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RESEARCHES CONCERNING THE DIFFERENTIATION OF THE GAMETOPHYTE OF THE FERN *ATHYRIUM FILIX-FEMINA* (L.) ROTH UNDER THE INFLUENCE OF A FUNGICIDE BASED ON FOSETYL-AL

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INTRODUCTION

Fosetyl-Al is an organophosphorus fungicide (with the formula $[C_2H_5OP(H)O_2]_3Al$, derived from ethylphosphite) having a systemic activity, which is used to combat fungal diseases produced by phycomycetes and ascomycetes in plants (Yancheva et al., 2016). It has been, and is still, used in quantities of the order of hundreds of tonnes/year in various countries (e.g. Japan), producing disturbances in aminoacid metabolism (Kaonga et al, 2017).

The aluminium contained in fungicides, medical drugs, cosmetics, food, etc. is one of the major contaminants of the environment with toxic effects on all living creatures (Kim et al. 2007, Fernández-Dávila et al. 2012, Krtková et al. 2012, Zhu et al. 2012a, b).

In plants, Al causes root growth inhibition, reduction in the uptake of mineral nutrients, epigenetic changes to DNA (Siecińska and Nosalewicz, 2016), altering plasma membrane, as well as altering activities of many enzymes and metabolic pathway involved in repair mechanisms (Rout et al., 2001).

In the endangered species *Arnica montana* and *Cirsium dissectum* Al in the concentration of 200-500 µmol/L caused growth reduction, yellowish leaves and reduced contents of Mg and P (De Graaf et al., 1997). Al^{3+} affects the rhizobia/legume symbiosis, which includes a decrease in root elongation and root hair formation, lowered soil rhizobial population, and suppression of nitrogen metabolism (Jaiswal et al., 2018).

In the *Salvinia natans* fern, Al caused a decrease in biomass, affected lipid peroxidation (MDA) and protein oxidation, and produced activation of enzymatic and non-enzymatic antioxidant pathways as a result of oxidative stress (Mandal et al., 2013). The goal of this paper was to observe the alterations induced by Fosetyl-Al on the differentiation of the gametophyte of *Athyrium filix-femina* (L.) Roth.

MATERIAL AND METHOD

The fungicide used, Fosetyl-Al, aluminum tris(O-ethylphosphonate), is a systemic one, conditioned in the form of water-dispersible granules. The commercial form used in the experiment contains 80% Fosetyl-Al.

The biological material used consisted in spores of *Athyrium filix-femina*, collected from plants in Valea Vâlsanului and dry-preserved in the refrigerator (Soare and Aldoiu, 2010) until cultivation.

Experimental variants. The spores were grown on Knop's solution having the following fungicide content: Control (C): 0%, A1-0.002%, A2-0.02%, A3-0.2%.

Growing the spores and monitoring the plant material. The culture vessels were placed in the EKO POL KK growth chamber at 25°C during the day, and 15°C overnight, for a photoperiod of 16 hours of light and 8 hours of darkness. After 9, 15, 30, 50, 60, and 140 days from spore cultivation, microscopic observations were conducted on the gametophytic differentiation stages. Photographs were made using the Optika B275 microscope with the Canon Power Shoot A630, and the Optika SZR stereomicroscope.

RESULTS AND DISCUSSIONS

Throughout the monitoring period there were differences in the gametophyte formation process, which were recorded (Table 1). Thus, 9 days into the experiment, the C gametophyte was in the 2-3 cellular filament stage, and in the A1-A2 variants, besides the stage similar to the control, germinated spores were also observed (Fig. 1).

In the A3 variant, in which the spores were exposed to the highest fungicide concentration, their germination was not recorded throughout the experiment (140 days), the dose being fatal in this case (Fig. 2). After 15 days the differences were maintained, and the stage of the prothalian blade formation was observed for C (Fig. 3), and much

earlier stages for A2 (germinated spores and 3-4 cellular filaments – Fig. 4.).

In variant A2, the gametophyte at different stages of development, after 15, 50 (Fig. 5) and 60 (Fig. 6) days, had short rhizoids, and necrotic prothalian cells were observed at 50-60 days. At the end of the monitoring period for the C (Fig. 7) and A1 (Fig. 8-9) variants, the gametophyte reached the elongated stage with anteridia, for A2 (Fig. 10) the corded prothalian stage with anteridia and arhegones, while the A3 spores were not germinated.

The continuous Fosetyl-Al exposure of the spores and the gametophyte formed by their germination resulted in a series of morpho-physiological modifications that are non-specific biomarkers (Vangronsveld et al., 2000), which may be useful in assessing environmental risk:

- total inhibition of spore germination when exposed to a concentration of 0.2% fungicide;
- delay of stages of differentiation of gametophyte;
- affecting the extension of rhizoid exposure when exposed to a concentration of 0.02% fungicide;
- necrosis of prothalian cells when exposed to a concentration of 0.02% fungicide.

The spores and the gametophyte tolerated concentrations of 0.002% and 0.02% of fungicide by stress compensation mechanisms, while the 0.2% fungicide concentration was 100% lethal. In the variant exposed to 0.02% of the fungicide, some symptoms of toxicity, namely the disturbance of the rhizoid stretching as well as areas of prothal necrosis, were recorded. Research on algae showed that Fosetyl-Al also inhibits the growth of some algae species, thus the EC50 for *Chlorella pyrenoidosa* was 6.7945 mg/L at 96 hours, while for *Scenedesmus obliquus* EC50 was 34.2194 mg/L at 96 hours of static exposure (Ma et al., 2002).

Concentrations of 10 and 20 mg/l Al in the culture medium reduced *Salvinia* growth. The reduction in plant growth was greater with the 20 mg/l Al variant compared to the 10 mg/l variant. Al also reduced the *a* and *b* chlorophyll and carotenoids contents (Gardner and Al-Hamdani, 1997).

Similar changes have been reported in the case of spore and gametophyte exposure to other pesticide-based substances such as copper-based fungicides, bifenthrin, thiamethoxam and acetamiprid insecticides (Soare et al., 2013a,b,c), the glyphosate herbicide (Droste et al., 2010; Aguilar-Dorantes et al., 2015). Pesticides affect non-target organisms in the ecosystems they pollute by inducing morphological, physiological and biochemical changes in the latter (Soare et al., 2019).

Table 1. Stage* of development of gametophyte of *Athyrium filix – femina* under the influence of the fungicide on the experimental variants in solution

Time of exposure (days)	**Experimental variants**			
	C	**A1**	**A2**	**A3**
9	prothallic filaments (2-3 cells)	germinated spores and filaments (2-4 cells)	germinated spores, without rhizoids and prothallic filaments (2 cells) with rhizoids	ungerminated spores
15	formation of prothallic blade	filaments → prothallic blade	germinated spores, short rhizoids and prothallic filaments (3-4 cells)	ungerminated spores
30	prothallic blades, some of them with anteridia	prothallic blades with anteridia	elongated prothalli	ungerminated spores
50	elongated prothalli with anteridia and cordate prothalli	cordate prothallus and prothallic blades with anteridia	elongated prothalli with short rhizoids, necrotic filaments and gametophytic cells	ungerminated spores
60	elongated prothalli with anteridia and cordate prothalli	cordate prothallus and prothallic blades with anteridia	elongated prothalli with short rhizoids, necrotic filaments and gametophytic cells	ungerminated spores
140	elongated prothalli with anteridia	elongated prothalli with anteridia	cordate prothallus with anteridia and archegonia	ungerminated spores

Stage for most analysed gametophytes

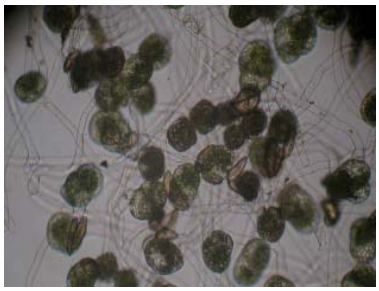

Fig. 1. A1 - 9 days (x100)

Fig. 2. A 3 - 9 days (x100)

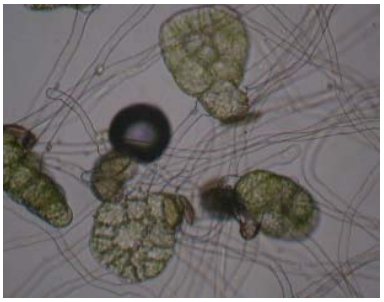

Fig. 3. Control – 15 days (x100)

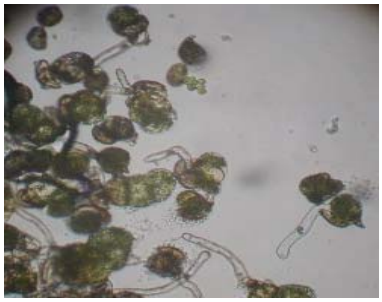

Fig. 4. A2 – 15 days (x100)

Fig. 5. A2 50 days (x100)

Fig. 6. A2 60 days (x100)

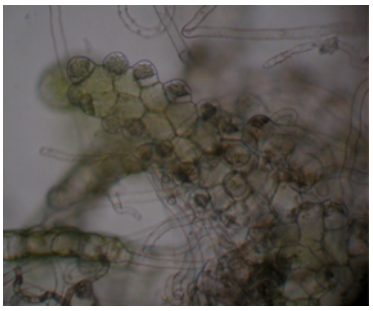

Fig. 7. Control-140 days (x100)

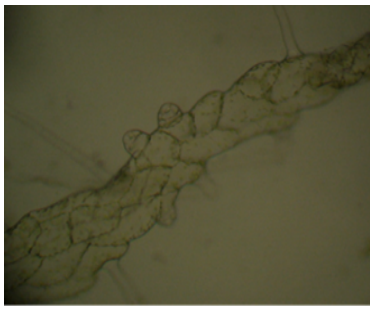

Fig. 8. A1 - 140 days (x100)

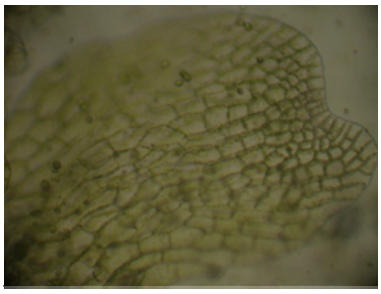

Fig. 9. A1 - 140 days (x100)

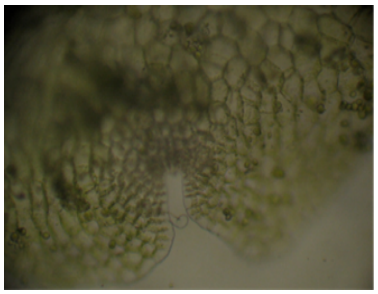

Fig. 10. A2 - 140 days (x100)

Other pollutants, such as heavy metals, affect spore germination and differentiation of gametophytes of ferns. Some species of ferns are tolerant to different metals, or they can hyperaccumulate metals.

For example, *Dicranopteris linearis* can accumulate up to 0.25% dw aluminum (Chour et al., 2018).

The study of the homeostatic mechanisms of metals/metalloids in plants indicates the involvement of small molecule organic acid (Verbruggen et al., 2009; Sharma et al., 2016), histidine (Shan et al. 2003), complexation of Al with fluoride (F) in vacuoles, and binding to cell walls and chloroplasts (Gao et al.2014), formation of amorphous phytolith particles (Coskun et al. 2018), etc.

CONCLUSIONS

The lethal dose of Fosetyl-Al for the spores of *Athyrium filix-femina* was 0.2%. At concentrations of 0.02% and 0.002%, changes were noted such as delayed gametophytic differentiation stages, disturbance of rhizoid elongation, necrosis of prothal cells, and these changes can be useful in assessing environmental risk.

ABSTRACT

The aim of this paper is to present the changes observed in gametophyte differentiation in the fern *Athyrium filix-femina* under the action of a fungicide containing fosetyl-aluminium. The biologic material consisted of spores collected from plants found in the Vâlsan Valley. The following experimental variants of culture media were prepared to cultivate the spores: Control (Knop solution), A1 (0.002% fungicide in Knop solution), A2 (0.002% fungicide in Knop solution), and A3 (0.2% fungicide in Knop solution). The spores were cultivated in 100 ml of solution, in culture vessels that were placed in the EKO POL KK growth chamber at 25°C during the day and 15°C during the night with a photoperiod of 16 hours of light and 8 hours of darkness. Microscopic observations on gametophyte differentiation were conducted periodically, after 9, 15, 30, 60 and 140 days from the cultivation of spores. The A1 and A2 variants showed a slower gametophyte differentiation than that in the Control variant. No spore germination or gametophyte differentiation was observed in the A3 variant. Thus, at the end of the experiment, the most affected was the A3 variant, with ungerminated spores, followed by the A2 variant, which was at the stage of elongated lamellate prothalli with short rhizoids or necrotic prothallial cells and filaments, while in the C variant, the gametophyte was at the stage of elongated prothalli with antheridia and cordiform prothalli. The least affected variant was A1, where the gametophyte exposed to the lower concentration of fungicide reached the end of the experiment at a stage close to that in the Control variant.

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