

Antifungal activity of cinnamon and clove essential oils against button mushroom pathogens *Cladobotryum dendroides* (Bull.) W. Gams & Hooz and *Lecanicillium fungicola* var. *fungicola* (Preuss) Hasebrauk

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SUMMARY

Antifungal activity of two essential oils, cinnamon (*Cinnamomum verum* J. Presl) and clove (*Syzygium aromaticum* (L.) Merrill & Perry), was evaluated against *Cladobotryum dendroides* (Bull.) W. Gams & Hooz, and *Lecanicillium fungicola* var. *fungicola* (Preuss) Hasebrauk, the causal agents of cobweb and dry bubble disease of cultivated mushroom. Inhibitory and fungicidal activity of the selected essential oils was assayed using three methods: microdilution, macrodilution fumigant and macrodilution contact method. Comparing all three methods, clove essential oil showed stronger activity than cinnamon against both fungi, having minimum inhibitory concentration (MIC) at the lowest concentrations tested (1.56, 0.02 and 0.1 $\mu\text{l ml}^{-1}$, respectively). However, cinnamon oil was more toxic to *L. fungicola* var. *fungicola* than to *C. dendroides* in all three methods. Both oils exhibited stronger antifungal effects when used in the macrodilution fumigant than in contact method. The results showed that both cinnamon and clove essential oils have the potential for further *in vivo* experiments against *L. fungicola* var. *fungicola* and *C. dendroides* and indicated a possible use of these oils in integrated disease management in mushrooms.

Keywords: Button mushroom; Essential oils; Cinnamon; Clove; Fungal pathogens; Antifungal activity

INTRODUCTION

Button mushroom, *Agaricus bisporus* (Lange) Imbach is the most common cultivated mushroom species in Serbia (Bugarski & Gvozdenović, 1998). The production of this species is severely afflicted by fungal, bacterial and viral pathogens that can cause diseases resulting in significant yield losses. The causal agents of dry bubble and cobweb disease, *Lecanicillium fungicola* var. *fungicola* (Preuss) Hasebrauk and *Cladobotryum dendroides* (Bull.) W. Gams & Hooz, are important fungal pathogens of button mushroom worldwide and in Serbia (Grogan & Gaze, 2000; Gea et al., 2003; Potočnik et al., 2008; 2010a). Symptoms of dry bubble, caused by *L. fungicola* var. *fungicola* and *L. fungicola* var. *aleophyllum*, vary depending on the time of infection. Undifferentiated masses of fruiting bodies develop under infection at an early stage of mushroom development, while spotting symptoms appear when maturing mushrooms are infected (Grogan et al., 2000; Potočnik et al., 2008). Cobweb disease of button mushroom is caused by three *Cladobotryum* species: *C. dendroides* (Bulliard: Fries) W. Gams & Hoozemans, *C. mycophilum* (Oudemans) W. Gams & Hoozemans, and *C. varium* Nees: Fries. All three species cause more or less similar symptoms: cottony fluffy white/greyish colonies on mushroom casing, rapid colonization of casing surface and covering of host basidiomata with mycelia, and ultimately decay. With time, mycelium becomes yellowish or redish/pink in colour (McKay et al., 1999).

Disease control on mushroom farms in Serbia and worldwide is commonly based on the use of fungicides, cultural practice and sanitation. The fungicides that are officially recommended in mushroom industry are: prochloraz and metrafenone in the EU countries, and chlorothalonil, thiabendazol and tiophanate-methyl in North America (Beyer & Kremser, 2004; Grogan, 2008). The most effective fungicide in mushroom disease control is prochloraz. Prochloraz is still effective against fungal pathogens in Serbia (Potočnik et al., 2008, 2009, 2010a, 2010b; Kosanović et al., 2013, 2015), though decreased sensitivity of pathogenetic fungi of the genera *Lecanicillium* and *Cladobotryum* has been already reported in Spain and Great Britain (Grogan & Gaze, 2000; Gea et al., 2003). Considering such resistance evolution, harmful impact on the environment and human health, special attention should be focused on biofungicides, microbiological products and various natural substances of biological origin as alternatives to synthetic fungicides, and consistent with good programs of hygiene (Potočnik et al., 2015).

Many compounds have been tested as control agents against edible mushroom diseases. Plant extracts, essential oils and their components have demonstrated strong fungistatic effects on mushroom pathogens. They could be used as supplements to commercial products in 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. 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 addition 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; Soković et al., 2009; Angelini et al., 2008; Kosanović et al., 2013). Thyme essential oil has demonstrated effective control of dry and wet bubble disease of button mushroom (Potočnik et al., 2005; 2008; 2010b; Regnier & Combrinck, 2010). The essential oils of cinnamon (*Cinnamomum verum* J. Presl) clove [*Syzygium aromaticum* (L.) Merrill & Perry], tea tree, oregano and geranium (*Pelargonium graveolens* L'Her) have shown high *in vitro* activity against *Cladobotryum* sp., *L. fungicola* var. *fungicola* and *Mycogone perniciosa* (Tanović et al., 2006, 2009).

Cinnamon and clove have been acknowledged as herbs with a plenty of pharmacological properties, they have been used in herbal medicine, as flavouring and antimicrobial agents. *C. dendroides* and *L. fungicola* var. *fungicola* are economically important causal agents of cobweb and dry bubble diseases of button mushroom in Serbia. The aim of this study was to assess the antifungal activity of cinnamon and clove essential oils against *C. dendroides* and *L. fungicola* var. *fungicola* using three *in vitro* methods: microdilution, macrodilution fumigant and macrodilution contact method.

MATERIAL AND METHODS

Test organisms

Isolates of *Lecanicillium fungicola* var. *fungicola* NS1V6 (2006, Novi Sad, Serbia) and *Cladobotryum dendroides* C8 (2004, Belgrade, Serbia), identified during a survey of mushroom farms in Serbia (Potočnik et al., 2008; 2009), were chosen for this study (culture collection of the Institute of Pesticides and Environmental Protection, Belgrade, Serbia).

Test substances

Commercially available essential oils of cinnamon bark (*Cinnamomum verum*) and clove (*Syzygium aromaticum*) were provided by Herba, d.o.o., Belgrade, Serbia.

Inoculum preparation

The fungal pathogens *Cladobotryum dendroides* and *Lecanicillium fungicola* var. *fungicola* were prepared as conidial suspensions (approximately 10^6 conidia ml^{-1}). The isolates were initially grown for three and 14 days, respectively, 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%), (REANAL Finomvegyszergy Rt., Hungary, No.: 805383) followed by filtration through a double layer of cheesecloth.

Screening of antifungal activity of essential oils in vitro

Antifungal activity was tested using three methods: macrodilution fumigant, macrodilution contact, and microdilution. Five concentrations of the oils were used. The same range of essential oil volumes was used for both macrodilution methods and microdilution: 1.56, 3.12, 6.25, 12.5 and 25 μl . Respective concentrations were calculated when these volumes of oils were added to agar medium (macrodilution contact), air phase (macrodilution fumigant), or microtiter well (microdilution method). Both macrodilution tests were repeated three and microdilution five times.

Macrodilution fumigant method - Mycelial fragments of each investigated strain ($R=6$ mm) were placed at the center of PDA medium in each glass Petri plate ($R=90$ mm). The isolates were exposed to the volatile phase of essential oils for three days at 22°C. The oils of cinnamon and clove were dripped onto the inner side of plate covers on filter paper cuttings at a range of oil volumes: 1.56, 3.12, 6.25, 12.5 and 25 μl . The concentrations of the essential oils were calculated considering the volume evaporated from the air phase volume inside Petri dishes above the PDA medium (78 ml). Final concentrations of the oils obtained in the air phase were (volume of the essential oil divided by volume of the air phase in Petri dish): 0.02, 0.04, 0.08, 0.16 and 0.32 $\mu\text{l ml}^{-1}$ of air. Plate bottoms were immediately placed on the covers. Control plates were without the essential oils. The plates were left upside down and sealed with parafilm to prevent gas exchange with the outside environment. Inhibition of mycelial growth was estimated three days after treatment by measuring radial growth of the isolates

treated with different oil concentrations and compared to the control. Oil concentrations that completely inhibited mycelial growth after the three-day exposure period at 22°C were considered to be fungistatic and the lowest of these concentrations was determined as the Minimum Inhibitory Concentration (MIC). Afterwards, mycelial fragments without visible growth were reisolated on PDA medium and incubated for three days at 22°C. The lowest concentration with fungicidal effect was defined as the Minimum Fungicidal Concentration (MFC). Three replicates per treatment were used for all concentrations of oils.

Macrodilution contact method - The isolates were exposed to contact with the essential oils for three days at 22°C. Cinnamon and clove oil volumes of 1.56, 3.12, 6.25, 12.5 and 25 μl were mixed with 1 ml of Tween 20 (0.01% v/v) and 14 ml of PDA medium to obtain final concentrations (volume of the essential oil divided by 15 ml volume of PDA medium): 0.1, 0.21, 0.42, 0.83 and 1.67 $\mu\text{l ml}^{-1}$ of medium. Mycelial fragments of the investigated isolates ($R=6$ mm) were placed at the center of each plate. Two controls were used: PDA medium and PDA medium with Tween 20. Inhibition of mycelial growth was estimated three days after treatment by measuring radial growth of the isolates treated with different oil concentrations and compared to the two controls. Fungal growth (colony diameter) was measured and the percentage of growth inhibition (PGI) was calculated according to formula:

$$\text{PGI (\%)} = [(C-T)/C] \times 100$$

where C is colony diameter (mm) in the control plate, and T is colony diameter (mm) in the tested plate (Kaiser et al., 2005).

Oil concentrations that completely inhibited mycelial growth after three-day exposure at 22°C were considered to be fungistatic and the lowest of these concentrations was determined as the Minimum Inhibitory Concentration (MIC). Afterwards, mycelia fragments without visible growth were transferred to PDA medium without essential oils and incubated at 22°C for three days. The lowest concentration with fungicidal effect on the transferred mycelia fragment was defined as the Minimum Fungicidal Concentration (MFC). Three replicates per treatment were used for all concentrations of oils.

Microdilution method - Antifungal activity was tested on potato-dextrose-broth (PDB) medium in microtiter plates with 96 wells. The conidial suspensions of the tested fungi were added by pipetting 10 μl of conidial suspension to a total volume of 100 μl . For negative

control 90 μl PDB medium and 10 μl of conidial suspension was used; for positive control 80 μl PDB medium, 10 μl of control fungicide prochloraz solution (adjusted to final concentration of 10 $\mu\text{l ml}^{-1}$) and 10 μl of conidial suspension was used, while 80 μl PDB medium, 10 μl solution of cinnamon or clove oil and conidial suspension was set for antifungal tests. Oil stock solution was prepared by dissolving 5 μl of each essential oil in 15 μl of Tween 20. Stock solution was further diluted with Tween 20 (1:1) to achieve the final range of concentrations: 1.56, 3.12, 6.25, 12.5 and 25 $\mu\text{l ml}^{-1}$. Inhibition of mycelial growth was estimated three days after treatment by visual inspection of fungal growth. Concentrations of oil which completely inhibited the mycelial growth after three-day exposure at 22°C were considered to be fungistatic and the lowest of these concentrations was determined as the Minimum Inhibitory Concentration (MIC). Minimum Fungicidal Concentration (MFC) was determined by sub-cultivation of 2 μl suspension without visible growth in 100 μl of PDB medium in microtiter plates and further incubation for three days. The lowest concentration without any visible growth was defined as the MFC, indicating 99.5% inhibition of spore germination, compared to the original inoculum (Soković & van Griensven, 2006). Five replicates per treatment were used for all concentrations of oils.

RESULTS

Growth of the isolates was partially or completely inhibited by both essential oils tested by all three methods (Table 1 and 2). Clove essential oil caused 100% inhibition of both pathogenic fungi at the lowest tested concentration in all three methods: macrodilution fumigant, macrodilution contact and microdilution at 0.02, 0.1 and 1.56 $\mu\text{l ml}^{-1}$, respectively. Cinnamon oil completely inhibited *L. fungicola* var. *fungicola* growth at 0.08 $\mu\text{l ml}^{-1}$ of air in the macrodilution fumigant test, 0.21 $\mu\text{l ml}^{-1}$ in macrodilution contact test, and 3.12 $\mu\text{l ml}^{-1}$ in microdilution test. Cinnamon essential oil inhibited *C. dendroides* growth at 0.32 $\mu\text{l ml}^{-1}$ of air in the macrodilution fumigant test, 0.42 $\mu\text{l ml}^{-1}$ in macrodilution contact test and 6.25 $\mu\text{l ml}^{-1}$ in microdilution test. The percentage of growth inhibition by cinnamon oil at different oil concentrations in tests with both macrodilution methods are shown in Figures 1 and 2. Clove essential oil inhibited the mycelial growth of both pathogens 100% with the lowest concentrations tested in all three methods and therefore data are not shown graphically.

Clove essential oil exhibited fungicidal effects against both pathogens at 0.02 $\mu\text{l ml}^{-1}$ concentration in the macrodilution fumigant test and at 1.56 $\mu\text{l ml}^{-1}$ in microdilution test, while fungicidal concentrations differed in the macrodilution contact test (0.21 $\mu\text{l ml}^{-1}$ for *L.*

Table 1. Effective concentrations of essential oils ($\mu\text{l ml}^{-1}$) against *Cladobotryum dendroides* C8

Essential oils	Macrodilution fumigant method		Macrodilution contact method		Microdilution method	
	MIC ^a	MFC ^b	MIC ^a	MFC ^b	MIC ^a	MFC ^b
Cinnamon (<i>Cinnamomum verum</i> J. Presl)	0.32	0.32	0.42	>1.67	6.25	6.25
Clove (<i>Syzygium aromaticum</i> (L.) Merrill & Perry)	0.02	0.02	0.1	0.83	1.56	1.56

^a Minimal concentration of essential oil causing complete inhibition of pathogen growth after three-day exposure (Minimum Inhibitory Concentration)

^b Minimal concentration of essential oils showing lethal effect on the pathogen (Minimum Fungicidal Concentration)

Table 2. Effective concentrations of essential oils ($\mu\text{l ml}^{-1}$) against *Lecanicillium fungicola* var. *fungicola* NS1V6

Essential oils	Macrodilution fumigant method		Macrodilution contact method		Microdilution method	
	MIC ^a	MFC ^b	MIC ^a	MFC ^b	MIC ^a	MFC ^b
Cinnamon (<i>Cinnamomum verum</i> J. Presl)	0.08	0.16	0.21	0.21	3.12	3.12
Clove (<i>Syzygium aromaticum</i> (L.) Merrill & Perry)	0.02	0.02	0.1	0.21	1.56	1.56

^a Minimal concentration of essential oil causing complete inhibition of pathogen growth after three-day exposure (Minimum Inhibitory Concentration)

^b Minimal concentration of essential oils showing lethal effect on the pathogen (Minimum Fungicidal Concentration)

fungicola var. *fungicola* and 0.83 $\mu\text{l ml}^{-1}$ for *C. dendroides*). Cinnamon essential oil exhibited fungicidal effect against *L. fungicola* var. *fungicola* at lower concentrations than against *C. dendroides* in all three methods (Table 1 and 2). Clove essential oil showed higher toxicity than cinnamon

essential oil against both pathogens in all three methods, except for *L. fungicola* in macrodilution contact test where the MFCs for both oils were the same. Nevertheless, sensitivity to cinnamon essential oil was higher in *L. fungicola* var. *fungicola* than in *C. dendroides*.

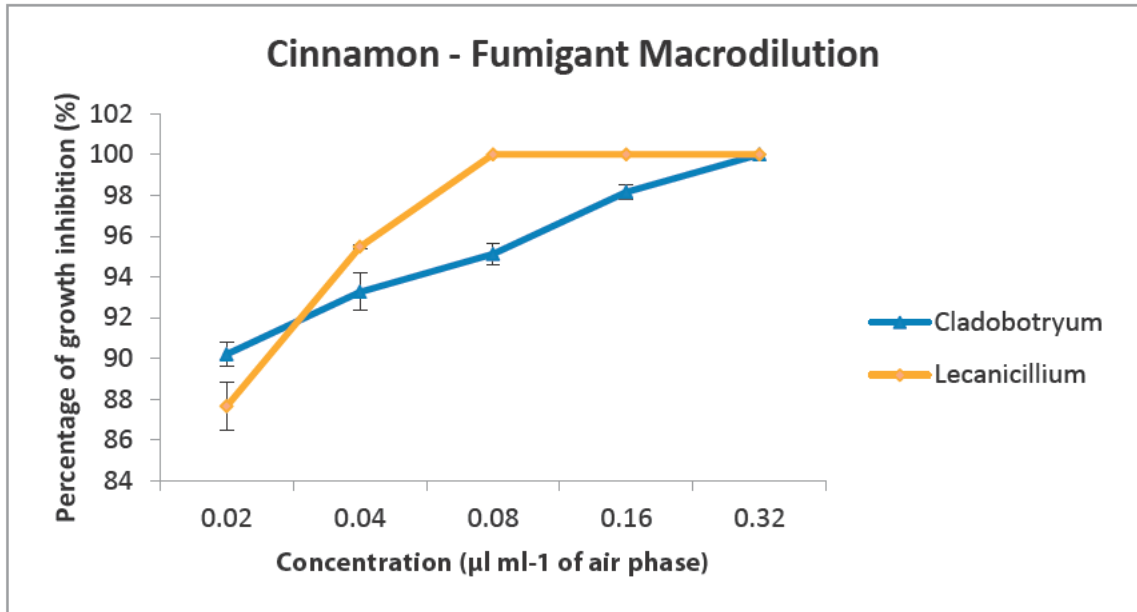


Figure 1. The effect of cinnamon essential oil on the growth of *Cladobotryum dendroides* and *Lecanicillium fungicola* var. *fungicola* *in vitro* using macrodilution fumigant method after three-day exposure (Mean \pm SE, inhibition of mycelial growth)

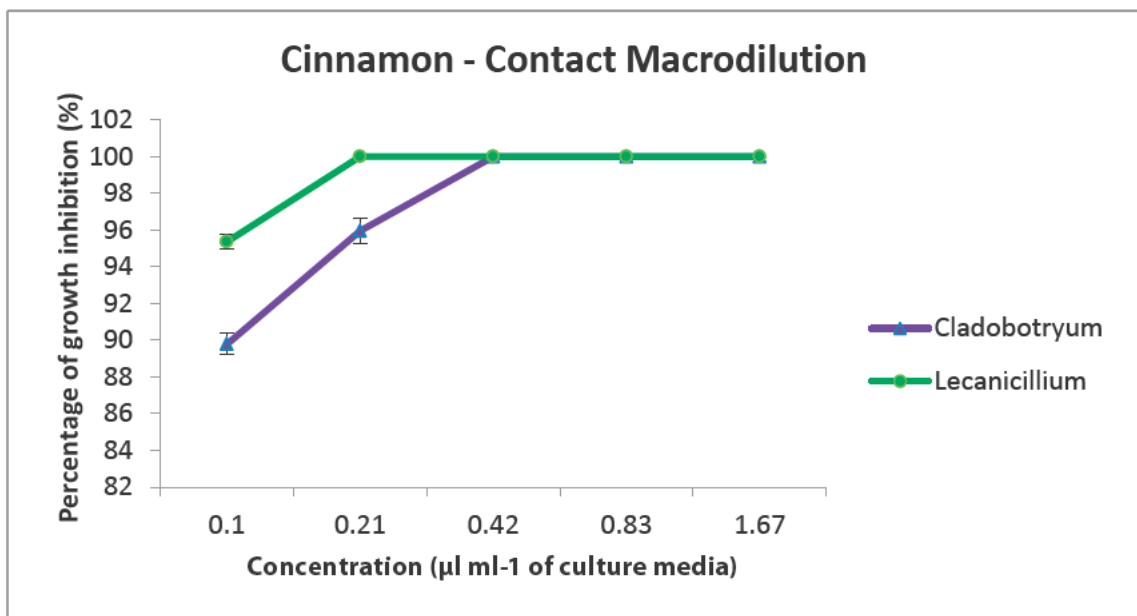


Figure 2. The effect of cinnamon essential oil on the growth of *Cladobotryum dendroides* and *Lecanicillium fungicola* var. *fungicola* *in vitro* using macrodilution contact method after three-day exposure (Mean \pm SE, inhibition of mycelial growth)

DISCUSSION

The results of the present study indicate that cinnamon and clove essential oils have an ability to suppress the growth of *L. fungicola* var. *fungicola* and *C. dendroides* isolates *in vitro*. The activity was confirmed by three distinctive methods. Cinnamon bark has cinnamaldehyde as its major component, while cinnamon leaf oil has eugenol rather than cinnamaldehyde. Cloves are the aromatic flower buds of a clove tree. Clove oil is dominated by eugenol (70-85%), eugenol acetate (15%) and beta-caryophyllene (5-12%) (Orwa et al., 2009). Eugenol is a phenolic compound, and several authors have reported its high antimicrobial activity in phenolic structured compounds (Suhr & Nielsen, 2003).

Many publications have reported about significant antimicrobial effects of cinnamon and clove essential oils against important fungal pathogens: *Botrytis cinerea* (Wilson et al., 1997), *Fusarium* spp., *Alternaria alterata* (Byron & Hall, 2002), *Pythium* sp., *Verticillium albo-atrum*, and *Rhizoctonia* sp. (Tanović et al., 2004; 2007) on different crops. Oils of oregano (*Origanum vulgare* L.), common thyme (*Tymus vulgaris* L.) and peppermint (*Mentha piperita* L.) have shown high *in vitro* activity against green mould *Trichoderma* species (Soković & van Griensven, 2006; Đurović-Pejčev et al., 2014), while essential oils of cinnamon, clove, tea tree, oregano and geranium have shown high *in vitro* activity against *Cladobotryum* sp., *L. fungicola* var. *fungicola* and *Mycogone perniciosa* (Tanović et al., 2006, 2009). Tanović et al. (2006) reported the highest values of both MIC and MFC of clove, cinnamon, thyme and tea tree (BeoLab Co., Belgrade), 0.02 µl ml⁻¹, against *L. fungicola* and *Cladobotryum* spp. using the fumigant macrodilution method, and the same activity of clove oil and cinnamon (Herba d.o.o., Belgrade) was found in the current test. Furthermore, research on the effectiveness of essential oils as crop protectants supports further research of their practical uses in crop protection. The application of thyme essential oil at the concentration of 1000 µl ml⁻¹ effectively controlled dry bubble disease of button mushroom *in vivo* (Potočnik et al., 2005). Regnier & Combrinck (2010) established a suitable application regime for thyme and lemongrass oils to control wet bubble disease caused by *M. perniciosa* in commercial production of button mushrooms. Also, essential oils, such as thyme (Regnier & Combrinck, 2010) or tea tree oil (Potočnik et al., 2010a) have been confirmed as nontoxic to *A. bisporus* and did not have a negative impact on mushroom yield.

As for the different methods of comparison, it was found that the volume of essential oils spent in both macrodilution tests was 48.43, while it was only 5 µl per microdilution test. Moreover, microdilution method enables the testing of spore germination, while macrodilution methods render effects on spore germination and mycelial growth. Clove oil was shown to be more toxic than cinnamon to both tested mycopathogenic fungi by all three methods. The isolate of *L. fungicola* was more sensitive to cinnamon essential oil than *C. dendroides*. Therefore, it is recommended to use microdilution method in preliminary screening of antimicrobial activity of essential oils considering oil consumption in the test and the fact that a significant amount of plant material is required to produce even a small quantity of essential oils. For example, cinnamon essential oil distilled from cinnamon bark yielded only 0.1% while clove oil from clove bud yielded 14-21% (Putievsky et al., 1986). Macrodilution methods are also suitable for antimicrobial activity assessments and for prediction of further fumigant or contact practical applications of essential oils. Both tested essential oils exhibited stronger antifungal effects when used in macrodilution fumigants than by contact. The results showed that cinnamon and clove essential oils have a potential for further *in vivo* experiments against *L. fungicola* var. *fungicola* and *C. dendroides*, and indicated a possible use of these oils in integrated management of mycopathogenic fungi.

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Antifungalna aktivnost etarskih ulja cimeta i karanfilića prema patogenima šampinjona: *Cladobotryum dendroides* (Bull.) W. Gams & Hooz i *Lecanicillium fungicola* var. *fungicola* (Preuss) Hasebrauk

REZIME

Ispitana je antifungalna aktivnost etarskih ulja cimeta i karanfilića prema *Cladobotryum dendroides* (Bull.) W. Gams & Hooz, i *Lecanicillium fungicola* var. *fungicola* (Preuss) Hasebrauk, prouzrokovanih paučinaste plesni i suve truleži šampinjona. Antifungalna aktivnost odabranih etarskih ulja testirana je primenom tri različite metode: mikrodilucione, makrodilucione fumigantne i makrodilucione kontaktne metode. Etarsko ulje karanfilića je ispoljilo jači efekat od ulja cimeta na obe gljive primenom sve tri metode, sa minimalnim inhibitornim koncentracijama pri najnižim testiranim koncentracijama (1,56, 0,02 i 0,1 $\mu\text{l ml}^{-1}$). Ulje cimeta je pokazalo veću toksičnost prema *L. fungicola* var. *fungicola* nego prema *C. dendroides*. Ispitivana etarska ulja ispoljila su jače antifungalno dejstvo primenjena fumigantno nego kontaktno u makrodilucionoj metodi. Antifungalno delovanje etarskih ulja cimeta i karanfilića *in vitro*, ukazuje na potencijal za dalja ispitivanja njihove efikasnosti *in vivo*, kao i mogućnosti uključivanja ovih etarskih ulja u integralnu zaštitu jestivih gljiva od bolesti prouzrokovanih mikopatogenim gljivama *L. fungicola* var. *fungicola* i *C. dendroides*.

Ključne reči: Šampinjon; Etarska ulja; Cimet; Karanfilić; Patogene gljive; Antifungalna aktivnost