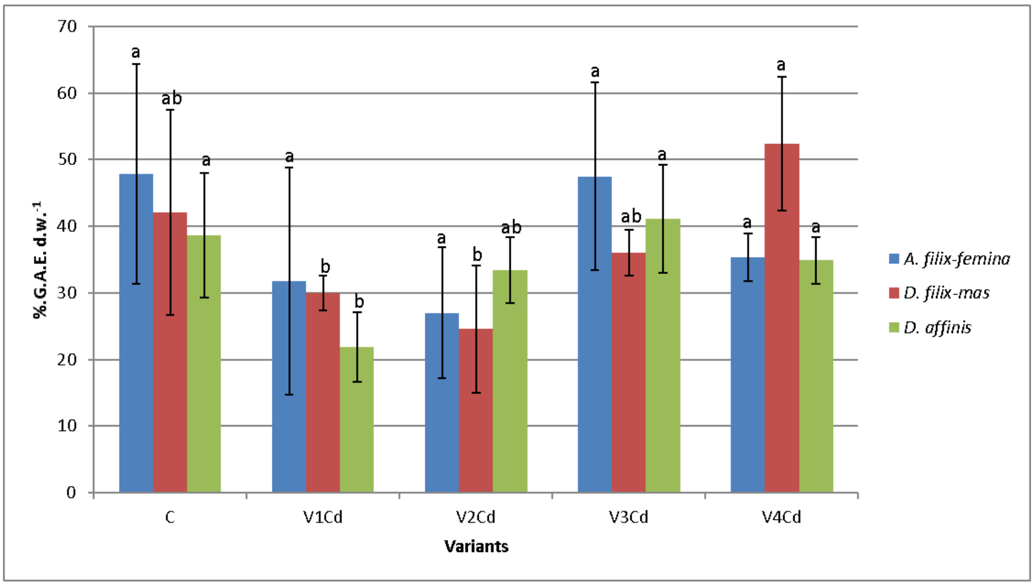

Fig. 17. Total polyphenol content after 4 months of exposure (%GAE/dry weight)

A similary situation was observed by Sivaci et al. (2008): in *Myriophyllum heterophyllum* and *Potamogeton crispus* the content of polyphenols increased with the concentration of Cd.
The increase of the polyphenols content can be seen as a protective reaction of the plant against stress caused by Cd (Dai et al., 2006). Márquez-García et al. (2012) recorded the increase of the polyphenols content in *Erica andevalensis* after Cd exposure. In *Eichornia crassipes* the content of polyphenols increased with Cd concentration, in the variants with 25 ppm to 50 ppm, and decreased slightly in the variant with 75 ppm. The smallest value for the polyphenols was determinated at Control. $CdCl_2$ induces the accumulation of secondary metabolites in these species to tolerate high concentration of metal (Borker et al., 2013). In *Matricaria chamomilla* the content of soluble phenolic compounds in leaf rosettes increased due to the application of high concentration of metals; at Cd the highest increases 23% and 41% were registered at the variants with 60 şi 120 µM Cd concentrations (Kováčik et al., 2008).

4. CONCLUSIONS

Over time, the differences between the variants regarding the differentiation of gametophyte were reduced, and the sporophyte appeared in all variants. The smallest concentration of Cd stimulate the amount of assimilatory pigments and the content of polyphenols increased at the highest concentrations of Cd.

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