Pestic. Phytomed. (Belgrade), 38(1), 2023, 11–21
DOI: https://doi.org/10.2298/PIF2301011M

UDC 632.4:632.952:632.937:632.95.028
Original scientific paper

Can Sclerotinia stem and root rot be managed effectively without causing environmental imbalance in soil?

Milica Mihajlović[1]*, Emil Rekanović[1], Jovana Hrustić[1], Mila Grahovac[2], Marija Stevanović[1] and Brankica Tanović[1]

[1] Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Belgrade, Serbia
[2] Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia
** Corresponding author: milica.mihajlovic@pesting.org.rs*

Received: 5 December 2022
Accepted: 13 January 2023

SUMMARY

Sclerotinia stem and root rot, caused by *Sclerotinia sclerotiorum*, is considered to be an important soil-borne disease of over 400 plant species, including a wide range of species important for agriculture. *In vitro* and *in vivo* sensitivity of *S. sclerotiorum* to several commercial fungicides and biofungicides was studied. The highest efficacy was achieved by boscalid (98%) and fluopyram (80%), and the lowest by a *B. subtilis*-based product (5%). The isolate was sensitive to all tested products *in vitro*. Considering the tested synthetic fungicides, fluopyram exhibited the highest toxicity (EC_{50}=0.003 mg/l), while captan exhibited the lowest (EC_{50}=8.94mg/l). Even lower efficacy was achieved by tea tree oil and *B. subtilis*-based products. The environmental impact of pesticides and biopesticides used for *Sclerotinia* control was assessed. Modeling of predicted environmental concentrations in soil (PECsoil), coupled with literature toxicity data, served for assessment of pesticides soil risks. A high long-term risk for earthworms was revealed for captan and thiophanate-methyl. Based on both efficacy and risk assessment results, fluopyram was found to have the best properties of all tested conventional pesticides, while tea tree oil exerted better performance than the *Bacillus* product. Further investigation of combined use of conventional and biopesticides might reveal new perspectives regarding effective *Sclerotinia* control, while simultaneously reducing negative environmental impact.

Keywords: soil-borne pathogen, fungicides, biocontrol, antagonistic activity, soil risk assessment

INTRODUCTION

Sclerotinia stem and root rot (syn. white rot), caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is considered to be an important soil-borne disease of over 400 plant species, including a wide range of open field and greenhouse crops of agronomic importance (Boland & Hall, 1994). A typical early symptom of the disease is the appearance of brown, more or less wet rot, often accompanied by masses of white fluffy mycelia, especially during humid conditions (Purdy, 1979). The pathogen produces over-wintering vegetative structures, composed of massive

mycelia protected by a well-developed and differentiated rind, known as sclerotia. The sclerotia of *S. sclerotiorum* reside in soil as inoculum source for several years. Under appropriate environmental conditions sclerotia may germinate either in a myceliogenic manner, giving rise to infective hyphae, or by carpogenic germination to produce apothecia, which release millions of sexually produced, air-borne ascospores (Coley-Smith & Cooke, 1971).

The extensively and effectively used preplant soil fumigant methyl bromide as an ozone-depleting supstance was gradually withdrawn from use after 1992 (UNEP, 2006). As fumigation with dasomet instead of methyl bromide was found significantly less effective in reducing the survival of resting fungal structures in soil, including sclerotia, several strategies have been proposed.

The application of fungicides from dicarboximide, benzimidazole and triazole groups to soil or to plants could be an effective disease management measure against soil-borne pathogens, including cyprodinil and fludioxonil registered against *S. sclerotiorum* (Mueller et al., 2002; Matheron & Porchas, 2004; Benigni & Bompeix, 2010). The implication of residues of chemical fungicides in soil and water as pollution has mandated a search for alternative approaches to disease management. Furthermore, rapid rise in demand for organically produced fruits and vegetables has increased the interest in biopesticide development and utilization. Therefore, interest in secondary metabolites from plant extracts (mainly essential oils) and antagonistic microorganisms as potential antimicrobial agents for use in food preservation, crop protection and pharmacological applications has increased over the past decades (Broadbent et al., 1977). As a promising alternative to synthetic pesticides, many essential oils have been studied for their antifungal activity (Kalemba & Kunicka, 2003). However, very few studies have focused on their effects on *S. sclerotiorum* (Edris & Farrag, 2003; Soylu et al., 2007). Although there have been numerous reports on antifungal activities of essential oils under *in vitro* conditions, there is no accessible research on the antifungal activity of essential oils against other soil-borne fungal pathogens under *in vivo* conditions. Beneficial bacterial species, particularly *Bacillus* spp., are known for their antifungal properties. Even though their importance for the control of numerous plant and animal diseases has been well-documented (Broadbent et al., 1977; Kim & Chung, 2004), their effectiveness against *S. sclerotiorum* remains indistinctive.

Modern agricultural practice relies on the use of synthetic pesticides or biopesticides, taking care that soil health and preservation of soil micro- and macro-organism communities are of utmost importance. Incorporation of pesticides or biopesticides into soil can potentially lead to severe changes in the distribution and abundance of soil organisms, causing long-term disturbance of the soil food web (Fließbach & Mäder, 2004; Sánchez-Bayo, 2011). Earthworms and microorganisms are an integral part of soil ecosystems, and they have a key role in organic matter degradation, transformation and provision of available nutrients to other soil organisms and plants (Arora & Sahni, 2016; Bart et al., 2019).

The objectives of this study were to: a) investigate the possibility of Sclerotinia stem and root rot control by using conventional fungicides with different modes of action and biofungicides based on either an essential oil or an antagonistic bacterial strain; b) assess environmental risks of their incorporation into the soil; c) gain a complete insight into the effects of the studied fungicides and biofungicides on *S. sclerotiorum* by *in vitro* sensitivity tests.

MATERIAL AND METHODS

S. sclerotiorum isolate

A *S. sclerotiorum* isolate was derived from infected pepper plants grown in the vicinity of Šabac, Serbia, using a standard phytopathological method (Dhingra & Sinclair, 1995). Briefly, small pieces of diseased root tissue were cut, washed under running water for 15 min, surface disinfected in a 1% sodium hypochlorite solution for 2 min, placed on sterile potato dextrose agar (PDA) and incubated at room temperature for seven days. The obtained hyphal tip isolate was identified based on pathogenic and morphological traits (Waterhouse & Waterston, 1966). Colony texture, color and shape, the appearance of margin and presence of sclerotia were observed in a 7-day-old colony. The appearance of hyphae was observed microscopically, using a compound microscope (Olympus Cx41, Japan). The identity of the isolate was confirmed by polymerase chain reaction (PCR) using the universal primer pair ITS1/ITS4 (White et al., 1990). The isolate was maintained on slants at 4°C in the Culture Collection of the Institute of Pesticides and Environmental Protection, Belgrade, Serbia.

Pathogenicity test

S. sclerotiorum inoculum was prepared by growing the 7-day-old culture in 500 ml glass bottles containing 150 g of sterilized barley grains at 25°C for 21 days. Then, the inoculum was mixed thoroughly with sterilized clay soil at the rate of 5% and then put in pots. Uninoculated pepper plants (treated with sterile barley grains) served as control. The pots were kept in

a greenhouse (24±2°C) and watered regularly until sympthoms appeared (Budge & Whipps, 2001).

Fungicides and biofungicides

Commercial formulations of boscalid (Cantus, 500 g/kg, WG, Bayer CropScience, Germany), fluopyram (Luna Privilege, 500 g/L, SC, Bayer CropScience, Germany), captan (Agrokaptan, 500 g/kg, SC, Agromarket, Serbia), prochloraz (Spartak 450-EC, 450 g/l, EC, Sinochem Ningbo, China), thiophanate-methyl (Funomil, 700 g/kg, WP, Agromarket, Serbia), tea tree oil (Timorex Gold, Stockton, Israel) and *B. subtilis* (Extrasol, Bisolbi Inter, Russia) were tested in this study.

Greenhouse experiment

Sclerotia of the *S. sclerotiorum* isolate that were used as inoculum source in potting greenhouse experiments were produced on double autoclaved wheat grain (25 g of wheat grain and 50 ml of water in 250 ml conical flasks were autoclaved for 15 min at 121°C on two successive days). Each grain flask was inoculated with three 10-mm-diameter agar plugs cut from the edge of *S. sclerotiorum* plate cultures grown for 7 days on PDA. After 3 weeks of incubation at 20°C, sclerotia were collected and graded, and only those between 2 and 4 mm in diameter were used for inoculum preparation (Budge & Whipps, 2001). The inoculum was prepared by mixing sclerotia with sterilized clay soil at the rate of 3%.

Three-week-old pepper seedlings (cv. 'Novosadska babura') were used as the model plant. Seedlings were transplanted into pots filled with 400 ml of plant growth substrate (Floragard, Germany). The prepared inoculum was then added into each pot, and 100 ml of each fungicide/biofungicide dispersion was applied at label rate. Seedlings inoculated and watered with 60 ml of sterile distilled water served as a positive control (K). Seedlings inoculated with sterile barley grains, watered with 100 ml sterile distilled water, served as a negative control (AK). The pots were kept in a greenhouse (24±2°C) and watered regularly. Infection degree was recorded daily until the final evaluation 15 days after inoculation by visual observation and by measurement of plant height and fresh weight. Disease severity was evaluated based on a scale ranging from 0 to 5, where 0 = no symptoms, 1 = chlorosis of leaves, 2 = slight wilting with pronounced chlorosis, 3 = slight wilting and necrosis, 4 = pronounced wilting and necrosis, and 5 = death of plant (D'Ercole et al., 2000; EPPO, 1997). The experimental design was a complete randomized block with five replicates per treatment and five plants per replicate. The experiment was conducted twice. Infection degree (ID) was calculated using the Townsend-Heuberger formula (Swiader et al., 2002):

$$ID = (nv)100/NV$$

where: n = degree of infection rated on a 1-5 scale, v = number of plants in a category, N = highest degree of infection rate, and V = total number of plants screened. The efficacy was evaluated using Abbott's formula (Abbott, 1925). Data were analyzed separately for each experiment using ANOVA, and the means were separated by Duncan's multiple range test.

In vitro sensitivity tests

The sensitivity of the isolate to fungicides and tea tree oil *in vitro* was determined in radial growth assays on PDA medium as it was described by Leroux and Gredt (1972) and Löcher and Lorenz (1991).

Based on preliminary concentrations of all investigated fungicides and tea tree oil, ranging from 0.003 to 1000 mg/l of active ingredient (a.i.), the following final a.i. concentrations in the medium were used: boscalid - 0.05, 0.1, 1 and 10 mg/l; fluopyram - 0.0125, 0.2, 0.1, 0.05 and 0.025 mg/l; captan - 3.12, 6.25, 12.5 and 25 mg/l; prochloraz - 0.003, 0.006, 0.0125, 0.025 and 0.05 mg/l; thiophanate-methyl - 1.12, 2.5 and 5 mg/l, and tea tree oil - 62.5, 125, 250, 500 and 1000 mg/l.

Fungicide-amended media were prepared by adding fungicides from dilution series, prepared in sterile distilled water, to the molten PDA medium (50°C) at 1:9 ratio. In the fungicide-free control medium, sterile distilled water was added instead of fungicide dilution.

Mycelial plugs (3 mm diameter) were cut from the edge of 7-day-old cultures of *S. sclerotiorum* grown on PDA medium at 22°C and used for inoculation of fungicide-amended and fungicide-free media. The experiment was conducted in three independent replications, using two Petri dishes per replicate, each containing one mycelial plug. After incubation for four days at 22°C, mycelial growth was measured. Growth on the fungicide-amended media was presented as the percentage of control value. Since experimental conditions were identical in all replications, the obtained data were pulled together and the fungicide concentration that inhibited mycelial growth by 50% (EC_{50}) and regression coefficient (b), expressing relative toxicity of the fungicide, were determined using probit analysis (Finney, 1971).

In vitro effect of the antagonistic *B. subtilis* strain Č13 was checked using the well difusion method on double-layer PDA. The strain was isolated from the commercial biofungicide Ekstrasol (Bisolbi Inter, Russia) using the plating method. The derived strain was grown in submersed culture in Erlenmeyer flasks on a shaker (200 rpm)

at 28°C for three days in Meynell medium: molasses – 20.0, K_2HPO_4 – 7.0; KH_2PO_4 – 3.0; $MgSO_4$ – 0.1; sodium citrate – 0.5; $(NH_4)_2SO4$ – 1.0; H_2O – adjusted to 1 l; pH 7.0 (Meynell et al., 1967). The *S. sclerotiorum* isolate was grown in a 300 ml shake flask containing 100 ml of the potato-dextrose-broth medium (PDB, *Sigma-Aldrich*, *Germany*) for 48 h at 25°C. The rotary shaker, set to 150 rpm, was used to mix the fluid during cultivation.

The double-layer PDA medium was made in 90-mm petri dishes. The first layer consisted of 20 ml of 2% PDA medium. The second layer, composed of 7 ml of *S. sclerotiorum* suspension and 3 ml of molten 1.2% PDA, was spread homogeneously over the first layer. After solidification, one well (10 mm in diameter) was inserted in the central part of each plate. The treatments (100 µl volume) included: a) prepared suspensions of the tested antagonistic strain; b) the fungicide prochloraz at a label rate (reference product); c) sterile distilled water (control treatment). The experiment was conducted in four replicates and repeated twice. The assessment of antagonistic activity was performed after incubation for 48 h at 25°C by measuring the diameter of inhibition zones (mm), i.e. zones around wells with no visible mycelial growth.

Soil risk assessment

Risk assessment was performed using literature toxicity data for earthworms and microorganisms, and predicted environmental concentrations in soil (PEC_{soil}) were modeled. PEC_{soil} values were modeled using the Dutch Board for Authorization of Plant Protection Products and Biocides (Ctgb) PEC soil calculator (https://english.ctgb.nl/documents/assessment-framework-ppp/2017/12/22/calculation-of-pec-soil-values). PEC values were calculated based on active ingredient properties and application rates recommended in Serbia. In addition to the studied fungicides, dazomet PEC values were also calculated, as it is the only fungicide registered for bare soil application as a for methyl-bromide.

RESULTS

S. sclerotiorum isolate

The *S. sclerotiorum* isolate, grown on PDA at 25°C in darkness, formed a well-developed white mycelium, initially homogeneous and airy, and later exhibiting radially distributed cottony clusters of hyphae, indicating initiation of sclerotia formation. Five days after inoculation, the sclerotial primordials turned gray and had water drops on the surface. Sclerotia turned dark after 7 days and assumed hard consistency, irregular shape, 1.5-7 x 2-15 mm size and mostly circular arrangement. No water secretion was observed. Based on the studied morphological characteristics, it was determined that the isolate belonged to the species *S. sclerotiorum*. This identification was confirmed by the sequence of approx. 500 bp amplicon, obtained using the universal primer pair ITS1/ITS4. A BLAST analysis showed that the ITS sequence of the studied isolate had 94% nucleotide identity with five *S. sclerotiorum* isolates (accession Nos. GQ375746, MK527225, MF408284, MZ540878 and MH137960) deposited in GenBank. Therefore, molecular analysis confirmed that the studied isolate belongs to *S. sclerotiorum*.

Pathogenicity test

Pepper plants inoculated with the tested *S. sclerotiorum* isolate exhibited wilting symptoms 15 days after inoculation. The fungus was successfully re-isolated from the inoculated plants, completing Koch's postulates in both assays. No symptoms were observed on the water-inoculated plants 15 days post inoculation.

Greenhouse experiment

The highest efficacy was recorded for boscalid (98%) and fluopyram (80%), and the lowest for the product containing *B. subtilis* as an active ingradient (5%). Table 1 summarizes the results of infection degree and efficacy of the products applied immediately after inoculation. Compared to the height of inoculated control pepper plants K (0.04 cm), all inoculated treatments other than treatment with *B. subtilis* (0.28 cm) exhibited significantly higher values. Table 2 summarizes the height and fresh weight of inoculated pepper plants treated immediately after inoculation. Maximum plant height was recorded in treatments with boscalid (5.64 cm) and captan (4.04 cm), respectively. Conversely, the lowest values of plant height were recorded in treatments with products based on *B. subtilis* (0.28 cm) and tea tree oil (0.56 cm).

The highest plant fresh weight was found in treatments with boscalid (0.29 g) and prochloraz (0.25 g), and the lowest in treatments with *B. subtilis* (0.07g) and tea tree oil (0.1 g). However, data were statistically significant only when compared with inoculated control pepper plants (0.01 g).

A strong positive correlation was revealed between fungicide efficacy and plant height ($r = 0.88$), as well as between efficacy and fresh weight of pepper plants ($r = 0.92$).

In vitro tests

Sensitivity of the isolate to conventional fungicides was much higher than it was to the product based on tea tree oil (Table 3). Of all tested products, fluopyram exhibited

the highest toxicity. Its calculated EC_{50} for inhibition of hyphal growth was 0.003 mg/l. The isolate also showed high susceptibility to boscalid (EC_{50}=0.1 mg/l); it was able to grow well at 0.1 mg/l of boscalid whereas it was severely inhibited by its higher concentrations. Considering the synthetic fungicides, captan showed the lowest toxicity to the tested isolate (EC_{50}=8.94 mg/l). The isolate demonstrated an ability to tolerate the concentration of 250 mg/l of tea tree oil. The calculated EC_{50} value for inhibition of hyphal growth was high, 70.28 mg/l.

Table 1. Infection rate and treatment efficacy on pepper plants inoculated with *Sclerotinia sclerotiorum* 15 days after the application of fungicides and biofungicides

Fungicide/ biofungicide	Rate (%)	Infection degree (%)		Efficacy (%)
		Ms	Sd	
K*	-	100.00 d	0.00	0.0
AK**	-	0.00 a	0.00	100.0
Prochloraz	0.08	24.00 b	16.40	76.0
Captan	0.30	50.00 c	17.70	50.0
Boscalid	0.15	2.00 ab	4.50	98.0
Thiophanate-methyl	0.10	55.00 c	11.20	45.0
Fluopyram	0.10	20.00 ab	20.90	80.0
Tee tree oil	1.00	57.00 c	33.50	43.0
B. subtilis	1.00	95.00 d	11.20	5.0
		LSD_{005} = 21.33	LSD_{001} = 28.58	

K* - Inoculated plants
AK* - Uninoculated and untreated plants

Table 2. Height (cm) and fresh weight (g) of pepper plants inoculated with *Sclerotinia sclerotiorum* 15 days after the application of fungicides and biofungicides

Fungicide/ biofungicide	Rate (%)	Height (cm)		Fresh weight (g)	
		Ms	Sd	Ms	Sd
K	-	0.04 a	0.09	0.01 a	0.00
AK	-	6.54 g	0.47	0.37 g	0.06
Prochloraz	0.08	2.64 c	0.17	0.25 ef	0.05
Captan	0.30	4.04 e	0.36	0.23 de	0.05
Boscalid	0.15	5.64 f	0.61	0.29 f	0.04
Thiophanate-methyl	0.10	2.64 c	0.22	0.21 de	0.02
Fluopyram	0.10	3.14 d	0.34	0.19 d	0.02
Tee tree oil	1.00	0.56 b	0.09	0.10 c	0.02
B. subtilis	1.00	0.28 ab	0.13	0.07 b	0.01
		LSD_{005} = 0.42	LSD_{001} = 0.57	LSD_{005} = 0.05	LSD_{001} = 0.06

Table 3. *In vitro* sensitivity of *Sclerotinia sclerotiorum*

Fungicide	EC_{50} (mg/l)		b*	
	Value	Range	Value	Range
Prochloraz	1.01	0.007-0.11	0.78	0.65-0.91
Captan	8.94	7.85-10.20	2.47	2.24-2.70
Boscalid	0.17	0.08-0.31	0.50	0.43-2.57
Thiophanate-methyl	1.32	1.07-1.55	2.64	2.97-2.31
Fluopyram	0.003	0.0002-0.02	0.60	0.09-1.11
Tee tree oil	70.28	23.78-118.17	0.63	0.48-0.78
B. subtilis	not applicable			

*b – Regression coefficient

Table 4. Risk assessment of conventional and biofungicides for earthworms (*Eisenia fetida*)

Active ingredient (a.i.)	Endpoint (mg a.i/kg soil)		PEC_{soil} (mg a.i/kg)	TER_A	Acute risk	TER_{LT}	Chronic risk
	LD_{50}	NOEC					
Boscalid	>500*	12.5*	1.583	315.9	↓	7.9	↓
Fluopyram	>500*	5.71*	0.645	775.2	↓	8.9	↓
Captan	>259.65*	5.8*	2.0	129.3	↓	2.9	↑
Prochloraz	>500*	4.2*	0.6	833.3	↓	7.0	↓
Thiophanate-methyl	-	0.8	0.653	-	-	1.23	↑
Tee tree oil	50*	-	0.778	64.3	↓	-	-
Bacillus subtilis	>5x10^9 CFU/ kg dws	1.58x10^9 CFU/ kg dws	2.67x10^5 CFU/ha	76404.5	↓	76404.5	↓
Dazomet	6.5*	-	800.0	0.1	↑	-	-

*Corrected values (LD_{50corr} and $NOEC_{corr}$) are derived by dividing endpoint by 2 for substances with logKow>2, in accordance with EPPO earthworm scheme 2002
TER_A acute toxicity exposure ratio
TER_{LT} chronic toxicity exposure ratio
Trigger value for acute toxicity is 10, and for chronic 5
↓ Low risk
↑ High risk

Table 5. Risk assessment of conventional and biofungicides for soil microorganisms

Active ingredient (a.i.)	Endpoint (mg a.i/kg soil)		PEC_{soil} (mg a.i/kg)	Risk
	N-transformation	C-transformation		
Boscalid	>8	>8	1.583	↓
Fluopyram	>3.3	>3.3	0.645	↓
Captan	>6	-	2.0	↓
Prochloraz	>5.4 kg a.i./ha	>5.4 kg a.s./ha	0.6	↓
Thiophanate-methyl	35	-	0.653	↓
Tee tree oil	>250 (α-terpinen, α-pinene, limonene)	-	0.778	↓
Bacillus subtilis	-	-	**2.67x10^5 CFU/ha**	-

↓ Low risk
↑ High risk

Assessment of *B. subtilis* antagonistic activity *in vitro*

Antagonistic activity of the *B. subtilis* strain Č13 was assessed by measuring the diameter (mm) of inhibition zone around each well. Complete absence of inhibition zone was observed in the treatment with *B. subtilis*, suggesting its weak antagonistic activity or complete lack of activity. The inhibition zone in prochloraz treatment (90 mm diameter) was completely clear without any mycelial growth.

Soil risk assessment

A comparison of toxicity exposure ratios (TER) and trigger values for earthworm in acute and long-term toxicity studies revealed an unacceptable risk for earthworms in two out of eight investigated fungicides (captan and thiophanate-methyl), and dazomet as an additional fungicide authorized for soil application (Table 4).

Comparing the available literature data for fungicide effects on soil nitrogen and carbon transformation and predicted environmental concentrations in soil, no adverse effect on soil microorganisms was revealed (Table 5).

DISCUSSION

Chemical control of the Sclerotinia white rot disease may approach the relevant pathogens either directly, by focusing on fungicide toxicity, or indirectly

by focusing on plant defense activation. Our results provide novel information on the efficacy of the succinate dehydrogenase inhibitor (SDHI) fungicides boscalid and fluopyram in controlling Sclerotinia stem and root rot disease. Boscalid, a fungicide in the carboxamide group, targets the succinate dehydrogenase enzyme as a functional part of the tricarboxylic cycle and mitochondrial electron transport chain (Matsson & Hederstedt, 2001). In our greenhouse experiment, boscalid provided 98% reduction in disease severity. Our results are consistent with those of Liu et al. (2018) where the efficacy of boscalid treatments in field experiments was 81%. A significant reduction in Sclerotinia stem rot of canola by boscalid application was also achieved in experiments by Bradley et al. (2006).

Since boscalid has not been registered or used in Serbia for the control of soil-borne plant diseases so far, *in vitro* testing of *S. sclerotiorum* sensitivity to boscalid could represent valuable initial findings. The EC_{50} value recorded in our experiment was similar to those reported by Hu et al. (2018). In their study, effective concentrations causing 50% mycelial growth inhibition of tested *S. sclerotiorum* isolates ranged from 0.0383 to 0.0395 mg/l.

Besides boscalid, fluopyram is another SDHI fungicide, and it is registered in Serbia against *S. sclerotiorum* in field crops (rapeseed, sunflower and soybean), while its efficacy against *S. sclerotiorum* in vegetables is unknown. Our results represent the first report on fluopyram efficacy in Scletorinia white rot control in pepper, which was also confirmed by *in vitro* sensitivity tests. Of all tested products, fluopyram exhibited the highest toxicity to the tested *S. sclerotinia* isolate under laboratory conditions. The calculated EC_{50} for inhibition of hyphal growth was 0.003 mg/l. These results are in agreement with those reported by Huang et al. (2019) where fluopyram had a strong inhibitory activity on the mycelial growth of *S. sclerotiorum* isolates and had EC_{50} values ranging from 0.02 to 0.30 mg/l.

In recent years, many studies have demonstrated deleterious effects of fungicides on the environment. Using microbial agents to control plant pathogens can be an eco-friendly and cost-effective component of an integrated management programme. Numerous biocontrol agents have been studied for the control of *S. sclerotiorum*. *Bacillus* species, including *B. subtilis,* are known for their antifungal properties, hence their importance for biological control of many plant and animal diseases (Broadbent et al., 1977). Our treatment with the *B. subtilis*-based product was neither sufficiently effective in the greenhouse, nor was the antagonistic activity of the *B. subtilis* strain satisfactory *in vitro*. On the other hand, Yang et al. (2009) reported that the efficacy of their tested *B. subtilis* strain was 77% against Sclerotinia stem rot of rape in a field experiment.

Essential oils from aromatic and medicinal plants have been known to possess biological activity, i.e. notable antibacterial, antifungal, and antioxidant properties (Bounatirou et al., 2007), mainly due to the presence of active monoterpene constituents (Knobloch et al., 1989). Tea tree oil has a long history of use in human pharmacology (Markham, 1999; Carson et al., 2006). Under laboratory conditions, the biofungicide based on tea tree oil exhibited low toxicity to the tested isolate of *S. sclerotiorum*, reaching the EC_{50} of 70.28 mg/l, which was confirmed by its partial efficacy (43%) in the greenhouse experiment.

Although high efficacy rate of a fungicide is the most significant aspect from a crop protection standpoint, information regarding its environmental impact should never be neglected (Kedia et al., 2015). In the present study, three fungicides with the highest efficacy (>75%) – boscalid, prochloraz and fluopyram, also exerted positive environmental properties and posed low risk to earthworms. In order to discuss our results with regard to regulatory-approved substances, risk assessment was extended to include the fungicide dazomet. Both boscalid and fluopyram exerted high efficacy, low earthworm toxicity and low risk of adverse effects, while dazomet, aside of being effective and nonpersistent, was found to pose a high risk to earthworms (EFSA, 2010). However, due to soil recolonization within a period of one year, the risk was concluded as environmentally acceptable. Studies of disturbance of soil microbial communities caused by fungicides are few but they give an insight into potentially adverse side effects. Although boscalid was found to have insignificant effect on nitrogen and carbon transformation, there was an indication of its adverse effects on the P-cycle (Xiong et al., 2014). Fluopyram application may also result in harmful effects on microbial community structure and functional diversity (Zhang et al., 2014; Santísima-Trinidad et al., 2018), while prochloraz exerts positive environmental properties – it poses low risk for earthworms, but also for soil microorganisms. According to Tejada et al. (2011), prochloraz applied at recommended doses, increased soil enzymatic activity and caused no negative effect on bacterial biodiversity.

Thiophanate-methyl and captan were medium effective in our investigation but environmental risk assessment indicated a high long-term risk for earthworm community and as such its use for soil application for

Sclerotinia control could not be recommended. Studies focusing on the effects of thiophanate-methyl on soil microbial population (*Azospirillum* spp., nitrifiers and soil bacteria, *Trichoderma* spp.) detected no adverse effects of the recommended application rates (Singh et al., 2014; Ramanamma et al., 2017). Interestingly, Joshi et al. (2021) reported significant reductions in bacterial, *Trichoderma* and other fungal populations, and simultanious stimulation of actinomycete population. Available literature suggests similar results for captan (Martínez-Toledo et al., 1998). The study found that captan, applied at agriculture-relevant concentrations, caused negative effects on nitrifying bacteria and aerobic diazotrophs, and had a potential for disturbing microbial equilibrium. These findings support a conclusion on ineligibility of thiophanate-methyl and captan for soil-borne disease control. It is interesting to point out that the environmentally-friendly tee tree oil formulation demonstrated efficacy that is comparable to the fungicides captan and thiophanate-methyl. Environmental conservation and research of potentials of this essential oil in disease and pest control has been at the focus of a number of researchers (Pavela et al., 2019, 2020; Mihajlović et al., 2020; Raveau et al., 2020; Tanović et al., 2020; Žabka et al., 2021). Even though assessment of toxic effects of various plant essential oils for earthworms has been recorded, the same information for tea tree oil is not available, neither in relevant literature, nor in regulatory documentation (EFSA, 2012). The tea tree oil product used in our study contained terpinen-4-ol (48%), γ-terpinene (28%) and α-terpinene (13%), while 10 other substances were present in lower shares (<10%). Some biologically active substances, such as γ-terpinene, have been detected in essential oils of various aromatic plants (Benelli et al., 2018, 2019; Pavela et al., 2019, 2020). Toxicological assessment of essential oils extracted from *Cuminum cyminum* (Benelli et al., 2018) and *Satureja sahendica* (Pavela et al., 2020) for the eartworm *Eisenia fetida* revealed no adverse effects (LD-50 > 100 mg/kg soil). Since tea tree oil and extracts from *C. cyminum* and *S. sahendica* have similar contents, i.e. all have high γ-terpinene and share six other components (alpha-terpinene, p-cymene, alpha-pineneterpinen-4-ol, alpha-terpineol), the results of Benelli et al. (2018) and Pavela et al. (2020) were considered as being representative for tea tree oil in this study. A similar aproach in soil microorganism risk assessment, based on the effects of leading tea tree oil components (terpinen, limonene and α-pinen), was also used for regulatory purposes (EFSA, 2012). The other ecologically-friendy product tested in our study, *B. subtilis*, demonstrated low efficacy (5%) in the control of *S. sclerotiorum* and, consequently, is not recommended as a promissing control option.

Our study demonstrated three highly efficient and ecologically acceptable fungicides for *Sclerotinia* disease control. Taking into consideration that disease management cannot rely on the application of a single compound, further investigation should focus on a more integrated approach, using the potential of combined applications of synthetic and eco-friendly compounds, as well as application of some other soil amendments, such as composts and compost extracts or measures like crop rotation and green manure application. It would ensure that efficacy is maintained at a high level, while disturbance of soil communities and amounts of pesticides in the environment could be significantly reduced.

ACKNOWLEDGEMENT

The study was funded by the Ministry of Science, Technological Development and Innovation (contract 451-03-9/2021-14/200214).

REFERENCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, *18*(2), 265-267.

Arora, S., & Sahni, D. (2016). Pesticides effect on soil microbial ecology and enzyme activity – An overview. *Journal of Applied and Natural Sciences*, *8*(2), 1126-1132.

Bart, S., Pelosi, C., Barraud, A., Péry, A.R.R., Cheviron, N., Grondin, V. ... Crouzet, O. (2019). Earthworms mitigate pesticide effects on soil microbial activities. *Frontiers in Microbiology 10*, 1535.

Benelli, G., Pavela, R, Maggi, F., Wandjou, J.G.N., Fofie, G.B.Y., Koné-Bamba, D. ... Caprioli, G. (2019). Insecticidal activity of the essential oil and polar extract from *Ocimum gratissimum* grown in Ivory Coast: Efficacy on insect pests and vectors and impact on non-target species. *Industrial Crops and Products, 132*, 377-385.

Benelli, G., Pavela, R., Petrelli, R., Cappellacci, L., Canale, A., Senthil-Nathan, S., & Maggi, F., (2018). Not just popular spices! Essential oils from *Cuminum cyminum* and *Pimpinella anisum* are toxic to insect pests and vectors without affecting non-target invertebrates. *Industrial Crops and Products, 124*, 236-243.

Benigni, M., & Bompeix, G. (2010). Chemical and biological control of *Sclerotinia sclerotiorum* in witloof chicory culture. *Pest Management Science, 66*(12), 1332-1336.

Boland, G.J., & Hall, R. (1994). Index of plant hosts of *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology, 16*(2), 93-108.

Bounatirou, S., Smiti, S., Miguel, M.G., Faleiro, L., Rejeb, M.N., Neffati, M. ... Pedro, L.G. (2007). Chemical composition, antioxidant and antibacterial activities of the essential oils isolated from Tunisian *Thymus capitatus* Hoff. et Link. *Food Chemistry, 105*(1), 146-155.

Bradley, C.A., Lamey, H.A., Endres, G.J., Henson, R.A., Hanson, B.K., McKay, R. ... Porter, P.M., (2006). Efficacy of fungicides for control of Sclerotinia stem rot of canola. *Plant Disease, 90*(9), 1129-1134.

Broadbent, P., Baker, K. F., Franks, N., & Holland, J. (1977). Effect of *Bacillus* spp. on increased growth of seedlings in steamed and in nontreated soil. *Phytopathology, 67*, 1027-1034.

Budge, S.P., & Whipps, J.M. (2001). Potential for integrated control of *Sclerotinia sclerotiorum* in glasshouse lettuce using *Coniothyrium minitans* and reduced fungicide application. *Phytopathology, 91*(2), 221-227.

Carson, C.F., Hammer, K.A., & Riley, T.V. (2006). *Melaleuca alternifolia* (tea tree) oil: a review of antimicrobial and other medicinal properties. *Clinical Microbiology Reviews Journal 19*, 50-62.

Coley-Smith, J.R., & Cooke, R.C. (1971). Survival and germination of fungal sclerotia. *Annual Review of Phytopathology, 9*, 65-92.

D'Ercole, N., Nipoti, P., Di Pillo, L., & Gavina, F. (2000). *In vitro* and *in vivo* tests of *Trichoderma* spp. as a biocontrol agent of *Verticillium dahliae* Kleb. in eggplants. In: Tjamos, E. C., Rowe, R. C., Heale, J. B., Fravel, D. R. (Eds.), *Advances in Verticillium research and disease management* (pp 260-263). St. Paul, MN, USA:. APS Press.

Dhingra, O.D., & Sinclair, J.B. (1995). *Basic plant pathology methods*. Boca Raton, FL, USA: CRC Press.

Edris, A.E., & Farrag, E.S. (2003). Antifungal activity of peppermint and sweet basil essential oils and their major aroma constituents on some plant pathogenic fungi from the vapor phase. *Food/Nahrung, 47*(2), 117-121.

EFSA (2010). Conclusion on the peer review of the pesticide risk assessment of the active substance dazomet. *EFSA Journal, 8*(10), 1833.

EFSA (2012). Conclusion on the peer review of the pesticide risk assessment of the active substance extract from tea tree. *EFSA Journal, 10*(2), 2542.

EPPO (1997). Soil fungi attacking ornamental plants – PP 1/40(2). In: *EPPO Standards - Guidelines for the efficacy evaluation of plant protection products* (pp 62-66). Paris, France, OEPP/EPPO.

Finney, D.J. (1971). *Probit analysis: a statistical treatment of the sigmoid response curve* (3rd ed.). Cambridge, UK_ Cambridge University Press.

Fließbach, A., & Mäder, P. (2004). Short- and long-term effects on soil microorganisms of two potato pesticide spraying sequences with either glufosinate or dinoseb as defoliants. *Biology and Fertility of Soils, 40*, 268-276.

Hu, S., Zhang, J., Zhang, Y., He, S., & Zhu, F. (2018). Baseline sensitivity and toxic actions of boscalid against *Sclerotinia sclerotiorum*. *Crop Protection, 110*, 83-90.

Huang, X.P., Luo, J., Li, B.X., Song, Y.F., Mu, W., & Liu, F. (2019). Bioactivity, physiological characteristics and efficacy of the SDHI fungicide pydiflumetofen against *Sclerotinia sclerotiorum*. *Pesticide Biochemistry and Physiology, 160*, 70-78.

Joshi, D., Kumar, S., & Kumar, M. (2021). Changes in soil microbial population dynamics in response to application of selected pesticides under a sugarcane agro-ecosystem. *Journal of Eco-Friendly Agriculture, 16*(2), 28.

Kalemba, D.A.A.K., & Kunicka, A. (2003). Antibacterial and antifungal properties of essential oils. *Current Medicinal Chemistry, 10*(10), 813-829.

Kedia, A., Prakash, B., Mishra, P.K., Singh, P., & Dubey, N.K. (2015). Botanicals as eco friendly biorational alternatives of synthetic pesticides against *Callosobruchus* spp. (Coleoptera: Bruchidae) – a review. *Journal of Food Science and Technology, 52*(3), 1239-1257.

Kim, P.I, & Chung, K.C. (2004). Production of an antifungal protein for control of *Colletotrichum lagenarium* by *Bacillus amyloliquefaciens* MET0908. *FEMS Microbiology Letters, 234*(1), 177-183.

Knobloch, K., Pauli, A., Iberl, B., Weigand, H., & Weis, N. (1989). Antibacterial and antifungal properties of essential oil components. *Journal of Essential Oil Research, 1*(3), 119-128.

Leroux, P., & Gredt, M. (1972). Etude de l'action in–vitro des fongicides, methode de l'incorporation ou milieu (pp 1-10). Versailles, France: Laboratorie de Phytopharmacie–INRA.

Liu, S., Fu, L., Hai, F., Jiang, J., Che, Z., Tian, Y., & Chen, G. (2018). Sensitivity to boscalid in field isolates of *Sclerotinia sclerotiorum* from rapeseed in Henan Province, China. *Journal of Phytopathology, 166(4)*, 227-232.

Löcher, F.J., & Lorenz, G. (1991). Methods for monitoring the sensitivity of *Botrytis cinerea* to dicarboximide fungicides. *EPPO Bulletin, 21*(2), 341-354.

Markham, J.L. (1999). Biological activity of tea tree oil. In I. Southwell & R. Lowe (Eds.), *Tea tree, the genus Melaleuca* (pp. 169-190). Amsterdam, Netherlands: Harwood Academic Publishers.

Martínez-Toledo, M.V., Salmerón, V., Rodelas, B., Pozo, C., & González-López, J. (1998). Effects of the fungicide Captan on some functional groups of soil microflora. *Applied Soil Ecology, 7*(3), 245-255.

Matheron, M.E., & Porchas, M. (2004). Activity of boscalid, fenhexamid, fluazinam, fludioxonil, and vinclozolin on growth of *Sclerotinia minor* and *S. sclerotiorum* and development of lettuce drop. *Plant Disease, 88*(6), 665-668.

Matsson, M., & Hederstedt, L. (2001). The carboxin-binding site on *Paracoccus denitrificans* succinate: quinone reductase identified by mutations. *Journal of Bioenergetics and Biomembrane, 33*(2), 99-105.

Meghana, D., Ramanamma, P., Rangaswamy, V., & Jaffer Mohiddin, G. (2017). Influence of novaluron and thiophanate methyl on microbial population in Groundnut (Arachis hypogaea. L) Soils. *IJAR, 3*(4), 566-571.

Meynell, G.G., Meynell, E., Mekler, L.B., Kriviskij, A.S., & Urbah, V.J. (1967). *Experimental microbiology*. Moscow, USSR: Mir. (In Russian)

Mihajlović, M., Rekanović, E., Hrustić, J., Grahovac, M., Stevanović, M., & Tanović, B. (2020). Effects of fungicides and biofungicides on *Rhizoctonia solani*, a pathogen of pepper. *Pesticides and Phytomedicine, 35*(2), 97-104.

Mueller, D.S., Dorrance, A.E., Derksen, R.C., Ozkan, E., Kurle, J.E., Grau, C.R. ... Pedersen, W.L., 2002. Efficacy of fungicides on *Sclerotinia sclerotiorum* and their potential for control of Sclerotinia stem rot on soybean. *Plant Disease, 86*, 26-31.

Pavela, R., Benelli, G., Pavoni, L., Bonacucina, G., Cespi, M., Cianfaglione, K. ... Maggi, F. (2019). Microemulsions for delivery of Apiaceae essential oils – Towards highly effective and eco-friendly mosquito larvacides? *Industrial Crops and Products, 129*, 631-640.

Pavela, R., Morshedloo, M. R., Mumivand, H., Khorsand, G. J., Karami, A., Maggi, F. ... Benelli, G. (2020). Phenolic monoterpen-rich essential oils from Apiaceae and Lamiaceae species: insecticidal activity and safety evaluation on non-target earthworms. *Entomologia Generalis, 40*(4), 421-435.

Purdy, L.H. (1979). *Sclerotinia sclerotiorum*: history, diseases and symptomatology, host range, geographic distribution, and impact. *Phytopathology, 69*(8): 875-880.

Raveau, R., Fontaine, J., & Lounés-Hadj Sahraoui, A. (2020). Essential oils as potential alternative biocontrol products against plant pathogens and weeds: A review. *Foods 9*(3), 365.

Sánchez-Bayo, F. (2011). Impacts of agricultural pesticides on terrestrial ecosystems. In: Sánchez-Bayo, F., Van den Brink, P. J., Mann, R. M. (Eds.), *Ecological impacts of toxic chemicals* (pp 63-87). USA: Bentham Science Publishers.

Santísima-Trinidad, A.B.L., del Mar Montiel-Rozas, M., Diéz-Rojo, M.A., Pascual, J.A., & Ros, M. (2018). Impact of foliar fungicides on target and non-target soil microbial communities in cucumber crops. *Ecotoxicology and Environmental Safety, 166*, 78-85.

Singh, M., Sharma, O.P., & Bhagat, S. (2014). Compability of promising *Trichoderma* spp. with pesticides. *Pesticide Research Journal, 26*(2), 217-220.

Soylu, S., Yigitbas, H., Soylu, E. M., & Kurt, Ş. (2007). Antifungal effects of essential oils from oregano and fennel on *Sclerotinia sclerotiorum*. *Journal of Applied Microbiology, 103*(4), 1021-1030.

Swiader, M., Pronczuk, M., & Niemirowicz-Szczyt, K. (2002). Resistance of Polish lines and hybrids of watermelon [*Citrullus lanatus* (Th unb.) Matsum et Nakai] to *Fusarium oxysporum* at the seedling stage. *Journal of Applied Genetics, 43*(2), 161-170.

Tanović, B., Hrustić, J., Mihajlović, M., Grahovac, M., Stevanović, M., & Gašić, S. (2020). Effects of developed thyme and oregano essential oil formulations on *Monilinia laxa* and *Monilinia fructicola*. *Pesticides and Phytomedicine, 35*(1), 49-56.

Tejada, M., Gómez, I., García-Martínez, A.M., Osta, P., & Parrado, J. (2011). Effects of prochloraz fungicide on soil enzymatic activities and bacterial communities. *Ecotoxicology and Environmental Safety, 74*(6), 1708-1714.

United Nations Environment Programme (UNEP) Ozone Secretariat (2006). Handbook for the Montreal protocol on substances that deplete the ozone layer (3th Edition). UNEP/Earthprint.

Waterhouse G.M., &.Waterston J.M., (1966). *Phytophthora cactorum*. Descriptions of pathogenic fungi and bacteria No. 111. Wallingford, UK: CAB International.

White, T.J., Bruns, T., Lee, S.J.W.T., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: A guide to methods and applications, 18*(1), 315-322.

Xiong, D., Li, Y., Xiong, Y., Li, X., Xiao, Y., Qui, Z., & Xiao, Y. (2014). Influnce of boscalid on the activities of soil enzymes and soil respiration. *European Journal of Soil Biology 61*(4), 1-5.

Yang, D., Wang, B., Wang, J., Chen, Y., & Zhou, M. (2009). Activity and efficacy of *Bacillus subtilis* strain NJ-18 against rice sheath blight and Sclerotinia stem rot of rape. *Biological Control, 51*(1), 61-65.

Žabka, M., Pavela, R., Kovařiková, K., Tříska, J., Vrchotová, N., & Bednář, J. (2021). Antifungal and insecticidal potential of the essential oil from *Ocimum sanctum* L. against dangerous fungal and insect species and its safety for non-target useful soil species *Eisenia fetida* (Savihny, 1826). *Plants, 10*(10), 2180.

Zhang, Y., Xu, J., Dong, F., Liu, X., Wu, X., & Zheng, Y. (2014). Response of microbial community to a new fungicide fluopyram in the silty-loam agricultural soil. *Ecotoxicology and Environmental Safety, 108*, 273-280.

Da li je moguće efikasno suzbijanje *Sclerotinia sclerotiorum* bez narušavanja mikrobiološke ravnoteže u zemljištu?

REZIME

Sclerotinia sclerotiorum je kosmopolitski, polifagni patogen, koji parazitira preko 400 biljnih vrsta. U radu je ispitana osetljivost izolata *S. sclerotiorum* na nekoliko komercijalnih fungicida i biofungicida u laboratorijskim i uslovima *in vivo*. Najviša efikasnost je zabeležena u tretmanu boskalidom (98%) i fluopiramom (80%), a najniža u varijanti sa biološkim preparatom na bazi antagonističke bakterije *B. subtilis* (5%). U uslovima *in vitro*, izolat *S. sclerotiorum* je ispoljio osetljivost na sve testirane fungicide. Fluopiram (EC_{50}=0.003 mg/l) je bio najtoksičniji za odabrani izolat, od svih preparata korišćenih u istraživanju, dok je kaptan (EC_{50}=8.94mg/l) ispoljio najnižu toksičnost među sintetičkim fungicidima. U tretmanima u kojima su primenjivani biološki preparati na bazi ulja čajnog drveta i *B. subtilis* zabeleženo je najslabije dejstvo na patogena *in vitro*. Još jedan od aspekata koji je proučavan u radu, bio je uticaj odabranih fungicida i biofungicida na životnu sredinu. Modelovanje očekivanih koncentracija u zemljištu (PEC soil), uz literaturne podatke o toksičnosti, korišćeni su u proceni rizika od pesticida za organizme u zemljištu. Visok dugotrajan rizik za kišne gliste utvrđen je kod izloženosti kaptanu i tiofanat-metilu. Na osnovu rezultata efikasnosti i procene rizika utvrđeno je da fluopiram ima najbolja svojstva od svih ispitivanih konvencionalnih pesticida, dok su kod biopesticida na bazi ulja čajnog drveta utvrđena bolja svojstva u odnosu na preparat koji sadrži sporogenu bakteriju *B. subtilis*. Dalja ispitivanja efekata kombinovane primene konvencionalnih preparata i biopesticida daće smernice za efikasnije suzbijanje *S. sclerotiorum*, uz smanjenje negativnih efekata na životnu sredinu i neciljne organizme.

Ključne reči: zemljišni patogeni, fungicidi, biološko suzbijanje, antagonističko delovanje, procena rizika za zemljište