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# *IN VITRO* EFFECTIVENES OF DIFFERENT ESSENTIAL OILS IN CONTROL OF *ALTERNARIA ALTERNATA*

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The effectiveness of volatile phase of essential oils of *menthe*, *eucalyptus* and *rosmarinus* in control of *A. alternata*, a postharvest pathogen on fruits and vegetables, expressed through inhibition of mycelium growth, in vitro, has been tested. The inhibitory effect of tested oils has been determined four days after setting the trial by calculating the percentage of inhibition of radial growth of pathogen mycelium (PIRG), while the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) have been determined seven i.e. fourteen days later. The highest value of PIRG (100%) was found in essential oil of menthe when applied in concentration of 0,15μl/ml of air and essential oil of rosmarinus applied in 0,6μl/ml of air.

Essential oil of eucalyptus had the highest PIRG value (89,74%) when applied in concentration of 0,6μl/ml of air. MIC of essential oil of menthe was 0,3μl/ml of air while other two essential oils did not show total inhibitory effect in tested concentrations (MIC >0,6μl/ml of air). Essential oil of menthe did not have a fungicide effect on pathogen, not even in the highest concentration (MFC > 0,6μl/ml of air). In other two oils MFC has not been determined since they did not express the inhibitory effect in the first. Therefore, menthe essential oils could be an alternative to chemicals to control *A. alternata*, a postharvest pathogen on fruits and vegetables, and can control this pathogen in vitro. These results will help in further testing of effectiveness of essential oils in vivo.

*Key words: mentha*, *eucalyptus*, *rosmarinus*, inhibition, biological control.

## INTRODUCTION

*Alternaria alternata* is one the most common pathogens in the vegetable crop production pre and post harvest. This pathogen jeopardizes production of different vegetable crops in all phases of production, starting from seed to plants in vegetation to fruits in storage (Agrios, 2005; Abd-Alla et al., 2009; Bulajić et al., 2009). As a postharvest pathogen, *A. alternata* causes Alternaria rot that causes great losses. These losses go up to 30% per year and even up to 43% of the total production of tomatoes (Abd-Alla et al., 2009). It is important to note that storage conditions necessary to preserve the quality of fruits for a long time are also favorable for the development of this pathogen. Particularly, the increased humidity which is necessary in order to prevent the occurrence of fruit shrivel is also very good for the development of the pathogen, and the fact that *A. alternata* develops even at low temperatures supports the fact that this is a very important pathogen (Barkai-Golan and Pasteur, 2008a; El-Sheshtawi et al., 2010).

Apart from direct economic considerations, diseased fruits pose a potential health risk. *A. alternata* is known to produce mycotoxins under certain conditions. The occurrence of Alternaria mycotoxins has been recorded in tomatoes, peppers, melons as well as in several processed fruits including tomato products (Barkai-Golan, 2008b). The major mycotoxins that can appear in the fruits of vegetables when stored are: alternariol ($C_{14}H_{10}O_5$), alternariol methyl ether ($C_{15}H_{12}O_5$) and altenuene ($C_{15}H_{16}O_6$), which are benzopirone derivates; also tenuazonic acid ($C_{10}H_{15}NO_3$), which is a tetramic acid derivate; and altertoxin-I ($C_{20}H_{16}O_6$), a perylene derivate (Andersen and Frisvad, 2004).

Several kinds of synthetic fungicides have been used to control the postharvest decay caused by Alternaria rot. Using fungicides for a long time results in the development of resistant strains (Rosslenbroich and Stuebler, 2000). At the same time, many of these fungicides gradually become ineffective (Spotts and Cervantes, 1986). As a result, it is easy to conclude that this pathogen is difficult to control. With the continued loss of currently used postharvest decay control measures (fungicides), there is a perpetual need to search for alternatives (Abd-Alla et al., 2009). Given the trend of modern plant protection to limit the use of fungicides, in order to protect consumers and the environment, it is very important to find new methods and environmentally friendly ways to control pathogens that will be equally effective (Djordjevic et al ., 2010a, 2010b). One possible solution is to apply the essential oils of some plants. The impact of these oils on certain pathogenic fungi of plants and fungi important in the food industry was investigated by several authors (Abd-Alla et al., 2009; Aslam *et al*., 2010; Parven *et al*., 2010; Simić *et al.*, 2008; Soković *et al*., 2008, 2009a, 2009b; Veljić *et al*., 2009; Zhang *et al.*, 2009). Given that plants are rich sources of antifungal compounds, they could be an appropriate alternative to conventional fungicides if these compounds are formulated correctly (Tanovic et al., 2005, Wilson et al., 1997). Before any pesticide's application *in vivo*, biological or conventional, it is first necessary to determine its toxicity, i.e. its efficiency *in vitro*.

The aim of this study is to determine the antimicrobial activity, i.e. the *in vitro* efficiency of some essential oils in controlling the postharvest pathogen of vegetable crops, *Alternaria alternata*, to determine their toxicity and determine the approximate concentration as the starting point for *in vivo* studies.

## MATERIALS AND METHODS

The pathogen identification required isolation of pure fungi culture by repetitive single-spore transfers followed by microscopic examination.

The antifungal activity of essential oils of *Mentha piperita*, *Eucaliptus citriodora*, *Rosmarinus officinalis* was investigated to *Alternaria alternata*, by expossing mycelium of pathogen to volatile phase of these oils (Soylu *et al.,* 2006; Tanović *et al.*, 2009). Mycelial plug (5x5mm) was transferred to the center of the Petri plate (R=9cm), after which the plates were turned upside down. The oils were applied as a drop onto the inner side of the plate covers on the sterile filter paper (R=0,5cm), which was placed in the center on the cover glass, at the concentrations of 0.04, 0.06, 0.1, 0.15, 0.3 i 0.6 µl/ml of air inside the Petri plates using micropipette. In order to enable the contact of volatile phase of oils and pathogen, the Petri plates were kept upside down. The plates were sealed with self-adhesive foil in order to prevent release of oil vapors out of the plates. The Petri plates were also kept at 23 °C. As control, a Petri plate with a drop of sterile distilled water instead of oil was used.

After four, seven and fourteen days, the radial growth of pathogen mycelium in the treated plates and control were measured. After four days of exposure to oil vapor, the percent of inhibition of radial growth of pathogen mycelium (PIRG) was calculated. After seven days from the exposure, the plates were observed for initial growth of mycelium without measuring. The concentration of oil which completely inhibited mycelium growth after seven-day exposure was considered fungistatic and the lowest of these concentrations were determined as the minimum inhibitory concentration (MIC). After that, the plates were opened and ventilated in the sterile laminar flow for 30 minutes in order to remove volatiles of oils in order to determine fungicidal effect of oils. The fungicidal concentrations were those which suppress mycelial growth even after seven days from ventilation. The lowest concentrations were considered as minimal fungicidal concentration (MFC).

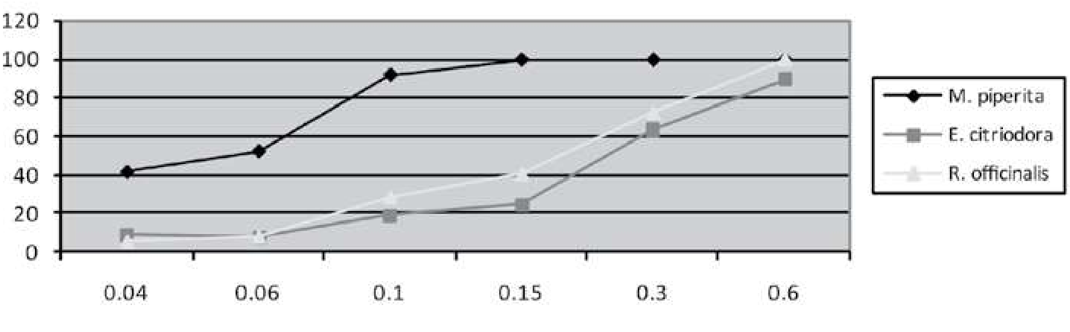
All experiments were performed twice with five replications of each oil concentration. The percentage of inhibition of radial growth of pathogens mycelia were calculated using the following formula:

$$PIRG(\%) = \{g_c - g_t / g_c\} \text{ x } 100 ,$$

where ***gc*** is the growth of mycelium in control plates, ***gt*** the growth of mycelium in treated plates. The analysis of variance and significance of differences using Duncan's Multiple Range Test (P=0.05) was done by using mathematical program MATLAB Ver. 7.0.

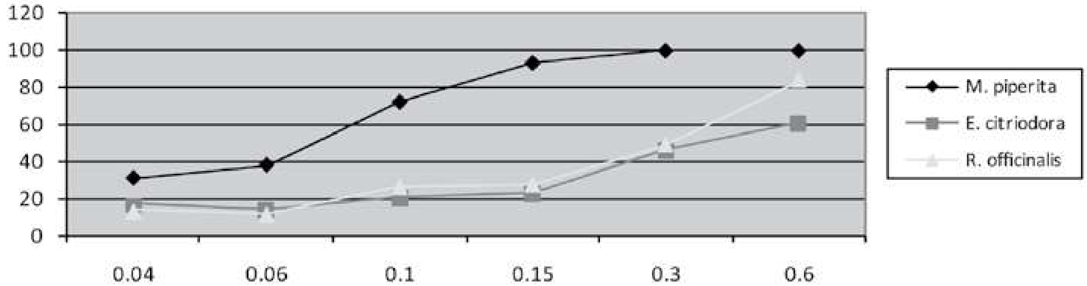
## RESULTS

The antifungal activity of essential oils of *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis* against postharvest pathogen *Alternaria alternate* were investigated. The oils were treated to each bioassay plate which allows only volatiles to be causative agents for any microbial inhibition. Four days after exposure of pathogen to oils, the highest percentage of inhibition was demonstrated in the essential oil of *M. piperita*, which showed total inhibition at a concentration of 0.15 µl/ml of air. The essential oil of *R. officinalis* expressed the same level of inhibition of *A. alternate*, applied at a concentration of 0.6 µl/ml of air. Meanwhile, the essential oil of *E. citriodora* demonstrated the highest percentage of inhibition in the concentration of 0.6 µl/ml of air and the value of inhibition was 89.74% (Graph. 1).




**Graf. 1 -** Inhibitorno dejstvo isparljive faze eteričnih ulja *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis* prema skladišnom patogenu Alternaria alternata nakon 4 dana.

**Graph. 1 -** Inhibitory activity of volatile phase of essential oils of *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis* toward postharvest pathogen Alternaria alternata after 4 days of exposure.




**Graf. 2 -** Inhibitorno dejstvo isparljive faze eteričnih ulja *Mentha piperita, Eucaliptus citriodora* and *Rosemarinus officinalis* prema skladišnom patogenu Alternaria alternata nakon 7 dana.

**Graph. 2 -** Inhibitory activity of volatile phase of essential oils of *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis* toward postharvest pathogen Alternaria alternata after 7 days of exposure.

Seven days after the exposure of pathogen to the effects of volatiles of essential oils, minimum inhibitory concentration (MIC) based on the percentage of inhibition was determined (Graph. 2). The lowest value of MIC was detected with the *M. piperita* oil and 0.3 µl/ml of air, while the other two oils did not have this value in the observed range of concentrations and the MIC was higher than 0.6 µl / ml of air (MIC> 0.6 µl/ml of air). Since the oil of *M. piperita* showed total inhibition of growth after seven days, MFC is determined only for this oil seven days after removing the oil phase, i.e. seven days after the determination of MIC and the Petri plates ventilation. After this period MFC was not determined because of the occurrence of growth of mycelium of *A. alternata* (MFC > 0,6 µL/ml of air) (Table 1.).

**Table 1 -** Values of MIC and MFC of *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis*

**Tabela 1 -** MIC i MFC eteričnih ulja *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis*

| **Essential oils (Eterična ulja)** | **MIC*** | **MFC**** |
|---|---|---|
| *M. piperita* | 0,3µl/ml of air | > 0,6µl/ml of air |
| *E. citriodora* | > 0,6µl/ml of air | > 0,6µl/ml of air |
| *R. officinalis* | > 0,6µl/ml of air | > 0,6µl/ml of air |

*MIC minimal inhibitory concentration,
**MFC minimal fungicidal concentration
*MIC minimalna inhibitorna koncentracija
**MFC minimalna fungicidna koncentracija

## DISCUSSION

Biological control has been consider as one of the most promising alternatives to fungicides (Abd-Alla et al., 2009; Feng and Zheng, 2007; Đorđević et al., 2010). Natural pesticides based on plant-essential oils may represent alternative crop protectans whose time has come, but according to some this claim has yet to be substantiated through controlled experiments and scientific investigation (Isman, 2000). Several studies have explored the potential of essential oils as antifungal agents (Abdolahi et al., 2010; Tanović et al., 2005; Lee et al., 2007; Fawzi et al., 2009). In this study, we investigated the antifungal activities of essential oils of *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis* against postharvest pathogen *Alternaria alternata* by exposure to volatile phases of the oils, *in vitro*. All of the oils had the inhibition effect on mycelial growth of *A. alternate*, more or less. As observed, the antifungal activities of essential oils were dependent on the type of essential oil and oil concentration. *In vitro* tests showed that the essential oil of *Mentha piperita* had the highest inhibition effect. However, the antifungal properties of this essential oil at used concentrations were fungistatic, not fungicidal. The oil of *R. officinalis* showed strong inhibition but not strong enough to have fungistatic or fungicidal effects on this pathogen. The weakest inhibition effect was seen in the essential oil of *E. citriodora*. In the research of Abd-Alla et al. (2009), the essential oil of *M. piperita* showed the inhibition of mycelial growth of *A. alternata* at maximal rate of application with growth reduction of 46%. The method in that research considers incorporation of oils in medium and, therefore, the differences ensue from that fact. Because of that, the essential oil of *E. citriodora* did not show any inhibitory effect on *A. alternate* in the research of Feng and Zheng (2007) while in this research it had growth reduction of 89,74% when applied in 0,6μl/ml of air. In other studies conducted to test antifungal effects of *E. citriodora* oil, evaluating the effect of volatile phase, Lee et al. (2007) proved that this phase has a strong inhibitory effect on *Botritis cinerea* and *Rhizoctonia solani*. In the same study, the essential oil of *R. officinalis* did not have any inhibitory effect on investigated pathogens while in our study it had 100% inhibition rate applied at 0,6μl/ml of air. Also, the essential oil of *M. piperita* in the study of Lee et al. (2007) did not show any fungistatic effect whatsoever, but in our study it had a very strong fungistatic effect. In the research of Tanović et al. (2005), which investigated antifungal effect of volatile phase of these oils on *Botritis cinerea*, it had a strong inhibition effect. These differences are showing that comparing results of different studies is difficult because of the differences in plant extract composition and in methodologies of assessments of microbial activity (Arslan and Dervis, 2010).

Although some studies have reported on the antifungal activity of essential oils, the mechanism(s) of action of such oils is (are) poorly understood. However, some researchers reported that there is a relationship between the chemical structure of the most abundant compounds in the essential oils and the antimicrobial activity. According to Faid et al. (1996), the antimicrobial activity of major oil compounds happens in the following order: phenols (highest activity) > alcohols > aldehydes > ketones > ethers > hydrocarbons. The chemical composition of these essential oils was the subject of several studies (Soylu et al., 2006; Lee et al., 2007). Based on the research of Pitarokili et al. (2002), the composition of oil may vary on different localities. Because of that, the analysis of composition of these oils will be the subject of further research.

*Alternaria alternata* is a postharvest pathogen that causes severe losses of vegetables in storage. Due to the fact that chemical compounds are becoming ineffective and that they are harmful for consumers, control strategy for this pathogen in the future is applying biological means of control. Using essential oils of different plant species is one of these potential control strategies. Based on the results of this study, we can confirm this statement and encourage future research of implementation of these and other potential essential oils *in vitro* as well as *in vivo*.

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# *IN VITRO* EFIKASNOST POJEDINIH ETERIČNIH ULJA U SUZBIJANJU *ALTERNARIA ALTERNATE*

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## REZIME

Ispitivana je efikasnost isparljive faze etarskih ulja *mente*, *eukaliptusa* i *ruzmarina* u suzbijanju *A. alternata*, patogena uskladištenih plodova povrtarskih i voćarskih kultura, izraženo kroz inhibiciju porasta micelije, *in vitro*. Inhibitorni efekat posmatranih ulja izračunavan je četiri dana nakon postavljanja ogleda i izražen je procentom inhibicije radijalnog porasta micelije patogena (PIRG) dok su minimalna inhibitorna koncentracija (MIC) i minimalna fungicidna koncentracija (MFC) izračunavane nakon sedam odnosno četrnaest dana. Etarsko ulje *mente* imalo je najveću vrednost PIRG (100%) pri koncentraciji ulja od 0,15µl/ml vazduha. Stoprocentni inhibitorni efekat (PIRG) ulje *ruzmarina* ispoljilo je pri koncentraciji 0,6µl/ml vazduha, dok je ulje *eukalipusa* imalo najvišu vrednost PIRG (89,74%) pri koncentraciji 0,6µl/ml vazduha. Najnižu vrednost MIC imalo je ulje *mente* (0,3µl/ml vazduha) dok ulja *eukaliptusa* i *ruzmarina* nisu pokazala totalni inhibitorni efekat u ispitivanim koncentracijama (MIC > 0,6µl/ml vazduha). Etarsko ulje *mente* nije pokazalo fungicidni efekat prema posmatranom patogenu ni u najvišoj koncentraciji (MFC > 0,6µl/ml vazduha). Kod druga dva ulja MFC nije ni određivana jer nisu imala ni inhibitorni efekat prema miceliji patogena. Na osnovu ovih rezultata možemo zaključiti da je primena etarskog ulja *mente* u cilju kontrole *A. alternata*, patogena uskladištenih plodova, opravdana i da se na taj način može kontrolisati ovaj patogen, *in vitro*. Ovi rezultati će poslužiti kao polazna tačka za dalja ispitivanja u cilju primene etarskih ulja i *in vivo*.

*Key words:* menta, eukaliptus, ruzmarin, inhibicija, biološka kontrola.

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