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IN VITRO EFFECT OF ESSENTIAL OILS FROM AROMATIC AND MEDICINAL PLANTS ON MUSHROOM PATHOGENS: *VERTICILLIUM FUNGICOLA* VAR. *FUNGICOLA*, *MYCOGONE PERNICIOSA*, AND *CLADOBOTRYUM* SP.

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Abstract — Lavender, anise, chamomile, fennel, geranium, oregano, parsley, and sage essential oils were tested for their effectiveness against mushroom pathogens: *Verticillium fungicola* var. *fungicola*, *Mycogone perniciosa*, and *Cladobotryum* sp. Isolates were exposed to the volatile phase of the oils and then ventilated in order to determine if the effect of the oil was lethal to the pathogen. Oregano and geranium oils were the most toxic, having a fungicidal effect at 0.02-0.08 µl/ml of air, depending on the pathogen. Oregano oil was characterized by high content of carvacrol and thymol, while citranelol and geraniol were the main components of geranium oil.

Key words: Essential oils, lavender, anise, chamomile, fennel, geranium, oregano, parsley, sage, antimicrobial activity

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INTRODUCTION

Verticillium fungicola var. *fungicola* (Preuss) Hassebrauk, *Mycogone perniciosa* (Magnus) Delacroix, and *Cladobotryum* spp. (Cooke) – the causal agents of dry bubble, wet bubble, and cobweb disease – are important fungal pathogens of the button mushroom, *Agaricus bisporus* (Lange) Imbach (Grogan and Gaze, 2000; Umaret et al., 2000; Gea et al., 2003). Symptoms of dry bubble, caused by *V. fungicola* var. *fungicola*, vary depending on the time of infection. Infection at an early stage in mushroom development results in the production of undifferentiated masses of mushrooms. If maturing mushrooms are infected, then spotting symptoms develop (Grogan et al., 2000; Potocnik et al., 2008). *Agaricus bisporus* fruit bodies infected with *M. perniciosa* become large and irregular, and tumorous fungal masses are formed (Umar et al., 2000). Exudation of accumulated extracellular fluid is observed on the surface of diseased mushrooms (Stanunton and Dunne, 1990). Caused by *Cladobotryum* spp., cobweb disease is characterized by growth of coarse mycelium covering affected mushrooms. As it ages, the white mycelium becomes pink (Fletcher et al., 1989).

Control of mycopathogens is based on the use of chemicals, cultural practices, and sanitation. The pesticides most commonly used on mushroom farms in Serbia are: benomyl, zinc-ethylenbisdithiocarbamate, and prochloraz-manganese. According to Fletcher and Yarham (1976), *V. fungicola* var. *fungicola* is resistant to benomyl, a fungicide which is frequently used to control *M. perniciosa*. By the mid-1980´s, the first sign of resistance of *Cladobotryum* spp. to benomyl was recorded in Ireland and Great Britain (Gaze, 1995). Prochloraz-manganese is widely used in Europe (Bonnen and Hopkins, 1997), although moderately resistant isolates of *V. fungicola* var. *fungicola* have been found in Great Britain (Grogan et al., 2000) and Spain (Gea et al., 2003). Resistance to commonly used pesticides and the presence of residues of pesticides in food

impose the necessity of finding a suitable alternative. Application of substances of natural origin as crop protectants could be a convenient solution, safe for both human health and the environment.

Antimicrobial properties of certain essential oils have already been known for a long time (Chamberlain, 1887), but their efficacy against mycopathogenic fungi has not been well documented. Essential oils isolated from savory (*Satureja thymbra*) and sage (*Salvia pomifera* ssp. *calycina*) were investigated for antifungal activity against *M. perniciosa*; the oil of *S. thymbra* expressed better antifungal activity against *M. perniciosa* than *S. pomifera* oil (Glamoclija et al., 2006). Previous *in vitro* experiments (Tanovic et al., 2007) showed that the volatile phase of certain essential oils such as those of Scots pine, eucalyptus, juniper, orange, rosemary, and thyme, applied at a concentration of 0.65 µl/ml of air, inhibited mycelial growth of soilborne pathogens: *Fusarium* spp., *Rhizoctonia* sp., and *Pythium* sp. In addition, application of thyme essential oil at a concentration of 1000 µl/l effectively controlled cucumber dumping-off disease (Tanovic et al., 2004) and dry bubble disease of button mushrooms caused by *V. fungicola* var. *fungicola* (Potocnik et al., 2005).

Accordingly, the objectives of this study were to investigate antimicrobial activity of several essential oils against *V. fungicola* var. *fungicola*, *M. perniciosa*, and *Cladobotryum* sp. *in vitro* and determine chemical composition of the oils expressing the highest antimicrobial activity.

MATERIALS AND METHODS

Test organisms

Isolates of *V. fungicola* var. *fungicola*, *M. perniciosa*, and *Cladobotryum* sp. identified during a 2002-2003 survey on mushroom farms in Serbia were chosen for this study.

Test substances

Essential oils of plants including lavender (*Lavandula officinalis*), anise (*Pimpinella anisum*), fennel (*Foeniculum vulgare*), geranium (*Pelargonium graveolens*), oregano (*Origani heracleotici*), parsley (*Petroselini aetheroleum*), and sage (*Salvia officinalis*) were provided by the Dr. Josif Pančić Institute for Medicinal Plant Research in Belgrade, Serbia.

Inoculum preparation

The fungal pathogens *V. fungicola* var. *fungicola*, *M. perniciosa*, and *Cladobotryum* sp. were prepared as a conidial suspension (approximately 10^6 conidia/ml). The isolates were initially grown for 14 days on potato-dextrose-agar (PDA) plates. Conidia were harvested by flooding the plates with 10 ml of sterile distilled water and Tween 20 (v/v 0.01%), followed by filtration through a double layer of cheesecloth.

Toxicity of essential oils 'in vitro'

Antifungal activity was tested on PDA in glass Petri plates (R = 90 mm) inoculated with the investigated strains by pipetting 20 µl of the conidial suspension into a well cut in the center of the plate (R = 10 mm). The isolate was exposed to the volatile phase of essential oils for seven days at 20°C. The oils were applied as a drop onto the inner side of plate covers at concentrations of 0.02, 0.04, 0.08, 0.16, and 0.32 µl/ml of the air inside the Petri plates using a micropipette. The bottoms of the plates were immediately placed on the covers. The plates were left up-side-down and sealed by parafilm to prevent gas exchange with the outside environment. Inhibition of the mycelial growth was estimated four days after the treatment by measuring radial growth of the isolate treated with different concentrations of the oils and compared to the control. Seven days after the treatment, the plates were observed for initial mycelial growth without measuring. Concentrations of an oil which completely inhibited mycelial growth after seven-day exposure at 20°C were considered to be fungistatic and the lowest of these concentrations was determined as the minimum inhibitory concentration (MIC). Afterwards, the plates were opened and ventilated in a laminar flow hood for 30 min in order to remove volatiles and determine the fungicidal effect. The concentrations of oil were considered to be fungicidal if microbial growth was not observed seven days after ventilation. The lowest concentration with fungicidal effect was defined

as the minimum fungicidal concentration (MFC). Four replicates per treatment were used and the experiment was repeated twice.

Analysis conditions

Qualitative and quantitative analysis of essential oils was performed by gas chromatography using two detector types. A Hewlett-Packard gas chromatograph (HP-5890 Series II) was equipped with a split-splitless injector, an HP-5 capillary column (25 m, 0.32 mm i.d., 0.52 µm film thickness), and a flame ionization detector (FID). Injector and detector temperatures were set to 250 and 300°C, respectively, while the hydrogen flow rate was 1 ml/min^{-1}. Column temperature was programmed linearly in the 40-260 °C temperature range at 4°C/min. Analyses with a mass spectrometer (MS) as detecting device were conducted using an HP G 1800C Series II analytical system. The same temperature program was used, while separation was performed by an HP-5MS column (30 m, 0.25 mm i.d., 0.25 µm). Helium was utilized as a carrier gas and the MS transfer line temperature was set to 260°C. The mass detector was operated in the electron impact (EI) mode (70 eV; 40-400 m/z range). Ethanol solutions (1%) of oil samples (1 µl) were injected in a split mode (split ratio of 1: 60). Identification of essential oil components was performed using various mass spectral libraries (NIST/Wiley).

RESULTS

Antimicrobial activity of essential oils

The growth rate of the isolates was partially or completely inhibited by all tested essential oils applied at 0.02-0.32 µl/ml of air. A 100% growth inhibition of these species was achieved by several oils at 0.32 µl/ml of air after four-day exposure. The most sensitive species was *M. perniciosa*; the growth of this pathogen was fully inhibited by all the oils at 0.02 µl/ml air after four-day exposure. Among the investigated oils, the most effective was oregano oil, which totally inhibited the growth of all three mycopathogenic isolates at 0.02 µl/ml of air, while the same concentration of the other oils caused only partial inhibition of the pathogens. The only exception was geranium

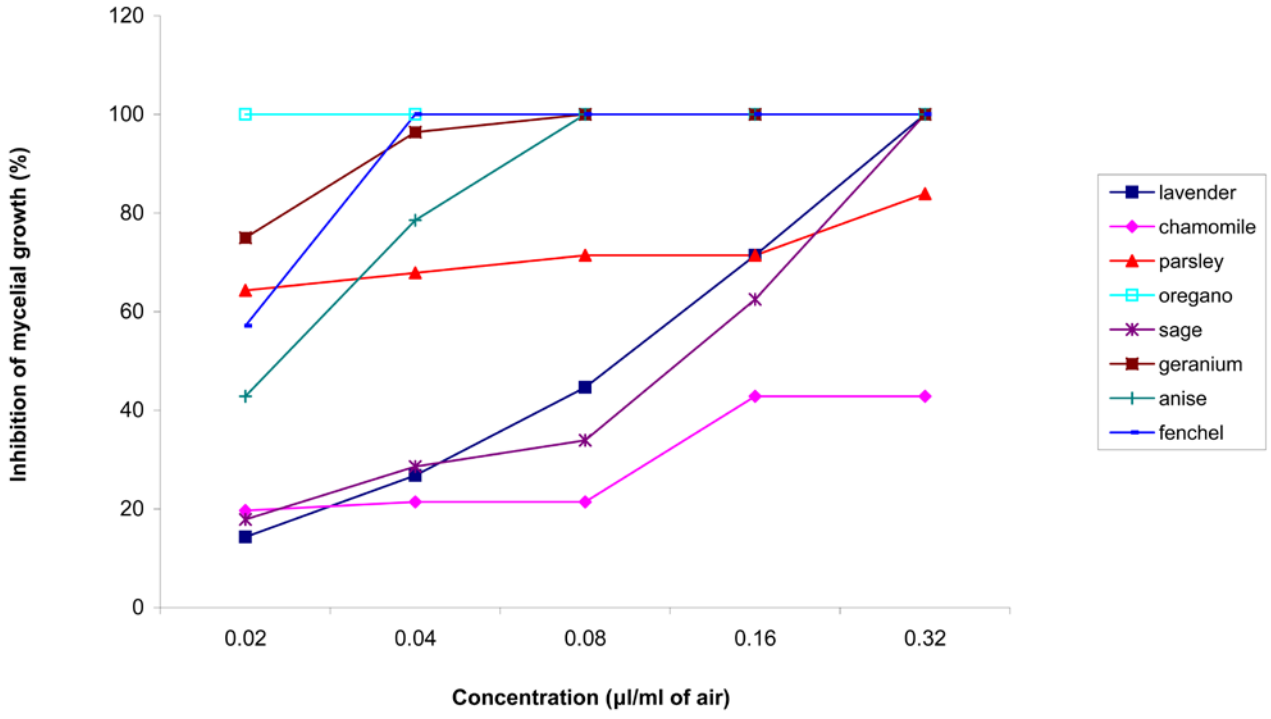

Fig. 1. Effect of the volatile phase of essential oils on growth of *Verticillium fungicola* var. *fungicola in vitro* after four-day exposure.

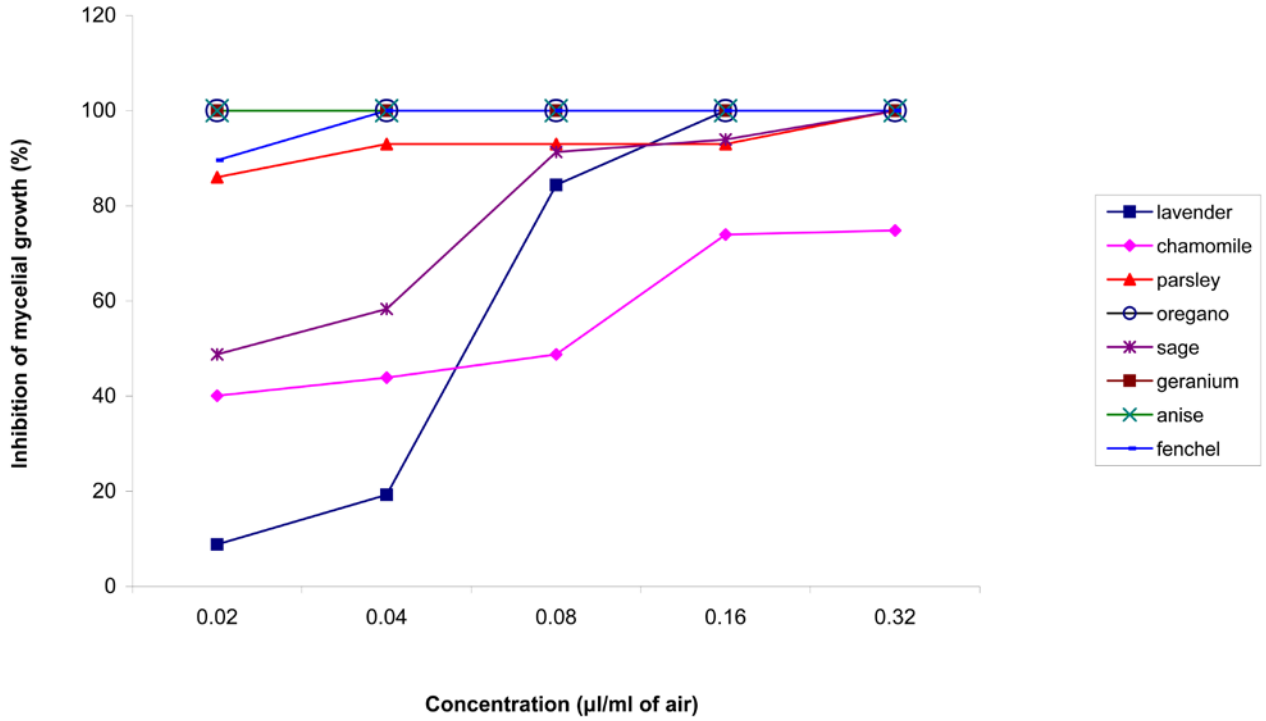

Fig. 2. Effect of the volatile phase of essential oils on growth of *Cladobotryum* sp. in vitro after four-day exposure.

oil, which was lethal to *M. perniciosa* at 0.02 µl/ml of air. Inhibitory effects of the other tested oils varied depending on the pathogen (Figs. 1 and 2).

Toxicity of essential oils

The results obtained seven days after oil application confirmed that the oils varied in their toxicity to *V. fungicola* var. *fungicola*, *M. perniciosa*, and *Cladobotryum* sp. isolates (Table 1). The obtained MIC and MFC values of the investigated oils ranged from 0.02 to more than 0.32 µl/ml of air. Oregano essential oil exhibited the highest level of toxicity to

Table 1. Toxicity of essential oils to *Verticillium fungicola* var. *fungicola*, *Mycogone perniciosa*, and *Cladobotryum* sp. [1]The minimal concentration of the essential oil causing complete inhibition of mycelial growth after seven-day exposure (minimum inhibitory concentration). [2]The minimal concentration of the oil showing a lethal effect on the pathogen (minimum fungicidal concentration).

Essential oils	Effective concentrations of essential oils (µl/ml of air)					
	Verticillium fungicola var. *fungicola*		*Mycogone perniciosa*		*Cladobotryum* sp.	
	MIC[1]	MFC[2]	MIC[1]	MFC[2]	MIC[1]	MFC[2]
Oregano (*Origani heracleotici*)	0.02	0.02	0.02	0.02	0.02	0.02
Geranium (*Pelargonium graveolens*)	0.08	0.16	0.02	0.02	0.04	0.08
Fenchel (*Foeniculum vulgare*)	0.08	0.32	0.04	0.04	0.16	0.32
Lavender (*Lavandula officinalis*)	0.32	0.32	0.08	0.32	0.32	> 0.32
Anise (*Pimpinella anisum*)	0.16	> 0.32	0.04	0.04	0.08	> 0.32
Parsley (*Petroselini aetheroleum*)	> 0.32	> 0.32	0.02	0.04	0.32	0.32
Sage (*Salvia officinalis*)	> 0.32	> 0.32	0.08	0.16	> 0.32	> 0.32
Chamomile (*Chamomilae aetheroleum*)	> 0.32	> 0.32	0.16	0.16	> 0.32	> 0.32

Table 2. Chemical composition of oregano essential oil.

Component	Composition (%)
Carvacrol	65.0
Thymol	14.8
β-Phellandrene	4.3
p-Cymene	1.9
α-Pinene	1.4
β-Caryophyllene	1.4
α-Terpinene	1.2
γ-Terpinene	0.6
1-Octen-3-ol	0.6
β-Myrcene	0.6
Linalool	0.6
α-Thujone	0.6
Terpinen-4-ol	0.6
β-bisabolen	0.6
Borneol	0.4
Total	94.6

Table 3. Chemical composition of geranium essential oil.

Component	Composition (%)
Citronelol	24.4
Geraniol	14.3
Linalool	12.5
Citronellyl formiate	8.5
Menthone	6.2
Geranyl-formiate	4.4
3,7-gvaiedien	4.0
α-Terpineol	3.5
Izomenthon	3.4
β-Burbonen	1.1
Tetrahydrogeraniol	1.1
α-Pinene	1.0
Geranyl-butyrate	0.8
Linalyl-propionate	0.8
Cis-rozokside	0.8
Geranyl-tiglate	0.7
β-Caryophyllene	0.7
Citronellyl-propionate	0.6
Citronellyl-butyrate	0.6
Calamenen	0.6
Neryl-propionate	0.5
Benzylidene camphor	0.4
Geranyl-propionate	0.4
δ-Gvaien	0.4
Total	91.7

all isolates tested, with an MFC value of 0.02 µl/ml, following by geranium oil, whose MFC value ranged from 0.02 to 0.16 µl/ml of air. Lavender, chamomile, and sage oils, applied at 0.16 µl/ml of air, did not exert a lethal effect on any of the isolates (Table 1).

Essential oil composition

Among 45 components detected in oregano essential oil, 15 constitute almost 95% of the oil mass (Table 2), while concentrations of the others are in the 0.06-0.7% range. Carvacol and thymol were the dominant components, exeeding 79% of the oil.

In geranium oil, 54 components were identified, 27 of which were present in low concentration (less than 0.3%). Table 3 presents only components which participated in the mixture in percentages higher than 0.4%, such components constituting 91.7% of the oil.

DISCUSSION

Our results indicated that some essential oils had an ability to suppress growth of *V. fungicola* var. *fungicola*, *M. perniciosa*, and *Cladobotryum* sp. isolates *in vitro*. Among the eight essential oils analyzed, those of oregano and geranium expressed the strongest antifungal activity against all of the investigated mycopathogens. Essential oils had previously been reported to have antimicrobial effects (Wilson et al., 1997; Suhr and Nielsen, 2003; Tanovic at al., 2004; Knezevic-Vukcevic et al., 2005; Mitic-Culafic et al., 2005; Ciric et al., 2008; Dzamic et al., 2008; Stanojevic et al., 2008). For instance, Daferera et al. (2003) reported strong activity of essential oils against the phytopathogenic fungi *Botrytis cinerea* and *Fusarium* sp. A fungistatic effect of certain oils on *Fusarium culmorum* and *Alternaria alternata* has also been demonstrated (Byron and Hall, 2002). Studies of the antifungal activity of several essential oils (Tanovic et al., 2004, 2007) against soil-borne plant pathogens including *Pythium* sp., *Verticillium albo-atrum*, and *Rhizoctonia* sp. showed that some of them had a strong inhibitory effect. Wilson et al. (1997) recorded that among the 49 essential oils tested, red thyme, cinnamon leaf, and clove bud oils showed the highest antifungal activity against *B. cinerea*, followed by oregano oil with a satisfactory effect. Oregano oil was also effective in suppress-

ing fumonisin B_1 production by *F. proliferatum* in maize grain (Velluti et al., 2003). Furthermore, this oil was highly effective in controlling internal wheat fungi in *in vivo* experiments (Paster et al., 1995). Screening experiments with 11 essential oils including those of oregano and sage against *Bacillus cereus*, a pathogen associated with food-borne disease of humans caused by toxins, showed oregano oil to be an effective growth inhibitor (Valero and Salmeron, 2003). These results demonstrated a broad spectrum of activity of oregano oil in suppressing microbial growth and indicated possible use of the oil in integrated management of various diseases.

Natural plant-derived fungicides should provide a wide variety of compounds as alternatives to synthetic fungicides, ones safe for both human health and the environment (Cutler and Hill, 1994; Daferera et al., 2003). Although the cost-effectiveness of oil application must be taken into account, this study justifies further research on practical use of essential oils for the control of *V. fungicola* var. *fungicola*, *M. perniciosa*, and *Cladobotryum* sp.

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ДЕЛОВАЊЕ ЕТАРСКИХ УЉА ИЗ АРОМАТИЧНИХ И ЛЕКОВИТИХ БИЉАКА НА МИКОПАТОГЕНЕ ГЉИВЕ: *VERTICILLIUM FUNGICOLA* VAR. *FUNGICOLA*, *MYCOGONE PERNICIOSA* И *CLADOBOTRYUM SP. IN VITRO*

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У раду су приказани резултати *in vitro* тестирања ефеката етарских уља: лаванде, аниса, камилице, морача, геранијума, оригана, першуна и жалфије, на микопатогене гљиве: *Verticillium fungicola* var. *fungicola*, *Mycogone perniciosa* и *Cladobotryum* sp. Изолати су излагани дејству пара проучаваних уља, а затим проветравани, након чега је оцењиван ефекат њиховог деловања. Уља оригана и геранијума испољила су највећу токсичност за све проучаване патогене, са вредностима минималне фунгицидне концентрације између 0.02-0.08 μl/ml ваздуха, зависно од патогена. Уље оригана имало је висок садржај карвакола и тимола, док су цитранелол и гераниол биле доминантне компоненте уља геранијума.