

Antifungal and synergistic activity of five plant essential oils from Serbia against *Trichoderma aggressivum* f. *europaeum* Samuels & W. Gams

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SUMMARY

Five essential oils isolated from plants originating from Serbia and ten combinations of the selected essential oils were assayed to test their inhibitory and fungicidal activity against *Trichoderma aggressivum* f. *europaeum* Samuels & W. Gams using two distinctive methods: microdilution and fumigant macrodilution methods. The strongest activity was demonstrated by spearmint (*Mentha spicata* L.) and thyme (*Thymus serpyllum* L.) oils at the minimum inhibitory concentration (MIC) of 6.25 $\mu\text{l ml}^{-1}$ using microdilution, and 0.16 $\mu\text{l ml}^{-1}$ of air using fumigant macrodilution method. The antifungal activity of basil (*Ocimum basilicum* L.) and peppermint (*Mentha piperita* L.) was medium, while the oil extracted from St. John's wort (*Hypericum perforatum* L.) exhibited the lowest activity. None of the selected essential oils exhibited fungicidal effect at minimal fungicidal concentrations (MFC) of over 25 $\mu\text{l ml}^{-1}$ or 0.32 $\mu\text{l ml}^{-1}$ of air, using micro- and macrodilution, respectively. When microdilution was used, the strongest antifungal activity was demonstrated by two oil combinations: spearmint-thyme and spearmint-peppermint, having MIC and MFC values of 3.75 $\mu\text{l ml}^{-1}$. The lowest activity was demonstrated by the basil-St. John's wort essential oil combination, at 30 $\mu\text{l ml}^{-1}$ MIC, and MFC exceeding 30 $\mu\text{l ml}^{-1}$. The obtained results indicate possible synergistic effects of essential oils and their components.

Keywords: green mould disease, button mushroom, biofungicides, essential oils

INTRODUCTION

Fungal pathogens have a significant negative effect on button mushroom (*Agaricus bisporus* (Lange) Imbach) quality and yield (Grogan, 2008). The most important fungal diseases of button mushroom and their causal agents are: wet bubble caused by *Mycogone perniciosa* (Magnus) Delacroix, dry bubble caused

by *Lecanicillium fungicola* var. *fungicola* (Preuss) Hassebrauk, cobweb disease caused by *Cladobotryum* spp. and green mould disease caused by *Trichoderma* spp. The most devastating disease is green mould, caused by *Trichoderma harzianum* Rifai, *Trichoderma aggressivum* f. *europaeum* Samuels & W. Gams and *Trichoderma aggressivum* f. *aggressivum* Samuels & W. Gams, which account for 60-100% of mushroom

yield losses (Seaby, 1996; Kredics et al., 2010). Green mould is characterized by the presence of white mycelia of fast-growing colonies that change their colour to dark green after extensive sporulation on the substrate. Brown necrotic spots and lesions may also appear on mushroom fruiting bodies as accompanying symptoms (Seaby, 1996). The predominant fungal pathogen of button mushroom in Europe is *T. aggressivum* f. *europaeum*, which has been transmitted from the British Isles to many countries, including Serbia (Kosanović et al., 2013).

Disease control in button mushroom farms worldwide usually includes a complex of preventive measures: strict hygiene, treatments with disinfectants, and application of fungicides and biofungicides. Only a few fungicides are currently available and officially recommended in mushroom industry: prochloraz and metrafenone in the EU countries, and chlorothalonil and thiabendazol in North America (Beyer & Kremser, 2004; Grogan, 2008; Anonymous, 2020). Recently, metrafenone has been introduced to control the fungal pathogens *Cladobotryum* spp. and *L. fungicola* in Spain, France, Belgium and the UK after reports of their decreased sensitivity to prochloraz (Carrasco et al., 2017; Anonymous, 2020). However, there is still no alternative regarding green mould control, and data on *Trichoderma* sensitivity to fungicides are scarce. Luković et al. (2020) found the fungicide prochloraz to be highly toxic to several *Trichoderma* species (*T. harzianum*, *T. aggressivum* f. *europaeum*, *Trichoderma pleuroti* S.H. Yu & M.S. Park and *Trichoderma pleuroticola* S.H. Yu & M.S. Park) isolated from edible mushrooms (button mushroom, oyster mushroom and shiitake), while the fungicide metrafenone was considerably toxic to the same pathogens. Metrafenone could also be recommended for the control of green mould disease in mushroom farms after additional *in vivo* trials.

In recent years, special attention has been dedicated to alternative measures, such as the use of microbiological products and various natural substances of biological origin (Potočnik et al., 2015). Antifungal activity of essential oils (EOs) and their components against the causal agents of green mould disease of edible mushrooms has been demonstrated mainly *in vitro*. Oils showing very strong activity may be promising but they require further extensive research and *in vivo* testing. The oils of oregano (*Origanum vulgare* L.), common thyme (*Thymus vulgaris* L.) and peppermint (*Mentha piperita* L.) have demonstrated very high *in vitro* activity against

T. aggressivum f. *europaeum*, *T. harzianum*, *Trichoderma atroviride* P. Karsten and *Trichoderma viride* Tul. (Soković & Van Griensven, 2006; Đurović-Pejčev et al., 2014). The addition of tea tree oil (*Melaleuca alternifolia* [Maiden & Betche]) to oyster mushroom substrate (Angelini et al., 2008) or button mushroom casing (Kosanović et al., 2013) resulted in considerable *in vivo* inhibition of *T. harzianum*.

Peppermint, spearmint, thyme, basil and common St. John's wort have been acknowledged as herbs with plenty of pharmacological properties that are used in herbal medicine, as flavoring herbs and antimicrobial agents. The present study focused on testing *in vitro* the antifungal activity of five selected essential oils originating from Serbia, and their ten combinations, against *T. aggressivum* f. *europaeum*, using two distinctive methods: microdilution and fumigant macrodilution methods.

MATERIAL AND METHODS

Plant samples and preparation of essential oil

Five essential oils (EOs) were isolated from different plants originating from Serbia: peppermint (*Mentha piperita* L.) herbal tea leaves collected from Kovin (Bilje Borča d.o.o., Borča, Serbia); spearmint (*Mentha spicata* L.) herbal tea leaves and thyme (*Tymus serpyllum* L.) herbal tea leaves collected from Vrnjačka Banja (Lekovito bilje sa Goča d.o.o., Mt. Goč, Serbia, June 2016); basil (*Ocimum basilicum* L.) flowers and leaves collected from Mali Mokri Lug, Belgrade, Serbia (July 2016) and common St. John's wort herb (*Hypericum perforatum* L.) collected at Divčibare, Serbia (May 2016).

Plant samples were air-dried at room temperature in the shade for 20 days and then subjected to hydro-distillation for 2.5 h in a Clevenger type apparatus. The obtained essential oils were dried over anhydrous sodium sulphate and preserved in sealed vials at 4°C until further analysis.

Combinations were prepared by mixing each essential oil with each other at 1:1 ratio. Out of the selected five EOs, a total of ten combinations were obtained and tested: spearmint-peppermint, spearmint-thyme, spearmint-basil, spearmint-St. John's wort, peppermint-thyme, peppermint-basil, peppermint-St. John's wort, thyme-basil, thyme-St. John's wort and basil-St. John's wort.

Test organism and inoculum preparation

The strain of *Trichoderma aggressivum* f. *europaeum* T77 used in the study was obtained from button mushroom compost containing mycelia characteristic for green mould (2010, Barajevo-Lisovići, Serbia), previously identified by Kosanović et al. (2013). The isolate was maintained on potato dextrose agar (PDA) medium at 20°C for 72 hours. 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. Conidial suspension was prepared daily in sterile distilled water and adjusted to a concentration of approximately 10^6 conidia ml⁻¹.

Screening of antifungal activity of essential oils *in vitro*

Antifungal activity was tested using two methods: fumigant macrodilution and microdilution. Five concentrations of the tested EOs were applied. The same volume range of the five EOs was used for macrodilution and microdilution methods: 1.56, 3.12, 6.25, 12.5 and 25 µl. The range of volumes of the selected oil combinations using the microdilution method was: 1.76, 3.75, 7.5, 15 and 30 µl. Respective concentrations were calculated when these volumes of oils were added to air phase (fumigant macrodilution) or microtiter wells (microdilution). The macrodilution test was repeated three, while microdilution test was repeated five times.

In the fumigant macrodilution test, antifungal activity was tested on PDA medium in glass Petri plates (R=90 mm) inoculated with mycelial fragments (R=6 mm) of the investigated strain placed at the plate center. The isolate was exposed to the volatile phase of essential oils for three days at 22°C. The selected oils were pipetted onto the inner side of plate covers on filter paper cuttings in a range of oil volumes: 1.56, 3.12, 6.25, 12.5 and 25 µl. The EO concentrations were calculated by considering the volumes evaporated in the air phase volume of Petri plates above PDA medium (78 ml). The final oil concentrations obtained in the air phase were (volume of essential oil divided by volume of air phase in Petri plate): 0.02, 0.04, 0.08, 0.16 and 0.32 µl ml⁻¹ of air. Plate bottoms were immediately placed on top of the covers. The plates were left upside down and sealed with parafilm to prevent gas exchange with

the outside environment. Control plates were without essential oils added. Inhibition of the mycelial growth was estimated three days after treatment by measuring the radial growth of the isolate treated with different oil concentrations and comparison with control plates. Fungal growth (colony diameter) was measured and the percentage growth inhibition (PGI) was calculated using the formula:

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

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

Concentrations of EOs which 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, mycelial fragments without visible growth were transferred to 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 each oil.

Antifungal activity was tested on a malt-extract-broth (MEB) medium in microtiter plates with 96 wells, using the microdilution method. Conidial suspension of the test fungus was added by pipetting 10 µl of conidial suspension into a total volume of 100 µl. A negative control was made by mixing 90 µl MEB medium and 10 µl conidial suspension, while a positive control included 80 µl MEB medium, 10 µl control fungicide prochloraz solution (adjusted to final concentration of 10 µl ml⁻¹) and 10 µl conidial suspension, and antifungal tests were performed with 80 µl MEB medium, 10 µl solution of selected oils and 10 µl of conidial suspension. Stock solution of each essential oil was prepared by solubilizing 5 µl of essential oil in 15 µl of Tween 20, while stock solution of a combination of two oils was prepared by adding 6 µl of mixed essential oil (3 µl per oil) to 14 µl of Tween 20. Stock solution was further diluted with Tween 20 (1:1) to achieve a final range of concentrations of 1.56, 3.12, 6.25, 12.5 and 25 µl ml⁻¹ for each oil, and 1.76, 3.75, 7.5, 15 and 30 µl ml⁻¹ for oil mixtures. Inhibition of mycelial growth was estimated seven days after treatment by visual inspection of fungal growth. Oil concentrations which completely inhibited mycelial growth after

seven-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 of suspension without visible growth in 100 µl of MEB medium in microtiter plates and further incubation for three days. The lowest concentration without any visible growth was defined as MFC, indicating a 99.5% inhibition of spore germination, compared to the original inoculum (Soković & Van Griensven, 2006). Five replicates per treatment were used for all oil concentrations.

RESULTS

In vitro antifungal activity of essential oils from Serbia

Pathogen growth was inhibited by four of the five essential oils applied in a concentration range from 0.02 to 0.32 µl ml⁻¹ of air using the fumigant macrodilution method, and from 1.56 to 25 µl ml⁻¹ using the microdilution method (Table 1). Growth inhibition of the test pathogen after three days was achieved by spearmint, thyme, peppermint and basil oils using

macrodilution. The same essential oils inhibited the test pathogen in the microdilution test after seven days. Only common St. John's wort oil showed neither inhibitory nor fungicidal effect on the isolate using either method. The strongest growth inhibition of *T. aggressivum* f. *europaeum* (T77) was demonstrated by the oils of spearmint and thyme, showing a MIC of 6.25 µl ml⁻¹ when microdilution was applied and 0.08 µl ml⁻¹ of air when the fumigant macrodilution method was used. Also, medium antifungal activity of basil and peppermint oils was recorded under both methods. None of the selected oils exhibited a fungicidal effect, having MFCs of over 25 µl ml⁻¹ (microdilution) or 0.32 µl ml⁻¹ of air (fumigant macrodilution). The percentage of mycelial growth inhibition of the test pathogen caused by five tested essential oils using the fumigant macrodilution method is shown in Figure 1.

In vitro antifungal activity of ten combinations of selected essential oils from Serbia

Pathogen growth was inhibited by all ten tested essential oil combinations which were applied in a concentration range from 1.76 to 30 µl ml⁻¹ using

Table 1. Effective concentrations of essential oils against *Trichoderma aggressivum* f. *europaeum* T77

Essential oil	Fumigant macrodilution method (µl ml ⁻¹ of air)		Microdilution method (µl ml ⁻¹)	
	MIC ^a	MFC ^b	MIC ^a	MFC ^b
Peppermint (<i>Mentha piperita</i> L.)	0.32	>	12.5	>
Spearmint (<i>Mentha spicata</i> L.)	0.08	>	6.25	>
Thyme (<i>Thymus serpyllum</i> L.)	0.08	>	6.25	>
Basil (<i>Ocimum basilicum</i> L.)	0.32	>	12.5	>
Common St. John's wort (<i>Hypericum perforatum</i> L.)	>	>	>	>

^aMIC – minimum inhibitory concentration

^bMFC – minimum fungicidal concentration

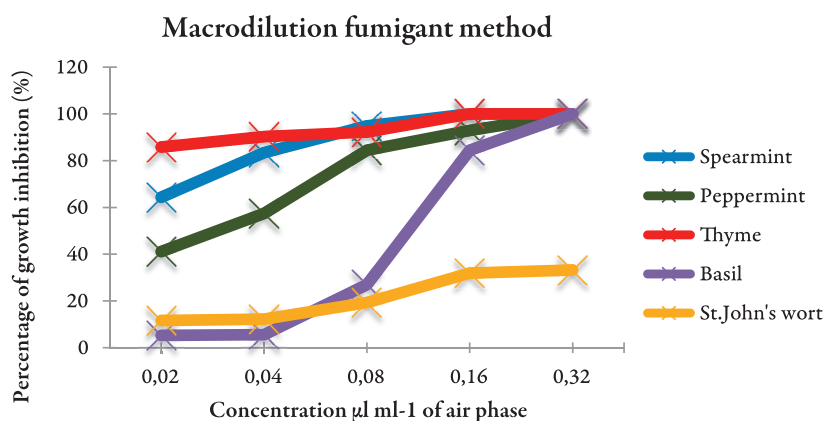


Figure 1. *In vitro* effects of the volatile phase of essential oils on the growth of *Trichoderma aggressivum f. europaeum* T77 after three-day exposure

the microdilution method (Table 2). The best results were recorded using mixtures that contained either spearmint or thyme oil, slightly weaker were mixtures with peppermint or basil oil, and the weakest were combinations with common St. John's wort oil. Of the ten tested essential oil combinations, three mixtures that contained St. John's wort (spearmint-St. John's wort, peppermint-St. John's wort and basil-St. John's wort) did not exhibit any fungicidal effect.

The strongest growth inhibition of *T. aggressivum f. europaeum* T77 was demonstrated by two essential oil combinations: spearmint-thyme and spearmint-peppermint, having MIC and MFC values of 3.75 µl ml⁻¹. The lowest activity was demonstrated by the combination of basil-St. John's wort oils, whose MIC was 30 µl ml⁻¹ and MFC exceeded 30 µl ml⁻¹.

Table 2. Effective concentrations of essential oil combinations (µl ml⁻¹) against *Trichoderma aggressivum f. europaeum* T77 using microdilution method

EO combination	MIC ^a	MFC ^b
Spearmint-peppermint	3.75	3.75
Spearmint -thyme	3.75	3.75
Spearmint-basil	7.5	7.5
Spearmint-common St. John's wort	15	>
Peppermint-thyme	7.5	7.5
Peppermint-basil	7.5	7.5
Peppermint- common St. John's wort	15	>30
Thyme-basil	7.5	7.5
Thyme-common St. John's wort	7.5	7.5
Basil-common St. John's wort	30	>30

^aMIC – minimum inhibitory concentration

^bMFC – minimum fungicidal concentration

DISCUSSION

The inhibitory and fungicidal activity of five selected EOs (peppermint, spearmint, thyme, basil and common St. John's wort), isolated from plants originating from Serbia, against *T. aggressivum* f. *europaeum* was tested using two distinctive methods: microdilution and fumigant macrodilution, while the antifungal activity of ten combinations (spearmint-peppermint, spearmint-thyme, spearmint-basil, spearmint-St. John's wort, peppermint-thyme, peppermint-basil, peppermint-St John's wort, thyme-basil, thyme-St. Johns' wort and basil-St. John's wort) was tested using only microdilution. Four of the five tested EOs (all except common St. John's wort) and their ten combinations inhibited the growth of *T. aggressivum* f. *europaeum*, while seven of the ten EO combinations (all except spearmint-St. John's wort, peppermint-St. John's wort and basil-St. John's wort) showed fungicidal effects on the pathogen.

Antimicrobial effects of essential oils against myco- and phytopathogens (especially fungi and bacteria) have been evaluated in many studies. Analysing 22 EOs from Germany and Albania by fumigant macrodilution, Todorović et al. (2016) found that wintergreen, lemongrass and oregano demonstrated the strongest activity against three bacteria (*Xanthomonas campestris* pv. *phaseoli*, *Clavibacter michiganensis* subsp. *michiganensis* and *Pseudomonas tolaasii*) that are pathogens of common bean, tomato and cultivated mushroom. On the other hand, two mint oil samples showed the strongest activity at 0.02 $\mu\text{l ml}^{-1}$ of air (followed by eucalyptus, black pine and cade) against *Verticillium dahliae*, a pathogenic fungus of pepper (Luković et al., 2019a), and against *Chryphonectria parasitica* (followed by black pine, eucalyptus, cade, sage and silver fir), a pathogenic fungus of chestnut (Luković et al., 2019b). Many publications have reported significant antifungal activity of various EOs against important button mushroom pathogens: *L. fungicola* var. *fungicola*, *M. perniciosus* and *Cladobotryum* spp. (Tanović et al., 2006; Glamočlija et al., 2006; Džamić et al., 2008; Soković et al., 2009; Luković et al., 2018). Testing 18 EOs, Tanović et al. (2006) found that thyme, cinnamon, clove and tea tree oils had the highest antifungal activity against these mycopathogenic fungi, while Luković et al. (2018) found that clove

and cinnamon completely inhibited the growth of *L. fungicola* and *Cladobotryum dendroides* in tests that used three distinctive methods. Various EOs have been tested against various *Trichoderma* species, pathogens of cultivated mushrooms, and they demonstrated inhibitory effects *in vitro* and *in vivo*. Oils of oregano, common thyme and peppermint have shown very high *in vitro* activity against *T. aggressivum* f. *europaeum*, *T. harzianum*, *T. atroviride* and *T. viride* (Soković & Van Griensven, 2006; Đurović-Pejčev et al., 2014), while tea tree oil added to oyster mushroom substrate or button mushroom casing resulted in considerable *in vivo* inhibition of *T. harzianum* (Angelini et al., 2008; Kosanović et al., 2013). Đurović-Pejčev et al. (2014) analyzed six essential oils originating from Serbia (peppermint, basil, yarrow, walnut, juniper and St. John's wort), using fumigant macrodilution. They found basil and peppermint oils to have the strongest activity against *T. aggressivum* f. *europaeum* at 0.02 and 0.04 $\mu\text{l ml}^{-1}$ of air, respectively, and only peppermint oil showed lethal effect at 0.64 $\mu\text{l ml}^{-1}$ of air. The main components of that peppermint EO were menthone (37.02%), menthol (29.57%) and isomenthone (9.06%). The strongest activity of peppermint and thyme essential oils were confirmed in the current study, but lethal effect was not recorded at the tested concentrations and it was higher than 0.32 $\mu\text{l ml}^{-1}$ of air. Essential oils of different *Mentha* species had a satisfactory antimicrobial potential, especially peppermint and spearmint EOs, which inhibited the growth of *T. viride* at 2.5 $\mu\text{l ml}^{-1}$ and two other pathogens of cultivated mushrooms: *T. harzianum* and *P. tolaasii* (Soković & Van Griensven, 2006; Soković et al., 2009). Saroglou et al. (2007) found that St. John's wort EO originating from Serbia exhibited antibacterial activity against *P. tolaasii*, which causes bacterial blotch on button mushroom, while the current study did not show St. John's wort EO to exhibit antifungal activity against *T. aggressivum* f. *europaeum*. Using the macrodilution method, Abdolahi et al. (2010) confirmed the antifungal activity of basil EO against the phytopathogenic fungus *Botrytis cinerea* at 0.5 $\mu\text{l ml}^{-1}$ with a mycelial growth inhibition of 42.5%. In the study, which used the fumigant macrodilution method, basil essential oil completely inhibited the growth of tested pathogenic fungi at 0.32 $\mu\text{l ml}^{-1}$ of air. Comparing different methods, microdilution enables the testing of spore germination, while macrodilution

shows effects on spore germination and mycelial growth. However, the volume of EO spent in a macrodilution test is higher than in microdilution test. Therefore, it is recommended to use the microdilution method in preliminary screening of antimicrobial activity of EOs, while macrodilution is also suitable for antimicrobial assessment and for prediction of further practical applications of EOs as fumigants. In the previous study, Luković et al. (2018) described and compared in detail three distinctive methods, including the two used in the current study.

Although studies of combinations and synergistic antifungal effects of EOs and their components are scarce compared to those focusing on the effects of oils applied individually, they have become more frequent in recent years. Stević et al. (2014) described a synergy between thymol and carvacrol in thyme and oregano EOs against fungi isolated from medicinal plants: *Aspergillus niger*, *Aspergillus flavus*, *Alternaria alternata* and *Fusarium* spp. A similar synergistic effect of thymol and carvacrol (thyme-oregano EO combination) has been reported against some *Aspergillus* spp. and *Penicillium chrysogenum* (Hossain et al., 2016) and against *B. cinerea* and *Penicillium expansum* (Nikkhah et al., 2017) as the combined treatments caused a more significant decrease in fungal growth than each oil individually. Similar findings of improved activities of combined oils were confirmed in the current study, i.e. that antifungal activity of the EO combinations thyme-spearmint and spearmint-peppermint were more efficient than the activity of each oil individually.

The obtained results indicate possible synergistic effects of essential oils and their components. Also, the study showed that four tested essential oils (peppermint, spearmint, thyme and basil) completely inhibited the growth of *T. aggressivum* f. *europaeum*, while their combinations had fungicidal effects against it, indicating that they could be considered as eligible candidates for further *in vivo* experiments.

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Antifungalno i sinergističko delovanje pet etarskih ulja poreklom iz Srbije na *Trichoderma aggressivum* f. *europaeum* Samuels & W. Gams

REZIME

Primenom mikrodilucione i makrodilucione fumigantne metode ispitana je inhibitorna i fungicidna aktivnost pet etarskih ulja izdvojenih iz biljaka poreklom iz Srbije i njihovih deset kombinacija prema *Trichoderma aggressivum* f. *europaeum* Samuels & W. Gams. Najjače delovanje ispoljila su ulja majčine dušice (*Thymus serpyllum* L.) i divlje nane (*Mentha spicata* L.) sa minimalnim inhibitornim koncentracijama (MIK) od 6,25 $\mu\text{l ml}^{-1}$, primenom mikrodilucione metode, i 0,16 $\mu\text{l ml}^{-1}$ vazdušne faze, primenom makrodilucione fumigantne metode. Slabije delovanje zabeleženo je kod ulja pitome nane (*Mentha piperita* L.) i bosiljka (*Ocimum basilicum* L.), dok je najslabije delovanje ispoljilo ulje kantariona (*Hypericum perforatum* L.). Testirana ulja nisu pokazala fungicidno delovanje. Minimalne fungicidne koncentracije (MFK) za sva testirana ulja su bile veće od 25 $\mu\text{l ml}^{-1}$ i 0,32 $\mu\text{l ml}^{-1}$ vazdušne faze nakon mikro- i makrodilucione metode. Primenom mikrodilucione metode utvrđeno je da su kombinacije etarskih ulja divlja nana-majčina dušica i divlja nana-pitoma nana ispoljile najjače delovanje sa vrednostima za MIK i MFK od 3,75 $\mu\text{l ml}^{-1}$. Najslabije delovanje ispoljila je kombinacija bosiljak-kantarion sa MIK od 30 $\mu\text{l ml}^{-1}$ i MFK većom od 30 $\mu\text{l ml}^{-1}$. Dobijeni rezultati ukazuju na moguće postojanje sinergističkog dejstva etarskih ulja i njihovih komponenti.

Ključne reči: zelena plesan, šampinjon, biofungicidi, etarska ulja