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Impact of *Origanum vulgare* subsp. *hirtum* (Link) Ietswaart derived extracts and essential oil on plant pathogens from genus *Phytophthora*

A. Lyubenova[1*], M. Nikolova[2] and S. B. Slavov[3,4]

[1] *Agricultural Academy, Department of Agrobiotechnology, AgroBioInstitute, 1164 Sofia, Bulgaria*
[2] *Bulgarian Academy of Sciences, Institute of Biodiversity and Ecosystem Research, Department of Plant and Fungal Diversity and Resources, 1113 Sofia, Bulgaria*
[3] *University of Forestry, Department of Plant Protection, Faculty of Agronomy, 1797 Sofia, Bulgaria*
[4] *Agricultural Academy, Department of Functional Genetics, Abiotic and Biotic Stress, AgroBioInstitute, 1164 Sofia, Bulgaria*
[*]*Corresponding author:* anetalyubenova@abi.bg

Abstract

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In the present study we evaluate the potential of essential oil and extracts obtained from Greek oregano (*Origanum vulgare* ssp. *hirtum* (Link) Ietswaart) to inhibit the mycelial growth of economically important plant pathogens from genus *Phytophthora*. The metabolite profiles of the essential oil, exudate, extract and methanolic polarity-based fractions were analyzed by GC/MS. The inhibitory activity of the essential oil and extracts against *P. nicotianae* var. *nicotianae*, *P. rosacearum, P. citricola, P. plurivora* and *P. cryptogea* was evaluated in an *in vitro* bioassay. Carvacrol (55.48%), p-cymene (11.06%) and γ-terpinene (15.04%) were the most abundant in the essential oil composition. In the acetone exudate and non-polar fraction, the main compound was carvacrol (48.15% and 50.34% respectively). In least amount carvacrol was found in the methanolic extract (13.24%) and completely absent in the polar fraction, where carbohydrates were the most abundant. All *Phytophthora* isolates responded to extracts and essential oil treatments with a decline of the vegetative growth to a different extend. A complete inhibitory effect was observed with *P. nicotianae* var. *nicotianae* in the essential oil, nonpolar fraction, acetone exudate and methanol extract variants, while the mycelial growth of *P. cryptogea* and *P. plurivora* was inhibited to a moderate degree. *P. rosacearum* was the most tolerant among tested oomycete representatives in these variants. We conclude that the economically important *Phytophthora* species, investigated in this study are susceptible to the impact of essential oil, methanol extract, the methanol's non-polar fraction, and acetone exudates of the aromatic plant *O. vulgare* subsp. *hirtum*. Essential oil, methanol extract, acetone exudate and non-polar fraction of Greek oregano are rich of carvacrol and could find further application in different sustainable crop protection systems.

Keywords: GC/MS analysis; carvacrol; agar diffusion method; mycelial growth area

Introduction

One of the main challenges faced by the contemporary agriculture is the urgent need for alternative and more sustainable approach for overcoming the ecological and health risks associated with the use of chemical pesticides (Nicolopoulou-Stamati et al., 2016). Plant-derived natural products, generally safe to humans and environment can be a part of the integrated pest management systems, as an effective alternative of synthetic chemical pesticides (Isman, 2000). In the last decades, a significant number of studies have been devoted on the potential use of natural plant derived compounds for the

control of different plant pathogenic microorganisms (Isman, 2000; Santra & Banerjee, 2020). Furthermore, some essential oils have demonstrated a strong inhibitory effect on seed germination of economically important weeds (Nikolova et al., 2021). Together with other natural products, plant extracts and essential oils are recognized as promising tools with a high antimicrobial potential on the fungal diseases control arena (Santra & Banerjee, 2020).

Origanum vulgare L. (*Lamiaceae*) is an aromatic perennial herb widely used throughout the world as spice and medicinal plant. The species is considered as perspective source of bioactive natural products, possessing the potential as a protective agent in a variety of human diseases, based on the anticancer, anti-inflammatory, antioxidant and antimicrobial activities of its bioactive phytochemicals (Pezzani et al., 2017). A significant number of studies demonstrate promising applications of oregano botanical compounds in plant protection. Multitude reports on the microbial inhibitory effects of oregano essential oil, as well as oregano extracts are summarized by (Kintzios, 2012). *O. vulgare* ssp. *hirtum* is characterized with a high essential oil yield (> 2%), compared to the other subspecies (Alekseeva et al., 2020). In a recent study the essential oil of *O. vulgare* and two of its main components thymol and carvacrol, have shown strong antifungal activity against *Botrytis cinerea*, both *in vitro* and *in vivo* (Zhao et al., 2021). It was also found that Greek oregano essential oil have strong inhibiting effect on a number of fungal pathogens – *Fusarium* spp., *Alternaria alternata*, *Neocosmospora* sp. and *Botrytis cinerea* (Krumova et al., 2021).

Species from genus *Phytophthora* include some of the most economically important plant pathogens on agricultural and forestry crops (Erwin & Ribeiro, 1996). The majority of *Phytophthora* species are soil born root pathogens, but some of them can infect the above ground plant parts. Their spread is favoured by the availability of free water in the soil. *P. nicotianae* infects a wide variety of host species and causes diseases such as root and crown rot, as well as blight of fruit or foliar tissue. The host range includes tobacco, citrus crops, cloves, cotton and much more other plant species (Erwin & Ribeiro, 1996). *P. plurivora* and *P. citricola* are close relative species, which occupy similar niches causing fine root destructions, collar rots and aerial bark cankers on oaks and other tree species (Jung & Burgess, 2009). *P. cryptogea* and *P. rosacearum* are both economically important pathogens on horticulture crops (Erwin & Ribeiro, 1996). *P. rosacearum* host range is restricted to family *Rosacea* (Hansen et al., 2009), while *P. cryptogea* is characterized with a wide host range including orchard, ornamental and forest plants from a variety of botanical families (Pérez-Sierra & Jung, 2013). In general, all *Phytophthora* species are difficult to control, due to their ability to evolve rapidly and to develop tolerance to chemical treatments (Callaghan & Guest, 2015; Hunter et al., 2018).

In this study was investigated the potential of *O. vulgare* subsp. *hirtum* derived extracts and essential oil to impair the development of economically important plant pathogens from genus *Phytophthora* under *in vitro* conditions. The obtained metabolite profiles of the essential oil, exudate, extract and fractions derived from Greek oregano plants are discussed in relation with their antifungal activity. The possible use of these plant derived components for *Phytophthora* diseases control is considered.

Materials and Methods

Plant material and preparation of the extracts

Aerial parts of *Origanum vulgare* ssp. *hirtum* were collected during the flowering stage from natural population at the Struma valley, Bulgaria. Voucher specimen was deposited at the Herbarium, Institute of Biodiversity and Ecosystem Research (SOM), Bulgaria (CO1409).

Methanol extract and fractions

Crude methanol extract was prepared from air-dried, powdered aerial parts of the species macerated with methanol at room temperature for 24 h. After filtration, the organic phase was evaporated to dryness. Part of the extract was left for phytopathogenic activity analyses and another was continued to be processed to obtain fractions. The extract was dissolved in distilled water and chloroform in a ratio of 1:3 and was processed in a separatory funnel several times to discoloration of the chloroform layer. The combined chloroform extracts were evaporated to dryness to produce a non-polar fraction. The aqueous phase was also evaporated to dryness and represents the polar fraction.

Acetone exudates

Exudate was prepared from air dried, not ground aerial parts by rinsing with acetone for several minutes to dissolve compounds accumulated on the surface of plant tissue.

Essential oil

Essential oil was extracted on a Clevenger apparatus by water distillation from dry aerial parts of the species.

GC/MS analysis

The GC–MS spectra of the used plant products were recorded on a Termo Scientific Focus GC coupled with Termo Scientific DSQ mass detector operating in EI mode at 70 eV. Before GC-MS analysis 50 mg of the extract, exudate and fractions were processed with derivatization reagent

N-O-bis(trimethylsilyl)trifluoroacetoamide (BSTFA). The chromatographic conditions for essential oil and the rest plant products are described by Traykova et al. (2019) and by Berkov et al. (2021) respectively. Retention Indices (RI) of the compounds were determined on the basis of homologous n-alkane hydrocarbons under the same conditions. The components were identified by comparing their mass spectra and retention indices (RI) with those of authentic standards and the National Institute of Standards and Technology (NIST) spectra library

Phytophthora spp. isolates

The *Phytophthora* isolates used in this study originate from agricultural or forestry ecosystems in Bulgaria, except one from imported plant. More detailed information is presented in Table 1.

The *Phytophthora* isolates were obtained by baiting rhizosphere soil samples (Jung et al., 2002) from various plants, exhibiting dieback symptoms to different extents. The taxonomic identification of the isolates was performed using both classical methods and molecular techniques (Lyubenova et al., 2015)

Bioassay

For the bioassay we used the Agar disk-diffusion method (Balouiri et al., 2016). Small agar blocks (2×2 mm) with mycelium of the corresponding isolate were cultured in the center of 9 mm Petri dishes. We grew the isolates from genus *Phytophthora* on carrot juice agar (16 g agar, 3 g $CaCO_3$, 100 mL HiPP Organic Pure Carrot Juice, and 900 mL distilled water). The Petri dishes were incubated in dark for 12 h for synchronize onset of growth before the drip of the tested oil and extracts. Each one of the tested extracts was dissolved in the corresponding solvent – methanol (for the methanol extract) or DMSO (for the acetone exudate, polar and non-polar fractions).

Five variants were performed with all isolates: with essential oil, methanol extract, acetone exudate and with the two polarity-based fractions of the methanol extract. Two drops with a volume of 15 µl of the extracts (100 mg/ml) were dripped into each plate at equal distances from its centre. In the same way the essential oil was applied with a drop volume of 2 µl. Four replicates were made for each variant, and control variants without treatment and with the solvent for each isolate. The Petri dishes were cultivated in a climatic chamber in darkness at 20°C. The results were documented after 6 days. Photographs (Canon Power shot A610) of all mycelial colonies were taken, and their mycelial growth areas were measured, using the image analysis program ImageJ (Schneider et al., 2012). On the basis of the obtained data (average mycelial growth area for each variant) was calculated the percentage of inhibition (Zygadlo et al., 1994) by the equation:

$$IMG = 100(C - T)\ C^{-1},$$

where *IMG* is percentage of inhibition of the mycelial growth, *C* is the area of the fungal colony without treatment (control) and *T* is the area of the fungal colony with treatment.

Results

GC/MS analysis

The used plant products of *O. vulgare* ssp. *hirtum* – essential oil, exudate, extract and fraction were analysed for their metabolite profile by GC/MS. The results are presented at Table 2. Carvacrol (55.48%), p-cymene (11.06%) and γ-terpinene (15.04%) were identified as the most abundant in the essential oil composition. Carvacrol was found as main compound in the acetone exudate (48.15%) and non-polar fraction (50.34%) also. In methanolic extract carvacrol was found in less amount (13.24%) and completely absent in the polar fraction. In the last carbohydrates were found as the most abundant.

Bioassay

All *Phytophthora* spp. responded with a decline of the mycelial growth to the application of all extracts used in the study, except of the polar fraction. The mycelial growth of *P. nicotianae* var *nicotianae* was reduced with 96% in the methanol extract variant, 95% in the essential oil, 85% in the non-polar

Table 1. List of isolates used in the study

Phytophthora sp.	Source of isolation	Origin, year of isolation
P. nicotianae Breda de Haan var. *nicotiana*e	Rhizosphere soil of *Euphorbia pulcherrima*	Garden center in Sofia, Bulgaria, plant imported from Netherlands, 2020
P. rosacearum Hansen et Wilcox	Rhizosphere soil of *Prunus domestica*	Orchard in Tulovo, Bulgaria, 2011
P. citricola Sawada	Rhizosphere soil of *Rubus idaeus*	Raspberry plantation in Gotse Delchev, Bulgaria, 2012
P. plurivora T. Jung & T.I. Burgess	Rhizosphere soil of *Alnus glutinosa*	Along Erma River near Tran, Bulgaria, 2011
P. cryptogea Pethybr. & Laff.	Rhizosphere soil of *Rubus idaeus*	Raspberry plantation in Troyan, Bulgaria, 2012

Table 2. Identified compounds (%) in the studied *O. vulgare* ssp. *hirtum* products (RI – retention indices, EO – essential oil; Me – methanolic extract; AF – Non-polar fraction; PF – polar fraction; AE – acetone exudate)

Compounds	RI	EO	Me	AF	PF	AE
β-Thujene	930	1.71				
α-Pinene	932	1.91				
Camphene	946	0.41				
β-Thujene	978	0.6				
β-Myrcene	988	2.91				
α-Terpinene	1015	3.28				
p-Cymene	1023	11.06				
Terpineolcis-β-	1144	0.25				
γ-Terpinene	1059	15.04				
Linalool	1094	0.16				
Isoborneol	1169	0.37				
Terpinen-4-ol	1175	0.38				
Carvacrol methyl ether	1231	2.21				
Thymoquinone	1249			3.29		4.16
Succinic acid	1310		0.6			
Carvacrol	1339	55.48	13.24	50.34		48.15
Carvacrol acetate	1372	0.13				
β-Bourbonene	1388	0.1				
Caryophyllene	1401	1.52				
Humulene	1457	0.16				
Malic acid	1473		1.09			
Erythritol	1493		0.56		0.23	
β-Bisabolene	1531	0.41				
Hydroquinone deriv.	1559		2.75			15.22
Caryophyllene oxide	1590	0.16		1.42		
4-Hydroxybenzoic acid	1637		0.53			
Octanoic acid	1575		0.39			
Vanilic acid	1753		0.14			
Fructose	1803		10.32		7.54	
Quinic acid	1840		4.43		3.22	
Glucose	1889		11.65		4.17	
Hydroxycinnamic acid	1934		0.5			
Hexadecanoic acid	2041			1.1		
Myo inositol	2080		1.76		2.63	
Linolenic acid	2090		0.78	1.34		0.67
Sucrose	2628		10.51	0.55	35.26	
Naringenin 7-methyl ether	2669			0.1		0.41
Catechine	2863		0.23			
Catechinegalate	2080				11.2	
β-Sitosterol	3335		0.58	0.21		
Rosmarinic acid	3454		2.1			
Triterpene acid	2928					1.8
Ursolova acid	3657		0.24			4.41

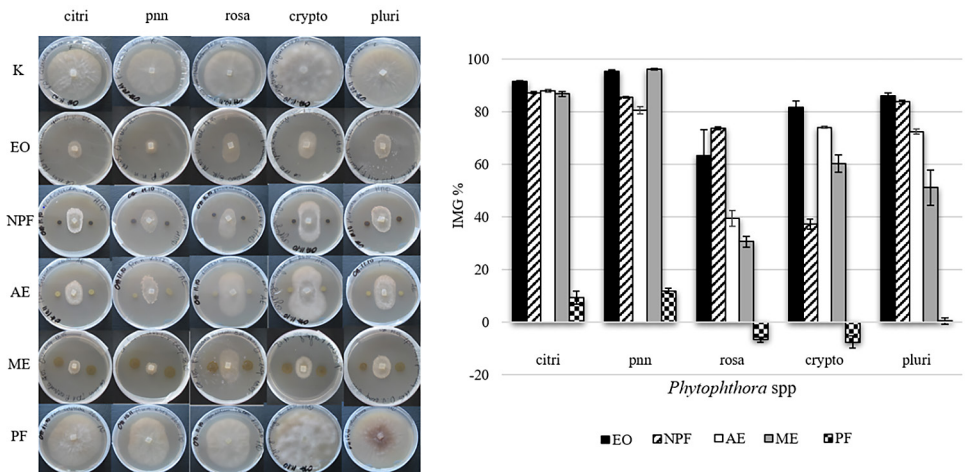

Fig. 1. Inhibition of the Mycelial Growth (IMG) of five *Phytophthora* spp. under the impact of *O. vulgare* subsp. *hirtum* essential oil (EO), methanolic extract (ME), acetone exudate (AE) methanolic polar fraction (PF) and non-polar (NPF) fraction under *in vitro* conditions after 6 days at 20°C. -; citri – *P. citricola*; Pnn – *P. nicoianae* var. *nicotianae*; crypto – *P. cryptogea*; rosa – *P. rosacearum*; pluri – *P. plurivora*. Bars represent the standard errors of the mean

fraction and 80% in the acetone exudate variant (Figure 1). The tendency is similar in the rest of the *Phytophthora* isolates – between 91 and 81% in *P. citricola*, 86 and 51% in *P. plurivora* and 81 and 60% in *P. cryptogea*. *P. rosacearum* was the most tolerant among tested oomycete species – between 73% IMG in the non-polar fraction variant and 37% IMG in the acetone exudate variant. The polar fraction had no significant inhibitory effect – between 11% and 9% IMG in *P. nicotianae* var *nicotianae* and *P. citricola* variants respectively. Furthermore – enhancement in the growth of *P. cryptogea* and *P. rosacearum*, compared to the control was recorded (Figure 1).

Discussion

The results from the bioassay point out a correlation between the % IMG and the percent content of carvacrol – the highest in oil, followed by the non-polar fraction and the acetone exudate (Table 2). The lack of inhibiting effect on the mycelial growth of the isolates in the polar fraction variants was expected, since the sucrose is present in the polar fraction at the highest rate (35.26%) (Table 2). There are many reports about antimicrobial activity of carvacrol (Friedman, 2014) and other monoterpenes (Marchese et al., 2017), which gives us reason to assume that the identified antifungal activity is determined mainly by these compounds.

The inhibitory effects of the fractions were more pronounced in the slow growing isolates: *P. citricola*, *P. nicotianae* and *P. plurivora* compared to the fast-growing *P. cryptogea* and *P. rosacarum* (Figure 1).

Recent scientific reports on the use of the plant essential oils as potent biocontrol agents were summarized by (Raveau et al., 2020), although the targeted plant pathogens were restricted mainly to *Alternaria*, *Botrytis*, *Fusarium*, *Penicillium* and *Rhizoctonia* spp. Authors emphasize out the potential of essential oils to target fungi causing diseases both during the cultivation or occurring during the storage (post-harvest diseases). Possibilities for control of diseases caused by *Phytophthora* plant pathogens with botanical extracts are an experimental field that is yet to grow.

Conclusions

The economically important *Phytophthora* species investigated in this study are susceptible to the impact of essential oil, methanol extract, the methanol non-polar fraction and acetone exudates of the aromatic plant *O. vulgare* subsp. *hirtum*. The susceptibility is expressed as inhibition of the mycelial growth of the isolates under *in vitro* conditions. The main bioactive compound in the tested oil and plant extracts is their polyphenolic constituent carvacrol. Oregano oil, methanol extract, acetone exudate and non-polar fraction of Greek oregano are rich on carvacrol and could find further application in different sustainable crop protection systems.

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