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Original scientific paper

Antifungal activity of plant essential oils and selected *Pseudomonas* strains against *Phomopsis theicola*

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SUMMARY

Development of natural plant protection products as an alternative to synthetic fungicides is of significant importance regarding the environment. This study was carried out with an objective to investigate *in vitro* antifungal activities of several essential oils extracted from oregano, basil, myrtle and Turkish pickling herb, and the plant growth-promoting rhizobacteria in the genus *Pseudomonas,* against the phytopathogenic fungus *Phomopsis theicola*. Microdilution methods were used to determine the minimum inhibitory concentrations (MIC) of selected antimicrobial essential oils (EOs). All EOs exhibited significant levels of antifungal activity against the tested fungal isolates. The oregano EO was found the most potent one (MIC – 5.5 µg/mL), followed by basil (MIC – 75.0µg/mL), myrtle (MIC – 775 µg/mL) and Turkish pickling herb (MIC - 7750 µg/mL). Inhibition of *Ph. theicola* mycelial growth was observed for all tested *Pseudomonas* spp. strains. K113 and L1 strains were highly effective and achieved more than 60% of fungal growth inhibition using the overnight culture and more than 57% inhibition by applying cell-free supernatants of both strains. A future field trial with K113 and L1 cultures and cell-free supernatants, containing extracellular metabolites toward *Ph. theicola*, will estimate their effectiveness and applicability as an alternative to chemical protection of apple trees.

Keywords: Essential oils; *Pseudomonas* spp; PGPR; *Phomopsis theicola*; Antifungal activity

INTRODUCTION

The genus *Phomopsis* (Sacc.) Bubák has a wide geographical distribution in the world, with over 800 species that occur as endophytes, saprotrophs and parasites in a very diverse range of host plants, including woody and herbaceous hosts. Pathogenic *Phomopsis* spp. can cause considerable economic losses in different crops and they are often associated with shoot blights, leaf spots, fruit rots, stem cankers, and dieback (Udayanga et al., 2011). Species of *Phomopsis* and their *Diaporthe* sexual states are very dangerous pathogens of young apple trees. *Phomopsis* spp. produce cankers and dieback shoot blight on apple trees, and it may take years before damage to internal wood is severe enough to kill a fruit tree. Stem canker and dieback are important factors that limit the longevity of apple trees and reduce their yield in Serbia. *Phomopsis* spp. have been described as a cause of dieback and canker in apple fruit growing regions of South Africa (Van Niekerk et al., 2004; Cloete et al., 2011).

Diaporthe canker is controlled by sanitation along with chemical treatment. Sprays of thiophanate-methyl, applied three times after petal fall (10, 20 and 30 days), are recommended to control the diseases on European pear in Japan. Apple cultivars vary in their susceptibility to *Diaporthe* canker. 'Jonagold' and 'Jonatan' are more susceptible than 'Tsugaru', 'Starking Delicious' and 'Indo' (Sutton et al., 2014).

Over the years, chemical pesticides have made a great contribution to efforts to control plant diseases. However, intensive applications of pesticides have resulted in a development of fungal resistance and extensive damage to the environment. Therefore, an eco-friendly alternative is required to preserve the quality and generate quantity of agricultural products.

Essential oils (EOs) of plant origin are one of the significant products of agriculture-based industry. They have a wide application in folk remedies but in recent years their potential antimicrobial activity has been increasingly recognized. Numerous studies have documented the antifungal properties of plant products (Carmo et al., 2008; Tavassoli et al., 2011). A few of them have confirmed their antifungal properties against fungal pathogens of fruit.

Plant growth-promoting rhizobacteria (PGPR) colonize plant roots and promote growth of diverse plant species. The PGPR include diverse genera but *Bacillus* and *Pseudomonas* are predominant (Podile & Kishore, 2006). Pathogen control capacity has been attributed to several substances produced by antagonistic rhizobacteria. The bacteria perform antagonistic activity toward pathogens using several mechanisms: synthesis of hydrolytic enzymes that can lyse pathogenic fungal cells; competition for nutrients; colonization of niches at the root surface or production of siderophores and antibiotics (Kamilova et al., 2005; Neeraja et al., 2010; Maksimov et al., 2011). Antibiotics that have diverse mechanisms of action, including the inhibition of synthesis of pathogen cell walls and interference with membrane structures of cells have been produced by antagonistic bacterial strains (Maksimov et al., 2011). Several indigenous *Pseudomonas* spp. from the rhizosphere of different plants have been confirmed as PGPRs in Serbia (Jošić et al., 2012; Pivić et al., 2015; Jošić et al., 2015).

The aim of this study was to investigate the possibility of biological control of the phytopathogenic fungus *Ph. theicola* using several essential oils and indigenous *Pseudomonas* spp. strains.

MATERIALS AND METHODS

Antifungal activity of EOs

The phytopathogenic fungus *Ph. theicola* was isolated from sunken canker tissue of apple trees cv. 'Golden Delicious' (Figure 1) in the locality Trstenik, Serbia (43° 37' N, 21° 00' E, and 164 m above the sea level). Essential oils (EOs) extracted by hydro-distillation from several medicinal plants: Turkish pickling herb (*Echinophora tenuifolia*), oregano (*Origanum vulgare*), basil (*Ocimum basilicum*) and myrtle (*Myrtus communis*) (Table 1), were used in antagonistic assays with a *Ph. theicola* isolate from the collection of the Institute for Plant Protection and Environment, Belgrade, Serbia (Figure 2).

Table 1. Essential oils used in this study

No	Essential oil	Plant origin	Origin
1	Turkish pickling herb	*Echinophora tenuifolia*	(Turkey)
2	Oregano	*Origanum vulgare*	(Turkey)
3	Basil	*Ocimum basilicum*	(Turkey)
4	Myrtle	*Myrtus communis*	(Turkey)

Fungal spores were washed from the surface of potato dextrose agar (PDA) plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted to a concentration of approximately $5.0x10^4$ in a final volume of 100 µl per well.

Figure 1. Symptoms of dieback and canker on an apple tree

Figure 2. Colony morphology of *Ph. theicola* on PDA

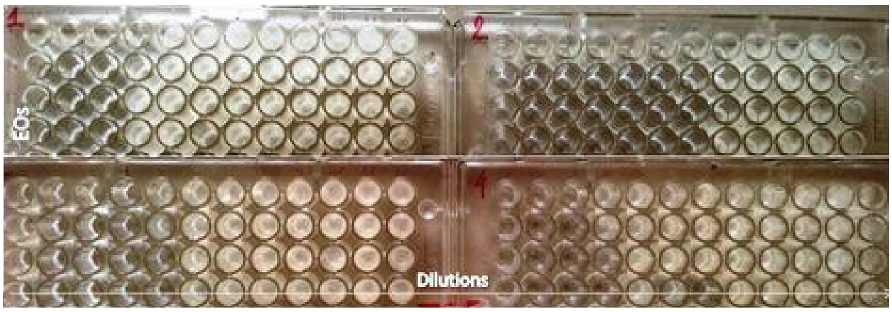

Figure 3. Microdilution method: antifungal effects of tested essential oils

Minimum inhibitory concentrations (MIC) were determined by microdilution method in 96 well microtiter plates (Daouk et al., 1995). Microtiter plates were incubated for 5 days at 28°C. The experiment was repeated four times. Fluconazole was used as a positive control. The lowest concentrations without visible growth were defined as the minimal concentrations which inhibited fungal growth (Figure 3).

Separation of minimal inhibitory concentration (MIC) means was carried out by Duncan's multiple range tests. An analysis of variance was performed on MIC data for four EOs applied to *Ph. theicola*. Significance was evaluated at $p<0.05$. STATISTICA v.7 (StatSoft, Inc.) was used for statistical analyses.

Antifungal activity of Pseudomonas spp.

The assay for antagonism *in vitro* was performed on Waksman agar medium by dual culture method (Wolf et al., 2002). Overnight cultures (ONC) of bacteria were optimized to 10^7 $CFUmL^{-1}$ and used for preparation of 3 fractions: cell-free supernatant (CFS), CFS treated with EDTA (ethylenediaminetetraacetic acid disodium salt dehydrate) (CFS-EDTA) and heat-treated cell-free supernatant (HS-CFS). ONCs were centrifuged twice at 13000 rpm for 5 min., without and with filtration (filter tubes with microporous membrane 0.22 µm) (Merck Millipore Ltd.); one aliquot was treated with 1mM EDTA, while another aliquot was heated at 70ºC for 30 min. Fungal mycelium was placed as a 6 mm plug in the center of each Petri dish, while bacteria (10 µL) were placed on its edges. Control variants contained only mycelia of *Ph. theicola* and the fungus with 1mM EDTA added instead of bacterial culture/fraction. The cultures were incubated at 25°C for 9 days. Morphological changes of *Ph. theicola* were observed in dual culture. The percentage inhibition (PI) of *Ph. theicola* growth was calculated using the following formula: $PI=100 \times (1-R2/R1)$, where R1 was the radial distance growth of the fungus in the control plate, and R2 was the radial distance growth of the fungus in bacterial treatment. All fungal inhibition assays were performed in four replicates and repeated three times.

RESULTS

Antifungal activity of EO

The results of the antimicrobial activity tests using microdilution method are summarized in Table 2. The EOs showed a wide range of antifungal acitivity against *Ph. theicola*. The oregano EO proved to be the most potent one (MIC – 5.5±0.5 µg/mL), then basil (MIC - 75±5.7 µg/mL), myrtle (MIC - 775±45.0 µg/mL) and Turkish pickling herb (MIC – 7750±4.5 µg/mL). Among all oils tested, oregano proved to be the best inhibitor of the apple pathogen *Ph. theicola,* followed by basil EO.

Table 2. Antifungal activity of essential oils expressed as minimal inhibitory concentrations (µg/mL) to *Ph. theicola*

Essential oils	MIC (µg/mL)
Echinophora tenuifolia	7750.0 ± 4.5 a
Origanum vulgare	5.5 ± 0.5 c
Ocimum basilicum	75.0 ± 5.7 bc
Myrtus communis	775.0 ± 45.0 b

Duncan's multiple range tests ($p<0.05$)

Antifungal activity of Pseudomonas spp.

All tested *Pseudomonas* spp. strains showed inhibition of *Ph. theicola* growth (Table 3). Morphological abnormalities of *Ph. theicola,* such as mycelial deviations, were observed in a dual culture using different fractions of *Pseudomonas* spp. strains. All treatments of E65 strain, as well as the CFS -EDTA and HS-CFS of M1 and K113 caused mycelial deformation and color change from dark brown to dark green. The same effects were observed in the HS-CFS of L1 and CFS-EDTA of B25 strains.

Differences in the effectiveness of *Pseudomonas* spp. strains on *Ph. theicola* growth inhibition are shown in Figure 4. The percentage of growth inhibition ranged from 13.5 (HS-CFS of E65) to 62.5% (ONC of K113). The highest inhibition was observed in all three fractions of K113 strain, while the CFS-EDTA fraction of L1 strain was the more effective inhibitor than the same fraction of K113 strain.

Table 3. *Ph. theicola* growth (mm) affected by *Pseudomonas* spp. strains

Ph. theicola	Bacterial culture fraction	*Pseudomonas* spp. strain				
		L1	M1	B25	E65	K113
Control	ONC [a)]	19.5 ± 1.9*	24.5 ± 1.3	27.5 ± 1.3	24.5 ± 1.7	18.8 ± 1.3
50 ± 0	CFS [b)]	21.2 ± 1.5	30.2 ± 0.5	28.2 ± 1.9	27.0 ± 0.8	20.5 ± 1.3
Control-EDTA	CFS-EDTA [c)]	30.2 ± 0.9	34.5 ± 1.3	35.0 ± 0,8	34.2 ± 1.5	32.8 ± 0.9
50 ± 0	HS-CFS [d)]	38.0 ± 1.1	38.5 ± 3.1	38.8 ± 1.3	43.2 ± 1.3	35.2 ± 0.9

*Values are means of three experiments, each with four replicates ± S.E
a) overnight cultures of bacteria
b) cell-free supernatant
c) CFS treated with EDTA
d) heat-treated cell-free supernatant

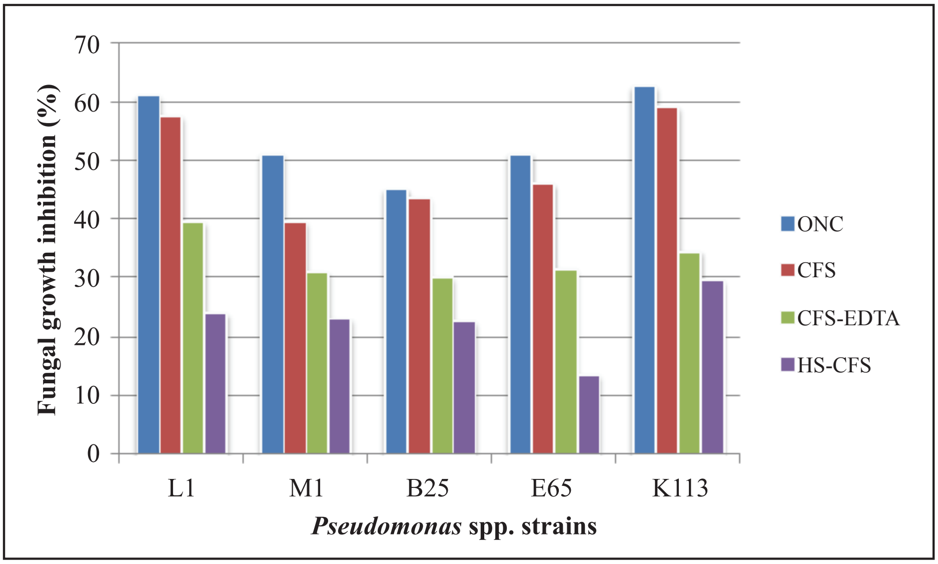

Figure 4. Inhibition of *Ph. theicola* growth achieved by *Pseudomonas* spp. strains

ONC = overnight cultures of bacteria, CFS = cell-free supernatant; CFS-EDTA = cell-free supernatant treated with EDTA; HS-CFS = heat-treated cell-free supernatant

DISCUSSION

The results obtained *in vitro* could be useful from the practical point of view. Turkish pickling herb, oregano, basil and myrtle essential oils used in this study can be used in future field trial evaluation of the efficacy of control of *Ph. theicola*.

The results of this study may serve as a guide for selecting essential oils and their concentrations in further *in vivo* trials aimed at fungicide development. The data obtained in the present work suggest that the selected essential oils originating from Turkey can be applied as inhibitors to prevent growth of phytopathogenic fungi. In accordance with numerous previous reports, oregano and basil oils originating from Serbia had been found to inhibit the growth of *Phomopsis* species at concentrations of 70 µg/mL and 5950.00 µg/mL, respectively (Stević et al., 2014). Myrtle oil demonstrated bioactive properties, especially antifungal activity to *Fusarium* sp., *Drechslera* sp. and *Macrophomina phaseolina* (Starović et al., 2016). Oregano oil was found to control *Botrytis cinerea* and *Monilinia laxa* growth on stone fruit and *Phomopsis* sp. (Lopez-Reyes et al., 2013), while basil oil controlled *Phomopsis* sp., *Fusarium* spp., and *Phoma* sp. (Stević et al., 2014) using concentrations of 750µg/mL and 5.1-7.65 mg/mL, respectively.

Morphological deformation of the phytopathogenic fungus *Ph. theicola* was a significant feature of the activity of *Pseudomonas* spp. strains tested in this study. *Bacillus subtilis* that produces diffusible and volatile compounds had been reported earlier to induce structural deformations in phytopathogenic fungi (Chaurasia et al., 2005). Ethyl acetate extracts of *P. aeruginosa* and *B. subtillis* culture filtrates caused similar effects on the germination and morphology of *Ph. azadirachtae* conidia (Girish et al., 2009).

In this study, the width of inhibition zones ranged from about 7 to 30 mm, which is similar to the results of Zalewska et al. (2004), who reported 8.6-23.5 mm inhibition zones of *Ph. viticola* caused by different *Pseudomonas* spp. A *P. putida* strain caused inhibition of *Ph. viticola* mycelial growth of 6-12 mm (Haggag et al., 2013), which is lower than the results for *Ph. theicola* inhibition by *Pseudomonas* spp. in this present study. The highest inhibition effect on *Ph. theicola* growth was shown by K113, followed by L1 strain, exceeding 60% and 57% for ONC and CFS, while the lowest inhibition values were observed for the ONC of B25 strain (45%) and CFS of M1 (39.5%). PI value decreased with EDTA treatment of CFS (30-39.5%), as well as with heat treatment of CFS (13.5-29.5%), suggesting that all tested strains produced thermo-sensitive extracellular metabolites. These results are consistent with the report by Girish et al. (2009), where ethyl acetate extract of *P. oleovorans* caused 42% of growth inhibition of *Ph. azadirachtae*. The same authors reported that *Ph. azadirachtae* inhibition by the same extract of *P. aeruginosa* reached the maximal PI value.

Biological control activity of *Pseudomonas* spp. and *Bacillus* sp. against several *Phomopsis* species have been reported. Srinivas et al. (2005) reported higher effectiveness of *P. fluorescens* than *T. harzianum* and fungicide treatments in reducing *Ph. vexans* infection and increasing brinjal seed germination, vigor index and field emergence. Combinations of two systemic fungicides and *P. aeruginosa* culture filtrate were effective in *in vitro* growth inhibition of *Ph. azadirachtae* and had no significant negative effect on neem seed germination (Girish et al., 2012). Patkowska and Błażewicz-Woźniak (2013) applied post-culture liquids of antagonistic bacteria *Pseudomonas* sp. Ps 255 and *Bacillus* sp. B 73 to the surface of soybean seeds and limited plant infection with *Ph. sojae* and other fungi previously isolated from seeds. *P. putida*, the producer of fluorescent siderophore pseudobactin, was very effective as a biocontrol agent in reducing the dieback and phomopsis diseases of grapevine (Haggag et al., 2013). To the best of our knowledge, this is the first report on growth inhibition of *Ph. theicola* and biological control of this pathogen by indigenous *Pseudomonas* spp. in Serbia.

Pseudomonas spp. strains K113 and L1 were moderately effective in inhibiting *Ph. theicola* mycelial growth, showing more than 60% and 57% inhibition when ONC and CFS were used, respectively. Further assessments of *in vivo* effectiveness of K113 and L1 cultures in protecting apple from *Ph. theicola* will be useful for estimation of their applicability as an alternative to chemical protection.

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Antifungalana aktivnost biljnih etarskih ulja i odabranih sojeva *Pseudomonas* spp. na *Phomopsis theicola*

REZIME

U novije vreme intezivno se radi na razvoju bioloških sredstava za zaštitu bilja, koja bi se uvodila kao zamena za sintetičke fungicide. U ovom radu ispitivan je *in vitro* antifungalni uticaj nekih etarskih ulja (EU) i odabranih rizobakterija koje stimulišu rast biljaka iz roda *Pseudomonas* na fitopatogenu gljivu *Phomopsis theicola*. Minimalne inhibitorne koncentracije (MIC) etarskih ulja su određene mikrodilucionom metodom. Sva primenjena EU su ispoljila značajni antifungalni efekat na ispitivani izolat gljive. EU origana je ispoljilo najnižu MIC od 5.5±0.51 µg/mL, zatim ulje bosiljka od 75.0±5.7 µg/mL, mirte 775±45.0 µg/mL i turske kisele biljke od 7750±4.5 µg/mL. Proučavan je stepen inhibicije porasta micelije *Ph. theicola* primenom različitih sojeva *Pseudomonas* spp. sojevi K113 i L1 su ispoljili visoku efikasnost inhibicije od preko 60% primenom dvadesetčetvoročasovnih kultura i preko 57% primenom filtrata supernatanta. U narednim ogledima u polju primenom kulture i supernatanta K113 i L1, koji sadrže ekstracelularne metabolite, proceniće se njihova efikasnost i mogućnost korišćenja kao alternative hemijskim sredstvima u zaštiti jabuke od *Ph. theicola*.

Ključne reči: Etarska ulja; *Pseudomonas* spp; PGPR; *Phomopsis theicola*; Antifungalna aktivnost