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The Effects of Some Pesticides on Spore Germination and Gametophyte Differentiation in *Athyrium filix-femina* (L.) Roth. and *Polypodium vulgare* L.

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Abstract

The aim of this study was to assess the effects of a fungicide (copper hydroxide with 50% metallic copper) (Co) and of an insecticide (bifenthrin 100 g/l) (B) on spore germination and gametophyte development in the fern species *Athyrium filix-femina* (L.) Schott. and *Polypodium vulgare* L. The experimental variants were: V1Co: 0.1 gr fungicide/100 ml Knop solution, V2Co: 0.2 gr fungicide/100 ml Knop solution, V3Co: 3 gr fungicide/100 ml Knop solution, V1B: 0.01 ml insecticide/100 ml Knop solution, V2B: 0.02 ml insecticide/100 ml Knop solution, V3B: 0.04 ml insecticide/100 ml Knop solution and Control (C): 100 ml Knop solution. Co inhibited spore germination in all the experimental variants tested on the species *Athyrium filix-femina*. In the V3Co variant, after 24 days, no spore germinated. B also inhibited spore germination in all the experimental variants. In *Polypodium vulgare,* Co significantly inhibited spore germination. In the experimental variants containing B, only in the V3B variant the germination is significantly inhibited. Calculations showed a significant negative correlation between the germination percentage and the concentration of pesticides. The fungicide also affected gametophyte differentiation, which happened much more slowly in both species. The rhizoids of the gametophytes of *Polypodium vulgare* showed modifications in their differentiation and morphology that could also be related to alterations in their biochemical composition. The experimental variants with the highest concentration of insecticide resulted in the differentiation of abnormal gametophytes growing in a tridimensional cellular mass with callus morphology. The responses of plants to the induced stress produced during the testing period may be used as biomarkers of environmental pollution caused by pesticides.

Keywords: biomarkers, fungicide, gametophyte, spores, tridimensional cell mass, unelongated rhizoids

Introduction

Ferns are the second largest group of cormophytes with over 250 different genera and about 9,000 living species (Smith *et al*., 2006). Fern spores and gametophytes have been recognized as useful models for plant research in different areas, such as biodiversity conservation, response to environmental factors etc. (Banks, 1999; Dyer, 1979; Fernández *et al*., 1999; Miller, 1968; Rybczyński and Mikuła, 2011; Zenkteler, 2002). The assessment of the ecotoxicological risks caused by pesticides to ecosystems is based on toxicity data and the effects of pesticides on non-target organisms (Velisek *et al*., 2009). Test protocols of terrestrial plants are developed for crop species and evaluate seed germination, root elongation, and early seedling and vegetative growth (Catalá *et al*., 2011). Today, fern spores and gametophytes are used for acute and chronic toxicity tests, as these experimental systems are easy to put into practice (Catalá *et al*., 2009; Marugán *et al*., 2012; Rodriguez-Gil *et al*., 2010; Singh and Devi, 1989). Thus, spores can be collected in large amounts; they can be preserved using appropriate methods for long periods of time, so that they are available throughout the year, and they need small storage spaces; germination tests are not expensive and require common lab equipment; the conclusions are relevant for eukaryotic organisms, especially for higher plants (Catalá *et al*., 2011). Gametophytes can easily be obtained in the lab through spore cultivation in different nutritional media. The aim of this study was to assess the effects of one fungicide with copper hydroxide and bifenthrin insecticide on spore germination and gametophyte development in two non-target species: *Athyrium filix-femina* (L.) Schott. and *Polypodium vulgare* L.

Materials and methods

Biological materials

Spores collected from the fern species *Athyrium filix-femina* and *Polypodium vulgare* (Vâlsan Valley, Argeș county, Romania). Mature leaves with mature sporangia were collected from three different populations. For each population leaves were collected from 5 individuals. The leaf samples were wrapped in paper, labelled and transported in polythene bags. In the laboratory, the leaves were washed in tap water, put with their lower side downwards on sheets of paper to dry and release the spores from sporangia. The leaves were dried for 1 week under ambient conditions (22±2 °C). After a week the spores obtained on the sheet of paper looked like a fine powder. The collected spores were put into the paper envelope, wrapped

in an aluminium foil, and kept under ambient condition (22±2 °C) for 2 months.

Pesticides used

The insecticide used (B) contain bifenthrin (100g/l) that is a member of the pyrethroid chemical class (moderately toxic - toxicity class II). It is characterized by strong environmental persistence and high insecticidal activity (Mokry and Hoagland, 1989). It affects the nervous system of vertebrates and invertebrates and cellular ATPase production (Roberts and Hutson, 1999). In the soil, bifenthrin is stable over a wide pH range and degrades at a slow rate. Bifenthrin has a high bioconcentration potential. The fungicide used (Co) comprises copper hydroxide (50% metallic cooper) (toxicity class III). This compound determines morphological and histological modifications in the organs of different non-target organisms such as the amphibians (Păunescu and Ponepal, 2011; Păunescu *et al*., 2011).

Experimental variants

The mineral culture medium was Knop (1865), one of the most popular media used for spore germination (Fernández and Revilla, 2003). The composition of the culture media was 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. For each experimental variant we used 100 ml Knop solution in which we added fungicide or insecticide (Tab. 1) in the concentrations that are commonly used in agriculture.

Spore germination and gametophyte differentiation

Spores were spread on the surface of the culture media; the average density of the spores on the culture media was 200 cm^{-2}. The culture vessels were closed with cover, sealed with Parafilm, and placed in the growth room at 25°C during the day and 15°C during night, under a 16-hour photoperiod (8 hours of darkness). The experiment was done in triplicate. After 24 days we randomly selected 100 spores for which we determined the germination percentage. A spore is considered to germinate when the spore wall has ruptured and when the first cell begins to develop. Statistical analysis was performed with SPSS (version 16 for Windows). We used One-Way ANOVA tests with Fisher's LSD (least significant difference) to make pairwise comparisons as a way of monitoring significant changes within a group. We also employed correlation analysis to determine statistical significance. For this purpose we conducted the Pearson correlation coefficient test between the concentration of pesticides and the germination percentage.

Tab. 1. Experimental variant

Variants	Pesticide concentration
V1Co	0.1gr fungicide /100 ml Knop solution
V2Co	0.2 gr fungicide /100 ml Knop solution
V3Co	0.3 gr fungicide /100 ml Knop solution
V1B	0.01ml insecticide /100 ml Knop solution
V2B	0.02 ml insecticide /100 ml Knop solution
V3B	0.04 ml insecticide /100 ml Knop solution
Control (C)	100 ml Knop solution

Microscopic observations on gametophyte differentiation were made 50 days after sowing the spores. Microphotographs were taken through the OPTIKA B 275 microscope using a Canon Power Shoot A630 digital camera.

Results and discussion

A series of endogenous and exogenous factors affects spore germination in ferns, among which mention should be made of the degree of ploidy (Kott and Peterson, 1974) and their age (Smith and Robinson, 1975), temperature (Pangua *et al*., 1994), light (Sugai and Furuya, 1967; Sugai *et al*., 1977, 1984), density of cultivated spores per medium (Smith and Rogan, 1970), nutritional conditions (Fernández *et al*., 1999) etc. In the last period pesticides like asulam and glyphosat have been cited among the exogenous factors that influence spore germination (Droste *et al*., 2010; Keary *et al*., 2000; Sheffield, 2002).

Spore germination under the influence of the tested pesticides in Athyrium filix-femina

The results obtained (Tab. 2) show that the fungicide inhibited spore germination in all tested experimental variants. In the case of the V3Co variant we noticed that no spore germinated within 24 days. Insecticide also inhibited spore germination in *Athyrium filix-femina* in all experimental variants; the most inhibitory treatment was V3B. A significant negative correlation was determined between the germination percentage and the concentration of pesticides (R= -0.953 for insecticide; R= -0.998 for fungicide, $p<0.01$).

Spore germination under the influence of tested pesticides in Polypodium vulgare

In *Polypodium vulgare*, the fungicide inhibited spore germination (Tab. 2). Thus, in comparison with the control sample where 87% of the spores germinated, in the experimental variants with Co we obtained significantly lower germination percentages. In the case of the experimental variants with B, in the V3B variant we recorded a significant decrease in the germination percentage. Calculations showed a significant negative correlation between the germination percentage and the concentration of pesticides (R= -0.866 for insecticide; R= -0.924 for fungicide, $p<0.01$).

The influence of the pesticide on gametophyte differentiation in the fern species Athyrium filix-femina and Polypodium vulgare

The characteristics of the gametophyte, differentiated under the influence of the two pesticides, are presented synthetically in Tab. 3. The normal gametophyte develop-

Tab. 2. The influence of pesticides on spores germination (Mean±SD)

Species	Experimental variants						
	Control	V1Co	V2Co	V3Co	V1B	V2B	V3B
	Germination percent (Mean±SD)						
Athyrium filix-femina	76.3±1.5^{a}	51.0±1.7^{d}	29.3±1.5^{e}	0^{f}	59.0±1.0^{c}	61.6±2.0^{b}	28.6±1.5^{e}
Polypodium vulgare	87.0±0.0^{bc}	61.0±1.0^{d}	62.3±1.5^{d}	46.0±1.00^{e}	86.0±1.0^{c}	88.3±1.1^{ab}	38.6±1.5^{f}

Notes: The values are mean of three replicates ± standard deviations. Values with different superscripts within the same row show significant differences (p<0.05)

Tab. 3. Gametophyte differentiation under the influence of pesticides (50 days after sowing the spores)

Experimental variants	Species	
	Polypodium vulgare	*Athyrium filix-femina*
	Stage* of gametophyte differentiation	
Control	young chordate prothallia	chordate prothallia with antheridia and archegonia
V1Co	prothallic blade	prothallic filament, necrotic chlorocytes
V2Co	prothallic filament, rhizoids with sinuous cell walls, more intensely coloured than those in V1C	prothallic filament, necrotic chlorocytes and unelongated rhizoids
V3Co	prothallic filament, unelongated rhizoids	prothallic filament, necrotic chlorocytes and unelongated rhizoids
V1B	young chordate prothallia	chordate prothallia, rhizoids surrounded by crystal-shaped deposits
V2B	young chordate prothallia	chordate prothallia, rhizoids surrounded by crystal-shaped deposits
V3B	tridimensional cell mass	tridimensional cell mass

* stage for most gametophytes

ment in leptosporangiate ferns involves the following stages: 1. germination of spores and formation of a prothallic filament; 2. differentiation of the prothallic blade; 3. differentiation of the chordate prothallus with gametangia (antheridia and archegonia) (Ehrendorfer, 1999).

The factors that influence spore germination also influence further differentiation of the gametophyte. In *Athyrium filix-femina* C variant, most gametophytes were still in the chordate stage, with antheridia and archegonia (Fig. 1 a, b); gametophyte differentiation in this case followed the characteristic stages. In all the other experimental variants, the pesticides added to their culture medium affected normal gametophyte development.

Thus, in the V1Co and V2Co variants, most gametophytes remained stagnant at the prothallic filament stage (Fig. 1e). Although spores had germinated, gametophyte development was much slower and prothallic chlorocytes were necrotic. The unequal division that results in the appearance of rhizoids took place normally but their further differentiation was affected and the rhizoids remained unelongated (Fig. 1e). The gametophytes from the V1B and V2B variants had a similar development to that of C, but their rhizoids were surrounded by crystal-shaped deposits (Fig. 1c). In the V3B variant, the normal differentiation of the gametophyte was affected and it developed as a tridimensional cellular mass, which lacked rhizoids (Fig. 1d). Tridimensional cellular masses, also known as callus, are found in abnormal gametophytes that developed from aged spores (Ballesteros *et al*., 2011, 2012; Smith and Robinson, 1975).

Fifty days after sowing the spore, the gametophyte of *Polypodium vulgare* C was still in the chordate stage (Fig. 2a). In the V1Co variant most gametophytes were in the less evolved stage of prothallic blade (Fig. 2e). Rhizoids had cell walls more intensely coloured than those of the C sample. In V2Co, most gametophytes, which were still in the stage of prothallic blade, had rhizoids with sinuous cell walls, more intensely coloured than those in V1Co (Fig. 2f). In the V3Co variant, most gametophytes were forming their prothallic blade; the rhizoids remained unelongated, in the stage of rhizoidal initial (Fig. 2g). The more intense colouring of the rhizoid cell walls may be the result of the accumulation of some polyphenolic compounds whose synthesis was stimulated by the stress induced by toxic substances added to their medium. Bialonska *et al*. (2007) showed that the leaves of *Vaccinium myrtillus* polluted with Zn and Pb suffer structural and biochemical changes. The level of phenols in the polluted leaves was 80-90% higher than that in the unpolluted leaves. The gametophyte developed in the V1B and V2B variants was in the chordate stage (Fig. 2 b, c), while in V3B a tridimensional cell mass (Fig. 2d) formed from the germinated spores, this concentration affecting the normal morphogenesis of the gametophyte, as in the case of the species *Athyrium filix-femina*.

The tested pesticides affect other non-target organisms (Păunescu and Ponepal, 2011; Păunescu *et al*., 2011). Other pesticides, such as asulam, have produced similar effects in the fern species *Cryptogramma crispa, Dryopteris filix-mas* and *Pteridium aquilinum* (Keary *et al*., 2000).

The gametophyte of 8 fern species, the gemmae of *Trichomanes speciosum* and the bulbils of *Huperzia selago* were affected when exposed to asulam (Sheffield, 2002). The same herbicide affects the growth of 18 species of moss (Rowntree *et al*., 2003). The herbicide glyphosat affects megaspore germination and sporophyte formation in the

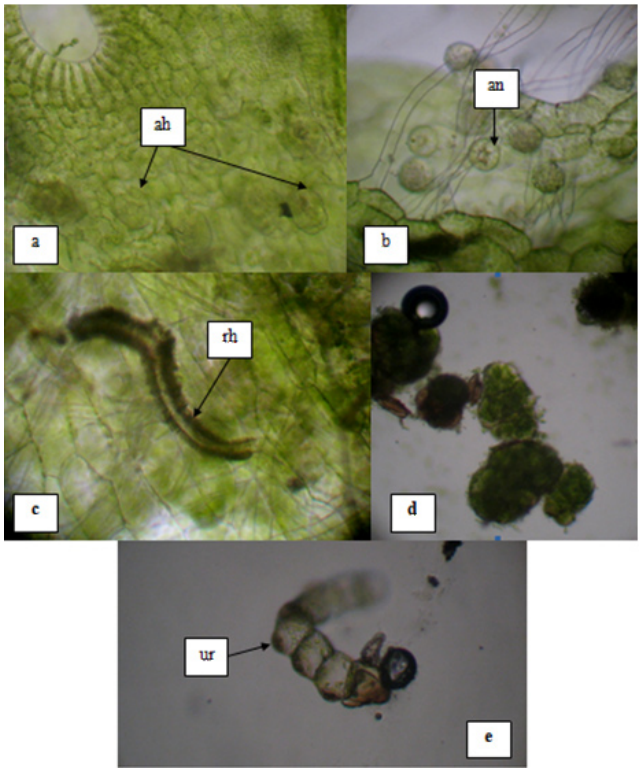

Fig. 1. Gametophyte of *Athyrium filix-femina* (L.) Roth - 50 days after sowing the spore: a, b-control, c-V2B, d-V3B, e-V2Co, ah-arhegonia, an-anteridia, rh-rhizoid surrounded by crystal-shaped deposits, us-unelongated rhizoid (a, b, c, d, e: 100×)

fern species *Regnellidium diphyllum* (Droste *et al*., 2010). The responses of the tested plants to the stress induced by pesticides may function as biomarkers of environmental pollution caused by pesticides (the biomarker concept includes not only biochemical, but also morphological, structural and ultrastructural markers) (Gostin, 2007). In their natural environment, pteridophyte populations can be affected by the pesticides applied either to the ecosystems in which they grow (e.g. forests, crop-growing areas) or to adjacent ecosystems.

Conclusions

The tested fungicide influences spore germination. The germination percentages in both tested species were significantly lower than in the control variant or the experimental variants in which the insecticide was used. The fungicide also affected gametophyte differentiation, which was much slower. In addition, rhizoids suffered modifications related to differentiation, morphology and biochemical composition. In the experimental variants with the highest concentration of insecticide, the gametophyte differentiated as a tridimensional cell mass (callus). Plant responses to the stress induced by tested pesticides may function as biomarkers of environmental pollution.

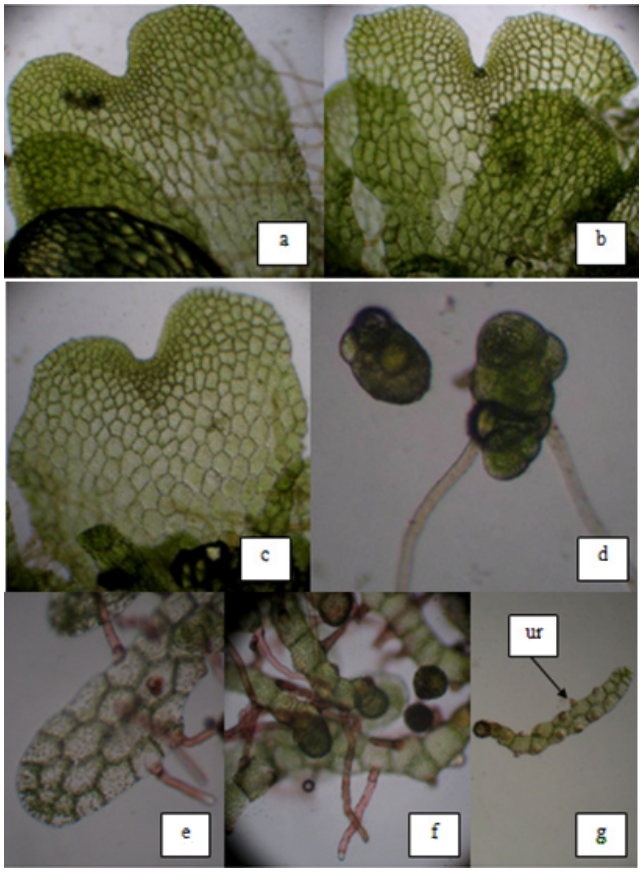

Fig. 2. Gametophyte of *Polypodium vulgare* L. - 50 days after sowing the spore: a-control, b-V1B, c-V2B, d-V3B, e-V1Co, f-V2Co, g-V3Co, ur-unelongated rhizoid (a, b, c, d, e, f: 100×; g: 40×)

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