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THE INFLUENCE OF A COPPER-CONTAINING FUNCICIDE ON THE GAMETOPHYTE OF SOME NON-TARGET PTERIDOPHYTE SPECIES

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Abstract: The aim of this study was to assess the effects of a fungicide with 20% metallic copper content on spore germination and gametophyte development in two non-target pteridophyte species: *Asplenium scolopendrium* and *Athyrium filix-femina*. The experimental variants were: V1 - 0.1% fungicide in Knop solution, V2 - 0.5% fungicide in Knop solution, V3 - 0.7% fungicide in Knop solution, and the Control variant (C) – Knop solution. The fungicide used affected spore germination in all the tested variants. The lowest germination percentages were registered in the species *Asplenium scolopendrium:* V1 - 69.33%, V2 - 65.66%, V3 - 51.33%. In terms of gametophyte differentiation, the experiments led to delays in developmental stages, absence of rhizoids and necrosis of prothallic cells. The results of the study can be used to assess the impact of the fungicide on the ecosystems in which it is applied, ecosystems where pteridophytes are also present or ecosystems adjacent to them.

Key words: *Asplenium scolopendrium*, *Athyrium filix-femina*, copper, gametophyte, germination, fungicide, pteridophytes

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Introduction

Pesticides have been cited among the factors that influence spore germination, gametophyte and sporophyte differentiation (Keary et al. 2000, Sheffield 2002, Luo & Ikeda 2007, Cassanego et al. 2010, Droste et al. 2010). The gametophytic generation or the gametophyte phase of pteridophyte species begins with the spores (usually haploid), produced in sporangia and ends with the formation of the zygote. During its differentiation, the gametophyte follows the stages of prothallic filament, prothallic blade and cordate prothallus with gametangia on which the zygote is formed. (Ehrendorfer 1999, Fernández & Revilla 2003). The gametophyte, independent from the sporophyte with a short lifespan, has been used in experiments designed to study the response of plants to the influence of different stress factors present in their environment, such as temperature (Pangua et al. 1994), light (Mohr 1963, Tomizawa et al. 1983, Sugai et al. 1984), nutrient elements (Fernández et al. 1999), salinity (Pangua et al. 2009), acid rain (Evans & Bozzone 1978) etc.. Gametophyte differentiation begins

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with spore germination. Gametophytes are that generation in the life cycle which is sensitive to stress factors in their environment. In the case of terrestrial plants, germination tests are among those used to assess the ecological risks caused by pesticides or other pollutants in ecosystems (Catalá et al. 2011). Pteridophytes are non-target organisms currently used in toxicity tests needed to assess the ecological risks associated with pollutants.

The aim of this study was to assess the effects of a fungicide with metallic copper content on spore germination and gametophyte development in two non-target pteridophyte species: *Asplenium scolopendrium* L. and *Athyrium filix-femina* (L.) Schott.

Material and methods

Biological material: spores collected from *Asplenium scolopendrium* L. and *Athyrium filix-femina* (L.) Schott. Spores were collected from individuals in the Vâlsan Valley (Argeş county, Romania).

The fungicide used contains 20% metallic copper; it is supplied as wettable powder and included in toxicity class IV. The experimental variants are presented in Table 1.

Table 1

Experimental variants

Experimental variants	Fungicide concentration
Control (C)	Knop solution
V1	0.1% fungicide in Knop solution
V2	0.5% fungicide in Knop solution
V3	0.7% fungicide in Knop solution

Fungicide concentrantions applied during the experiment were those indicated for phytosanitary treatments. The Knop solution (1865) used had the following composition: 1.00 $g \cdot l^{-1}$ $Ca(NO_3)_2$, 0.25 $g \cdot l^{-1}$ $MgSO_4$, 0.25 $g \cdot l^{-1}$ KH_2PO_4, 0.25 $g \cdot l^{-1}$ KNO_3. Spores were cultivated in 50 ml of prepared solutions, in culture vessels sealed with Parafilm and kept in the Sanyo growth chamber under controlled temperature (25/15°C day/night), humidity and light conditions (16-hour photoperiod, 16:8 h L:D). After a week the spore germination percentage was determined by counting under the microscope 100 spores for each experimental variant. For each experimental variant we calculated the average and the standard deviation. After 3, 6 and 14 weeks, respectively from the cultivation of spores, we performed microscopic observations on gametophyte development. The photos were taken through an Optika B-275 microscope, using a Canon PowerShot A630 camera. The experiment was conducted in triplicate.

Results and discussion

Spore germination under the influence of the tested fungicide. The results obtained in the spore germination test show that the fungicide used had influenced the germination process. The species *Asplenium scolopendrium* was more affected than *Athyrium filix-femina* (Fig. 1, 2). Thus, in the species *Athyrium filix-femina* there is

a17.33% difference between the germination percentages obtained for the C variant and for the V3 variant, while in *Asplenium scolopendrium* the difference is 37.66%. The tests also revealed that there is a negative correlation between the germination percentage and the fungicide concentration. The calculated correlation index was -0.8955 in *Asplenium scolopendrium* and -0.9264 in *Athyrium filix-femina*.

The influence of the pesticide on gametophyte differentiation. In leptosporangiate pteridophytes (as gametophyte differentiation begins from spores) the formation of the prothallic filament, of the prothallic blade and of the cordate prothallus on which the embryo is formed takes place in approximately 3 months. This period varies, depending on the species (Fernandez & Revilla 2003).

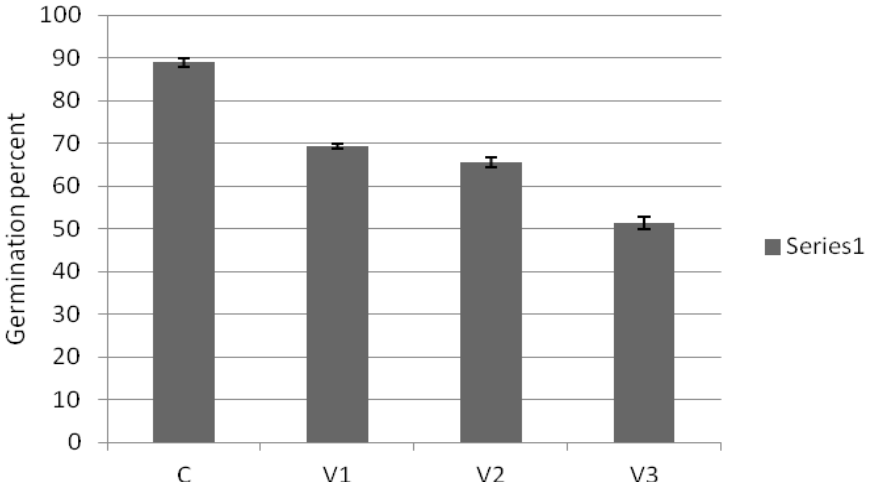

Fig. 1. The germination of *Asplenium scolopendrium* spores under the influence of the fungicide

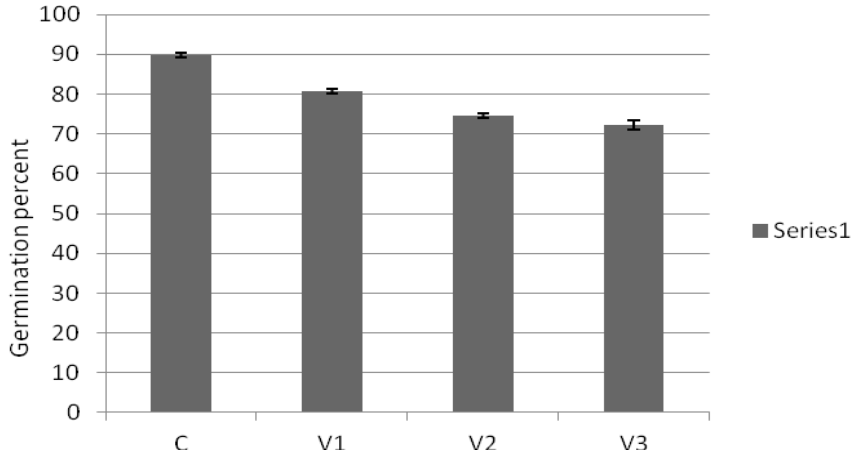

Fig. 2. The germination of *Athyrium filix-femina* spores under the influence of the fungicide

Periodic microscopic analyses of the gametophytes obtained from spores showed that, unlike the C variant, the differentiation process was affected in the experimental variants with fungicide treatment (Table 2). Thus, after 3 weeks, most gametophytes in the C variant of *Asplenium scolopendrium* were at the stage of forming prothallic blades (Plate I.1), while the V2 and V3 variants contained only germinated spores. After 6 weeks from the initiation of the experiment, the cordate prothallus was about to be formed in the C variant (Plate I.4). The V1-V3 variants had formed prothallic filaments. We noticed unelongated rhizoids or even the absence of rhizoids (Plate I.5-6). After 14 weeks, cordate prothalli were present in the C, V1 and V2 variants (Plate I.7). Needle-like crystals were also located at the tip of the prothallic trichomes in V1(Plate I.8). V3 remained at the stage of prothallic filaments with necrotic cells (Plate I.9).

Table 2

Gametophyte differentiation under the influence of the fungicide

Experimental variants	***Stage of gametophyte differentiation**		
	Asplenium scolopendrium		
	3 weeks	**6 weeks**	**14 weeks**
C	formation of prothallic blade	prothallic blade → cordate prothallus	cordate prothallus with anteridia and archegonia
V1	filaments → prothallic blade	filaments, prothallic blades, unelongated rhizoids	cordate prothalli with anteridia, short rhizoids, crystal deposits at the tip of papillae
V2	germinated spores	branched prothallic filaments, lacking rhizoids or presenting unelongated rhizoids	cordate prothalli with anteridia, short and deformed rhizoids
V3	germinated spores	branched filaments lacking rhizoids or very few unelongated rhizoids	branched prothallic filaments, most of them necrotic
	Athyrium filix-femina		
C	prothallic blades → cordate prothallus	young cordate prothallus	cordate prothallus with anteridia and archegonia
V1	formation of prothallic blade, unelongated rhizoids	prothallic blades	cordate prothalli with anteridia
V2	filament → prothallic blade	filaments and prothallic blades with unelongated rhizoids	necrotic filaments and blades
V3	germinated spores (99%), filaments	prothallic filaments and prothallic blades	necrotic filaments and blades

* Stage for most gametophytes analysed.

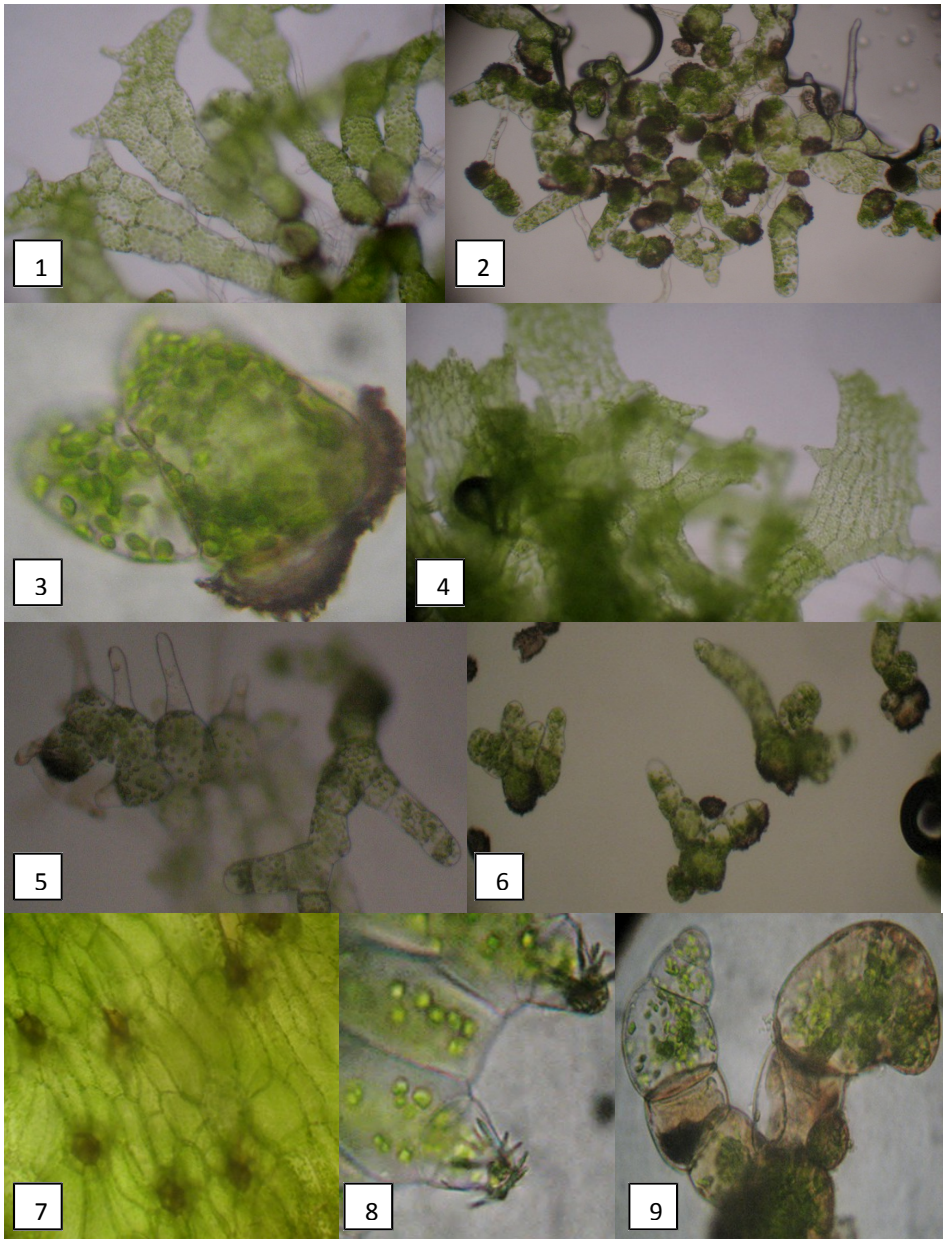

Plate I. Gametophyte differentiation in *Asplenium scolopendrium*. 1. C variant after 3 weeks from initiation of culture (x 100). **2.** V1 after 3 weeks (x 100). **3.**V2, V3 after 3 weeks (x 400). **4.** C variant after 6 weeks (x 40). **5.** V1 after 6 weeks (x 100). **6.** V2, V3 after 6 weeks (x 100). **7.** C variant after 14 weeks (x 400). **8.** V1 after 14 weeks (x 400). **9.** V3 after 14 weeks (x 400).

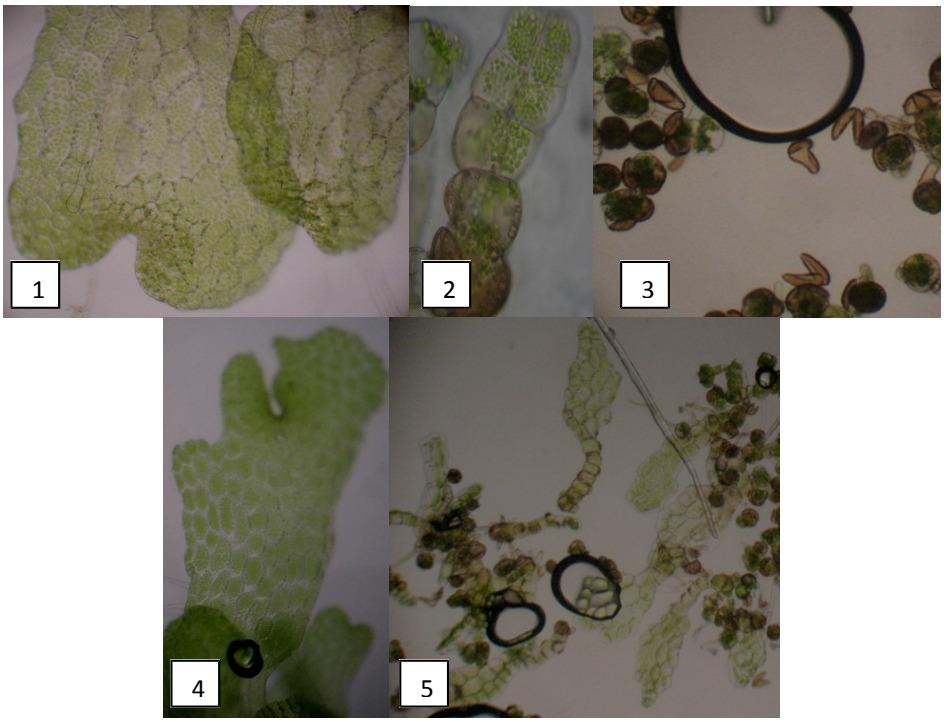

Plate II. Gametophyte differentiation in *Athyrium filix-femina*. **1.** C variant after 3 weeks of culture (x 100). **2.** V1 after 3 weeks (x 400). **3.** V3 after 3 weeks (x 40). **4.** C variant: young cordate prothallus after 6 weeks of culture (x 100). **5.** V1 after 6 weeks (x 40).

A delay in gametophyte differentiation was also registered in the species *Athyrium filix-femina* during the 14 weeks of culture (Table 2, Plate II.1-5). Thus, after 3 weeks, the cordate prothallus was about to be formed in the C variant (Plate II.1), while in the V3 variant most gametophytes were only at the stage of germinated spores (Plate II.3). After 6 weeks from the cultivation of spores, the cordate prothallus was present in the C variant. Prothallic blades and filaments had developed in the V1-V3 variants.

Different stages in gametophyte differentiation were also observed after 14 weeks from the initiation of the experiment (Table 2). In addition to slower gametophyte differentiation, mention should be made of the inhibition of the elongation process of rhizoids as well as the necrosis of prothallic cells.

The results obtained may be linked to the different capacity of plants to tolerate Cu and other metals in their environment. Some fern species are known as hyperaccumulators of metals (Nishizono et al. 1987a, Sela et al. 1989). Gametophytes of *Pteris vittata* and *Athyrium yokoscense* were shown to tolerate and accumulate lead (Kamachi et al. 2005). Gametophytes of *Pteris vittata* were also tolerant of high levels of arsenic and showed arsenic hyperaccumulation (Gumaelius et al. 2004, Kamachi et al. 2005). Copper had a greater affinity for the cell wall and was prevented from entering the cytoplasm. A large proportion of these heavy metals in the cell wall were

exchanged as ions (Nishizono et al. 1987b). Other compounds containing copper affected spore germination as well. Copper bromide induced changes in spore germination, growth and ultrastructure of *Polypodium cambricum* gametophytes (Muccifora 2008). The germination percentage was only 25% and the ultrastructure of gametophytes showed the absence of a vacuolar compartment.

Conclusions

The fungicide used in the experiment influenced the process of spore germination in the two non-target species tested, *Asplenium scolopendrium* and *Athyrium filix-femina*. As far as gametophyte differentiation is concerned, we registered not only a delay in developmental stages, but also the absence of rhizoids, negative effects on the elongation process of rhizoids and necrosis of prothallic cells. These changes may be used to assess the impact produced by the fungicide in the ecosystems where it is applied, ecosystems in which pteridophytes are also present or ecosystems adjacent to them.

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