Antifungal Activity and Chemical Composition of *Origanum Vulgare* L. Essential Oil

CRISTIAN FLORIN RUS[1], ERSILIA ALEXA[1], RENATA M. SUMALAN[1], ATENA GALUSCAN[2], ADINA DUMITRACHE[4]*, ILINCA M. IMBREA[1], IOAN SARAC[1], ANDREEA PAG[3], GEORGETA POP[1]

[1]Banat University of Agricultural Sciences and Veterinary Medicine King Michael I of Romania,119 Calea Aradului, 300645, Timisoara, Romania

2 University of Medicine and Pharmacy, Victor Babes, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

[3] Institute of Technical and Natural Sciences Research Development-Innovation of Aurel Vlaicu University, 77 Revolutiei Blvd. 310130, Arad, Romania

[4] University of Medicine and Pharmacy, Carol Davila, 37 Dionisie Lupu, 020021, Bucharest, Romania

This study is important because it provides information for obtaining a natural green compound to replace the toxic fungicides used in our days in crop and grain protection. The aim of this research is to establish the minimum concentration of essential oil (EO) extracted from Origanum vulgare L. which causes a fungi static and fungicidal effect. Origanum vulgare L. was cultivated in a temperate climate zone in Timisoara, Romania. The EO were obtained through hydrodistillation using Clevenger equipment. The chemical composition of the EO was determined using gas chromatography/mass spectrometry (GC/MS) analysis. The Microbiology Discipline from Horticulture and Forestry Faculty of Timisoara provided the fungal cultures used in this study. To point out the inhibition of the mycelium we used the poisoned medium method. Fungi plugs of 8 mm Ø from active mycelia were inoculated on CYGA medium amended with Origanum vulgare L. EO at the following concentrations (v/v); 0.25 , 0.5 , 1 ; 5 ; 10 15 ; 20 mg· L^{-1}. The growth of the mycelia was measured after 5 days. The readings were reduced by 8 mm representing the initial plug diameter. The main chemotypes identified were trans-Caryophyllene 30.729%, Sabinene-18.16%, Caryophyllene oxide-8.635%, Germacrene-D-8.159%. The MIC of the Origanum vulgare L. essential oil for both species of fungi was 0.5 mg·L^{-1}. The MFC of the Origanum vulgare L. EO for both species of fungi was 5 mg·L^{-1}. In the present research work, we demonstrated that Origanum vulgare L. essential oil can be very effective in fungal growth inhibition even at small concentrations like 0.5 mg·L^{-1}.

Keywords: Essential oil, minimum inhibitory concentration, minimum fungicidal concentration

Nowadays there is a growing interest to replace synthetic chemicals with natural products with bioactive properties. Aromatic plants are excellent sources for obtaining bioactive compounds that can be used as natural antifungal agents. [17, 4]. *Origanum vulgare* L. is originated from Mediterranean area and within the *Lamiaceae* family and it is probably one of the most used aromatic plant. The *Origanum vulgare* L. essential oil is rich in mono- and sesquiterpenes. [14].

Verticillium dahliae and *Penicillium aurantiogriseum* are very common in crop deposits because they are resilient to drying and preservatives. They are responsible for the degradation of the grains after the harvest. [10]. Big economic damages take place because of food deterioration caused by metabolic activity of fungi species. [6].

Verticillium wilt is hard to control and current strategies for the management of this disease are showing unsatisfactory results. Most of the synthetic compounds used are creating several side effects in the forms of residual toxicity. That is why biologically active antifungal compounds from plant origins are assumed to be more acceptable than synthetic compounds and represent a good source for antifungal agents [18].

The aim of this research is to establish the minimum concentration of the *Origanum vulgare* L. EO cultivated in the western part of Romania wich causes a fungistatic and fungicidal effect against *Verticillium dahliae* and *Penicillium aurantiogriseum* fungi. For a good comparation with other studies we will determine the chemical composition of this tested EO.

* email: dmtrchadina@yahoo.com

Experimental part

Isolation of EOs

At the time of harvest *Origanum vulgare* L. was in the 4th year of vegetation. The harvest took place in June 2014 during the blooming phase after a sunny period of days because the EO quantity is higher due to the influence of the sun light [7]. *Origanum vulgare* L. was cultivated in a temperate climate zone in Timisoara, Romania ($21^0 13^{'}$E longitude, $45^0 45^{'}$ N latitude). The fresh herb was dried in a room at a temperature between 20 and 22 °C with no sunlight access. We obtained the EO through hydrodistillation using a volatile oil distilling Clevenger equipment. The extracted EO was stored at +4°C until analysis.

Gas chromatography-mass spectrometry identification

The chemical composition of the EO was determined using gas chromatography/mass spectrometry (GC/MS) analysis. Agilent Technology 7820A (AGILENT Scientific, USA) coupled with mass spectrometer MSD 5975 and equiped with a capillary column DB 5: (30 m X 250 μm X 0.25μm, AGILENT, USA) was used. The carrier gas was helium with a mass flow of 1 mL· $min^{"1}$. In order to separate the compounds, the following GC oven program was used: 40 °C for 1 min, 5 °C min^{-1} to 210 °C for 5 min. The injector and ion source temperatures were 250 and 150°C, respectively. The injection volume was 1μL with a split ratio 1:20. The NIST spectra library has been used to identify the volatile compounds.

Antifungal avtivity

Microbiology Discipline from Horticulture and Forestry Faculty of Timisoara provided the fungal cultures used in this study. The *Verticillium dahliae* strain was isolated from sea bucktorn plants infected with *Verticillium dahliae*, preserved at -4°C on PDA medium with Va 09-13 index [5]. The *Penicillium aurantiogriseum* strain was isolated from the fungal microbiota of the wheat seeds preserved on PDA, at -4^{0}C, with the index Lv 07-11 [1].

The research method used was the poisoned medium disc method.

The young fungi cultures were obtained on CYGA (chloramphenicol - yeast- glucose agar, produced by SIGMA) by spread techniques with a spore suspension in melted agar 0.2% + TWEEN 80, 0.05% . After we stored them for 4 days in dark at a constant temperature, we cut plugs of 8 mm Ø from active mycelia and put them on CYGA medium amended with *Origanum vulgare* L. EO at the following concentrations (v/v); 0.25 mg·L^{-1}, 0.5 mg·L^{-1}, 1 mg·L^{-1}; 5 mg·L^{-1}; 10 mg·L^{-1}; 15 mg·L^{-1} ; 20 mg·L^{-1} and 0 for control. *Thiophanate-methyl*, a commercial agricultural fungicide, has been used as negative control for *Penicillium* and for *Verticillium* too.

Each Petri dish containing *Origanum vulgare* L. EO, at different concentrations, was inoculated with two plugs from young mycelia. After inoculations, dishes were kept in dark at 22±2 °C. After 5 days the radial mycelia growth was measured. The readings were reduced by 8 mm representing the initial plug diameter [16].

MIC (minimal inhibitory concentration) is the lowest concentration of oil where no visible fungal growth can be observed. For the establishment of MFC (minimal fungicidal concentration) we used Petri dishes with EOs which had no mycelium radial growth after 5 days. The mycelial plugs with no growth were re-inoculated on fresh CYGA medium in Petri dishes which were sealed with parafilm and incubated in the dark at 22±2^{0}C. The readings were made on the 5th and the 14th day. In the dishes where no mycelium plug growth was observed after 5 and 14 days respectively we considered that the initial concentration from which the reinoculated plug comes from has a fungicidal effect, representing minimal fungicidal concentration (MFC). For control and comparison we used a control dish with thiophanate-methyl (in the recommended dose for practical use).

Results and discussions

Chemical composition of Origanum vulgare L. EO

In table 1 we can find the chemical composition of the EO extracted from *Origanum vulgare* L. We took under consideration the chemical compounds that were found in a quantity over 0.2 % from the total amount. We identified 36 compounds, of which 33 major compounds (over 0.2%) totalling over 99 % of the total compounds.

The main chemotypes identified were trans-Caryophyllene 30.729%, Sabinene-18.16%, Caryophyllene oxide-8.635% and Germacrene-D-8.159% (table 1). The rest of the chemical compounds were found in a quantity under 10% of the total amount.

Mockute in 2004 obtained a similar chemical composition with our study: major compounds are sabinene, 1,8-cineole, β-ocimene, β-caryophyllene, germacrene D, bicylcogermacrene, β-bisabolene, and spathulenol [11].

Radudiene in 2005 also obtained a similar chemical composition regarding the major compounds: β-caryophyllene (4.7–25.0% in inflorescences and 5.4–24.5% in leaves), cis- and trans- β-ocimene (0.9–18.1% / inflorescences and 1.1–22.6% / leaves), sabinene (0.3–25.1% / inflorescences and 0.9– 18.3 / leaves) and germacrene-D (2.1–20.1% in inflorescences and 1.5–13.2% in leaves) [13].

Not all previous studies have shown the same information regarding the chemical composition of *Origanum vulgare* L. E.O.

For example Teixeira in 2013 found carvacrol, β-fenchyl alcohol, thymol, and γ-terpinene as major compounds [17]. Arslan found in 2012 carvacrol as the major component [3].

The chemical composition diferences shown in more than one research paper comfirm that the content of volatile compounds and the dominant chemotype varies with species, site of cultivation and the time of harvest [12].

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The inhibition of the mycelial growth of *Verticillium dahliae* was obvious for *Origanum vulgare* L. EO even at the concentrations of 0.25 mg·L^{-1} and 0.5 mg·L^{-1}. After 9 days *Verticillium dahliae* grew 6 mm in diameter at the concentration of 0.25 mg·L^{-1}. After 5, 7 and even after 14 days there was no growth of the fungus at the concentration of 0.5 mg·L^{-1}. Therefore the minimum inhibitory concentration for *Verticillium dahliae* was 0.5 mg·L^{-1}.

Penicillium aurantiogriseum grew only 2 mm in diameter after 9 days from the inoculation on the medium at concentration of 0.25 mg·L^{-1} but it had no visible growth at the concentration of 0.5 mg·L^{-1}. Therefore the minimum inhibitory concentration for *Penicillium aurantiogriseum* was also 0.5 mg·L^{-1}.

Both fungus species shown a high sensitivity to the *Origanum vulgare* L. EO. Even at small concentrations like 0.25 mg·L^{-1} the mycelial growth was strongly inhibited for both species of fungi. The difference between *Verticillium dahliae* and *Penicillium aurantiogriseum* was that *Verticillium dahliae* managed to grow 6 mm in diameter after 9 days at the concentration of 0.25 mg·L^{-1} while *Penicillium aurantiogriseum* grew only 2 mm in diameter at the same concentration of EO in 14 days.

We determined the MFC using the technique of mycelium re-inoculation from poisoned environment to fresh environment. In the dishes where no mycelium plug growth was observed after 5 and 14 days respectively we considered that the initial concentration from which the reinoculated plug comes from has a fungicidal effect, representing minimal fungicidal concentration (MFC). The mycelial plugs from 0.25 mg, 0.5 mg and 1 mg·L^{-1} concentrations after the reinoculation on fresh medium started to show hyphae revival after 5 days for both species of fungi. Therefore the MFC for both *Penicillium aurantiogriseum* and *Verticillium dahliae* was 5 mg·L^{-1}.

There were not too many studies found regarding the MIC value of *Origanum vulgare* L. EO over *Verticillium dahliae* and *Penicillium aurantiogriseum*.

One study [15], showed that the extract concentration of 0.15-10 mg·L^{-1} exhibited a strong inhibitory effect against *P. aurantiogriseum* (76.43%). At 0.25 mg·L^{-1}, the growth of *P. aurantiogriseum* was totally inhibited after 14 days of incubation.

Another study [8] tested some *Origanum* spp. EO on *Penicillium aurantiogriseum* fungi and determined a MIC value ranging from 0.25 to 10 mg·L^{-1}.

Two different research papers [9] and [2] proved the strong antifungal effect of *Origanum vulgare* L. EO over *Verticillium dahliae* fungi.

No.	RT (min)	Compounds	(%)
1	7.711	α-Thujene	0.32
2	7.893	α-Pinene	0.484
3	**9.064**	**Sabinene**	**18.16**
4	9.129	β-Phellandrene	0.652
5	9.22	1-Octen	1.181
6	9.432	3-Octanone	0.378
7	9.558	β -Myrcene	1.07
8	10.538	Benzene	3.741
9	10.659	d-Limonene	1.09
10	10.737	1,8-Cineole	0.331
11	10.95	cis-Ocimene	4.181
12	11.249	1,3,6-Octatriene	0.448
13	11.808	trans-Thujan-4-ol	0.455
14	15.059	3-Cyclohexene-1-ol	0.502
15	16.677	Thymol methyl eter	0.769
16	16.95	2-Cyclohexene-1-one	2.611
17	20.54	α-Copaene	0.245
18	20.791	β-Bourbonene	1.718
19	**21.791**	**trans-Caryophyllene**	**30.729**
20	21.927	β-Cubenene	0.376
21	22.547	α - Humulene	2.003
22	22.729	Allo-aromadendrene	0.601
23	**23.236**	**Germacrene-D**	**8.159**
24	23.604	Bicyclogermacrene	0.562
25	23.817	α-Farnesene	2.953
26	23.847	β-Bisabolene	3.416
27	24.229	delta - Cadinene	0.585
28	25.503	Germacradien-5-ol	1.282
29	25.555	Spathulenol	0.599
30	**25.698**	**Caryophyllene oxide**	**8.635**
31	26.292	o-Menth-8-ene	0.274
32	26.973	Germacrene-D 1,10-epoxide	0.657
33	27.289	T-Muurolol	0.367
Total (%)			**99.57**

Table 1
THE MAJOR CHEMICAL COMPOUNDS OF *Origanum vulgare L.* EO

Conclusions

This research showed that *Origanum vulgare* L. EO has a strong anti-fungal capacity and can be used as ecological fungicide against pathogen fungi or against spoilage fungi like *Verticillium dahliae* and *Penicillium aurantiogriseum.*

The MIC value for both *Verticillium dahliae* and *Penicillium aurantiogriseum* was 0.5 mg·L^{-1} *Origanum vulgare* L. EO.

The MFC value for both *Verticillium dahliae* and *Penicillium aurantiogriseum* was 5 mg·L^{-1} *Origanum majorana* L. EO.

In vitro research has to be made to determine a the MIC value of *Origanum vulgare* L. EO in normal deposit conditions and also to create a stable compound or emulssion that can be successfully used in grain deposites.

Acknowledgements: This study is a part of a PhD program, funded by the European Social Fund, the Pilot Program PhD Research Scholars Support Contract from the POSDRU/CPP107/DMI 1.5/S/80127, ID Project: 132765. Contract code: POSDRU/159/1.5/S/132765 This work was also supported by project Centru de Cercetare în Stiinte Tehnice si Naturale - CESTN co-funded by European Union through European Regional Development Fund Structural Operational Program Increasing of Economic Competitiveness Priority axis 2. Operation 2.2.1. POSCCE Nr. 621/2014 POS-CCE.

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Manuscript received: 15.03.2016