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Original scientific paper

Effects of developed thyme and oregano essential oil formulations on *Monilinia laxa* and *Monilinia fructicola*

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SUMMARY

Essential oils have been well-known for their antimicrobial properties for a very long time. Some of them have been effectively used in human medicine for decades. Our earlier investigation revealed a great potential of thyme and oregano essential oils as crop protectants against some postharvest fruit pathogens. The effects of formulated thyme and oregano essential oils on *Monilinia laxa* and *Monilinia fructicola* were studied *in vitro* and *in vivo*. *In vitro* antagonistic assays were performed on solidified PDA medium using a slightly modified agar overlay technique, while in vivo experiments were conducted on inoculated apple fruits. In vitro essays showed that the developed formulations (emulsifiable concentrates - EC) significantly inhibited the mycelial growth of *Monilinia* spp. Experiments *in vivo*, performed on inoculated apple fruits, revealed that the developed formulations provided a significant level of *Monilinia* spp. suppression. To our knowledge, another EC formulation of oregano essential oil intended for use in *Monilinia* spp. control has never been developed before. The presented results are initial findings and evaluation of the activity of the developed products should therefore proceed under field conditions to determine their efficacy and activity spectrum, and to estimate economic aspects of their use.

Keywords: essential oils, biopesticides, postharvest fruit pathogens, *Monilinia*

INTRODUCTION

Human needs for fresh high quality food products is constantly growing. During the second half of the 20th century, the use of synthetic fungicides along with improvements in storage technologies, have significantly amended the quality and extended the shelf life of fruit on the market. Nevertheless, postharvest losses caused by fungal diseases still vary from the estimated 5-20% in developed to more than 50% in developing countries, despite all applied control measures (Janisiewicz & Korsten, 2002). On the other side, increasing concerns about human health and environment have resulted in regulatory restrictions on synthetic fungicide use, as well as in growing public demands for safer alternatives (Lodovica Gullino &

Kuijpers, 1994; Daferera et al., 2003; Romanazzi et al., 2012). The application of substances of natural origin, such as essential oils, could provide a desired solution because they are safe both in terms of human health and the environment (Janisiewicz & Korsten, 2002; Zhang & Zheng, 2005). Antimicrobial effects of essential oils and their components have been investigated for a long time. Some of them, such as the essential oil of tea tree (*Melaleuca alternifolia* [Maiden & Betche] Cheel), have been used in human medicine for years (Hammer et al., 2002). Inhibitory effects of the volatiles of tea tree oil on postharvest fruit pathogens *in vitro* were reported by Tanović et al. (2005) and Hrustić et al. (2012). However, the succeeding two-year trials in raspberry fields, using a commercially available formulated product Timorex 66EC (Stockton Chemical Corporation, Israel), showed that only a partial grey mould control could be achieved, depending on disease pressure (Tanović et al., 2012). Our previous screening of 56 essential oils of different origin, revealed that the volatile phase of thyme (*Thymus vulgaris* L.) and oregano (*Origanum vulgare* L.) oils exerted the highest toxicity to some of the postharvest apple fruit pathogens *in vitro* (Tanović et al., 2010; Hrustić et al., 2012; Grahovac et al., 2012). Further research showed that the thyme oil itself, i.e. its emulsion containing the oil dissolved in ethanol, as well as its formulated product (emulsifiable concentrate - EC), were effective against *Monilinia fructigena*, an important postharvest pathogen of pome and stone fruits. Moreover, experiments *in vivo*, performed on inoculated apple fruit, revealed that the applied formulation process successfully decreased the evaporation of oil from treated area and provided a significantly higher level of *M. fructigena* control than the pure oil (Tanović et al., 2013). Taking into account that *Monilinia laxa* is a considerably more important pathogen of stone fruits than *M. fructigena* (Hrustić et al., 2015), and that *Monilina fructicola*, a significantly more agressive pathogen than the other *Monilinia* species, was recently introduced in Serbia (Hrustić et al., 2013), the objectives of the present study were:

- to determine the effects of previously developed thyme essential oil EC formulation on *M. laxa* and *M. fructicola in vitro* and *in vivo*,
- to develop an EC formulation of oregano essential oil and to test its effects on *M. laxa* and *M. fructicola in vitro* and *in vivo.*

MATERIALS AND METHODS

Test organism

Isolates of *M. laxa* and *M. fructicola* were derived from infected cherry and peach fruits, respectively, showing symptoms of brown rot. Small fragments were aseptically excised from the border between healthy and diseased parenchymal fruit tissue and placed onto potato-dextrose agar (PDA) in Petri plates. The obtained single-spore isolates were grown on PDA at 24°C and stored on PDA slants at 4°C for short-term storage and in 20% glycerol at -80°C for long-term storage. Patogenicity of the isolates was confirmed by inoculation of healthy apple fruits previously surface disinfected with 0.5% NaOCl. The derived isolates were identified based on morphological characteristics of the colony and conidia using the synoptic key described by Lane (2002). Identification was confirmed by multiplex PCR proposed by Côté et al. (2004), using the common reverse primer MO368-5 (5′-GCAAGGTGTCAAAACTTCCA-3), which is specific for *Monilinia* spp., and three species-specific forward primers: MO368-8R (5′ - AGATCAAACATCGTCCATCT - 3′, for *M. fructigena* and *M. polystroma*), MO368-10R (5′-AAGATTGTCACCATGGTTGA-3′, for *M. fructicola*) and Laxa - R2 (5′-TGCACATCATATCCCTCGAC- 3′, for *M. laxa*). Template DNA for multiplex PCR was extracted from 7-days-old mycelia of the isolates grown on PDA medium, according to a method described by Harrington & Wingfield (1995).

Inoculum preparation

Mycelial fragments (R=10 or 3 mm), cut from the edge of 10-day-old PDA culture of *M. laxa* and *M. fructicola* isolates were used in the experiments *in vitro* and *in vivo*, respectively.

Test substances

Commercially available thyme (*Thymus vulgaris* L.) and oregano (*Origanum vulgare* L.) essential oils were provided by Beolab Co., Belgrade, Serbia. As reference products, iprodione (Kidan 250 SC, Bayer CropScience, Germany) and tea tree essential oil (Timorex gold, Stockton Group, Israel) were used at label rates in all experiments.

An esterified rape seed oil was purchased for formulation development from a commercial source (Oleon, Belgium) and used without further purification. Surfactants of commercial quality (Rhodia, Italy and Ajinomoto OmniChem, Belgium) were used.

Emulsifiable concentrates (EC) of thyme and oregano essential oils were prepared the same way as described in a previous study (Tanović et al., 2013). Briefly, mixtures containing thyme or oregano essential oils (10%) with nonionic emulsifiers (10%) and esterified rape seed oil (80%) were homogenized for 30 min using a magnetic stirrer (IKA, RH basic 2). A blank formulation was prepared the same way as the EC formulation and it contained esterified rape seed oil instead of thyme or oregano essential oils.

Formulation activity

In vitro antagonistic assays were performed on 10 ml of solidified PDA medium in Petri plates (90 mm) using a slightly modified agar overlay technique (Cooper, 1963). The test substances (100 µl) were added into wells (10 mm in diameter) cut 1 cm from the edge of each Petri plate, while mycelial fragments (R=10 mm) of all test isolates were placed 1 cm from the opposite side of each plate. Treatments included: thyme oil formulation, oregano oil formulation, two reference treatments (commercial biofungicide based on tea tree essential oil and conventional fungicide iprodione) and sterile distilled water as a negative control. Assessments were made seven days after treatment by measuring mycelial growth towards each well. The experiment was done in three replications and was repeated twice. Since all experimental conditions in the repeated experiment were the same, all obtained data were pulled together and subjected to the analysis of variance (ANOVA) and Duncan's multiple range tests. The effect of blank formulation on mycelial growth was tested before the experiment.

In vivo assays were performed on mature apple fruits (cv. 'Idared') of similar size. Each fruit was surface disinfected and wounded with a sterile carpenter nail (4 mm diameter and 3 mm depth). A 10 µl drop of each treatment (thyme oil formulation, oregano oil formulation, commercial tea-tree-oil-based product, iprodione, and sterile distilled water) was added into each wound 15 minutes prior to inoculation, which was performed by placing a mycelial plug (R=3 mm) on each wound. Fruits inoculated with sterile PDA plugs were used as a negative control. The fruits were left to rest on two layers of moist paper towels in plastic containers at 24°C, 97% RH (relative humidity). The width and length of lesions on the inoculated fruits were measured seven days after inoculation. Each treatment was done in three replicates and the experiment was repeated twice. The results from both experiments were pulled together and subjected to the analysis of variance (ANOVA) and Duncan's multiple range tests. Prior to the experiment, phytotoxicity of a blank formulation was checked on wounded apple fruits using the same method as for treatments.

RESULTS

Test organism

After the incubation of stone fruit tissue fragments on PDA medium at 24°C for 4 days, colonies resembling those of *Monilinia* spp. developed. Conidia, if present, were unicellular, hyaline, ellipsoid or ovoid, arranged in chains. Based on morphological characteristics of the colonies and the presence, arrangement and shape of conidia, the isolates were preliminarily identified as *Monilinia* spp. The isolate derived from peach that developed hazel-colored zonate colony with more or less even margin and abundant sporulation with concentric rings of spores on the surface was identified as *M. fructicola*. The isolate derived from cherry fruit, forming light to dark gray colony with lobed margin and no sporulation, was identified as *M. laxa*. Identification of the isolates to the species level was confirmed by the resulting 535 and 351 bp PCR amplicons, which were reported by Côté et al. (2004) as the amplicon sizes specific to *M. fructicola* and *M. laxa*, respectively.

Formulation activity *in vitro*

Significant differences were found in the mycelial *in vitro* growth of both species ($p<0.01$) as a consequence of the activity of different treatments (Figure 1). Duncan's multiple range test showed that the thyme and oregano oil formulations exhibited significantly higher mycelial growth inhibition than tea tree oil, which was used as a reference substance. Regarding *M. fructicola,* both treatments were as effective against it as the fungicide iprodion, applied at label rate. In the case of *M. laxa*, the oregano oil formulation was significantly more effective than the thyme oil formulation and iprodione, which had the same inhibitory effect. The thyme oil formulation inhibited *M. fructicola* and *M. laxa* growth 73.9% and 68.4%, respectively, compared to the control. Blank preparation did not cause any inhibitory effect on mycelial growth, and its effect was therefore excluded from further analysis.

Formulation activity *in vivo*

Brown rot development in apple fruit inoculated with *M. fructicola* and *M. laxa* was significantly affected by different treatments ($p<0.01$) (Figure 2). The formulations of thyme and oregano oils exhibited significantly lower disease suppression than the fungicide iprodion, regardless of the pathogen species used for inoculation. However, the oregano essential oil formulation was significantly more effective than thyme oil against *M. laxa*, while the difference in their effects on *M. fructicola* was not significant. It was also observed that components of the formulated products, as well as the products themselves, did not cause any phytotoxic effects on apple fruit.

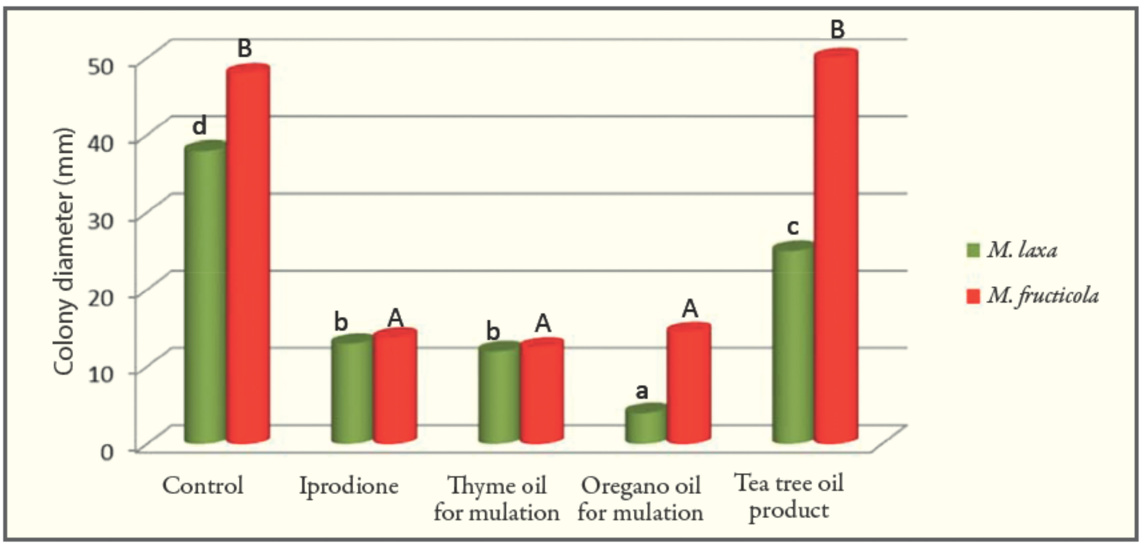

Figure 1. The effects of formulated thyme and oregano essential oils on mycelial growth of *Monilinia fructicola* and *Monilinia laxa in vitro*. Bars marked with the same letters are not significantly different (Duncan's test, $p > 0.01$).

DISCUSSION

The present study confirmed high potentials of the tested essential oils to be used as safe and environmentally-friendly alternatives to conventional synthetic fungicides against plant pathogens and food contaminants. Various essential oils and their active components have been evaluated so far against plant pathogenic microorganisms and found to be effective *in vitro* and *in vivo* (Soylu et al., 2007; Combrinck et al., 2011; Tian et al., 2011; Lu et al., 2013). Due to their strong inhibitory activity, essential oils are being extensively investigated for use in pharmaceutical, food, agricultural and cosmetic products (Burt, 2004; Bakkali et al., 2008). The Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) classified some essential oils, including thyme and oregano, as "generally regarded as safe" (GRAS), a category that includes food preservatives in the USA (Burt, 2004; Lu et al., 2013; Singh et al., 2019). Natural origin and low mammalian toxicity of essential oils (Isman, 2000), coupled with their low persistence in the environment and biodegradability to nontoxic products (Soylu et al., 2010), make the most attractive aspects in terms of their possible use as crop protectants. However, an important problem for practical application of essential oils in agriculture is their lack of persistent

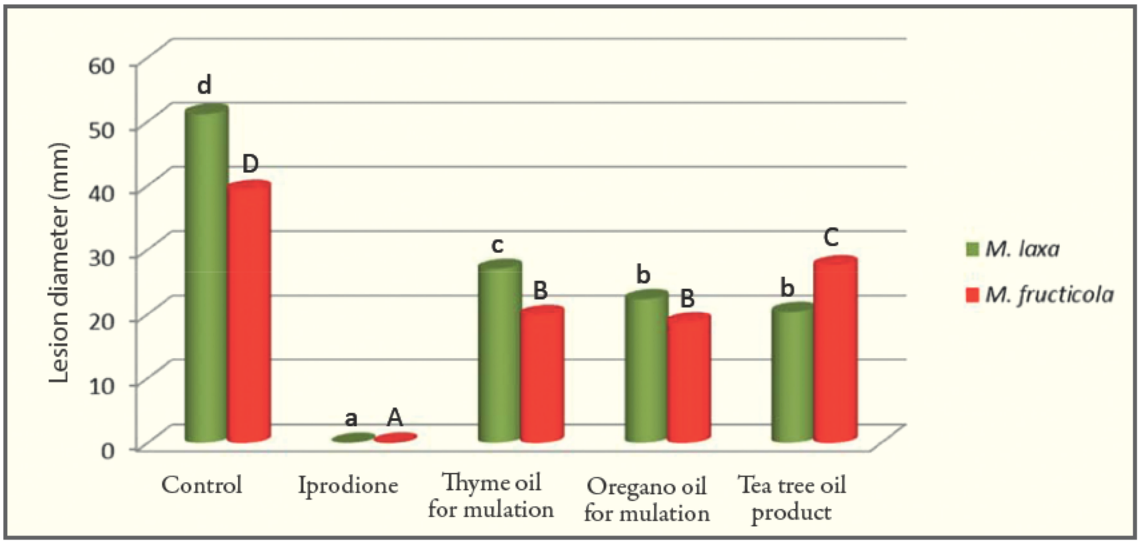

Figure 2. The effects of formulated thyme and oregano essential oils on brown rot development on apple fruits inoculated with *Monilinia fructicola* and *Monilinia laxa*. Bars marked with the same letters are not significantly different (Duncan's test, $p > 0.01$).

efficacy in the field (Chen et al., 2013). However, this drawback could be defeated by improved formulation. Newly-developed essential oil formulations aim to ensure stability during biopesticide production, processing and storage, to improve convenience for users, to protect the biopesticide from environmental conditions and to increase biopesticide activity against target organisms (Isman, 2000; Chen et al , 2013).

The inhibitory effects of most essential oils are for the most part nonselective. Generally, they are more effective against bacteria than fungi. However, oils of the Lamiaceae family have attracted considerable attention due to their strong antifungal activity (Adepu & Khandelwal, 2020). For example, thyme and oregano oils have exhibited very strong effects against *Monilinia* spp. *in vitro* (Hrustić et al., 2012), suggesting their possible practical use. To determine their full potential as crop protectants in the present study, we created EC formulations and tested their effects on *M. fructicola* and *M. laxa in vitro* and *in vivo*. In the *in vitro* experiment, the developed formulations allowed oil diffusion towards the test isolates in PDA Petri plates. Under such conditions, we observed a significant inhibition of mycelial growth of *Monilinia* spp. isolates (68.5-74%, depending on oil and fungal species). In addition, the oils provided significant suppression of brown rot development in the *in vivo* experiment on inoculated apple fruits. It is important to emphasize that phytotoxicity to apple fruits was not observed after the formulated essential oils were applied. This aspect should not be overlooked while evaluating natural products because some essential oils have been shown to cause considerable phytotoxic effects at concentrations used for the control of plant pathogenic fungi (Isman & Machial, 2006).

Since essential oils are complex mixtures of liquid volatile aromatic compounds, they easily evaporate from the system (Dayan et al., 2009; Singhet al., 2019). This problem can be solved in different ways during the formulation process (Moretti et al., 2002). To suppress essential oil volatilization, a carrier oil phase could be used. While vegetable oils are considered to be a good choice for carrier oil phase, because they are fully biodegradable and therefore environmentally friendly, their esterified derivates are even more appropriate since they are less viscous and consequently more convenient for formulation development than vegetable oils themselves. Low water solubility of essential oils could be overcome by adding surfactants. Besides improving poor water solubility of essential oils, surfactants also enable the spreading and penetration of formulated products (Wang & Liu, 2007). In the present study, the formulation process significantly improved the level of *M. fructicola* and *M. laxa* control by essential oils, confirming the results of our previous investigation (Tanović et al., 2013). However, the level of pathogen suppression *in vivo* was lower than it was in the experiment *in vitro*. Further research is needed to determine if that lower effectiveness *in vivo* was a consequence of experimental conditions or of partial oil volatilization from the treated apple fruits that was not sufficiently suppressed by this type of formulation. Some authors have suggested micro- and nanocapsules, as well as nanoemulsions as more promising types of essential oil formulations (Osman Mohamed Ali et al., 2017; Munhuweyi et al., 2018; Singh et al., 2019). Microencapsulation of essential oils with encapsulating agents such as β-cyclodextrin protects oils from oxidation, heat degradation and evaporation (Munhuweyi et al., 2018). On the other hand, reduced droplet size of nano-emulsions might enhance the transport of active molecules through biological membranes and so improve product efficacy (Solans et al., 2005). However, before drawing any conclusions, these speculations should be experimentally tested in comparative studies under uniform experimental conditions.

To the best of our knowledge, an EC formulation of oregano essential oil intended for *Monilinia* spp. control had never been developed before. Therefore, our *in vitro* and *in vivo* experiments present some initial findings, and product testing should be continued to determine its effects under field conditions, as well as the spectrum of activity and economic aspects of its use.

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Efekti razvijenih formulacija na bazi etarskih ulja timijana i origana na *Monilinia laxa* i *Monilinia fructicola*

REZIME

Antimikrobna aktivnost etarskih ulja dobro je poznata i opisana u literaturi, kako u humanoj medicini, tako i u poljoprivredi. Naša prethodna istraživanja pokazala su da etarska ulja timijana i origana ispoljavaju veliki potencijal u suzbijanju nekih biljnih patogena koji mogu prouzrokovati gubitke tokom perioda skladištenja. U radu su proučeni efekti razvijenih formulacija na bazi etarskih ulja timijana i origana na *Monilinia laxa* i *Monilinia fructicola*. Bioaktivnost razvijene formulacije testirana je u *in vitro* i *in vivo* uslovima. Ogledi *in vitro* izvedeni su po delimično modifikovanoj metodi testiranja antimikrobne aktivnosti na površini PDA podloge, dok su *in vivo* eksperimenti realizovani na inokulisanim plodovima jabuke. *In vitro* testiranja pokazala su da razvijene formulacije (koncentrat za emulziju – EC) značajno inhibiraju porast micelije *Monilinia* spp. U ogledima *in vivo*, na inokulisanim plodovima jabuke, pokazano je da testirane formulacije značajno inhibiraju razvoj mrke truleži ploda. Koliko nam je poznato, u ovom radu prvi put je razvijena EC formulacija etarskog ulja origana za upotrebu u suzbijanju vrsta roda *Monilinia* spp. Dobijeni rezultati predstavljaju početak istraživanja koje treba dopuniti rezultatima ispitivanja efekata razvijene formulacije u uslovima praktične primene, proučavanja spektra njenog delovanja, kao i ekonomske isplativosti njene primene.

Ključne reči: etarska ulja; biopesticidi; patogeni uskladištenog voća; *Monilinia*