

Antifungal activity of plant essential oils against *Verticillium dahliae* Klebahn, the causal agent of Verticillium wilt of pepper

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SUMMARY

Biofungicides based on plant oils have some advantages compared to chemical fungicides, especially considering their harmful effect on the environment. Twenty-two essential oils from Germany and Albania were assayed for inhibitory and fungicidal activity against *Verticillium dahliae* Klebahn, the causal agent of Verticillium wilt of pepper, using the macrodilution fumigant method. The concentrations of oils obtained in the vapour phase were: 0.02, 0.04, 0.08, 0.16 and 0.32 $\mu\text{l ml}^{-1}$ with determined minimum inhibitory and fungicidal concentrations. The strongest activity was shown by two samples of mint oil (*Mentha piperita* L.) at 0.02 $\mu\text{l ml}^{-1}$ of air, both from Germany and Albania, followed by plant essential oils of eucalyptus (*Eucalyptus globulus* Labillardie), black pine (*Pinus nigra* L.) and cade (*Juniperus oxycedrus* L.), and all of them were lethal to the pathogen. Nine oils: two samples of mint, cade, eucalyptus, black pine, lavender (*Lavandula angustifolia* Mill.), sage (*Salvia officinalis* L.) and rosemary (*Rosmarinus officinalis* L.) inhibited the growth of *Verticillium dahliae*, while five oils (two samples of mint, eucalyptus, black pine and cade) showed fungicidal effects on the pathogen. These results showed that mint, eucalyptus, black pine and cade essential oils have a potential for further *in vivo* experiments against *Verticillium dahliae*.

Keywords: essential oils; antifungal activity; Verticillium; pepper

INTRODUCTION

Verticillium wilt is one of the most important diseases of pepper, being present in all commercial

pepper-growing areas. The disease is also known as the green wilt of pepper, because of its characteristic symptoms. The causal agents of the disease are fungi of the genus *Verticillium* (Santamarina & Rosello, 2006),

namely *Verticillium albo-atrum* Reinke & Berthold and *Verticillium dahliae* Klebahn. *Verticillium dahliae* is a cosmopolitan soilborne fungus causing wilt diseases on more than 400 plant species (Pegg & Brady, 2002; Klosterman et al., 2009). Its hosts can be fruit plants, vegetables, forest trees, shrubs and flowers, as well as many weeds and some field crops. In the absence of a host plant, the pathogen may persist in soil for more than 10 years as melanised microsclerotia, the resting structure and primary inoculum of the pathogen in soil (Pegg & Brady, 2002). Infected plants show yellowing, chlorosis and necrosis of lower leaves and premature defoliation. Vascular tissues of the basal stem and tap roots have a brownish streak. Losses due to *Verticillium* wilt can reach more than 80% under favorable wet and warm conditions (Goldberg, 2003).

Control of *Verticillium* wilt is currently achieved by soil fumigation with methyl bromide, fungicides and by planting partially resistant or tolerant cultivars. The fungicides commonly used to control *Verticillium* wilt of pepper are benzimidazoles - carbendazim and thiophanate-methyl (Talboys, 1984; Tian et al., 1998). However, the use of soil fumigation and fungicides leads to ozone depletion and off-target pollution, and creates imbalance in the microbial community. Intensive research efforts have been focused on finding alternative ways to control *Verticillium* wilt. A significant amount of research work is currently focused on the use of mycopathogenic fungi (such as *Pythium oligandrum* Drechs., *Trichoderma harzianum* Rifai, etc.), and the use of chemicals which stimulate plant defense mechanisms or plant growth (Santamarina & Rosselo, 2006; Rekanović et al., 2007; Jayaraj et al., 2008; Ślusarski & Pietr, 2009) or those promoting native antagonists or introducing alien antagonists that are found in compost. *Bacillus* and *Pseudomonas* have been found to be effective in inhibiting many soil-borne pathogens (Abada & Ahmed, 2014; Abada & Hassan Eman, 2017; Abada et al. 2018; Milijašević-Marčić et al., 2018).

As part of evaluation of alternative means, antimicrobial properties of essential oils (EOs), as well as of their components, have been demonstrated. They could be used as supplements to commercial products for disease control, which would minimize the quantity of fungicides used. Oils, due to their very strong activity, could be promising control agents in future extensive research and *in vivo* examination (Luković et al., 2018). *In vitro* experiments have shown that the volatile phase of certain essential oils, such as those of Scottish pine, eucalyptus, juniper, orange, rosemary and thyme, applied

at the concentration of 0.65 $\mu\text{l ml}^{-1}$ of air, inhibits the growth of several soil-borne pathogens: *Fusarium* spp., *Rhizoctonia* sp. and *Pythium* sp. (Tanović et al., 2007). Oils of oregano (*Origanum vulgare* L.), common thyme (*Tymus vulgaris* L.) and peppermint (*Mentha piperita* L.) have shown high *in vitro* activity against *Trichoderma* green mould, while application of tea tree oil [*Melaleuca alternifolia* (Maiden and Betche) Cheel] to oyster mushroom substrate or button mushroom casing layer has resulted in considerable *in vivo* inhibition of *Trichoderma harzianum* Rifai (Soković & Van Griensven, 2006; Kosanović et al., 2013). Among the 22 essential oils analyzed, those of wintergreen (*Gaultheria procumbens* L.), lemongrass (*Cymbopogon flexuosus* Stapf.) and oregano have demonstrated the strongest antimicrobial activity against three pathogenic bacteria and parasites of common bean, tomato and cultivated mushroom (Todorović et al., 2016).

The objective of this study was to assess the vapor antifungal activities of 22 essential oils against *Verticillium dahliae* by evaluating their minimal inhibitory and fungicidal concentrations *in vitro*, using a macrodilution fumigant method, in an attempt to promote their use as alternative products for biological control.

MATERIAL AND METHODS

Essential oil samples

Twenty-two essential oils (EOs) were provided by the Institute for Medicinal Plant Research “Dr. Josif Pančić”, Belgrade, Serbia (Table 1). A part of the samples was obtained from the Institute’s collection containing 11 EOs produced by Frey & Lau, Ulzburg, F.R. Germany. The characteristics of samples in that group fully satisfied the quality control requirements prescribed by the Fifth European Pharmacopoeia (Ph. Eur. 5.0). The latter of the two listed oregano oil samples was declared as nature-identical. The other samples consisted of oils acquired from the Albanian companies Xherdo Co. sh.p.k., Tirana, and Agroherbal sh.p.k, Mamurras.

Test organism and inoculum preparation

Verticillium dahliae SV-2 was isolated from infected pepper plants in Serbia (Padinska Skela, 2003) using a method described by Dhingra and Sinclair (Dhingra & Sinclair, 1995). Small fragments of diseased xylem tissue were placed aseptically on potato dextrose agar (PDA)

and incubated at $25 \pm 1^\circ\text{C}$ for 10 days. The mycelium was transferred to fresh PDA medium to obtain a pure culture. Conidia were harvested by flooding the plates with 10 ml of sterile distilled water and Tween 20 (v/v 0.01%), which was followed by filtration through a double layer of cheesecloth. Conidial suspension was prepared daily in sterile saline and adjusted to a concentration of approximately 10^6 conidia ml^{-1} .

Antifungal activity of essential oils *in vitro*

Antifungal activity was tested on PDA medium in glass Petri plates (R=100 mm). The medium was inoculated with the investigated fungi by pipetting 20 μl of conidial suspension into each well cut at the centre of the plate (R=10 mm). The inoculum was then exposed to the volatile phase of EOs for 48 h at 20°C . The oils were applied as a single drop onto the inner side of each plate

cover on filter paper disc at concentrations of 0.02, 0.04, 0.08, 0.16 and $0.32 \mu\text{l ml}^{-1}$ of air inside Petri plates using a micropipette. The bottom of the plates was immediately placed upon the cover. The plates were sealed with parafilm to prevent gas exchange with the outside environment. Oil concentrations that completely inhibited fungal growth after two-day-exposure at 20°C were considered to be fungistatic and the lowest of these concentrations was determined as minimum inhibitory concentration (MIC). The plates were then opened and ventilated in a laminar flow hood for 30 min in order to remove volatiles and determine fungicidal effects. Oil concentrations were considered fungicidal if no fungal growth was observed two days after ventilation. The lowest concentration that had fungicidal effect was defined as minimum fungicidal concentration (MFC) (Tanović et al., 2006). Four replicates per treatment were used and the experiment was conducted twice.

Table 1. List of tested essential oils

Essential oils	Source*	Designation	Remark
Anise (<i>Illicium verum</i> Hooker)	Frey & Lau	S0100154	Ph. Eur. 5.0 ⁺
Cade (<i>Juniperus oxycedrus</i> L.)	Xherdo Co.	-	-
Eucalyptus (<i>Eucalyptus globulus</i> Labillardie)	Frey & Lau	S0100321	Ph. Eur. 5.0 ⁺
Lavender (<i>Lavandula angustifolia</i> Mill.)	Frey & Lau	P0123527	Ph. Eur. 5.0 ⁺
Lavender (<i>Lavandula angustifolia</i> Mill.)	Agroherbal	-	-
Lemon (<i>Citrus limon</i> L.)	Frey & Lau	P0119551	Ph. Eur. 5.0 ⁺
Lemongrass (<i>Cymbopogon flexuosus</i> Stapf.)	Frey & Lau	P0120241	Ph. Eur. 5.0 ⁺
Mint (<i>Mentha piperita</i> L.)	Agroherbal	No. 41/A	-
Mint (<i>Mentha piperita</i> L.)	Frey & Lau	P0123884	Ph. Eur. 5.0 ⁺
Oregano (<i>Origanum vulgare</i> L.)	Frey & Lau	P0125062	Ph. Eur. 5.0 ⁺
Oregano (<i>Origanum vulgare</i> L.)	Frey & Lau	P0125412	Ph. Eur. 5.0 ⁺
Pine (Black) (<i>Pinus nigra</i> L.)	Agroherbal	-	-
Pine (Maritime) (<i>Pinus pinaster</i> Aiton)	Frey & Lau	P0125332	Ph. Eur. 5.0 ⁺
Pine (Maritime) (<i>Pinus pinaster</i> Aiton)	Agroherbal	No. 2	-
Pine (Scotch) (<i>Pinus silvestris</i> L.)	Frey & Lau	P0124319	Ph. Eur. 5.0 ⁺
Rosemary (<i>Rosmarinus officinalis</i> L.)	Frey & Lau	P0124476	Ph. Eur. 5.0 ⁺
Rosemary (<i>Rosmarinus officinalis</i> L.)	Agroherbal	No. 1	-
Rosemary (<i>Rosmarinus officinalis</i> L.)	Agroherbal	No. 41	-
Rosemary (<i>Rosmarinus officinalis</i> L.)	Xherdo Co.	-	-
Sage (<i>Salvia officinalis</i> L.)	Xherdo Co.	-	-
Silver fir (<i>Abies alba</i> Mill.)	Xherdo Co.	-	-
Wintergreen (<i>Gaultheria procumbens</i> L.)	Frey & Lau	P0117394	Ph. Eur. 5.0 ⁺

* Sources:

Frey & Lau, Ulzburg (FR Germany).

Agroherbal sh.p.k., Mamurras (Albania).

Xherdo Co. sh.p.k., Tirana (Albania).

⁺ Conforms to the requirements of the 5th European Pharmacopoeia (Ph. Eur. 5.0).

Essential oil analysis

Analyses of the essential oils were performed by gas chromatography (GC) using two detector types. Analytical gas chromatography (GC/FID) analysis of the oils was carried out on a Hewlett-Packard HP-5890 Series II GC device equipped with split-splitless injector and automatic liquid sampler (ALS) attached to HP-5 column (25 m × 0.32 mm, 0.52 µm film thickness) and fitted to flame ionization detector (FID). Carrier gas flow rate (H₂) was 1 ml min⁻¹, split ratio 1:60, injector temperature was 250°C, detector temperature 300°C, while column temperature was linearly programmed from 40-260°C (at a rate of 4°C min⁻¹). Solutions of the essential oil samples in ethanol (~1%) were consecutively injected by ALS (1 µl, split ratio=1:20). Area percent reports, obtained as a result of standard processing of chromatograms, were used as a base for quantification purposes. The same analytical conditions as those mentioned for the GC/FID were employed for gas chromatography/mass spectrometry (GC/MS) analysis, along with column HP-5MS (30 m × 0.25 mm, 0.25 µm film thickness), using a Hewlett-Packard HP G 1800C Series II GCD system. Instead of hydrogen, helium was used as the carrier gas. Transfer line was heated to 260°C. Mass spectra were acquired in EI mode (70 eV), in *m/z* range 40-450. Sample solutions in ethanol (~1 %) were injected by ALS (200 nl, split ratio=1:20). Oil components were identified by comparison of their mass spectra to those from Wiley275 and NIST/NBS libraries, using different search engines. The experimental values for retention indices were determined by the calibrated Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver.2.1.), compared to those from available literature (Adams, 2007), and used as an additional tool to approve MS findings.

RESULTS

Isolate growth was inhibited by nine of 22 tested EOs applied in a concentration range from 0.02 to 0.32 µl ml⁻¹ of air (Table 2). Thirteen oils showed neither inhibitory nor lethal effects on the isolate. Growth inhibition of the tested pathogen after two days was achieved by the oils of two samples of mint, eucalyptus, black pine, cade, lavender, sage, pine and rosemary. Five oils (two samples of mint, eucalyptus, black pine and cade) exhibited lethal effect on the pathogen. The strongest growth inhibition of *Verticillium dahliae* was achieved by the two samples of mint (from Germany and Albania) with MIC and

MFC values of 0.02 µl ml⁻¹ of air. The following oils also exhibited good inhibitory effects on the isolate: eucalyptus and black pine, having MIC and MFC values of 0.16 µl ml⁻¹ of air, followed by cade with MIC of 0.08 µl ml⁻¹ of air and MFC of 0.32 µl ml⁻¹ of air, and lavender with MIC of 0.16 µl ml⁻¹ of air. Fungicidal effect was shown by five oils with various MFC values (0.02, 0.16 and 0.32 µl ml⁻¹ of air) after four-day exposure. The oils of one rosemary sample, pines and sage, having MICs of 0.32 µl ml⁻¹ of air, showed as weaker growth inhibitors. Thirteen oils (anise, lemon, lemongrass, two samples of oregano, silver fir, wintergreen, the remaining samples of lavender [1], rosemary [3] and pine [2]) did not inhibit pathogen growth at all.

Table 2. Effective concentrations of essential oils (µg ml⁻¹ of air) against *Verticillium dahliae* Klebahn

Essential oil	MIC ^a	MFC ^b
Anise S0100154	>0.32	>0.32
Cade Xherdo Co.	0.08	0.32
Eucalyptus S0100321	0.16	0.16
Lavender P0123527	>0.32	>0.32
Lavender Agroherbal	0.16	>0.32
Lemon P0119551	>0.32	>0.32
Lemongrass P0120241	>0.32	>0.32
Mint No. 41/A, Agroherbal	0.02	0.02
Mint P0123884	0.02	0.02
Oregano P0125062	>0.32	>0.32
Oregano P0125412	>0.32	>0.32
Pine (Black) Agroherbal	0.16	0.16
Pine (Maritime) P0125332	>0.32	>0.32
Pine (Maritime) No. 2, Agroherbal	>0.32	>0.32
Pine (Scotch) P0124319 Ph.	0.32	>0.32
Rosemary PO124476	0.32	>0.32
Rosemary No. 1, Agroherbal	>0.32	>0.32
Rosemary No. 41, Agroherbal	>0.32	>0.32
Rosemary Xherdo Co.	>0.32	>0.32
Sage Xherdo Co.	0.32	>0.32
Silver fir Xherdo Co.	>0.32	>0.32
Wintergreen P0117394	>0.32	>0.32

^aMinimal concentration of oil showing lethal effect on the pathogen (Minimum Lethal Concentration)

^bMinimal concentration of essential oil causing complete inhibition of bacterial growth after seven-day exposure (Minimum Inhibitory Concentration)

The most successful essential oil in assay was the mint oil with its highest inhibiting and lethal activity.

Mint oil composition was evaluated by Đurović-Pejčev et al. (2014). The main components of mint essential oil were: menthone (37.02%), menthol (29.57%) and isomenthone (9.06%). Eucalyptus essential oil, showing the second highest lethal effect after mint oil, was chosen for chemical composition analysis (Table 3). As a result, eleven components of eucalyptus essential oil were identified, and they accounted for 100% (v/w) of total mass. The dominant oil components were: 1,8-cineole (82.8%) and limonene (8%), followed by p-cymene (2.6%), γ -terpinene (2.6%) and α -pinene (2.3%). The components listed constituted 98.3% of total mass. Concentrations of all other components varied from 0.1 to 0.6% (Table 3).

DISCUSSION

The results of this study indicated that some essential oils had the ability to suppress the growth of *Verticillium dahliae* *in vitro*. Of the 22 essential oils analyzed, nine oils (two samples of mint, cade, eucalyptus, black pine, lavender, sage and rosemary) inhibited the growth of *V. dahliae*, while five oils (two samples of mint, eucalyptus, black pine and cade) showed fungicidal effects on the pathogen.

Various essential oils have been tested against *V. dahliae*. Arslan & Dervis (2010) tested antifungal activity of the essential oils of different plants against three

vegetative-compatibility groups of *V. dahliae* using *in vitro* volatile phase. Contrary to our findings, they found the highest activity of oregano oils: *Origanum syriacum* L. (wild marjoram), *O. onites* L. (Cretan oregano), *O. minutiflorum* (O. Schwartz & P.H. Davis) (Turkish oregano) and *O. vulgare* L. (oregano), having carvacrol as the main component, and *Thymus.vulgaris* L. (thyme) with p-cymene and thymol as the main components. Abou-Jawdah et al. (2002) tested petroleum or methanolic extracts of wild plants *in vitro* against *V. dahliae*. Petroleum extracts exhibited higher effects than methanolic. Complete inhibition of mycelial growth and spore germination was shown by *O. syriacum*, the wild marjoram. The oil of *Mentha longifolia* L. (wild mint) also was highly effective. Bayan et al. (2016) found that the essential oil of *Heracleum platytaenium* Boiss, tested by diffusion method using 10 μ l per well, significantly inhibited *V. dahliae* growth, though below 100% and with a LC₉₀ of 149.54 μ l. The main components of the oil were: myristicin 27.47 %, octyl acetate 25.1 % and 1-octanol 16.9 %. Hammami et al. (2015) found that a leaf essential oil of *Ruta montana* L, in concentration of 1000 μ g per filter paper disc showed 60 % inhibition, and its leaf extracts of 1500 μ g per filter paper disc showed a remarkable antifungal effect on spore germination. The main components of the oil were: 1-butene 38.33 %, methylcyclopropane 15.47 %, 2-butene 22.56 %, and caryophyllene oxide 8.18 %. The oil showed 600 μ g ml⁻¹ MIC for mycelial growth, and 125 μ l ml⁻¹ MIC for spore

Table 3. Chemical composition of the tested essential oil of eucalyptus (*Eucalyptus globulus* Labillardie) S010032 ^a

	Constituents	KI _E	KI _L	Content [%]	RRT	CI
1	α -Pinene	927	932	2.3	0.760	28
2	β -Pinene	970	974	0.4	0.866	4
3	Myrcene	987	988	0.6	0.891	7
4	α - Phellandrene	1000	1002	0.4	0.930	5
5	α -Terpinene	1012	1014	0.1	0.959	1
6	p-Cymene	1020	1020	2.6	0.979	31
7	Limonene	1023	1024	8.0	0.990	96
8	1,8-Cineole	1030	1026	82.8	1.000	1000
9	cis- β -Ocimene	1035	1032	0.1	1.029	2
10	γ -Terpinene	1054	1054	2.6	1.061	31
11	Terponolene	1086	1086	0.2	1.135	2
	Sum of contents			100.00		

^aKI_E=Kovats (retention) index, experimentally determined (AMDIS);

KI_L=Kovats (retention) index, literature data (Adams, 2007);

RRT=relative retention time of selected constituents (1,8-cineole =1.000);

CI=concentration index;

Content [%]=percentage relative to total essential-oil composition

germination. Garcia-Rellán et al. (2016) found that a *Satureja cuneifolia* L. (winter savory) essential oil from Spain inhibited *V. dahliae* 40 % with a concentration of 1000 µg ml⁻¹ using the macrodilution disc method. The main components were: camphor 47.6 % and camphene 13.6 %. Varo et al. (2017) reported that *Thymus* sp. inhibited mycelial growth and microsclerotia 100% *in vitro* and *in planta*, and achieved Verticillium wilt reduction in olive plants by 65% using the commercial product *Thymus* sp. 01, and by 42 % using *Thymus* sp. 04.

In vitro experiments showed that the volatile phase of certain essential oils, such as those of Scottish pine, eucalyptus, juniper, orange, rosemary and thyme applied at a concentration of 0.65 µl ml⁻¹ of air, inhibited the growth of soil-borne pathogens: *Fusarium* spp., *Rhizoctonia* sp. and *Pythium* sp. (Tanović et al., 2007). A study of antifungal activity of several essential oils against phytopathogenic soil-borne fungi, including *Pythium* sp., *Rhizoctonia* sp and *Verticillium albo-atrum*, showed that cinnamon and thyme oils were the most toxic (Tanović et al., 2007). Eucalyptus and clove oils showed fungistatic effects on *Fusarium culmorum* and *Alternaria alternata* in another study (Byron & Hall, 2002). Oils of mint, thyme and oregano have shown high *in vitro* activity against *Trichoderma* green mould, while tea tree oil added to oyster mushroom substrate or button mushroom casing layer has resulted in considerable *in vivo* inhibition of *Trichoderma harzianum* (Soković & Van Griensven, 2006; Kosanović et al., 2013; Đurović-Pejčev et al., 2014). Đurović-Pejčev et al. (2014) analyzed six essential oils, and found only peppermint oil to exhibit lethal effect on *Trichoderma aggressivum* f. *europaeum*. The main components of the peppermint essential oil were menthone (37.02%), menthol (29.57%) and isomenthone (9.06%). Analysing 22 essential oils from Germany and Albania, Todorović et al. (2016) found those of wintergreen lemongrass and oregano to demonstrate the strongest antimicrobial activity against three pathogenic bacteria (*Xanthomonas campestris* pv. *phaseoli*, *Clavibacter michiganensis* subsp. *Michiganensis* and *Pseudomonas tolaasii*, the parasites of common bean, tomato and cultivated mushroom).

The results of this study show that mint, eucalyptus, black pine and cade essential oils have a potential qualifying them for further *in vivo* experiments against *V. dahliae*. The strongest inhibitory and fungicidal effects were displayed by the oils of two samples of mint, eucalyptus, black pine and cade. In further investigation, it will be of great importance to test these oils *in vivo* as potential biofungicides for the control of Verticillium wilt in pepper and other host plants of *V. dahliae*.

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Antifungalno delovanje etarskih ulja na *Verticillium dahliae* Klebahn, prouzrokovavača verticilioznog uvenuća paprike

REZIME

Biofungicidi bazirani na biljnim uljima imaju prednost u odnosu na hemijske fungicide, posebno kada se uzme u obzir njihovo štetno dejstvo na životnu sredinu. Primenom makrodilucione fumigantne metode ispitana je inhibitorna i fungicidna aktivnost 22 etarska ulja iz Nemačke i Albanije na *Verticillium dahlia* Klebahn, prouzrokovavača verticilioznog uvenuća paprike. Efikasnost etarskih ulja testirana je na različitim koncentracijama (0,02; 0,04; 0,08; 0,16 i 0,32 $\mu\text{l ml}^{-1}$ vazdušne faze) i na osnovu njih su određene minimalne inhibitorne i fungicidne koncentracije. Najjače dejstvo ispoljila su dva uzorka etarskog ulja nane (iz Nemačke i Albanije), oba sa minimalnim inhibitornim i fungicidnim koncentracijama od 0,02 $\mu\text{l ml}^{-1}$ vazdušne faze, zatim etarska ulja eukaliptusa, crnog bora i crvene kleke. Sva nabrojana ulja bila su letalna za patogenu gljivu. Devet od 22 ispitivana etarska ulja ispoljila su inhibitorni efekat: dva uzorka nane, crvena kleka, eukaliptus, crni bor, lavanda, žalfija i ruzmarin, dok je pet etarskih ulja ispoljilo i fungicidni efekat (dva uzorka nane, eukaliptus, crni bor i crvena kleka). Rezultati ukazuju da su ulja nane, eukaliptusa, crnog bora i crvene kleke pogodna za dalja ispitivanja *in vivo* testovima protiv prouzrokovavača verticilioznog uvenuća paprike (*Verticillium dahlia*).

Ključne reči: etarska ulja; antifungalna aktivnost; *Verticillium*; paprika