Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. № 140, 9—19, 2021

UDC 633.63:632.26
UDC 633.63: 665.52/.54
https://doi.org/10.2298/ZMSPN2140009S
ORIGINAL ARTICLE

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ANTIFUNGAL ACTIVITY OF PLANT ESSENTIAL OILS AND *Pseudomonas chlororaphis* STRAINS AGAINST *Cercospora beticola* Sacc.

ABSTRACT: Leaf spot disease caused by *Cercospora beticola* Sacc. is the most destructive foliar disease of beet. *Cercospora* leaf spot is controlled primarily by fungicides because the non-chemical alternatives do not provide commercially viable control. One of the ways of reducing chemical application is the use of different essential oils (EOs) or antagonistic plant growth-promoting rhizobacteria (PGPB). This study evaluates several EOs and PGPB belonging to *Pseudomonas chlororaphis* as possible control agents of this pathogen. Antifungal properties were determined by *in vitro* microdilution method against five *C. beticola* monosporial isolates originated from the locality Brus, Serbia (53°53' N, 21°04'E and 429 m above sea level) using EOs from medicinal plants: Turkish pickling herb (*Echinophora tenuifolia*), oregano (*Origanum vulgare*), basil (*Ocimum basilicum*), and myrtle (*Myrtus communis*) obtained by a hydro-distillation method. All tested oils displayed some antifungal activity against the fungal isolates. *Origanum vulgare* EO demonstrated the strongest antifungal activity (MIC – 0.0055±0.0051mg/mL), *Ocimum basilicum* slightly lower (MIC – 0.075±0.045mg/mL), followed by *Myrtus communis* (MIC – 0.775±0.045 mg/mL) and *Echinophora tenuifolia* (MIC – 7.75±4.5 mg/mL). Five tested *P. chlororaphis* strains exhibited some antagonistic effect against *C. beticola*. Overnight culture (ONC) of *P. chlororaphis* strain E65 induced the highest percentage of inhibition (75.8%), followed by N3 (72.0%). A cell-free supernatant (CFS) and the CFS treated with EDTA (CFS-EDTA) of these strains showed similar inhibition of 60.2 and 56.0%, and both strains suppressed *C. beticola* growth. *P. chlororaphis* strains M1 and K113 also reduced the fungal growth by

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67–70% using ONC and between 48–57% using different CFS fractions. The strains L1 and B25 caused inhibition of 60% using ONC and 50% by CFS. The lowest inhibition (~40%) by CFS-EDTA and heat-treated cell-free supernatant (HT-CFS) was recorded for B25, which was used as a reference strain. The tested isolates of *C. beticola* were susceptible to all selected essential oils and *P. chlororaphis* strains E25, N3, M1, and K113 *in vitro*, making them a promising non-chemical control agent. It is recommended that these findings should be tested in field conditions.
KEYWORDS: antagonism, CLS, essential oils, MIC, *Pseudomonas* sp.

INTRODUCTION

Cercospora leaf spot (CLS) caused by the fungus *Cercospora beticola* Sacc. is the most important foliar disease of sugar beet (*Beta vulgaris*) worldwide (Holtschulte, 2000; Jacobsen and Franc, 2009; Skaracis et al., 2010). Yield reduction due to CLS can be over 50% (Kaiser and Varrelmann, 2008). CLS is known to cause economic damage in Serbia up to 50% (Trkulja et al., 2017). It is usually controlled by single-site fungicides, which can lead to a quite intensive application of pesticides resulting in the development of fungal resistance and environmental pollution. *C. beticola* quickly develops resistance to the fungicides, which makes protection difficult (Georgopoulos and Dovas, 1973). Consequently, an environmentally friendly alternative is required that can also ensure acceptable quality and quantity of agricultural yield (Ma and Michailides, 2005). Biological control agents and essential oils are an attractive and promising alternative for disease control due to their low environmental impact and their ability to slow down fungicide resistance in pathogen populations (Jochum et al., 2006). Biopesticides can be divided into three different types according to their active substance: microorganisms (bacteria, fungi, oomycetes, viruses, and protozoa), biochemicals (secondary metabolites), and semiochemicals (a chemical signal produced by one organism that causes a behavioural change in an individual of the same or a different species) (Chandler et al., 2011). A biopesticide can be defined as a mass-produced agent manufactured from a living microorganism or a natural product and sold for the purpose of controlling plant pests and diseases. This definition encompasses most entities classed as biopesticides within the Organisation for Economic Cooperation and Development countries (OECD, 2009).

Medicinal plants are a rich source of biologically active compounds. Different medicinal plant species have been used in traditional medicine because of their well-known antimicrobial properties. Essential oils (EOs) have been used for their bactericidal, virucidal, and fungicidal properties (Bakkali et al., 2008). There are numerous data on the high inhibitory effect of EOs from different plant species against many phytopathogenic fungal species (Özcan and Erkmen, 2010; Stevic et al., 2014; Combrinck et al., 2011), but there are very few studies on the control of *C. beticola* (Fatoung et al., 2011) using plant extracts.

Genus *Pseudomonas* comprises a number of plant growth-promoting (PGP) species that express characteristics that can lead to the improvement of plant growth and health. *P. putida* WCS358 was involved in inducing systemic

resistance in plants (Meziane et al., 2005). In the biological control of cotton seedling, damping-off disease caused by *R. solani*, *P. aureofaciens* (*chlororaphis*) and *P. fluorescens* were found to produce phenazine, siderophore, and volatile and non-volatile metabolites (Samavat et al., 2014).

Antagonistic activities of Serbian indigenous *Pseudomonas* spp. strains against different phytopathogenic fungi were reported by Djuric et al. (2011), and Jošić et al. (2015). *P. chlororaphis* strains harbouring multiple PGP traits, including antibiotics phenazine-1-carboxylic acid (PCA) and 2-hydroxy-phenazine-1-carboxylic acid (2-OH-PCA), successfully inhibited *Alternaria alternata* growth and disease incidence on cardoon (Jošić et al., 2012), *P. theicola*, and *F. oxysporum* (Starović et al., 2017; Poštić et al., 2019).

In this study, we have assessed the possibility of using Eos, as well as different fractions of bacterial culture of *P. chlororaphis* strains as antifungal agents in preventing the development of one of the most dangerous fungal beet diseases.

MATERIAL AND METHODS

Antifungal activity of EOs

In this study, Turkish pickling herb or Tarhana herb (*Echinophora tenuifolia*), oregano (*Origanum vulgare*), basil (*Ocimum basilicum*), and myrtle (*Myrtus communis*) were obtained from Mersin (Turkey) by hydro-distillation in a Clevenger-type apparatus, as previously reported (Özcan and Erkmen, 2001; Starović et al., 2016). The essential oils obtained this way were stored in sealed glass bottles, protected from the light by aluminium foil wrapping and stored at -18 ^{o}C.

Fungi. The antifungal activity was tested using fungal species identified as *Cercospora beticola* from the collection of the Institute for Plant Protection and Environment, Belgrade, Serbia, and sub-cultured on potato dextrose agar (PDA) at 28 °C. The suspension of aerial mycelia was prepared from a 20-day-old mycelium suspended in 0.9% (w/v) NaCl with 0.1% (v/v) Tween 20 by mixing thoroughly using a vortex mixer.

Antifungal effect *in vitro*. To investigate the antifungal activity of EOs, a micro-dilution method was used (Rodriquez-Tudela et al., 2008). The final volume of microtiter plate wells was 100 µl (90 µl/well potato dextrose medium with appropriate dilutions of EO and 10 µl/well mycelia suspension of *C. beticola*). The experiment was performed in four repetitions. Microtiter plates were incubated for 5 days at 28 °C.

The minimal inhibitory concentration (MIC) was defined as the lowest concentration of essential oils (EO) that completely inhibited the visible fungal growth (Figure 1). Fluconazole was used as a positive control.

The obtained values of MIC were processed by Duncan's multiple range tests. Analysis of the variance was performed on MIC data of four EOs on

C. beticola. The significance was evaluated at $p<0.05$ for all tests. Statistical analyses were done by procedures of STATISTICA v.7 (StatSoft, Inc.) and IBM SPSS Statistics v.20 (SPSS, Inc.).

Antifungal activity of *P. chlororaphis* strains

Five *P. chlororaphis* strains from the rhizosphere of different plants were tested for the antifungal effect on *C. beticola*. The *P. chlororaphis* B25 strain was included in this experiment as a reference strain, whose complete genome sequence has been deposited in DDBJ/503 EMBL/GenBank under the accession number CP027753 (Biessy et al., 2019).

The antagonistic activity of *P. chlororaphis* strains against *C. beticola* was tested on Waksman agar medium by dual culture method (Wolf et al., 2002). A bacterial overnight culture (ONC), extracellular metabolites in cell-free supernatant (CFS), CFS treated with EDTA (ethylenediaminetetraacetic acid disodium salt dehydrate), known as CFS-EDTA, and a heat-treated cell-free supernatant (HT-CFS) were tested for the inhibition of *C. beticola* growth. To obtain the supernatant fraction, an optimized ON culture (10^6 CFU mL^{-1}) was centrifuged twice at 13,000 rpm for 5 min, with or without filtration (filter tubes with microporous membrane 0.22 µm, Merck Millipore Ltd.). To test two additional fractions, CFS was treated with 1mM EDTA or heated at 70 ºC for 30 min.

P. chlororaphis culture and its fraction (10 µL) were placed on the edge of Petri dishes, 2.5 cm distance from fungal mycelia placed in the centre as 1 cm plug, while the control variant contained only mycelia of *C. beticola* on WA and WA with 1mM EDTA added instead of bacterial culture/fraction. Four replicates per variant were used and the test was performed twice. After incubation of the cultures at 25 °C for 7 days, the growth of fungus was measured. The percentage of the fungal growth inhibition was calculated using the following formula: 100*(1-R2/R1), where R1 was the radial distance growth of the fungus in a control plate and R2 was the radial distance growth of the fungus in the bacterial treatment. The results of the fungal growth inhibition by *P. chlororaphis* strains were compared using a statistically significant difference ($p\leq0.01$) from Duncan's test (STATISTICA v.7; StatSoft, Inc.).

RESULTS

Antifungal activity of EOs

All tested oils showed some antifungal activity against the tested fungal pathogen (Figure 1). *Origanum vulgare* EO showed the strongest antifungal activity (MIC – 5.5±0.0051µg/mL), with a slightly lower *Myrtus communis* (MIC – 325.5±0.045µg/mL), followed by *Ocimum basilicum* (MIC – 775.5±0.045µg/mL), and *Echinophora tenuifolia* (MIC – 3250.0±4.5 µg/mL) (Table 1).

Table 1. Antifungal activity of the essential oils expressed as the minimal inhibitory concentrations (µg/mL) against *Cercospora beticola*.

Essential oils	MIC (µg/mL)*
Echinophora tenuifolia	3250.0c
Origanum vulgare	5.5a
Ocimum basilicum	775.5b
Myrtus communis	325.0b

*Different letters in MIC column indicate a significant difference between means ($p<0.05$)

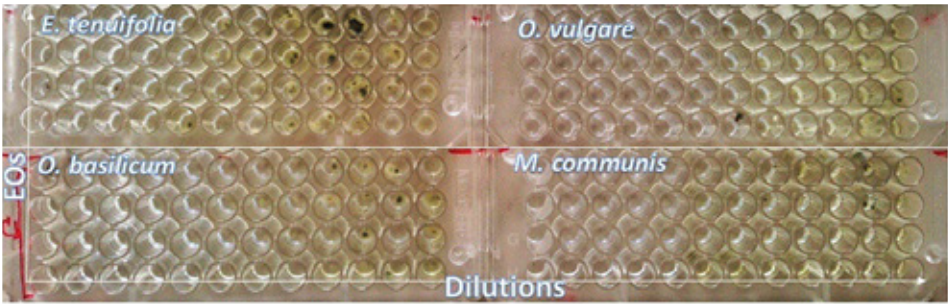

Figure 1. Micro-dilution method: antifungal effect of investigated essential oils.

Antifungal activity of *P. chlororaphis* strains

Our results have demonstrated that all *P. chlororaphis* strains showed an antagonistic effect on *C. beticola* (Table 2). The percentage of fungal growth inhibition by overnight culture of the tested strains ranged from 60.6 to 75.8%, and from 48.8 to 60.2% by cell-free supernatants. The fraction CFS-EDTA of the strain B25 induced the lowest inhibition rate of 39.4% (Figure 2). EDTA did not influence fungal growth at all, showing no statistically different values compared to the control.

Table 2. Growth of *C. beticola* (mm) affected by *P. chlororaphis* strains.

Fungus *C. beticola*	Bacterial strain	Bacterial culture* and fraction			
		ONC	CFS	CFS-EDTA	HT-CFS
C	B25	13.88±0.83	17.38±1.5	21.88±096	20.13±0.82
36.00±1.69	L1	14.25±1.16	18.5±1.07	19.13±1.64	18.63±1.3
C-EDTA	M1	11.88±0.64	15.5±1.26	16.13±0.5	16.88±0.82
36.25±1.28	N3	10.13±0.64	14.38±1.06	15.25±0.89	14.88±0.83
mean	E65	8.75±0.52	14.38±1.06	16.13±0.83	15.38±1.3
36.13±1.45	K113	10.75±0.76	16.63±0.74	18.75±0.89	17.38±1.06

*bacterial culture 10^6 CFU mL^{-1} and its fractions; C – Control; C-EDTA – Control supplemented with 1mM EDTA; ONC – overnight culture; CFS – cell-free supernatant; CFS-EDTA – CFS treated with 1mM EDTA; HT-CFS – heat-treated CFS. Values indicate mean values (±SD).

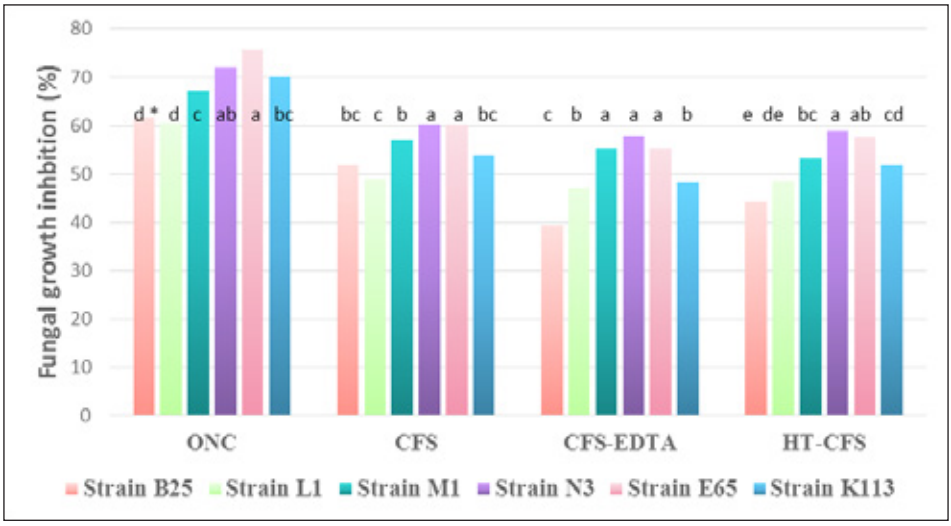

Figure 2. Growth inhibition of *C. beticola* affected by *P. chlororaphis* strains.
* The bars with the same letters are not significantly different at P < 0.01 using Duncan's test.

DISCUSSION

The selected essential oils, especially oregano, exhibited significant antifungal activity against *C. beticola*. These results are encouraging considering how resistance of *C. beticola* to the fungicides is widely established. Another advantage of using EOs is that they can be applied in organically grown crops. The results of this investigation support our earlier study of antifungal activity against *Phomopsis theicola*, as the most potent oil was oregano (MIC-5.5µg/mL) (Starović et al., 2017).

Numerous studies from other researchers have shown significant antifungal activity of oregano's essential oils, such as Kocić-Tanackov et al. (2012) which have demonstrated that the concentration of 25 µg/mL inhibits the growth of *Fusarium oxysporum*, *F. proliferatum*, *F. subglutinans*, and *F. verticilioides* by 81–88%. Bedoya-Serna et al. (2018) obtained the MIC of oregano essential oil of 0.20 µg/mL, 0.26 µg/m L, and 0.30 µg/mL against *Fusarium* sp., *Cladosporium* sp., and *Penicillium* sp. respectively. In another study, the MICs of oregano essential oil against *Botrytis cinerea* and *Colletrotrichum gleosporioides* were 0.8 and 1.0 mg/mL respectively (Cid-Pérrez et al., 2016). In addition, the MIC of myrtle essential oil against *Fusarium* spp. was 0.3–3.25 mg/mL (Starović et al., 2016). Citral, methyl anthranilate, and nerol (citrate EOs) in a concentration of 5.0 mL/L reduced *C. beticola* by 78–80% in field condition and manifested a very high antagonistic effect against *C. beticola in vitro* conditions (Fatouh et al., 2011).

Dual culture assay *in vitro* and inhibition zone area was taken as a measure of the antagonistic potential, which led to the selection of bacterial strains with a better inhibitory potential on the growth of *C. beticola*. ONC of *P. chlororaphis* strain E65 induced the highest percentage of inhibition (75.8%), followed by N3 (72.0%), showing a significant difference at $P<0.01$ using Duncan's test. The reverse significance was calculated for their HT-CFS fraction. No significant difference was observed in fungal inhibition of CFS and CFS-EDTA of these strains. Both strains were highly effective in antagonistic action toward *C. beticola* growth. The lowest percentage of inhibition – around 60% for ONC and 50% for CFS was caused by *P. chlororaphis* strains L1 and B25. B25 showed the lowest inhibition for CFS-EDTA and HT-CFS. *P. chlororaphis* strains M1 and K113 also reduced the fungal growth at a high percentage (67–70%) by ONC and between 48–57% by different CFS. No significant difference between strains E65, N3, and M1 in the inhibition of fungus comparing CFS-EDTA fractions was exhibited.

This finding is in agreement with the results obtained by other researchers. Arzanlou et al. (2016) reported that *C. beticola*, a causal agent of Cercospora leaf spot disease in sugar beet, was inhibited by strains from different genera isolated from the rhizosphere of sugar beet – *Bacillus*, *Pseudomonas*, and *Paenibacillus*. These strains significantly decreased the disease severity in laboratory and greenhouse assays. In their study, *Pseudomonas* strain inhibited two *C. beticola* strains by 53.13 and 64.84% in dual culture assay, which is in concordance with our results. Poornima et al. (2011) tested *Bacillus subtilis*, *P. fluorescens*, *Trichoderma harzianum*, *T. koningii,* and *T. viride* as bioagents, under *in vitro* and *in vivo* conditions, against *C. beticola* that caused CLS of palak (*Beta vulgaris* var. bengalensis Hort). Although *T. harzianum* was significantly superior under *in vitro* condition, *P. fluorescens* was the most effective under *in vivo* conditions showing the lower percent of disease severity.

To assess the effectiveness of biological control of another phytopathogen from genera *Cercospora*, the causal agent of frogeye leaf spot of soya bean – *C. sojina*, Simonetti et al. (2012) used one *P. fluorescens* and two *Bacillus amyloliquefaciens* indigenous strains. The fungal growth was inhibited by 52–53% using the culture of *Bacillus* strains and 32–34% with the *P. fluorescens* strain. Besides two different species of pathogen and an antagonist from the same genera, we noticed that *P. fluorescens* caused a smaller reduction in *C. sojina* growth than culture and three fractions of all *P. chlororaphis* strains of *C. beticola* in our study.

In an attempt to find alternatives to fungicides against *C. beticola*, Derbalah et al. (2013) tested several different formulations, including the biological agent containing *Bacillus subtilis*, *B. pumilus*, *P. fluorescens*, *Epicoccum nigrum*, and found that biological formulations were the most effective treatments against sugar beet leaf spot, followed by nanosilica and nanozinc oxide.

CONCLUSIONS

Our study provides sound scientific evidence of the practical use of traditional medicinal plants in biological control of fungal diseases and encourages selection and testing of other plant products as potential alternatives to pesticides. Based on our promising results on the effectiveness of the EOs from Turkish pickling herb, oregano, basil, and myrtle in *C. beticola* control, further studies could lead to the selection of essential oils and their concentrations for *in vivo* trials of products with fungicidal properties. Further studies should focus on the investigation of the most effective antagonistic *P. chlororaphis* strains E65, N3, and M1 in the control of CLS disease under greenhouse and field conditions.

ACKNOWLEDGMENTS

The authors are thankful to the Ministry of Education, Science and Technological Development of the Republic of Serbia for financial support during this study through the Projects grant TR31018 and III46007. A special thanks to Mrs. S. Bajic-Raymond, a Director of Raymond Educational Consultancy Ltd, and Professor G. Raymond, University of Bristol, UK, for proof-reading the paper and linguistic corrections.

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АНТИФУНГАЛНА АКТИВНОСТ БИЉНИХ ЕТАРСКИХ УЉА И СОЈЕВА *Pseudomonas chlororaphis* НА *Cercospora beticola* Sacc.

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РЕЗИМЕ: Пегавост лишћа шећерне репе чији је проузроковач *Cercospora beticola* Sacc. је врло деструктивно обољење ове биљне врсте. Хемијска средства се уобичајено користе за контролу ове болести, јер за сада алтернативне мере заштите нису комерцијализоване. У овом раду испитиван је *in vitro* антифунгални утицај неких етарских уља (ЕУ) и *Pseudomonas chlororaphis* сојева – ризобактерија стимулатора раста биљака, као могућих агенаса за контролу овог патогена. Микродилуционом методом су одређене минималне концентрације инхибиције (MIC) етарских уља. Сва примењена етарска уља су испољила задовољавајући степен инхибиције. Етарско уље оригана испољило је најјачи антифунгални ефекат (MIC – 0,0055±0,0051mg/mL), нешто нижи босиљка (MIC – 0,075±0,045 mg/mL), мирте (MIC – 0,775±0,045 mg/mL) и најслабији турске киселе биљке (MIC – 7,75±4,5 mg/mL). Ангонистички ефекат на пораст мицелије *C. beticola* детектован је код 5 сојева *P. chlororaphis*, а сој B25 коришћен је као референтни. Прекоћна култура *P. chlororaphis* соја E65 испољила је највиши степен инхибиције (75,8%), затим соја N3 (72,0), док су фракције супернатаната са и без EDTA изазвале инхибицију 60,2 и 56,0%. Прекоћне културе сојева M1 и K113 инхибирале су 67–70% пораст мицелије, а 48–57% различите фракције супернатаната. Инхибицију раста гљиве 60% условиле су прекоћне културе сојева L1 и B25, а 50% њихови супернатанти, док су супернатант обогаћен EDTA и термички третирани супернатант соја B25 остварили најнижи степен инхибиције (~40%). Сва примењена етарска уља и *P. chlororaphis* сојеви E25, N3, M1 и K113 испољили су значајан степен инибиције пораста мицелије изолата *C. beticola* пореклом са шећерне репе, што их чини потенцијално перспективним нехемијским агенсима, чији ефекат треба проверити и у пољским условима.

КЉУЧНЕ РЕЧИ: антагонизам, етарска уља, MIC, CLS, *Pseudomonas* sp.