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ACTIVITATEA ANTIFUNGICĂ A EXTRACTULUI VEGETAL OBȚINUT DIN SPECIA DE FERIGĂ *DRYOPTERIS FILIX-MAS (L.)*

ANTIFUNGAL ACTIVITIES OF VEGETAL EXTRACT OBTAINED FROM *DRYOPTERIS FILIX-MAS (L.)* FERN

Călinescu Mirela[1], Ungureanu Camelia[2*], Marin Florin Cristian[1], Militaru Mădălina[1], Soare Cristina[3], Fierăscu Radu Claudiu[4], Fierăscu Irina[4]
[1]Research Institute for Fruit Growing Pitești, Romania
[2]University "Politehnica" of Bucharest, Romania
[3]University of Pitești, România
[4]The National Institute for Research & Development in Chemistry and Petrochemistry, Bucharest, Romania
*Corresponding author: Camelia Ungureanu; e-mail: ungureanucamelia@gmail.com

Abstract

In this study, we tested the ability of alcoholic extract obtained from underground plants of *Dryopteris filix-mas* (L.) fern to inhibit the growth of apple scab - *Venturia inaequalis* and powdery mildew - *Podosphaera leucotricha (in vitro* and *in vivo)*. The obtained row alcoholic extracts were characterized by analytical techniques and in terms of phytochemical assays. After testing this alcohoolic extract it was obtained an inhibition percente (IP) =42.68 % in case of apple scab (*Venturia inaequalis)* and IP = 55.88 % for powdery mildew *(Podosphaera leucotricha).*

Cuvinte cheie: antimicrobian, extract alcoolic, rapănul mărului, făinarea mărului.
Key words: antimicrobial, alcoholic extract, apple scab, powdery mildew.

1. Introduction

Apple scab, which is caused by the pathogen *Venturia inaequalis* and powdery mildew produced by *Podosphaera leucotricha*, are the most important diseases of apple crop worldwide and it is no wonder that researches on these two pathogens are focused to achieve the health of apple orchards.

Apple scab, also known as black spot, caused by *Venturia inaequalis* ((Cook.) Wint.) is one of the most serious diseases of apple reported in almost all apple producing regions is causing huge economic losses (up to 70% reduction of apple production). Scab infection leads to deformation in shape and size of the fruits, premature leaves and fruits drop, and high susceptibility of tree to chilling and freezing injuries (McHardy, 1996).

Until recently, the use of the synthetic products to control the apple scab and powdery mildew was perceived as being the only measure of economic control but this perception is changed, because the high costs for the production of new molecules, increased the resistance of pathogens to the fungicides, inducing damages of the environment, and negative consumer perception on the use of fungicides.

To control the main apple diseases, apple scab [*Venturia inaequalis* (Cooke) G. Wint.] and powdery mildew [*Podosphaera leucotricha* (Ellis & Everh.) E.S. Salmon], standard strategies are based on intensive fungicide applications in orchard (Parisi, et al., 2013) and cultivars restistance.

However, this resistance is not able to supply a complete protection. Thus, the implementation of strategies is needed to associate different cultural practices and low-fungicides applications (Brun et al., 2010; Didelot et al., 2010).

Chemical control of apple scab caused by *Venturia inaequalis* represents a considerable part of the pest and diseases control measures necessary for apple orchard protection when it is planted with one or several cultivars susceptible to the disease.

For these reasons, the interest in developing alternative strategies to manage the two pathogens, apple scab - *Venturia inaequalis* and powdery mildew - *Podosphaera leucotricha* is increasing. In order to manage apple scab and produce a marketable crop, growers across the globe have relied on 10 to 18 applications of synthetic chemicals at an annual cost of US$202 to $506 per hectare. Nowdays there is an strong interest in increasing to develop alternative strategies to manage especialy apple scab.

These changes include redesigning orchards so that cultivars with differential susceptibility can be treated with fungicides based on different schedules and using post-harvest treatments, such as leaf shredding or application of biological control agents (Misba et al., 2017)

However, these intensive treatments have a negative impact on the beneficial fauna and can lead to the outbreak of certain pests (Gadoury et al., 1989; Cuthbertson and Murchie, 2003; Sauphanor *et al.*, 2005; Carisse and Jobin, 2012), as well as to problems of *V. inaequalis* resistance to the active ingredients used (Parisi et al., 1994; Steinfeld et al., 2001, Köller et al, 1994, 2004, 2005).

More recently, research has focused on the use of bio-fungicides to reduce the damage caused by these diseases. The use of plant extracts as natural bio-fungicides received forward by consumers as the plant extracts may be non-toxic even at concentrations above the concentrations of substances with fungicidal effect obtained by synthesis.

Natural extracts (including various fern extracts) were previously proven to have enhanced antimicrobial properties, against a series of pathogens (Sutan et al., 2016). The aim of the current research was to test the antifungal potential of ethanolic extracts of *Dryopteris* sp. ferns rhizomes against *Venturia inaequalis* and *Podosphaera leucotricha*.

Integrated Fruit Productions Programs encourage the reduction of fungicide sprays to control apple scab (Cooley and Autio,1997).

2. Material and methods

Natural extracts

The vegetal material was collected in 2018 from Valea Valsanului, Romania (N45°20'41.1"; E024°44'06,0", altitude 735 m). A voucher specimen was deposited in the Arges County Museum Herbarium.

The harvested plant material was cleaned of impurities and washed with tap water and then dry at room temperature on paper sheets. The dried vegetal material was grinded to fine powder and extracted using a classical extraction method (temperature extraction, at 68 °C, using ethanol (96%) as previously described (Fierascu et al., 2019); the extract was encoded “DE”. The ratio vegetal material to solvent was kept at 1:19 (w:v).

Evaluation of chemical composition of leaf extracts

In order to evaluate its chemical composition, the extract was characterized using specific procedures for the determination of total phenolics content (Maurya and Singh, 2010), and high-performance liquid chromatography (HPLC).

The quantification of the total phenolic content of the extracts was determined using Folin-Ciocalteu reagent assay as described by Fierascu et al. (Fierascu et al., 2015; Ortan et al., 2015).

Briefly, a pre-determined quantity of diluted Folin-Ciocalteu reagent and Na_2CO_3 solution were added to the corresponding quantity of extract dissolved in ethanol and the mixtures were incubated for 30 minutes with shaking at room temperature. The absorbance was measured at 765 nm, with the results being expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight using the formula:

$$\mathbf{C_{tp} = c \cdot \frac{V}{m}} \quad (1)$$

where C_{tp} - total phenolic content (mg/g) in GAE (gallic acid) equivalent

C - Concentration of gallic acid obtained from calibration curve in mg/mL

V- volume of extract in mL

m - mass of extract in gram

The calibration curves were constructed using gallic acid as standard (Sigma-Aldrich, Germany).

The experiments were carried out in triplicate and the results are presented as standard equivalents.

HPLC analyses were performed using a Varian system (solvent delivery pumps Prostar 410, DAD detector Prostar 335 and autosampler Prostar 410); the obtained data were analyzed using Varian Workstation 6.3 software. The standards used were quercetin and chalcone.

In vitro antimicrobial activity

Antifungal assay was evaluated by agar well diffusion method for antifungal susceptibility as described in detail in previous studies (Ungureanu and Ferdes, 2012; Barbinta-Patrascu et al., 2014). Briefly, agar plates are inoculated with 1 mL inoculum of the test fungal strain. Wells were made at the size of 6 mm diameter, in the agar plates using the sterile borer. The wells with 50 µL of fern extract were placed on the inoculated plates.

The Petri dishes were incubated under suitable conditions at 28 °C for 48 h.

The biological strains used for this investigation were *Venturia inaequalis* and *Podosphaera leucotricha.* The tested fungi were grown in Potato-Dextrose-Agar, PDA (Ph. Eur.) with medium composition: potato peptone 4 g L^{-1}, glucose 20 g L^{-1} and agar g L^{-1}. Similarly, each plate carried a blank well by adding solvent (ethanol) alone to serve as a negative control. Positive control was selected as the reference two chemical fungicidal substance, Captadin 80 WDG (Arysta LifeScience) for *Venturia inaequalis* and Sulfomat 80 PU (Mifalchim) for *Podosphaera leucotricha.* Each experiment was carried out in triplicate with three separate assay runs. The percent inhibition (I%) of the target microorganism was calculated according to the following formula:

$$\mathbf{I\,(\%) = [(ZI - NC)/ZI] \times 100} \quad (2)$$

where: ZI - zone of inhibition, NC - negative control (González-Domínguez et al., 2017).

***In vivo* antifungal activity**

Apple scab - *Venturia inaequalis*

For evaluation antifungal activity of the vegetal extracts against apple scab, young seedlings at stage 4-5 true leaves, originated from 'Idared' cv. were inoculated in greenhouse, in March, with a suspension of 4.5 x 10^5 conidia/mL of *Venturia inaequalis* fungi, applied using a manual atomizer under controlled conditions (temperature 18-20 °C, humidity 70-90 % and a photoperiod of 14 hours light and 8 hours dark). (Militaru et al., 2017). The suspension of conidia was provided by naturally infected leaves harvested from the susceptible varieties. In 2018, the leaves were collected in August - September period, were dried and kept in cool storage. In the spring, the vegetal material was hydrated in distilled water and the apple scab inoculum was prepared and brought to a concentration of 4.5 x 10^5 conidia /mL. After primary infection the treatments with vegetal extracts were applied. The experimental variants were tested in triplicate.

Experimental variants: V1 - Negative control (untreated); V2 - *Dryopteris* extract (DE); V3 - Captadin 80 WG (standard product).

Evaluation of the results was carried out 10-14 days after treatments, visual symptoms of scab infection was quantified according to the scale (Table 1) proposed by Chevalier et al. (1991).

Damage degree DD% was calculated according the formula:

$$DD\% = (F \cdot I) / 100 \quad (3)$$

where, DD (%) = damages degree; F (%) = attack frequency; I (notes) = attack intensity, estimated with notes between 1 and 4.

The antifungal efficacy of the vegetal extracts on the diseases was evaluated after 10 - 14 days, as inhibition percentage, calculated using Abbott formula:

$$\text{Inhibition percent (\%)} = [1-(A/B)] \times 100 \quad (4)$$

where, A represent treated variants and B represent the untreated control.

Powdery mildew - *Podosphaera leucotricha*

For powdery mildew (*Podosphaera leucotricha*), the same kind of the young seedlings, were kept under controlled conditions at a constant temperature of 20 °C and high humidity for the production of natural infection with powdery mildew. After infection occurrence, the treatments with vegetal extracts were applied. The experimental variants were tested in triplicate.

Experimental variants: V1 - Negative control (untreated); V2 - *Dryopteris* extract (DE); V 3 - Sulfomat 80 PU (standard product).

Visual assessment regarding powdery mildew - *Podosphaera leucotricha* leaf area infection were carried out with the aid of diseases rating scale (Table 2) described by Spencer (1977).

Damage degree DD% was calculated according the formula (3) and (4)

where, DD (%) = damages degree; F (%) = attack frequency; I (notes) = attack intensity, estimated with notes between 1 and 6.

Statistical analysis

All experiments were carried out in triplicate, and the data derived was analyzed for statistical significance using analysis of variance (one-way ANOVA) and Tukey test to determine significant differences among means. Significant differences were set at $p < 0.05$. The results presented represent the Mean ± standard error of mean (SEM) of independent replicates.

3. Results and discussions

Phytochemical constituents of the studied object were established by high performance liquid chromatography analysis. HPLC analyses revealed the presence of several active compounds presented in Table 3.

The results of the phytochemical screening revealed the presence of Quercetin and Chalcone; flavonoids like quercetin possess many biochemical effects (Lugasi and Hóvári, 2000; Lugasi and Hóvári, 2002), while the presence of chalcone, in a relatively high amount, would suggest a good antimicrobial potential of the extract, at this particular polyphenolic compound was previously presented as possessing a wide spectrum of biological activities (including antimicrobial, antioxidative, anti-inflammatory or anti-cancer properties) (Boyer-Orlikova, 2011). The total phenolic content of the extracts was 32.35 mg GAE/100 g dw.

The crude extract was found to be active against both fungal species used in this study (responsible for the occurrence of the most common apple diseases). After testing of alcoholic extract it was obtained an inhibition rate IR = 85% in case of apple scab (*Venturia inaequalis)* and IR = 76% for powdery mildew *(Podosphaera leucotricha),* (Table 4 and fig. 1.).

Assessment of the table 5 and fig. 2. reveals that the treatments with vegetal extract - DE - V2 in control of apple scab - *Venturia inaequalis* lead to an inhibition percent 42.68% compared to standard treatment (V3), where the inhibition percent was 85.06%.

Regarding the efficacy of treatments against powdery mildew - *Podosphaera leucotricha,* the inhibition percent in V2 (DE) was 55.88% compared to 94.64% in chemical standard treatment (V3) (Table 6 and Fig. 3.).

4. Conclusions

Dryopteris sp. ferns rhizomes are a valuable source of active ingredients useful for extraction and formulation of bio-fungicides.

Our lab researches highlighted that ferns rhizomes extracts are rich in quercetin and chalcone, two molecules with fungicidal effect.

Alcoholic extracts obtained from ferns rhizomes were effective against the main fungal pathogen of apple crop apple scab - *Venturia inaequalis* and powdery mildew *(Podosphaera leucotricha),* the obtained *in vitro* inhibition rates (IR) were 85% and respectively 76%.

Under greenhouse condition, the inhibation percent of apple scab - *Venturia inaequalis* was 42.68% and 55.88% for powdery mildew *(Podosphaera leucotricha)*

In order to improve the biofungicide formulation and subsequent efficacy, additional researches must be carried out into the orchard.

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Tables and figures

Table 1. Apple scab - *Venturia inaequalis* visual symptoms scale (Chevalier et al., 1991)

Attack intensity (Note)	Symptoms
0	No visible reaction
1	Pit-point symptom. Depression of 100-500 μm where the epidermal cells have collapsed. No subcuticular stroma
2	Wide but shallow depressions. Limited stroma formation. No sporulation
3	Epidermal cells collapsed over large areas. Close to the center the abundant mycelial stromata could produce conidiophores with a limited number of conidia; lesions are a network of mycelial strands. Aborted conidiophores are mixed with normal conidiophores. Sporulating chlorosis and sporulating necrosis occur
4	Numerous conidiophores are often grouped in clusters and sporulate abundantly

Table 2. Powdery mildew - *Podosphaera leucotricha* disease severity rating scale for leaves (Spencer 1977)

Attack intensity (Note)	Percent leaf area infected (%)
0	No infection
1	≤1% infection
2	2-5% infection
3	6-20% infection
4	21-40% infection
5	>40% infection
6	100% infection

Table 3. Composition of the obtained extracts (results are presented in mg L^{-1})

Standard	DE
Quercetin	0.356±0.22
Chalcone	40.549±0.31

Table 4. Inhibition rate of tested sample

Microorganism	Inhibition rate (%)	Zone of inhibition (mm)		
		Sample	NC	PC
Venturia inaequalis	85	32	5	49
Podosphaera leucotricha	76	41	10	67

Table 5. Efficacy of vegetal extract (DE) against apple scab - *Venturia inaequalis*

Variant	Replication	Assessed leaves (no)	Attacked leaves (no)	Frequency [F%]	Intensity (I) [scale1-4]	Damage degree DD [%]	Inhibition percent [%]
V1	R1_R3	46	42	92.59	3.50	3.28	0.00
V2	R1_R3	51	32	62.75	3.00	1.88	42.68
V3	R1_R3	48	15	32.36	1.50	0.49	85.06
	AVG_v	33.00	15.67	31.70	1.50	0.79	42.58
Indicators	STDEV	2.1213	12.0208	21.4890	1.0607	0.9847	29.9672
	VAR	6.4282	76.7286	67.7814	70.7107	124.7727	70.3785

Table 6. Efficacy of vegetal extract against (DE) powdery mildew- *Podosphaera leucotricha*

Variant	Replication	Assessed leaves (no)	Attacked leaves (no)	Frequency [F%]	Intensity (I) [scale1-6]	Damage degree DD [%]	Inhibition percent [%]
V1	R1_R3	47	45	96.29	6.00	5.78	0.00
V2	R1_R3	48	35	72.92	3.50	2.55	55.88
V3	R1_R3	49	12	23.84	1.33	0.31	94.64
	AVG_v	32.33	15.67	32.25	1.61	0.95	50.17
Indicators	STDEV	0.7071	16.2635	34.7060	1.5321	1.5805	27.4075
	VAR	2.1869	103.8093	107.6062	95.0937	165.5160	54.6255

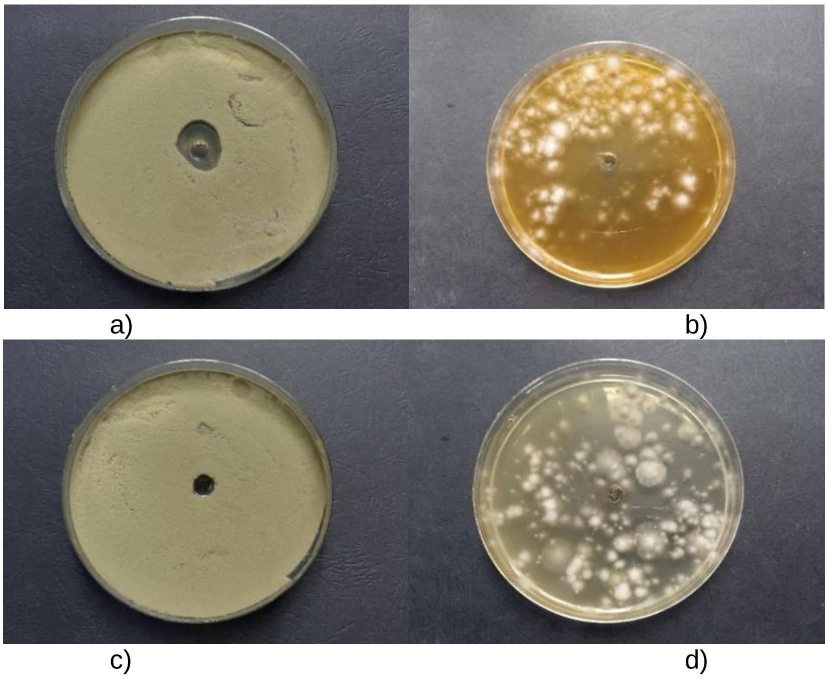

Fig. 1. Representative Petri dishes for antifungal activity of DE on a) *Venturia inaequalis.* b) *Podosphaera leucotricha* and antifungal activity of NC on c) *Venturia inaequalis.* d) *Podosphaera leucotricha*

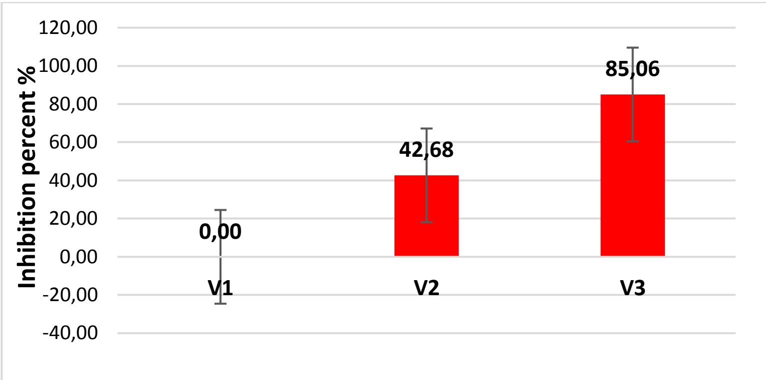

Fig. 2. Vegetal extracts effect in control of apple scab - *Venturia inaequalis* (inhibition percent)

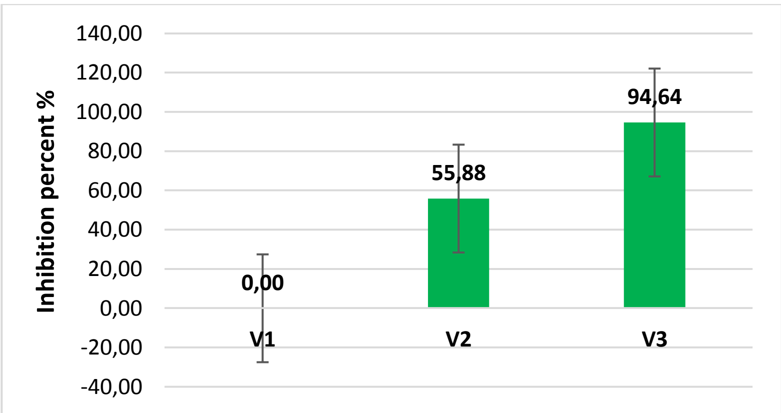

Fig. 3. Vegetal extracts effect in control of powdery mildew - *Podosphaera leucotricha* (inhibition percent)