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Response of *Verticillium fungicola* var. *fungicola, Mycogone perniciosa* and *Cladobotryum* sp. Mushroom Pathogens to Some Essential Oils

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SUMMARY

Antifungal activity of 18 essential oils was evaluated against *Verticillium fungicola* var. *fungicola*, *Mycogone perniciosa* and *Cladobotryum* sp., the causal agents of button mushroom diseases. Essential oils including: turpentine, basil, lemon, mint, fenchel, rose geranium, anise, cinnamon, scots pine, clove, thyme, juniper, lavender, orange, eucalyptus, rosemary, bergamot orange and tea tree, were screened for their effectiveness against the pathogens ***in vitro***. In order to investigate fungicidal activity, isolates were exposed to the volatile phase of the oils for seven days. Of the 18 essential oils analyzed, cinnamon, clove, thyme, and tea tree showed the highest antifungal activity against all investigated mycopathogens, with Minimum Fungicidal Concentration (MFC) being 0.02 µl/ml of air. Turpentine essential oil expressed the lowest antifungal effect to all isolates tested.

Keywords: Essential oils; *Verticillium fungicola* var. *fungicola*; *Mycogone perniciosa*; *Cladobotryum* sp.; Growth inhibition

INTRODUCTION

Verticillium fungicola var. *fungicola* (Preuss) Hassebrauk, *Mycogone perniciosa* (Magnus) Delacroix and *Cladobotryum* spp. (Cooke) causal agents of dry bubble, wet bubble and cobweb disease, are important fungal pathogens of button mushroom, *Agaricus bisporus* (Lange) Imbach (Umar et al. 2000; Grogan and Gaze, 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). *A. 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 present on the surface of diseased mushrooms (Staunton and Dunne, 1990). Cobweb disease caused by *Cladobotryum* spp. is characterized by coarse mycelium growth covering the 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. Most commonly used pesticides in 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 mid-1980s the first signs of resistance of *Cladobotryum* spp. to benomyl occurred 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 as well as residues of pesticides in food imply the necessity to find 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., 2004a) showed that the volatile phase of certain essential oils such as scots pine, eucalyptus, juniper, orange, rosemary and thyme, applied at concentration of 0.65 µl/ml of air, inhibited mycelial growth of soil borne pathogens: *Fusarium* spp., *Rhizoctonia* sp. and *Pythium* sp. In addition, application of thyme essential oil at concentration 1000 µl/l effectively controlled cucumber dumping-off disease (Tanovic et al., 2004b) and dry bubble disease of button mushrooms (Potocnik et al., 2005).

Therefore, the objectives of this study were to investigate antimicrobial activity of several essential oils against *V. fungicola* var. *fungicola*, *M. perniciosa* and *Cladobotryum* spp. *in vitro*.

MATERIAL AND METHODS

Test organisms

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

Test substances

Commercially available essential oils including turpentine (*Pinus terebinta*), basil (*Ocimum basilicum*), lemon (*Citrus limonum*), mint (*Menta piperita*), fenchel (*Foeniculum vulgare*), rose geranium (*Pelargonium graveolens*), anise (*Pimpinella anisum*), cinnamon (*Cinnamomi zeylanicum*), scots pine (*Pinus silvestris*), clove (*Eugenia caryophyllata*), thyme (Thymus vulgaris), juniper (*Juniperus communis*), lavender (*Lavandula officinalis*), orange (*Citrus aurantum*), eucalyptus (*Eucayiptus globulus*), rosemary (*Rusmarinus officinalis*), bergamot orange (*Citrus aurantium* ssp. *bergamia*) and tea tree (*Melaleuca alternifolia*) were provided by BeoLab Co., Belgrade.

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 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 double layer of cheesecloth.

Toxicity of the essential oils to *Verticillium fungicola* var. *fungicola*, *Mycogone perniciosa* and *Cladobotryum* sp. *in vitro*

Antifungal activity was tested on PDA medium in glass Petri plates (R=90 mm) inoculated with the investigated strains by pipetting 20 µl of the conidial suspension into a well cut at the centre 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 dripped onto the inner side of plate covers at concentra-

tions of 0.02, 0.04, 0.08, 0.16, 0.32, and 0.65 µl/ml of air inside the Petri plates by using a micropipette. Plate bottoms were immediately placed on the covers. The plates were left upside down and sealed by parafilm to prevent gas exchange with the outside environment. Inhibition of the mycelial growth was estimated four days after treatment by measuring the radial growth of the isolate treated with different oil concentrations and compared to the control. Seven days after treatment, the plates were observed for the initial mycelial growth without measuring. Concentrations of an oil which completely inhibited the 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 fungicidal effect. The oil concentrations were considered as fungicidal if microbial growth had not been 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.

RESULTS

Antimicrobial activity of essential oils

The growth rate of the isolates was partially or completely inhibited by all tested essential oils applied at 0.02-0.65 µl/ml of air. A 100% growth inhibition of these species was achieved by all oils at 0.65 µl/ml of air after four-day exposure. The most effective were basil, mint, fenchel, rose geranium, anise, cinnamon, clove, thyme and tea tree oils exhibiting total inhibition of all three mycopathogenic isolates at 0.04 µl/ml of air, while the same concentration of turpentine, scots pine, juniper, and rosemary oils caused only partial inhibition of the pathogens. The inhibitory effect of the other tested essential oils varied depending on the pathogen (Figure 1, 2 and 3).

Toxicity of the 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). MIC and MFC values of the investigated oils ranged from 0.02 to more than 0.65 µl/ml of air. Cinnamon, clove, thyme, and tea tree essential oils exhibited the highest level of toxicity with MFC value of 0.02 µl/ml, followed by rose geranium oil with a MFC value of 0.04 µl/ml of air for all isolates tested. Turpentine, juniper, lavender, bergamot orange, rosemary, and orange essential oils, applied at 0.08 µl/ml of air, did not prevent mycelial growth of the isolates. Turpentine, juniper and lavender oils, at the concentration of 0.65 µl/ml of air or lower, were not lethal to *Cladobotryum* sp. (Table 1).

DISCUSSION

Our results indicated that some essential oils had an ability to suppress growth of *V. fungicola* var. *fun-*

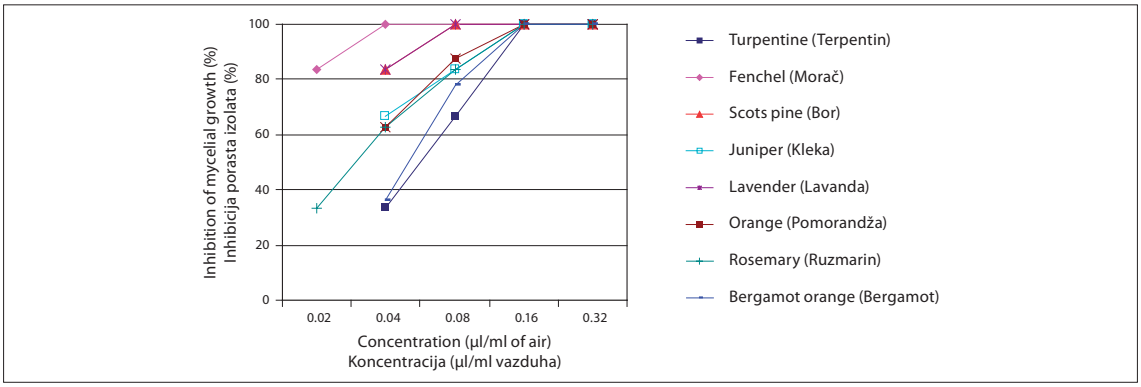

Fig. 1. The effect of volatile phase of essential oils on the growth of *Verticillium fungicola* var. *fungicola* isolate *in vitro* after four-day exposure
Sl. 1. Efekat gasovite faze etarskih ulja na por ast micelije izolata *Verticillium fungicola* var. *fungicola in vitro* nakon četiri dana izlaganja

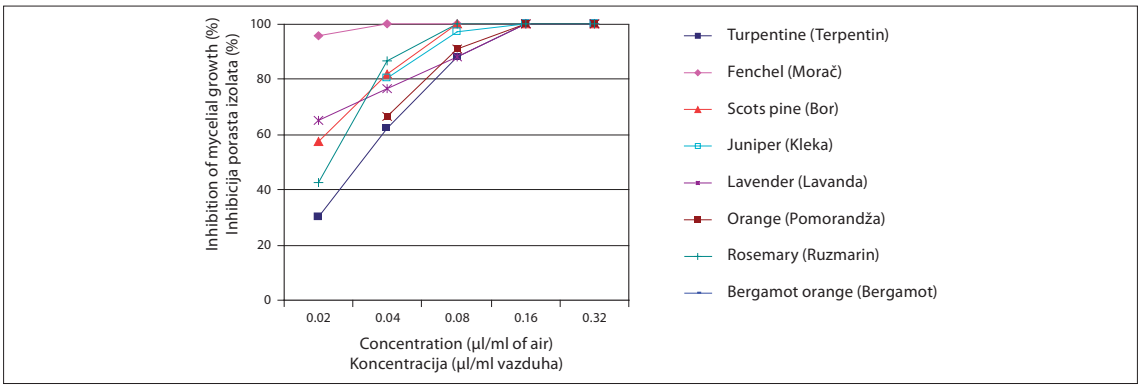

Fig. 2. The effect of volatile phase of essential oils on the growth of *Mycogone perniciosa* isolate *in vitro* after four-day exposure
Sl. 2. Efekat gasovite faze etarskih ulja na porast micelije izolata *Mycogone perniciosa in vitro* nakon četiri dana izlaganja

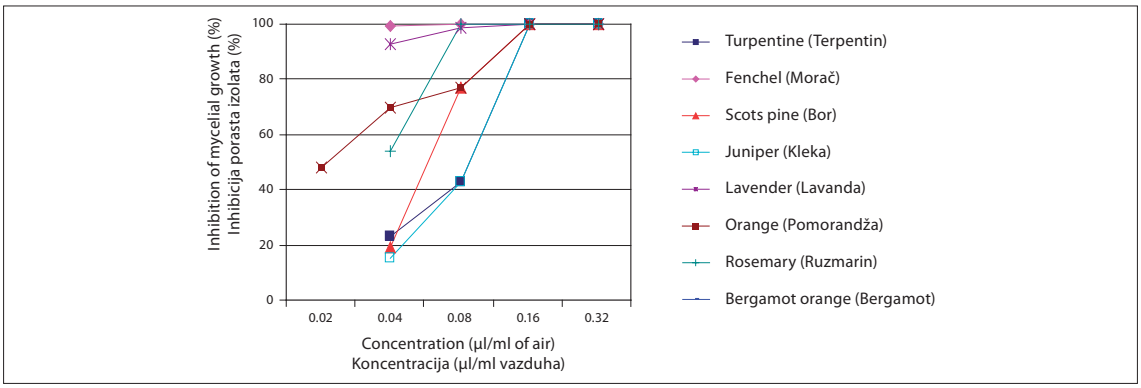

Fig. 3. The effect of volatile phase of essential oils on the growth of *Cladobotryum* sp. isolate *in vitro* after four-day exposure
Sl. 3. Efekat gasovite faze etarskih ulja na porast micelije izolata *Cladobotryum* sp. *in vitro* nakon četiri dana izlaganja

gicola, *M. perniciosa* and *Cladobotryum* sp. isolates *in vitro*. Of the 18 essential oils analyzed, the oils of cinnamon, clove, thyme, and tea tree expressed the strongest antifungal activity against the investigated mycopathogens. These essential oils had already been reported to have fungicidal and antibacterial effects (Wilson et al., 1997; Suhr and Nielsen, 2003; Tanovic at al., 2004a). Daferera et al. (2003) showed that thyme oil had strong activity against *Botrytis cinerea* and *Fusarium* sp. Eucalyptus and clove oils showed fungistatic effect on *Fusarium culmorum* and *Alternaria alternata* (Byron and Hall, 2002). A study of antifungal activity of several essential oils (Tanovic at al., 2004a; 2004b) against phytopathogenic fungi including *Pythium* sp., *Verticillium alboatrum* and *Rhizoctonia* sp. showed that cinnamon and thyme oils were the most toxic. Furthermore, screening experiments with thyme, cinnamon, clove, and orange oils against *Penicillium roqueforti, P. corylophilum* and *Eurotium* spp. reported thyme oil to be the best growth inhibitor, followed by clove and cinnamon, while orange oil had very limited effect (Suhr and Nielsen, 2003). Wilson et al. (1997) recorded that among 49 essential oils tested, red thyme, cinnamon leaf and clove bud oils demonstrated the highest antifungal activity against *Botrytis cinerea*. These results indicated a possible use of thyme, clove, tea tree, and cinnamon essential oils in integrated management of mycopathogenic fungi.

Natural plant-derived fungicides should provide a wide variety of compounds as alternatives to synthetic fungicides, safe both for humane health and the environment (Cutler and Hill, 1994; Daferera et al., 2003). Although cost-effectiveness of oil application should not be underestimated, this study supports further research of the practical uses of these essential oils for controlling *V. fungicola* var. *fungicola*, *M. perniciosa* and *Cladobotryum* sp.

Table 1. Toxicity of several essential oils to *Verticillium fungicola* var. *fungicola*, *Mycogone perniciosa* and *Cladobotryum* spp.
Tabela 1. Toksičnost etarskih ulja za *Verticillium fungicola* var. *fungicola*, *Mycogone perniciosa* i *Cladobotryum* spp.

Essential oils Etarska ulja	Effective concentrations of essential oils (µl/ml of air) Efektivne koncentracije etarskih ulja (µl/ml vazduha)					
	Verticillium fungicola var. *fungicola*		*Mycogone perniciosa*		*Cladobotryum* spp.	
	MIC[1]	MFC[2]	MIC[1]	MFC[2]	MIC[1]	MFC[2]
Tea tree - Čajno drvo (*Melaleuca alternifolia*)	0.02	0.02	0.02	0.02	0.02	0.02
Clove - Karanfilić (*Eugenia caryophyllata*)	0.02	0.02	0.02	0.02	0.02	0.02
Thyme - Timijan (*Thymus vulgaris*)	0.02	0.02	0.02	0.02	0.02	0.02
Cinnamon - Cimet (*Cinnamomi zeylanicum*)	0.02	0.02	0.02	0.02	0.02	0.02
Rose geranium – Geraniol (*Pelargonium graveolens*)	0.04	0.04	0.02	0.04	0.04	0.04
Mint - Pitoma nana (*Menta piperita*)	0.04	0.16	0.02	0.16	0.08	0.32
Fenchel - Morač (*Foeniculum vulgare*)	0.08	0.08	0.04	0.16	0.08	0.32
Lemon - Limun (*Citrus limonum*)	0.08	0.08	0.08	0.16	0.08	0.65
Anise - Anis (*Pimpinella anisum*)	0.08	0.32	0.02	0.16	0.08	0.16
Scots pine - Bor (*Pinus silvestris*)	0.08	0.65	0.16	0.16	0.65	0.65
Basil - Bosiljak (*Ocimum basilicum*)	0.16	0.16	0.08	0.16	0.16	0.65
Eucalyptus - Eukaliptus (*Eucayiptus globulus*)	0.16	0.16	0.32	0.32	0.32	0.32
Orange - Pomorandža (*Citrus aurantum*)	0.16	0.16	0.32	0.32	0.65	0.65
Rosemary - Ruzmarin (*Rusmarinus officinalis*)	0.32	0.32	0.32	0.16	0.32	0.65
Bergamot orange - Bergamot (*Citrus aurantium* ssp. *bergamia*)	0.32	0.32	0.16	0.65	0.32	0.65
Lavender - Lavanda (*Lavandula officinalis*)	0.32	0.32	0.16	0.16	0.16	>0.65
Juniper - Kleka (*Juniperus communis*)	0.32	0.32	0.16	0.16	0.32	>0.65
Turpentine - Terpentin (*Pinus terebinta*)	0.32	0.65	0.16	0.65	>0.65	>0.65

[1]The minimal concentration of essential oil causing complete inhibition of the mycelial growth after seven-day exposure (Minimum Inhibitory Concentration)
[1]Minimalna koncentracija etarskih ulja koja izaziva potpunu inhibiciju porasta izolata nakon ekspozicije od sedam dana (minimalna inhibitorna koncentracija)

[2]The minimal concentration of oil showing lethal effect on the pathogen (Minimum Fungicidal Concentration)
[2]Minimalna koncentracija etarskih ulja koja deluje letalno na patogena (minimalna fungicidna koncentracija)

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Osetljivost patogena šampinjona *Verticillium fungicola* var. *fungicola*, *Mycogone perniciosa* i *Cladobotryum* sp. na neka etarska ulja

REZIME

U radu je ispitivano delovanje 18 etarskih ulja na *Verticillium fungicola* var. *fungicola*, *Mycogone perniciosa* i *Cladobotryum* sp., prouzrokovače bolesti šampinjona. Ispitano je antifungalno dejstvo etarskih ulja: terpentina, bosiljka, limuna, pitome nane, morača, geraniola, anisa, cimeta, bora, karanfilića, timijana, kleke, lavande, pomorandže, eukaliptusa, ruzmarina, bergamota i čajnog drveta na porast izolata *in vitro*. Fungicidni efekat gasovite faze etarskih ulja na izolate je određen nakon ekspozicije od sedam dana. Među 18 proučenih etarskih ulja, cimet, karanfilić, timijan i čajno drvo su najtoksičniji za sve izolate mikopatogenih gljiva sa minimalnom fungicidnom koncentracijom od 0.02 µl/ml vazduha. Najslabije inhibitorno dejstvo ispoljila su ulja terpentina na sve testirane izolate.

Ključne reči: Etarska ulja; *Verticillium fungicola* var. *fungicola*; *Mycogone perniciosa*; *Cladobotryum* sp.; inhibicija porasta