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Antifungal activity of plant extracts against phytopathogenic fungi

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ABSTRACT
Aqueous-methanolic extracts and acetone exudates of 24 plant species were screened for their antifungal activity against four largely spread plant pathogens possessing a broad spectrum of plant hosts - *Alternaria alternata*, *Botrytis cinerea*, *Phytophthora cambivora* and *Fusarium oxysporum*. Examination of the effect of plant extracts on mycelium growth and development of plant pathogens was done by diffusion method in vitro on PDA medium. The highest antifungal activity with an impact on the most studied pathogens was found for the acetone exudates. Among the tested exudates that of *Salvia officinalis* showed the highest activity against three plant pathogens tested. The exudates of *Artemisia campestris, Artemisa absinthium* and *Clinopodium vulgare* displayed activity against *Fusarium oxysporum* and *Phytophthora cambivora*. Chemical profiles of the most active exudates were determined by HPTLC and GC/MS. Non-polar substances composed mainly of flavonoid aglycones, terpenes, sterols and fatty acids were identified.

Key words: *Artemisia*, *Clinopodium vulgare*, exudates, GC/MS, *Salvia officinalis*, plant pathogens

Introduction

Synthetic fungicides are still largely used in agriculture for the control of plant diseases. Recently there is a growing need for finding a reliable alternative control means in order to avoid the negative aspects of synthetic fungicides application as high levels of toxic residues, relatively quick emergence of resistant fungal plant pathogens to fungicides and high development cost of new chemicals (Lee et al., 2007; Anand & Bhaskaran, 2009; Ademe et al., 2013). Alternative substances derived from natural plant extracts are an object of more extensive research lately for antimicrobial activity (Proestos et al., 2008; Zaker & Mosallanejad 2010; Schrader et al., 2010; Soković et al., 2013; Pierre et al., 2015; Cordova-Albores et al., 2016). Plant extracts and essential oils show antifungal activity against a wide range of fungi and at the same time bioactive products of plants are considered as safe for the human beans, animals and non-target organisms and less persistent in environment (Davidson & Parish, 1989; Kurita et al., 1981; Wilson, et al., 1997; Meepagala et al., 2002; Sharma &Trivedi, 2002; Fokialakis et al., 2006; Barrera-Necha et al., 2008).

Among the very large numbers of phytopathogenic fungi that infect the agricultural crops and forests plants, *Alternaria alternata*, *Botrytis cinerea*, *Phytophthora spp.* and *Fusarium spp.* are largely spread plant pathogens possessing a broad spectrum of plant hosts.

Alternaria alternata has been recorded as a saprophytic or a weak pathogen on a number of crops (Nishimura, 1980; Abbas et al., 1995), but it also can seriously affect many crops, causing leaf spots and defoliation of plants. *Alternaria alternata* rarely kills plants, but can reduce quality and quantity of yield and commercial value of ornamental plants.

Fusariosis induced by *Fusarium oxysporum* is one of the most difficult to control and it is a severe disease of several crops, greenhouse plants and trees. *Fusarium oxysporum* is a broad spread species in the world and it causes significant losses in crop production and has been reported in many countries (Agrios, 1988; Jones et al., 1991).

Phytophthora cambivora (Petri) Buisman is widespread in regions with temperate climate of all continents and occurs in soils of natural forests, agricultural fields, and orchards. It is among the most important soilborne diseases of stone fruit trees (Browne et al., 1995; Browne & Mircetich, 1996) The pathogen causes of extensive root rot of several woody plants (Jönsson et al., 2003; Jung, 2009; Jung et al., 2000; Vettraino et al., 2002, 2005) in Europe and North America. The fungus along with *P. cinnamomi*, is known as the causal agent of ink disease, one of the most destructive diseases affecting chestnut (Castanea sativa Mill) (Day, 1938; Vannini & Vettraino 2001; Vettraino et al., 2005). Currently, no efficient

control strategies are available for ink disease (Vannini and Vettraino, 2011).

Botrytis cinerea is a fungal pathogen recorded on many plant species like grapevine, other fruit plants and vegetables. It can affect almost every part of plants, from the stems all the way to the leaves, flowers, or fruit.

The aim of present study was to screen aqueous-methanolic extracts and acetone exudates of plant species for their inhibition activity against four largely spread plant phytopathogenic fungi possessing a broad spectrum of plant hosts - Alternaria alternata, *Botrytis cinerea*, *Phytophthora cambivora* and *Fusarium oxysporum*. The most active samples were analyzed for chemical composition by HPTLC and GC/MS.

Materials and Methods

Plant material

Plant material was collected from natural populations or cultivated areas of 24 plant species of family. The species were identified according to the keys by Assyov et al. (2012).

Acetone exudates

Air-dried, but not ground plant material of studied species was briefly (2-3 min) rinsed with acetone at room temperature to dissolve the lipophilic components accumulated on the surface. The obtained acetone exudate was then dried using a rotary-evaporator to give a crude extract.

Metanolic extracts

Air-dried, ground plant material was extracted with 80% methanol by classical maceration for 24 h two times. After evaporation of the solvent, the crude extract was subject to subsequent analysis.

Antifungal activity

Examination of the effect of plant extracts on mycelium growth and development of plant pathogens was done by diffusion method with some modifications (Magaldi et al., 2004; Balouiri et al., 2016). Fungal mycelium growth was studied in vitro in Petri dishes on PDA agar in a laboratory. The media in Petri dishes were inoculated with a small amount of mycelium in the center of the Petri. 100 mg of the extracts of each plant was dissolved in 1 ml of the extraction solvent and four drops of 15 µL of each solution were placed on the surface of the media in Petri dishes, equally distant from each other and from the mycelium. The Petri dishes with the plant pathogens and applied exudates were incubated at temperature 25 °C in dark. The time of incubation was from 3 to 10 days depending on the growth speed of the fungal species. Each variant was maintained in four replicates. Three controls were parallel incubated – one with mycelium only and one with mycelium and applied on the media drops of extraction solvent. The restriction in the diameter of the colonies was estimated after the fungal colonies in the control without plant extracts covered completely the medium in Petri dish.

The inhibition effect of the exudates on the mycelia growth was evaluated using the following scale:

+++ - strong inhibition effect on mycelium growth;

++ - weak inhibition effect on mycelium growth;

+ - hardly noticeable inhibition effect on mycelium growth;

− - no inhibition observed on mycelium growth

Thin layer chromatographic analysis

The acetone exudates were examined for flavonoid aglycones by co-*TLC* with authentic standards. Two TLC sorbents and four mobile phases were used for the analysis of the flavonoid exudates. Toluene:dioxan:acetic acid (95:25:4, v/v/v) and toluene:methylethylketone (9:1, v/v) were used for the development of the aglycones mixture on silica gel plates Kiselgel 60 F254 (10x20 cm, 0.2 mm layer). Toluene: methylethylketon:methanol (60:25:15, v/v/v) and toluene : petrol ether : methylethylketone : methanol (60:30:10:5, v/v/v/v) were used for DCAlufolien Polyamid 11 F254 plates (10x20 cm, 0.15 mm layer). Chromatograms were viewed under UV=366 nm light before and after spraying with ‘‘Naturstoffreagenz A’: 1% solution of diphenylboric acid 2-aminoethyl ester complex in methanol.

Metabolite analysis

The GC–MS spectra were recorded on a Termo Scientific Focus GC coupled with Termo Scientific DSQ mass detector operating in EI mode at 70 eV. ADB-5MS column (30 m x 0.˙25 mm x 0.˙25 µm) was used. The temperature program was: 100-180 ^{o}C at 15 ^{o}C x min^{-1}, 180-300 20 at 5 ^{o}C x min^{-1} and 10 min hold at 300 ^{o}C. The injector temperature was 250 ^{o}C. The flow rate of carrier gas (Helium) was 0˙8 mL x min^{-1}. The split ratio was 1:10 1 µL of the solution was injected. The methanolic extract were silylated with 50 µL of N,O-bis-(trimethylsilyl)trifluoro-acetamide (BSTFA) in 50 µL of pyridine for 2 h at 50°C.

The metabolites were identified as TMSi derivatives comparing their mass spectra and Kovats Indexes (RI) with those of an on-line available plant specific database. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. RI of the compounds were recorded with standard n-hydrocarbon calibration mixture (C9-C36) (Restek, Cat no. 31614, supplied by Teknokroma, Spain) using AMDIS 3.6 software.

Results

RESEARCH ARTICLE

Six acetone exudates and eighteen aqueous-methanolic extracts were evaluated for antifungal activity against four plant pathogens - *Alternaria alternata*, *Botrytis cinerea*, *Phytophthora cambivora* and *Fusarium oxysporum*. Four of the six studied exudates showed activity (Table 1).

Among them that of *Salvia officinalis* showed the highest activity against three plant pathogens tested. The exudates of *Artemisia campestris*, *Artemisa absinthium* and *Clinopodium vulgare* displayed activity against *Fusarium oxysporum* and *Phytophthora cambivora*.

Among the studied eighteen aqueous-methanolic extracts six showed antifungal activity with different intensity. The extracts of *Artemisia annua* and *Achillea critmifolia* showed significant activity against *Alternaria alternata*. Additionally the exudate of *A. annua* exhibited activity against *Botrytis cinerea*. The extracts of *Plantago conutii*, *Elymus hispidulus*, *Arnica montana*, *Abies alba*, *Leuzea carthamoides* displayed low activity. Antifungal activity was no found for the extracts of the other species. None of the tested aqueous-methanolic extracts showed activity against *Phytophthora cambivora*.

The received results showed that the studied acetone exudates exhibited stronger antifungal activity and have an impact on a wider spectrum of pathogens than the aqueous-methanolic extracts; therefore the exudates became objects of chemical composition analysis. GC/MS and HPTLC based analysis of acetone exudate of the flower parts of *Salvia officinalis* showed presence mainly of terpenes and flavonoid aglycones (Table 2 and Table 3).

Thujone and camphor were identified in the largest amount by GC-MS. Flavone aglycones apigenin and luteolin and their methyl derivatives were identified by HPTLC. In the acetone exudates of *Artemisa campestris* phenylpropanoid - euvgenol was identified as main component. Flavonoid aglycones of flavones and flavonol classless were identified also. Highly methylated quertcetagetin derivatives (flavonol aglycones) were identified as main compounds in the acetone exudates of *Artemisia absinthium*. Triterpenoids - ursolic, oleanolic and betulinic acids were found as predominant compounds in the acetone exudate of *Clinopoium vulgare* by GC/MS.

Table 1. *Antifungal activity of plant extracts against phytopathogenic fungi.*

Plant species	Plant parts	Phytopathogens			
		Alternaria alternata	*Botrytis cinerea*	*Fusarium oxysporum*	*Phytophthora cambivora*
		Acetone exudates			
Artemisia absinthium	A	−	−	+++	+ ++
Artemisia campestris	A	−	−	+++	+
Achillea asplenifolia	A	−	−	−	−
Clinopodium vulgare	A	−	−	++	++
Stachys officinalis	A	−	−	-	-
Salvia officinalis	F	+++	+++	+ +	-
		Aqueous-methanolic extracts			
Erigeron canadensis	A	−	−	−	−
Erodium cicutarium	A	−	−	−	−
Hepatica nobilis	A	−	−	−	−
Forsicia viridissima	L	−	−	−	−
Rumex alpine	R	−	−	−	−
Hypericum perforatum	A	−	−	−	−
Euphorbia helioscopia	A	−	−	−	−
Plantago cornuti	L	−	−	++	−
Achillea critmifolia	A	+ ++	−	−	−
Artemisia annua	A	+ ++	+ +	−	−
Crithinum maritimum	L	−	−	−	−
Abies alba	N	+		−	−
Arnica montana	S	+	−		
Arnica montana	A	−	−		
Artemisia vulgaris	A	+	−	−	−
Elymus hispidulus	L	+ +	−	−	−
Stachys officinalis	A	−	−	−	−
Leuzea carthamoides	R	+	−	−	−

Legend: A – aerial; F – flower; L – leaves; N - needles; R – root; S – semen; +++ - strong inhibition effect on mycelium growth; ++ - little inhibition effect on mycelium growth; + - very little inhibition effect on mycelium growth; − - no inhibition observed on mycelium growth

Table 2. *Metabolites identified in the studied acetone exudates by GC-MS**

Compounds	RI	SO	AC	AA	CV
cis-Thujone	1102	7.2	0.1		
trans-Thujone	1114	24.2			
Camphor	1146	17.0			
Isobornerol	1162	0.7			
Glycerol	1260	2.3	4.7	6.0	
Bornyl acetate	1289	5.4			
α-Copaene	1377	0.4			
Eugenol	1359		44.0		
β-Cubebene	1388	0.9	2.6		
Farnesene	1457	2.5			
Germacrene D	1487	2.8			
Pentadecane	1500				
α-Bulnesene	1509			1.4	
α-Muurolene	1511	2.4			
Nonanedioic acid (azelaic acid)	1512				0.6
γ-Cadinene	1554	1.1			
trans-Calamenene	1529	2.0			
Spathulenol	1578		5.2		
Caryophyllene oxide	1583		2.9		
Cubenol <1,10-di-epi->	1619		4.7		
β-Eudesmol	1651		0.5		
1-Tetradecanol	1720		0.3		
Tetradecanoic acid	1788				2.2
Hexadecanoic acid (Palmitic acid, C16:0)	1922	4.8	14.8	12.6	
Abietatriene	2062	1.6			
Octadecadienoic acid (Linoleic acid, C18:2)	2089		2.1		
Octadecanoic acid (Stearic acid, C18:0)	2123	0.5	0.5		
Octadecanol	2152		0.2		
9-(E)-Octadecenoic acid	2227				0.7
Tricosane	2298		0.4		
Silane	2545			3.4	
Hexadecanoylglycerol	2583	1.3		4.4	1.2
Tetracosanol	2742			7.0	
1-Monooctadecanoylglycerol	2775	0.5	0.4	3.5	
Triterpene 1	3004				0.7
Triterpene 2	3035				5.6
Oleanolic acid	3062				15.5
Betulinic acid	3080				25.7
Ursolic acid	3115				30.9
Naphthalene derivative	3142				14.6
Triterpene 3	3164				0.6
Stigmasterol	3262		3.5		
Triterpene 4	3311				0.9
Quercetagetin 3,5,6,7,3',4'-pentamethyl ether	3318			61.7	
β-Sitosterol	3334		2.2		
Triterpene 6	3340				0.5
β Amyrin	3360.8	1.4	2.5		

Legend: *SO-Salvia officinalis, flower; AC Artemisia campestris, aerial parts; AA- Artemisia absinthium, aerial parts; CV-Clinopodium vulgare, aerial parts*

**Data are expressed as percentage of the total peak area of identified compounds [%]*

Discussion

The strongest and a broad spectrum antifungal activity were found for the acetone exudates. The most general exudates are contained of non-polar compounds that are accumulated on the surface of plants. The disposition of these compounds suggests that they have a protective role and allelopathic potential as having been confirmed by studies in this regard (Onyilagha and Grotewold 2004**;** Lahtinen et al., 2004). The literary review has showed that there are many

Table 3. *Flavonoid compounds identified of acetone exudates of studied species*.

Compounds	Acetone exudates of studied species			
	AA	AC	SO	CV
Apigenin		●	●	●
Apigenin 4‘-methyl ether			●	
Apigenin7-methyl ether		●		
6-Hydroxyapigenin (scutellarein) 6-methyl ether		●	●	
8-Hydroxyapigenin (isoscutellarein) 8- methyl ether			●	
Luteolin		●	●	●
6-Hydroxyluteolin-6-methyl ether		●	●	
Kaempferol 3,7-dimethyl ether	●			
Kaempferol 7- methyl ether		●		
Quercetin		●		
Quercetagetin 3,6,7-trimethyl ether	●			
Quercetagetin 3,6,7,3'-tetramethyl ether	●			
Quercetagetin3,6,7,3',4'-pentamethyl ether	●			

Legend: SO-*Salvia officinalis*, flower; AC *Artemisia campestris*, aerial parts; AA- *Artemisia absithium*, aerial parts; CV- *Clinopodium vulgare*, aerial parts

data on antifungal activity of essential oil (Ouadi et al., 2015; Rus et al 2015) and methanolic extract (Yanar Y et al., 2011) of *Salvia officinalis*, aqueous and alcoholic extracts of *Artemisia* species (Ali et al., 2015 Wang et al., 2001; Damian-Badillo et al., 2010) but not found evidence on the effect of exudates of plants on phytopathogenic fungi.

GC/MS and HPTLC analysis of exudates of *Salvia officinalis*, *Artemisia campestris* and *A. absinthium* showed that they rich on flavonoid aglycones and terpenes. The identification of the flavonoid aglycones was also confirmed by literature data (Valant-Vetschera et al., 2003; Nikolova M et al., 2006; Veličković D et al., 2007; Karabegović et al., 2011). These are secondary metabolites with great antifungal potential (Hadizadeh et al., 2009; Lourenço et al., 2013). More that there are data that flavonoid aglycones display stronger antifungal activity than flavonoid glycosides which are component of methanolic extracts (Picman et al., 1995). Triperpene derivatives were determined as a main component of acetone exudate of *Clinopodium vulgare* in the present study. This is consistent with the previously reported data for chemical composition of the species (Todorova et al., 2016). There is evidence that detected compounds of acetone exudate of *C. vulgare* exhibited antifungal activity (Favel et al., 1994; Rokade and Sayyed, 2009; Choi et al., 2017). Alcohol extracts of *C. vulgare* have been shown to display antibacterial activity (Opalchenova & Obreshkova 1999; Stefanovic et al., 2011).

Conclusion

Examination of the effect of acetone exudates of Salvia officinalis, Artemisa absinthium, A. campestris and Clinopodium vulgares as well as aqueous-methanolic extracts of Artemisa annua and Achillea critmifolia on mycelium growth and development of studied pathogens in the present study showed promising prospects for the utilization of exudates as “biological fungicide” for the control of plant pathogens.

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