ANTIFUNGAL ACTIVITY AND CHEMICAL COMPOSITION OF *ORIGANUM MAJORANA* L. ESSENTIAL OIL

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Abstract. *The importance of this study is to provide information for a future green solution to the toxic fungicides used in our days in crop and grain protection. Verticillium dahliae and Penicillium aurantiogriseum are very common in crop deposits and they are very hard to commbat even with synthetic fungicides. The aim of this research is to establish the minimum concentration of essential oil (EO) extracted from Origanum majorana L. which causes a fungistatic and fungicidal effect on Verticillium dahliae and Penicillium aurantiogriseum fungi. Previous research has shown the minimum inhibitory concentration of the Origanum majorana L. EO over Verticillium dahliae but not on Penicillium aurantiogriseum. Also we can say after reading more research papers that the chemical composition of the EO depends on the site of cultivation, climate conditions and the time of harvest. The tested medical plant,Origanum majorana L., was grown in a temperate climate zone in Timisoara, Romania ($21^0 13`$E longitude, $45^0 45`$ N latitude). At the time of harvest Origanum majorana L. was in the 4th year of vegetation. Identification of the species was confirmed by the department of Aromatic plants from USAMVB Timisoara. We obtained the EO from the dry herb using a volatile oil distilling Clevenger equipment. The chemical composition of the EO was determined using gas chromatography/mass spectrometry (GC/MS) analysis. The tests made in vitro on CYGA medium, with additional oil at 0.25 $mg \cdot L^{-1}$; 0.5 $mg \cdot L^{-1}$; 1 $mg \cdot L^{-1}$; 5 $mg \cdot L^{-1}$; 10 $mg \cdot L^{-1}$ and 15 $mg \cdot L^{-1}$and 20 $mg \cdot L^{-1}$ concentrations and inoculated with plugs harvested from a young mycelium, revealed a different reaction of the fungus depending on the oil concentration. The value of minimum inhibitory concentration (MIC) has been established after 14 days after verifying the restauration of hyphae and mycelium of fungi. The MIC value for Verticillium dahliae was 5 $mg \cdot L^{-1}$ Origanum majorana L. EO (essential oil). The MIC value for Penicillium aurantiogriseum was 1 $mg \cdot L^{-1}$ Origanum majorana L. EO (essential oil). The MFC(minimum fungicidal concentration) value for both Verticillium dahliae and Penicillium aurantiogriseum was 20 $mg \cdot L^{-1}$ Origanum majorana L. EO.*

Key words*. Origanum majorana L., mycelial growth inhibition, minimum fungicidal and fungistatic concentration.*

INTRODUCTION

Essential (volatile) oils research has received increased attention from both industrial and academic circles due to a growing interest in green consumerism. Along with other essential oils that may be useful as antifungal agents, majoram oil (*Origanum majorana* L.) may have a great potential for use in industrial applications (C. BUSATTAA & al [1]).

The essential oils are one of the most promising groups of natural products to replace the sinthetic chemical compounds used nowadays in crop protection. (BAŞER & AL [2], SINGH & AL [3] , LOPES-LUTZ & AL. [4], CHRISTAKI & AL. [5], ZOUBIRI AND BAALIOUAMER[6]).

Origanum majorana L. is an important aromatic plant which is commonly used in cookery as a spice or condiment. The volatile oil is employed as a food flavour, in cosmetics and in the production of vermouths and bitters. The plant is perennial, native to the

mediterranean region and it grows also in the south central region of Europe. (S.G. DEANS & al [7]).

Origanum majorana L. is cultivated for the aerial parts and it is processed only dried to obtain volatile oil. The volatile oil content can vary, depending on sowing and climate conditions, between 0.2% and 0.9% on raw material and between 0.5% and 2.65% for dry material. The sowing should begin before the blooming phase, when the volatile oil content is at it's peak. (L.S. MUNTEAN [8]).

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. (MAGAN & al. [9])

Because *Verticillium dahliae* and *Penicillium aurantiogriseum* are hard to combat even with synthetic anti-fungal substances and we are trying to find a green solution to the post-harvest mould spread on the grain it is very important to research the antifungal potential of *Origanum majorana* L. EO.

The main objectives of our research is to establish the minimum concentration of the *Origanum majorana* L. EO cultivated in the western part of Romania wich causes a fungistatic and fungicidal effect against *Verticillium dahliae* and *Penicillium aurantiogriseum* fungi. Also we will determine the chemical composition of this tested EO and compare it with other results shown in recent studies.

MATERIAL AND METHODS

1.Isolation of EOs

Origanum majorana L. was in the 4th year of vegetation at the time of harvest. The harvest took place in May 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. (Ianculov I. & al. [10]). *Origanum majorana* L. was cultivated in a temperate climate zone in Timisoara, Romania ($21^0 13`$E longitude, $45^0 45`$ N latitude) We dried the fresh herb 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.

2.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 0.25 µm, 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 1 µL with a split ratio 1:20. The NIST spectra library has been used to identify the volatile compounds.

3.Antifungal avtivity

The 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. (Cotuna O. & al. [11]). The *Penicillium aurantiogriseum* strain was isolated from the fungal microbiota of the wheat seeds preserved on PDA, at -4^0C, with the index Lv 07-11(ALEXA E. & al [12]).

We used the poisoned medium method to point out the inhibition of the mycelium.

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 majorana* L. EO at the following concentrations (v/v); 0.25 $mg \cdot L^{-1}$, 0.5 $mg \cdot L^{-1}$, 1 $mg \cdot L^{-1}$; 5 $mg \cdot L^{-1}$; 10 $mg \cdot L^{-1}$; 15 $mg \cdot L^{-1}$; 20 $mg \cdot L^{-1}$ 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 majorana* 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. (P. TAYLOR & al. [13]).

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 ± 2^0C. 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) (KIM & al.[14]). For control and comparison we used a control dish with thiophanate-methyl (in the recommended dose for practical use).

RESULTS AND DISCUSSIONS

1.Chemical composition of Origanum majorana L. *essential oil*

In table 1 we can find the chemical composition of the EO, extracted from *Origanum majorana* L. We took under consideration the chemical compounds that were found in a quantity over 0.2 % from the total amount. In the *Origanum majorana* L. EO we identified 44 compounds, of which 31 major compounds (over 0.2%) totalling over 97 % of the total compounds.

The main chemotypes identified were *Lynalyl acetate* - 17.4 %, *γ-Terpinene* - 14.6%, *Benzene* - 13.33 % (table 1). The rest of the chemical compounds were found in a quantity under 10% of the total amount.

Other studies didn't show *Lynalyl acetate* and *Benzene* as main chemotypes. The common main chemotype found in several studies was *γ-Terpinene*.

M. Arslan & al [15] found these 3 main chemotypes in *Origanum majorana* L. harvested from Turkey: *Trans-Sabinene hydrate* - 20.44 %, *Terpinene-4-ol* - 25.48 % and *γ-Terpinene* - 11.16 %. C. Busattaa & al [16] found *terpinen-4-ol*, as the major component, followed by *γ-terpinene*, *cis-sabinene hydrate*, *α-terpinene*, *sabinene* and *α-terpineol*. R.R. VERA & al [17] determined the following chemical composition: *terpinen-4-ol* (38.4%), cis-*sabinene hydrate* (15.0%), p-*cymene* (7.0%) and γ-*terpinene* (6.9%). S. RAMOS & al [18] found as main constituents *cis-sabinene hydrate* (30.2%), *terpinen-4-ol* (28.8%) and *γ-terpinene* (7.2%).These studies comfirm that the content of volatile compounds and the dominant chemotype varies with species, site of cultivation and the time of harvest. (L.S. MUNTEAN [8].

Table 1.

The major chemical compounds of *Origanum majorana* L. EO

No.	RT (min)	Compounds	*(%)*
1	7.711	α-Thujene	0.751
2	7.893	α-Pinene	0.362
3	9.042	Sabinene	2.575
4	9.224	1-Octene	0.749
5	9.562	β -Myrcene	1.409
6	10.308	α-Terpinene	1.380
7	**10.594**	**Benzene**	**13.338**
8	10.681	β-Phellandrene	0.789
9	10.984	cis-Ocymene	6.61
10	11.284	1,3,6-Octatriene	4.801
11	**11.639**	**γ-Terpinene**	**14.168**
12	12.454	trans-Linalool oxide	0.214
13	**12.914**	**Lynalyl acetate**	**17.407**
14	14.726	Borneol	0.272
15	15.064	3-Cyclohexene-1-ol	0.213
16	16.941	Thymol	1.886
17	18.563	Phenol	3.496
18	20.791	β-Bourbonene	0.671
19	20.951	β-Elemene	0.237
20	21.723	trans-Caryophyllene	7.440
21	22.542	α - Humulene	0.687
22	22.729	Neo-Allo-Ocimene	0.523
23	23.271	Germacrene-D	9.207
24	23.479	α-Bergamotene	0.324
25	23.613	Bicyclogermacrene	1.02
26	23.826	α-Farnesene	4.583
27	24.034	α-Amorphene	0.213
28	24.233	delta - Cadinene	0.646
29	25.495	endo-1-Bourbonanol	0.360
30	25.551	Spathulenol	0.551
31	25.69	Caryophyllene oxide	1.252
Total (%)			**97.95**

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

The inhibition of the mycelial growth of *Verticillium dahliae* is obvious for *Origanum majorana* L. EO even at the concentrations of 0.5 $mg \cdot L^{-1}$ and 1 $mg \cdot L^{-1}$. After 14 days the fungus grew 4 mm in diameter at the concentration of 0.5 $mg \cdot L^{-1}$ and 2 mm in diameter at the concentration of 1 $mg \cdot L^{-1}$. After 5, 7 and even after 14 days there was no growth of the fungus at the concentration of 5 $mg \cdot L^{-1}$. Therefore the minimum inhibitory concentration for *Verticillium dahliae* was 5 $mg \cdot L^{-1}$.

Table 2.

Average of fungal growth and signification of differences in case of *Verticillium dahliae*

Variants*	Average of radial growth (mm)	Differences from control
Control	44	0
0.25 mg·L^{-1}	14	-30
0.5 mg·L^{-1}	12	-28
1 mg·L^{-1}	10	-34

** We have taken in consideration only variants with MGI(minimum growth index) value below 100%*

Penicillium aurantiogriseum shown a higher sensitivity than *Verticillium dahliae* to the *Origanum majorana* L. essential oil. Therefore at the concentrations of 0.25 mg·L^{-1} and 0.5 mg·L^{-1} the fungus grew only 2 mm and 1 mm in diameter in 14 days. The minimum inhibitory concentration was 1 mg·L^{-1}.

Table 3.

Average of fungal growth and signification of differences in case of *P. aurantiogriseum*

Variants*	Average of radial growth (mm)	Differences**
Control	36	0
0.25 mg·L^{-1}	10	-26
0.5 mg·L^{-1}	9	-27

** We have taken in consideration only variants with MGI (minimum growth index) value below 100%*

Both fungus species shown a high sensitivity to the *Origanum majorana* L. EO. Even at small concentrations like 0.25 mg·L^{-1} and 0.5 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 2 mm in diameter after 14 days at the concentration of 1 mg·L^{-1} while *Penicillium aurantiogriseum* had no growth at that concentration of EO in 14 days.

Using the technique of mycelium re-inoculation from poisoned environment to fresh environment, we determined the MFC. 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). Therefore the MFC for both *Penicillium aurantiogriseum* and *Verticillium dahliae* was 20 mg·L^{-1}. The mycelial plugs from 5 mg·L^{-1}; 10 mg·L^{-1} and 15 mg·L^{-1} concentrations after the reinoculation on fresh medium started to show hyphae revival after 5 days for both species of fungi.

There are not many studies regarding the antifungal effect of *Origanum majorana* L. EO on *Penicillium aurantiogriseum* and *Verticillium dahliae*. One specific study regarding the antifungal activity of *Origanum majorana* L. EO over *Verticillium dahliae* shown that the

essential oil of *Origanum majorana* L. has a greater antifungal activity when its concentrations exceeded 3 mg·L^{-1}. (M. Arslan & al [15]).

Penicillium digitatum was inhibited completely by *Origanum majorana* L. EO at relatively low concentrations (0.25-0.40 mg·L^{-1}) (D.J. DAFERERA & al [19]).

Origanum majorana L. EO was proven to be effective in postharvest control of *Botrytis cinerea* and *Penicillium expansum* (J.G. LOPEZ-REYES & al [20]).

CONCLUSIONS

Analysis of EO obtained from *Origanum majorana* L. from west Romania showed a chemical composition wich is different from the results found in other studies. This comfirms that the content of volatile compounds and the dominant chemotype varies with species, site of cultivation and the time of harvest.

The EO has a high anti-fungal capacity and can be used as ecological fungicide against pathogen fungi or against spoilage fungi like *Verticillium dahliae* and *Penicillium aurantiogriseum.* Our study showed that *Penicillium aurantiogriseum* has a higher sensitivity to the *Origanum majorana* EO.

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

The MFC value for both *Verticillium dahliae* and *Penicillium aurantiogriseum* was 20 mg·L^{-1} *Origanum majorana* L. EO.

In vitro research has to be made to determine the optimal EO chemical compounds that inhibits the growth of seed borne fungi and to create a non volatile emulsion that can be used as a fungicide.

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