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***In vitro* EFFECTS OF ESSENTIAL OILS ON *Colletotrichum* spp.**

**SUMMARY**

Apple production is largely affected by the fungi *Colletotrichum gloeosporioides* and *C. acutatum*, causal agents of fruit bitter rot. Economic losses account for 30-80%. *Colletotrichum* spp. are the most destructive if the infection occurs after harvest and storage. The disease is managed by fungicide application during the season. Due to increased concern for human health and the environment, as well as to problems with pathogen resistance development, eco-friendly alternatives to chemical control measures, such as essential oils, became the object of many researches. The aim of the study was to investigate which essential oils have a potential to be used as control agents against *Colletotrichum* spp. Effects of volatile phase of 56 essential oils on *C. gloeosporioides* and *C. acutatum* were investigated. For both fungal species, oregano oils A, B, C and D were fungicidal at rates ranging from 0.02 to 0.04  $\mu\text{l/ml}$  of air, and oregano oil E at rate of 0.08  $\mu\text{l/ml}$  of air. Minimum inhibitory concentration for thyme oil ranged from 0.02 to 0.04  $\mu\text{l/ml}$  of air. Out of 56 tested essential oils, 47 oils did not stop mycelial growth even at concentration of 0.16  $\mu\text{l/ml}$  of air. The results indicate that oregano and thyme essential oils have a potential in management of investigated apple pathogens.

**Key words:** post-harvest diseases, apple, *Colletotrichum* spp., essential oils

**INTRODUCTION**

Fungi from the genus *Colletotrichum* are important plant pathogens worldwide. As anthracnose causal agents, *Colletotrichum* spp. cause significant economic losses in temperate, subtropical and tropical regions on a wide range of grains, legumes, vegetables and fruits (Freeman et al., 1998). These phytopathogenic fungi are also important causal agents of the diseases in field as well as in storages in Serbia (Trkulja, 2003; Grahovac et al., 2010; Živković, 2011). Although *Colletotrichum* species infect many plant species during vegetation, economically the most important losses occur after harvest. Many species cause latent infections, and the disease symptoms become visible only

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after the infected fruit ripens and the pathogen moves from latent to necrotrophic phase. The ability to cause latent infections makes fungi from *Colletotrichum* genus highly destructive postharvest pathogens, easily introduced to different parts of the world (Freeman et al., 1998).

Fruit species of temperate region are very susceptible to diseases caused by *Colletotrichum* spp., particularly species *Colletotrichum acutatum* J.H. Simmonds and *C. gloeosporioides* (Penz.) Penz. i Sacc. (Wharton and Dieguez-Uribeondo, 2004). Presence of the two mentioned pathogens in Serbia was confirmed on different fruit and vegetable species. Among fruit species, the most common hosts are sour cherry, apple, strawberry and pear (Ivanović et al., 2005; Grahovac et al., 2010; Živković, 2011).

According to Crusius et al. (2002), apple fruit losses in storages amount 40%, i.e. they range from 30 to 80%, as stated by Zhang et al. (2008).

Chemical protection is still a basic fruit protection measure applied against postharvest diseases (Barrera Necha et al., 2008). However, frequent fungicide application increases the share of resistant strains in pathogen populations (Tanović et al., 2005). Also, the use of conventional synthetic fungicides is not allowed in organic production. Therefore, producers are limited to alternatives to chemical control measures in management of disease causal agents. Among different alternatives to chemical control, natural plant products, including essential oils which are biodegradable and eco-friendly, are in spotlight of scientists worldwide.

It is known that some essential oils produced by plants have fungicidal properties, and are safer for consumers than synthetic fungicides (Chamberlain, 1887; Wilson et al., 1997; Pitarokili et al., 1999; Meepagala et al., 2002; Tanović et al., 2005; Duduk et al., 2010).

Kurita et al. (1981) conducted screening of activity of 40 different essential oils against seven different phytopathogenic fungi, while Muller Riebau et al. (1995) tested inhibitory effect of nine essential oils against four phytopathogenic fungal species. Wilson et al. (1997) investigated toxicity of 49, and Tanović et al. (2005) toxicity of 18 essential oils to *Botrytis cinerea*.

Antifungal activity of essential oils is closely related to content of monoterpenic phenols, particularly thymol, carvacrol and eugenol (Barrera-Necha et al., 2008). Effects of essential oils on *C. gloeosporioides* and especially on *C. acutatum*, were investigated on significantly lower scale compared to other phytopathogenic fungi (Palhano et al., 2004; Barrera-Necha et al., 2008; Bosquez-Molina et al., 2010; Duduk et al., 2010; Padman and Janardhana, 2011). There is almost no available data on effects of oregano essential oils on *Colletotrichum* spp.

The aim of the paper was to test activity of a large number of essential oils, and to single out the oils that exhibit the highest toxicity to *C. acutatum* and *C. gloeosporioides*.

## MATERIAL AND METHODS

### *Test organism*

Isolates of the species *C. acutatum* and *C. gloeosporioides* obtained from diseased apple fruits collected from storages in Serbia in 2010 and maintained on potato dextros agar medium (PDA) at 23 °C, were used in the investigation. The isolates were identified based on morphological and ecological characteristics, and the identification was confirmed by polymerase chain reaction (PCR).

### *Essential oils*

Effects of volatile phase of commercially available essential oils (Table 1) on two isolates of *C. acutatum* (CA1 and CA2) and one isolate of *C. gloeosporioides* (CG) species were investigated.

### *In vitro testing of antifungal activity*

Antifungal activity of essential oils was tested on PDA medium in 90 mm Petri-dishes. PDA medium was inoculated by mycelial fragments obtained from the colony margin of seven days old cultures. The oils were applied in the form of drop put on the inner side of Petri-dish cover at the following concentrations: 0.16; 0.08; 0.04 and 0.02 µl/ml of air inside Petri-dish. Immediately after oil application, Petri-dishes were placed in reverse position and sealed with parafilm. The isolates were exposed to volatile phase of tested oils for seven days, at temperature of 23 °C. The trial was set in four replicates. Isolates incubated under the same conditions, but without treatment were used as a control. Seven days after the treatment, it was registered whether essential oils applied at certain concentration caused complete inhibition of isolates growth. The lowest concentration that caused complete growth inhibition was defined as minimum inhibitory concentration (MIC). After assessment, Petri-dishes in which no growth of isolates was observed were opened and ventilated by air flow in laminar cabinet for 30 minutes, to remove volatile phase of oil and to determine lethal effect. The concentration of oil for which no initial growth of isolates was recorded seven days after ventilation was regarded as lethal. The lowest concentration with lethal effects was defined as minimum lethal concentration (MLC).

## RESULTS AND DISCUSSION

### *Inhibitory effects of essential oils*

Out of 56 tested essential oils, only six oils (five oregano oils (*O. vulgare* A, B, C, D and E) and one thyme oil (*T. vulgaris*) caused complete growth inhibition of all three tested isolates. Four oregano oils (A, B, C and D) caused complete inhibition of mycelial growth of all three isolates at concentration range 0.02-0.04 µl/ml of air, while for oregano E minimum inhibitory concentration amounted 0.08 µl/ml of air (Table 1). Minimum inhibitory concentration of thyme oil (*T. vulgaris*) for both *C. acutatum* isolates (CA1 and CA2) was 0.02 µl/ml of air, and for *C. gloeosporioides* isolate it was 0.04 µl/ml of air.



<i>Pinus nigra A</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Pinus nigra B</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Ocimum basilicum</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Mentha piperita</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Geraniol</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Pimpinella anisum A</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Pimpinella anisum B</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Cinnamomum verum A</i>	0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Cinnamomum verum B</i>	0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Cinnamomum camphora</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Syzygium aromaticum</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Thymus vulgaris</i>	0,02	0,16	0,02	0,08	0,04	>0,16
<i>Juniperus communis A</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Juniperus communis B</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Citrus sinensis</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Eucalyptus globulus</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Rosmarinus officinalis</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Melaleuca alternifolia A</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Melaleuca alternifolia B</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Aethum graveolens A</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Aethum graveolens B</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Aethum graveolens C</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Aethum graveolens D</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Ruta montana</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Laurus nobilis</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Lignum santali</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Leptospermum scoparium</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Cymbopogon citratus</i>	0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Carum carvi</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Coriandrum sativum</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16
<i>Helichrysum italicum</i>	>0,16	>0,16	>0,16	>0,16	>0,16	>0,16

<sup>1</sup>Minimum concentration of essential oil that causes complete mycelial growth inhibition after seven days of exposure (Minimum Inhibitory Concentration) – (µl/ml of air)

<sup>2</sup>Minimum concentration of essential oil that causes lethal effects on the pathogen (Minimum Lethal Concentration) – (µl/ml of air)

\*Essential oils of different producers originating from the same plant species are marked with different letters following the plant species name

Results of the investigation show that oregano and thyme essential oils show significantly higher toxicity to *C. acutatum* and *C. gloeosporioides* compared to other tested oils, as well as that oregano is more toxic than thyme to both fungal species. Pronounced antimicrobial activity with different spectrum is already known for essential oils of many plants (Wilson et al., 1997; Suhr and Nielsen, 2003; Tanović et al. 2004). Similarly, significant difference in sensitivity of different fungal species to the same essential oil has also been

proven (Muller Riebau et al., 1995; Soković et al., 2009). Regarding the results obtained by Tanović et al. (2004) that suggest that oils exhibiting lethal effects at concentration of 0.16 µl/ml of air could be selected for further investigations of possibilities of their use in practice, the mentioned concentration was the highest tested concentration in this paper. This enabled selection of six perspective oils, originating from two plant species – *O. vulgare* and *T. vulgaris*, out of 56 tested oils. It should be noted that among six oregano oils derived from different producers, one oil (oregano C) proved to be the most toxic. However, the remaining four oils also had satisfying efficacy. It is assumed that small differences in toxicity are the consequence of differences in chemical content of the oils which was also proven in previous researches (Soković et al., 2009; Vale-Silva et al., 2012).

Antifungal activity of thyme essential oil was recorded by many authors (Hammer et al., 1999; Soto-Mendivil et al., 2006; Yang and Clausen, 2007; Bosquez-Molina et al., 2010). Bosquez-Molina et al. (2010) proved that thyme oil inhibits mycelial growth of *C. gloeosporioides* when incorporated in PDA medium at concentration of 0.06%. Also, soaking of papaya fruits in thyme oil at concentration of 0.12% reduced fruit rotting for 50% compared to control. Oregano and thyme essential oils were applied as fumigants against *Aspergillus* spp. on stored wheat whereas only oregano oil had fungicidal effect (Paster et al., 1995). Wilson et al. (1997) found that oils of red thyme, cinnamon leaf and clove bud have strong antifungal activity against *B. cinerea*, while Daferera et al. (2003) registered satisfying effect of oregano oil against the same pathogen. Oregano oil reduced production of fumonisin B<sub>1</sub> by *F. proliferatum* in corn grains (Velluti et al., 2003). In screening tests with 11 essential oils oregano oil was the most efficient inhibitor of mycelial growth of *Bacillus cereus*, a food borne human pathogen (Valero and Salmeron, 2003).

## CONCLUSIONS

Fungicidal effect of oregano and thyme essential oils registered in the investigation points to the possibility of use of these substances produced by plants in control of causal agents of apple bitter rot.

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### ***In vitro* EFEKTI ETARSKIH ULJA NA *Colletotrichum* spp.**

#### **SAŽETAK**

Proizvodnju jabuke značajno ugrožavaju gljive *Colletotrichum gloeosporioides* i *C. acutatum*, prouzrokovajući gorke truleži plodova. Gubici prouzrokovani ovim patogenima kreću se od 30 do 80%. Vrste roda *Colletotrichum* su najdestruktivnije ukoliko se zaraza javi posle berbe i po iznošenju plodova iz skladišta. Prouzrokovatelj oboljenja se suzbija tretiranjem fungicidima u toku vegetacije. Zbog sve veće brige za ljudsko zdravlje i životnu sredinu, kao i zbog problema rezistentnosti patogena na fungicide, ekološki bezbedne alternative hemijskim merama zaštite, kao što su etarska ulja, privlače pažnju istraživača. Cilj istraživanja bio je da se ispita koja etarska ulja imaju potencijal da se koriste u suzbijanju *Colletotrichum* spp. Ispitano je delovanje isparljive faze 56 etarskih ulja na vrste *C. gloeosporioides* i *C. acutatum*. Za obe ispitivane vrste gljiva etarska ulja origana A, B, C i D ispoljila su fungicidno delovanje u opsegu koncentracija 0,02 - 0,04  $\mu\text{l/ml}$  vazduha, a ulje origana E pri koncentraciji od 0,08  $\mu\text{l/ml}$  vazduha. Minimalna inhibitorna koncentracija ulja timijana kretala se u opsegu od 0,02 do 0,04  $\mu\text{l/ml}$  vazduha. Od 56 ispitivanih ulja, 47 nije zaustavilo porast ispitivanih izolata ni pri koncentraciji od 0,16  $\mu\text{l/ml}$  vazduha. Dobijeni rezultati ukazuju da etarska ulja origana i timijana imaju potencijal za primenu u suzbijanju ispitivanih patogena jabuke.

**ključne reči:** skladišna oboljenja, jabuka, *Colletotrichum* spp., etarska ulja