ANTIFUNGAL ACTIVITY AND CHEMICAL COMPOSITION OF *SALVIA OFFICINALIS* L. ESSENTIAL OIL

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Abstract*. The aim of this research study is to establish the minimum inhibitory concentration (MIC) of the Salvia officinalis L. EO cultivated in the western part of Romania wich causes a fungistatic effect against Verticillium dahliae and Penicillium aurantiogriseum fungi. Also we will determine the chemical composition of this tested EO.Verticillium dahliae and Penicillium aurantiogriseum are very common in crop deposits and they are responsible for the degradation of the grains after the harvest.This research is carried for green solutions of crop protection, safe for human consumption and friendly for the environment.The antifungal activity of Salvia officinalis EO has been tested worldwide but the chemical composition and the MIC can vary due to the climate conditions, region of cultivation and the time of harvest. Further research has to be made to extract and create the optimal combination of chemical compunds from the EO that inhibits the growth of seed borne fungi. Salvia officinalis L. was grown in a temperate climate zone in Timisoara, Romania. The harvest took place in May 2014 during the blooming period after a sunny period of time. At the time of harvest Salvia officinalis L. was in the 4th year of vegetation. The tests made in vitro on CYGA medium, with additional oil at 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} concentrations and inoculated with harvested plugs from a young mycelium, pointed out a different reaction of the fungus depending on the oil concetration. The value of the minimum inhibitory concentration (MIC) has been established after 14 days. Restauration of hyphae and mycelium of fungi was verified. The MIC value for Verticillium dahliae was 10 mg·L^{-1} for Salvia officinalis L. EO. Penicillium aurantiogriseum had a very similar sensitivity to the Salvia officinalis L. EO, therefore the MIC value was 10 mg·L^{-1} guaranteed for a period of 14 days.*

Key words. *Salvia officinalis L., mycelial growth inhibition, fungistatic, minimum inhibitory concentration*.

INTRODUCTION

Salvia officinalis L.(sage, garden sage, or common sage) is a perennial, evergreen subshrub, with grayish leaves, woody stems and blue to purplish flowers. It is native to the Mediterranean region but currently it is cultivated in various countries around the world. (M. S. ABU-DARWISH & al [1]). *Salvia officinalis* 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 [2]).

In our days there is a worldwide concern about obtaining primary products with green origins and avoiding chemical compounds in crop treatments is the path to ensure products that are uncontaminated with fungicides residue. Research is carried for green solutions of crop protection, safe for human consumption and friendly for the environment. (GAN-MOR & al. [3]).

The essential oils are one of the most promising groups of natural products to replace the synthetic chemical compounds used nowadays in crop protection. (BAŞER & AL [4],

SINGH & AL [5] , LOPES-LUTZ & AL. [6], CHRISTAKI & AL. [7], ZOUBIRI AND BAALIOUAMER [8]).

The anti-fungal potential of *Salvia officinalis* L. EO was demonstrated by in vitro testing of *Fusarium* mycotoxins (R.SUMALAN & al. [9]). The *Salvia officinalis* L. E.O. proved the same effect for *Botritis cinerea* and *Fusarium sp.* (DAFERERA & al. [10]). Also (M. S. ABU-DARWISH & al [1]) proved that *Salvia officinalis* L. EO has antifungal activity against dermatophyte strains.

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. [11])

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 *Salvia officinalis* EO.

The main objectives of our research is to establish the minimum inhibitory concentration (MIC) of the *Salvia officinalis* L. EO cultivated in the western part of Romania wich induces a fungistatic effect against *Verticillium dahliae* and *Penicillium aurantiogriseum* fungi. Also we will determine the chemical composition of this tested EO.

MATERIALS AND METHODS

1.Isolation of EOs

Salvia officinalis L. was grown in a temperate climate zone in Timisoara, Romania ($21^0 13`$E longitude, $45^0 45`$ N latitude). The harvest took place in May 2014 during the blooming period after a sunny period of days because sun light has a positive influence on the synthesis of the volatile oil. (IANCULOV I. & al. [12]). At the time of harvest *Salvia officinalis* L. was in the 4th year of vegetation. Identification of the species was confirmed by the department of Aromatic plants from USAMVB Timisoara and a voucher specimen was preserved. The fresh herb was dried in a room with no sunlight access at a temperature between 20 and 22 °C. The EO was obtained through hydrodistillation using a volatile oil distilling Clevenger equipment. The EO was kept 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 fungal cultures used in this study were provided by the Microbiology Discipline from Horticulture and Forestry Faculty of Timisoara. 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. [13]). 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 [14]).

We used the poisoned medium method to determine the inhibition of the mycelium. First, 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 *Salvia officinalis* 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} 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 EO, at different concentrations was inoculated with two plugs from young mycelia. After inoculations, dishes were kept in dark at 22±2 °C. The radial mycelia growth was measured after 5 days at two perpendicular diameters. The plug diameter measured 8 mm so we reduced the average of the readings with 8mm. (P. TAYLOR & al. [15]).

MIC is the lowest concentration of oil where no visible fungal growth can be observed. The Petri dishes 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. 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 Salvia officinalis L. essential oil

In table 1 we can find the chemical composition of the EO, extracted from *Salvia officinalis* L. We took under consideration the chemical compounds that were found in a quantity over 0.2 % from the total amount. In the *Salvia officinalis* L. EO we identified 38 compounds, of which 24 major compounds (over 0.2%) totalling over 90% of the total compounds.

The main chemotypes identified were *Camphor* 20.4 %, *Eucalyptol*-11.7 %, *Camphene*-11.5 %, *α -Pinene* – 9.5 % and *Borneol* 8.8%. (table 1). The results obtained are similar with the data from the literature except that in our tested *Salvia officinalis* L. EO we can find *α-thujone* in a very small amount in comparison with other studies. The content of volatile compounds and the dominant chemotype varies with species, site of cultivation and the time of harvest. (L.S. MUNTEAN [2])

Therefore in a study from Jordan (M. S. ABU-DARWISh & al [1]) we can see that 1.8-*cineole* is shown as the major compound of *Salvia officinalis* L. EO followed by *camphor*. *Salvia officinalis* L. harvested in Brasil had *α-thujone* as the major compound (40%) followed by *camphor*-26%, β-*thujone*-5.62% and α-*pinene*-5.85%. (PORTE A. & al [16]). Also a study from Romania showed α-*thujone* as the major compound of *Salvia officinalis* L. EO followed by *camphor* and *viridiflorol*. (ONIGA I. & al [17]) which is very different from our studied EO because we have *α-thujone* in a quantity of only 0.23 %. In a mediterranean cultivated *Salvia officinalis* L. (RUSSO A & al [18]) found that *α-thujone* was also the major compound of the EO followed by *camphor, borneol, γ-muurolene* and *sclareol*.

In Lithuania G. BERNOTIENĖ & al [19] found *cis-thujone* (18.0–43.0%) as the major compound followed by *camphor* (4.5–24.5%), *1,8-cineole* (5.5–13.0%), *trans-thujone* (3.0–8.5%), *α-humulene* (≤12.0%), *α-pinene* (1.0–6.5%), *camphene* (1.5–7.0%).

LAKHAL H. & al [20] found the next chemical composition of *Salvia officinalis* L.: *α-thujone* (24.52%), *camphor* (16.86%), *1,8-cineole* (15.92%), *β-thujone* (6.50%) and *veridiflorol* (6.35%

Another study showed *r-thujone* (34.80%) as the major compound followed by *r-humulene* (14.55-33.24%). Further major compounds included *β-pinene*, *1,8-cineole*, and *camphor*. (A. BOSZORMENYI & al [21])

The main similarity between the research studies around the world regarding the *Salvia officinalis* L. EO is that we can find *α-thujone* as the major compound followed by *camphor* in 20-25 % of the total amount of chemical compounds.

Table 1.

The major chemical compounds of *Salvia officinalis* L. EO

No.	**RT (min)**	**Compounds**	***(%)***
1	5.765	3,4-Octadiene	0.222
2	7.555	Tricyclene	0.490
3	7.707	α-Thujene	0.213
4	**7.893**	**α -Pinene**	**9.597**
5	**8.309**	**Camphene**	**11.587**
6	9.124	β-Pinene	5.048
7	9.558	β -Myrcene	0.833
8	10.299	α-Terpinene	0.238
9	10.538	Para-Cymene	0.257
10	10.655	D-Limonene	1.455
11	**10.733**	**Eucalyptol**	**11.751**
12	11.578	γ-Terpinene	0.457
13	12.45	Terpinolene	0.515
14	12.779	Linalool	0.605
15	12.961	Cis-Thujone	1.987
16	13.278	Trans-Thujone	1.02
17	**14.106**	**Camphor**	**20.465**
18	14.73	Borneol	8.803
19	15.064	4-Terpineol	0.244
20	18.112	L- α -Bornyl acetate	5.09
21	21.684	trans-Caryophyllene	3.413
22	22.542	α -Humulene	4.991
23	25.885	Viridiflorol	1.043
24	35.457	Epimanool	0.398
Total (%)			**90.722**

2. Antifungal activity

The inhibition of the mycelia growth of *Verticillium dahliae* is obvious for *Salvia officinalis* L. EO at the concentration of 5 $mg \cdot L^{-1}$. After 5 days there was no growth of the fungus at the concentration of 5 $mg \cdot L^{-1}$, only a small amount of mycelia growth on the top of the plug. And after 14 days there was a growth of 2 mm in diameter. Therefore the minimum inhibitory concentration for *Verticillium dahliae* was 10 $mg \cdot L^{-1}$ guaranteed for a period of 14 days.

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	48	0
0.25 mg·L^{-1}	30	-18
0.5 mg·L^{-1}	28	-20
1 mg·L^{-1}	21	-27
5 mg·L^{-1}	2	-46

** We have taken into consideration only variants with MGI value below 100%*

P. aurantiogriseum had similar sensitivity to the *Salvia officinalis* L. EO as *Verticillium dahliae* because we had obvious growth of the fungus at 0.25 mg·L^{-1}; 0.5 mg·L; and 1 mg·L^{-1} concentrations and a small growth at the concentration of 5 mg·L^{-1} even after 14 days. The minimum inhibitory concentration was 10 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 from control
Control	38	0
0.25 mg·L^{-1}	22	-16
0.5 mg·L^{-1}	18	-20
1 mg·L^{-1}	12	-26
5 mg·L^{-1}	1	37

** We have taken into consideration only variants with MGI value below 100%*

Both fungus species had a strong growth at the concentrations of 0.25 mg·L^{-1}, 0.5 mg·L and 1 mg·L^{-1} even after 5 days, similar to the control medium with no EO. At the concentration of 5 mg·L^{-1} the studied EO strongly inhibited the growth of the 2 species of fungi (they had a growth of only 1-2 mm in diameter after 14 days).

Similar studies were made to observe the *Salvia officinalis* L. EO effect. A concentration of 6 mg·L^{-1} of *Salvia officinalis* L. EO was sufficient for inhibiting the growth of *Epidermophyton floccosum*. (M. S. ABU-DARWISH & al [1]).

E. MAHMOUDI & al [22] have had similar results in testing the *Salvia officinalis* L. EO on other species of fungus like *Alternaria alternata.* In their study the minimum inhibitory concentration was 5 mg·L^{-1}.

In a different study made on some *Aspergillus* fungi species, the *Salvia officinalis* L. EO fully inhibited the growth of the fungus at the concentration of 6.6 $mg \cdot L^{-1}$. (A. RASHIDI & al [23]).

CONCLUSIONS

Analysis of EO obtained from *Salvia officinalis* L. demonstrated that *camphor, camphene, eucalyptol, α –pinene* and *borneol* are the main chemotypes from *Salvia spp.* cultivated in west Romania. 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.*

Even if the chemical composition of *Salvia officinalis* L. EO from our study is not 100 % the same as found in other studies the antifungal activity is similar. Previous researches have shown that the anti-fungal effect is not caused only by one major compound but by the synergy of the other compounds found in smaller amounts. (Nakatsu & al. [24], Kivrak & al. [25]). The high proportion of *camphor, camphene, eucalyptol, α –pinene* and *borneol* totalling over 60 % of the total chemical composition indicates the fungus' s tolerance to these compounds.

Further research has to be made to extract and create the optimal combination of chemical compunds from the EO that inhibits the growth of seed borne fungi.

The MIC value for both *Verticillium dahliae* and *Penicillium aurantiogriseum* was 10 $mg \cdot L^{-1}$ guaranteed for a period of 14 days.

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 Ştiinţe Tehnice şi 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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