

Effects of fungicides and biofungicides on *Rhizoctonia solani*, a pathogen of pepper

Milica Mihajlović¹, Emil Rekanović¹, Jovana Hrustić¹, Mila Grahovac²,
Marija Stevanović¹ and Brankica Tanović¹

¹*Institute of Pesticides and Environmental Protection, Banatska 31b, Belgrade 11000, Serbia*

²*University of Novi Sad, Faculty of Agriculture, Trg Dositeja Obradovića 8, Novi Sad 21000, Serbia*

*Corresponding author: diplagronom@gmail.com

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SUMMARY

In vitro and *in vivo* sensitivity of *Rhizoctonia solani* to several commercial fungicides and biofungicides was studied. An isolate of *R. solani*, derived from diseased pepper plants originating from a greenhouse in Knjaževac, Serbia, was used. The highest efficacy in *R. solani* control under greenhouse conditions was achieved by iprodione (95.80%, compared to control), although differences in the effectiveness of iprodione, tea tree oil, azoxystrobin and thiophanate-methyl were not statistically significant. The isolate was sensitive to all tested products *in vitro*. The obtained EC₅₀s were: 0.43 mg/l for iprodione, 1.84 mg/l for thiophanate-methyl, 13.84 mg/l for prochloraz, 430.37 mg/l for fluopyram, 596.60 mg/l for azoxystrobin, and 496.79 mg/l for tea tree oil.

Keywords: *Rhizoctonia solani*, pepper, fungicides, biofungicides, sensitivity, efficacy

INTRODUCTION

Agricultural production in Serbia is central to the country's economy. It is also considered to be an engine for rural development. According to the 2012 Serbian Agriculture Census, solanaceous vegetable crops (potato, tomato, pepper and eggplant) were grown on more than 35 000 ha in that year with pepper, grown on 7 661 ha, being the most important in economic terms. Pepper production is severely affected by soil-borne disease outbreaks that occur almost every year both in open fields and in protected cultivation. One of

the most important soil-borne diseases is *Rhizoctonia* root and collar rot, caused by *Rhizoctonia solani* Kiihn species complex (teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk 1956 (Ogoshi, 1987)). Under favorable weather conditions (prolonged periods in cool, moist conditions), this pathogen causes root and crown rot, aerial blight of leaves, stems and fruit of pepper plants, as well as damping-off of seedlings. For instance, Rini and Sulochana (2006) reported damping-off of 33.2 and 40.2 % of pepper seedlings in protected and open field cultivation, respectively. Even though diseased plants often produce an abundance of

secondary roots above rotted taproot, wilting and death of plants scattered throughout a field are the most eye-catching above-ground symptoms of Rhizoctonia root rot (Coa et al., 2004). Additionally, the fungus has a tremendous capacity for saprotrophic growth and can survive in soil indefinitely in the absence of a host plant.

Similar to other soil-borne diseases, Rhizoctonia root rot is often more difficult to control than diseases that affect above-ground plant parts because they are very difficult to detect and identify before serious damage has already occurred (Pritchard, 2011). To control serious disease outbreaks, conventional synthetic fungicides and fumigants are applied at regular intervals before crop establishment or throughout the growing season. However, there are evident issues with synthetic fungicides, including ecological disturbance, human health hazard, damage to aquatic ecosystems, reduction of beneficial microorganisms in soil and even ozone layer depletion (UNEP, 1994). With increasing environmental constraints, alternatives to broad-spectrum fungicides and fumigants are being developed and put into use. However, these alternative disease-management methods either have inconsistent results (Gerik & Hanson, 2011) or show less effective results than the phased-out methyl bromide that had been used for decades. Biological control by using antagonists is an ecofriendly and promising alternative to the use of chemicals. The application of *Bacillus* species, including *Bacillus subtilis*, is an example of biocontrol agents with antagonistic effects on plant pathogens coupled with plant growth promoting ability (Ryder et al., 1998; Whipps & Lumsden, 2001). Likewise, essential oils have been investigated for their wide spectrum of antimicrobial activity. Tea tree oil has a long history of use as a topical microbicide in human pharmacology (Markham, 1999; Carson et al., 2006).

In Serbia, only a few products have been registered for the control of soil-borne pathogens. However, none have been registered for the control of *R. solani* in pepper production during the growing season, although some fungicides in the dicarboximide, benzimidazole and triazole groups have been recommended (Ivanović & Ivanović, 2007; Mijatović et al., 2007).

The objective of this study was to examine the possibility of Rhizoctonia root rot control in pepper by using conventional fungicides and biofungicides based on tea tree essential oil and *B. subtilis*. *In vitro* sensitivity

tests were conducted in order to determine if there is any correlation between the efficacy of pepper protection under greenhouse conditions and the sensitivity of a *R. solani* isolate to conventional fungicides and tea tree oil.

MATERIAL AND METHODS

R. solani isolate

A *R. solani* isolate, derived from infected pepper plants from Knjaževac in Serbia, was chosen for a study that used a method described by Dhingra and Sinclair (1995). The isolate was purified by the single hyphal tip method (Narayanasamy, 2001), identified based on morphological characteristics and maintained on slants at 5°C in the Culture Collection of the Department for Phytomedicine and Environmental Protection, University of Novi Sad, Serbia. Prior to greenhouse experiments, the identity of the isolate was confirmed based on morphological macroscopic and microscopic traits, and by sequencing of an amplicon obtained by polymerase chain reaction (PCR) using universal primer pair ITS1/ITS4 (White et al., 1990). Colony appearance and the presence of sclerotia were observed in 10-day old colonies growing on PDA (Sharma et al., 2005), while the hyphal branching pattern was observed microscopically using a compound microscope (Olympus, Japan).

Fungicides and biofungicides

Commercial formulations of thiophanate-methyl (Funomil, 700 g/kg, WP, Agromarket), iprodione (Kidan 250 SC, 255 g/l Bayer CropScience), prochloraz (Spartak 450-EC, 450 g/l, EC, Sinochem Ningbo), fluopyram (Luna Privilege, 500 g/l, SC, Bayer CropScience), azoxystrobin (Quadris, 250 g/kg, SC Syngenta), tea tree oil (*Stockton, Israel*) and *Bacillus subtilis* (Bisolbi Inter, Russia) were used in this study.

Greenhouse potting experiment

The inoculum of *R. solani* for a potting experiment was prepared by transferring a mycelial disk, cut from the edge of a 5-day-old colony, into a 500 ml glass bottle containing 150 g sterilized barley grains, and incubated at 25°C for 21 days. Then the inoculum was mixed

thoroughly with sterile growth substrate (Floragard®, Germany) at the rate of 5% (Gaskill, 1968). Five-week-old pepper plants (cv. Novosadska babura) were transplanted into 10 cm × 5 cm pots filled with 400 ml of inoculated growth substrate and 60 ml of each fungicide/biofungicide was added to pots at label rate. Plants inoculated and watered with 60 ml of sterile distilled water served as positive control (K-1). Uninoculated pepper plants, watered with 60 ml sterile distilled water, served as negative control (K-2). The pots were kept in a greenhouse (24±2°C) and watered regularly until final evaluation. The degree of wilting was observed daily with a final evaluation performed 22 days after inoculation by visual observation of symptoms and by measuring the height and fresh weight of plants. Infection was rated based on a 0-5 scale, where 0 = no symptoms, 1 = chlorosis of lower 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. Disease severity (infection degree, ID) was calculated using the Townsend and Heuberger formula (Swiader et al., 2002):

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

where: n = degree of infection rated on a scale of 1-5, 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. Data were analyzed separately for each trial using ANOVA and the means were separated by Duncan's multiple range test.

***In vitro* sensitivity tests**

Sensitivity of the isolate *in vitro* was determined in a radial growth assay on PDA medium as 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 0.10 to 1000 mg/l of active ingredient (a.i.), the following final concentrations of the fungicides in medium were used: thiophanate-methyl 0.6, 1.25, 2.5, 5 and 10 mg/l; iprodione 0.3, 0.6, 1.25, 2.5 and 5 mg/l; prochloraz 1.56, 3.12, 6.25, 12.5, and 25 mg/l; fluopyram 250, 350, 500, 700 and 1000 mg/l; azoxystrobin 10, 100,

500, and 1000 mg/l; tea tree oil 62.5, 125, 250, 500 and 1000 mg/l. Each fungicide-amended medium was made by adding a fungicide from appropriate dilution series prepared in sterile distilled water to the molten PDA medium (50°C). In the fungicide-free control media, sterile distilled water was added instead of fungicide dilutions. In order to inhibit an alternative respiratory pathway in the fungus that can interfere with the activity of azoxystrobin *in vitro* (Wood & Hollomon, 2003), salicylhydroxamic acid (SHAM), dissolved in ethanol, was added to azoxystrobin-amended and azoxystrobin-free media at a previously determined nontoxic concentration of 0.1 mg/l.

Mycelial plugs (3 mm diameter) were cut from the edge of 5-day-old *R. solani* culture grown on PDA medium at 25°C and used for inoculation of the fungicide-amended and fungicide-free media. The experiment was conducted in three independent replications using two petri dishes containing three mycelial plugs each, per replicate. After incubation for four days at 25°C, mycelial growth was measured. The growth on fungicide-amended media was presented as the percentage compared to the control. Since experimental conditions were identical in all replications, the obtained data were pulled together and fungicide concentrations that inhibited mycelial growth by 50% (EC50) and regression coefficients (b), expressing relative fungicide toxicity, were determined using probit analysis (Finney, 1971).

RESULTS

The isolate

The chosen isolate formed a round, fast-growing colony which was white as young and became partially brown colored with age (Figure 1). After 7-10 day incubation, small (0.5-1 mm in diameter), initially creamy and then light-brown superficial or partly immersed sclerotia were formed. The isolate exhibited a typical *Rhizoctonia*-like branching pattern with multinucleate hyphal cells able to anastomose with each other. The observed morphological features confirmed that the isolate belonged to *Rhizoctonia solani* species complex as it had been previously determined. This was also confirmed by the sequence of approx. 700 bp amplicon, obtained by using the universal primer pair ITS1/ITS4.



Figure 1. Colony morphology of *R. solani* (surface and reverse) on PDA medium

Greenhouse experiment

Table 1 summarizes the results of the disease severity and efficacy of the products applied after inoculation of pepper plants with *R. solani*. Under greenhouse conditions, the highest efficacy in *R. solani* control was achieved by iprodione (95.80% compared to control), although differences in disease severity between treatments with iprodione, tea tree oil, azoxystrobin and thiophanate-methyl were not statistically significant. Among the tested products, the lowest efficacy of 47.4% was achieved by fluopyram and the *B. subtilis*-based product.

Table 2 summarizes the effects of tested products on the height and fresh weight of pepper plants

inoculated prior to product application. Maximum height was achieved by plants treated with iprodione (5.96 cm), although the difference from treatments with *B. subtilis*, fluopyram and prochloraz was not statistically significant. The lowest plant height (3.40 cm) was observed after treatment with the tea tree oil product. Similarly, maximum fresh weight was recorded in plants treated with iprodione (1.56 g), while the other treatments were not statistically different from the inoculated untreated control (11.34 g).

A moderate positive correlation between fungicide efficacy and plant height ($r = 0.60$), and a weak positive correlation between fungicide efficacy and plant fresh weight ($r = 0.34$) were found.

Table 1. Rhizoctonia disease severity and treatment efficacy on pepper plants 22 days after treatments with fungicides and biocontrol agents

Fungicide/biofungicide	Rate (%)	Disease severity(%)		Efficacy (%)
		Ms	Sd	
Tee tree oil	1.00	9.00 a	10.20	90.50
<i>B. subtilis</i>	1.00	50.00 c	17.70	47.40
Thiophanate-methyl	0.10	15.00 ab	13.70	84.20
Iprodion	0.30	4.00 a	5.50	95.80
Fluopyram	0.10	50.00 c	17.70	47.40
Azoxystrobin	0.075	10.00 a	13.70	89.50
Prochloraz	0.08	32.00 b	17.50	66.30
K-1	-	95.00 d	11.20	0.00
K-2	-	0.00 a	0.00	100.00

LSD₀₀₅ = 17.27

LSD₀₀₁ = 23.14

Table 2. Height (cm) and fresh weight (g) of pepper plants 22 days after treatments with fungicides and biofungicides

Fungicide/biofungicide	Rate (%)	Height (cm)		Fresh weight (g)	
		Ms	Sd	Ms	Sd
Tee tree oil	1.00	3.40 bcd	0.70	1.04 ab	0.40
<i>B. subtilis</i>	1.00	4.30 de	1.20	1.00 ab	0.40
Thiophanate-methyl	0.10	3.48 cd	2.10	1.04 ab	0.30
Iprodion	0.30	5.96 e	1.30	1.56 b	0.40
Fluopyram	0.10	4.24 de	1.10	1.22 ab	0.60
Azoxystrobin	0.075	3.70 d	1.20	0.96 ab	0.50
Prochloraz	0.08	4.10 de	1.40	1.10 ab	0.80
K-1	-	1.40 a	2.20	0.48 a	0.20
K-2	-	10.50 f	1.90	14.24 c	1.28

LSD₀₀₅ = 1.93 LSD₀₀₁ = 2.59 LSD₀₀₅ = 0.82 LSD₀₀₁ = 1.10

In vitro tests

Sensitivity of the *R. solani* isolate to the tested fungicides and tea tree oil *in vitro* is presented in Table 3. The EC₅₀ value of iprodione (0.43 mg/l) was the lowest compared to the other tested fungicides. The isolate was capable to grow well at 0.3 mg/l, while severe inhibition was observed at 1.25 mg/l and higher concentrations of

iprodione. Azoxystrobin and fluopyram exhibited the lowest toxicity of all conventional fungicides; their EC₅₀ values were 596.60 mg/l and 430.37 mg/l, respectively. The tea tree oil product severely inhibited isolate growth at 1000 mg/l, while its inhibitory effect was significantly weaker at the lower studied concentrations. The EC₅₀ value of tea tree oil was 496.79 mg/l.

Table 3. *In vitro* sensitivity of *R. solani* to fungicides and tea tree oil

Fungicide	EC ₅₀ (mg/l)*		b**	
	Value	Range	Value	Range
Tea tree oil	496.79	371.19-742.20	1.15	1.00-1.30
Prochloraz	13.84	10.58-19.98	1.09	0.94-1.24
Fluopyram	430.37	301.92-555.57	1.03	0.76-1.30
Azoxystrobin	596.60	308.81-790.56	0.47	0.38-0.56
Iprodione	0.43	0.35-0.51	2.07	1.89-2.25
Thiophanate-methyl	1.84	1.57-2.18	1.91	1.70-2.12

*Concentration that inhibited mycelial growth by 50% (EC₅₀); ** Regression coefficient

DISCUSSION

The results of the present study provide new information on the efficacy of the Quinone outside Inhibitor (QoI) azoxystrobin in the control of

Rhizoctonia root rot. In our experiments under greenhouse conditions, azoxystrobin provided a significant reduction in disease severity of 89.50% compared to the control. However, it did not exhibit high toxicity to the same *R. solani* strain in laboratory

tests, where the EC_{50} value was 596.60 mg/l despite the use of SHAM, a specific terminal oxidase inhibitor that inhibits alternative respiration pathway which had been proven to interfere with the activity of strobilurins *in vitro* (Ziogas et al. 1997). As a QoI fungicide, azoxystrobin inhibits mitochondrial respiration by blocking electron transport. It binds at the quinol outer binding site of the cytochrome b-c1 complex, where ubiquinone (coenzyme Q10) would normally bind when carrying electrons to that protein. As a consequence, ATP production in fungi is prevented (Bartlett et al., 2002). The low toxicity of azoxystrobin found in our *in vitro* experiment using PDA medium could be either due to its mode of antifungal action or to the medium influence on its toxicity despite terminal oxidase inhibition. Anyway, QoI fungicides constitute one of the most significant classes of fungicides due to their broad-spectrum activity against major groups of plant pathogenic fungi, low application rates and some yield benefits (Bartlett et al., 2002). Taking into account the results of the glasshouse experiments in which azoxystrobin was highly effective against the same *R. solani* strain, further research is needed to determine whether *in vitro* sensitivity tests conducted on growth media should be used as a reliable information source for general conclusions, at least for the combination QoI fungicides-*R. solani*.

In our present study, iprodione was highly effective against *R. solani* on inoculated pepper plants (95.8% compared to control). It was also highly toxic to the *R. solani* isolate *in vitro* ($EC_{50} = 0.43$ mg/l), suggesting that it could be effectively used against this pathogen. Csinos and Stephenson (1999) reported that iprodione showed good *in vitro* activity against *R. solani* cultures isolated from diseased tobacco plants. Furthermore, their field studies suggested that iprodione reduced damage caused by *R. solani*, and had an excellent activity in reducing lesion development in naturally-infected seed beds of tobacco (Csinos & Stephenson, 1999).

In recent years, many studies have documented problems arising from the presence of pesticide residues in the environment, food and feed. This has led to restrictions and a reduction in the availability of some chemical fungicides previously used to control plant diseases and spoilage of their products used for food. Biological control of soil-borne plant pathogens by microorganisms has gained widespread acceptance as a potential tool in optimizing agricultural productivity.

Understanding the potential use of an antagonist for biological control of a disease depends on answers to a series of questions regarding interactions of the host (crop), pathogen, and antagonist. *Bacillus* species, including *Bacillus subtilis*, are known for their antifungal properties, hence their importance in the biological control of a number of plant and animal diseases (Pandey & Palni, 1997; Ryder et al., 1998). The *B. subtilis*-based product used in our experiment was partially effective in controlling Rhizoctonia root rot.

Besides microorganisms, plant extracts and especially volatile essential oils from medicinal plants, have been reported to possess antimicrobial activity against a variety of food-borne, human and plant pathogens and pests (Isman, 2000; Kalemba & Kunicka, 2003; Burt, 2004). A wide variety of essential oils are known for antimicrobial properties and in many cases their activity is due to the presence of active monoterpene constituents. Tea tree oil has a long history of use as a topical microbicide in human pharmacology (Markham, 1999; Carson et al., 2006). The biofungicide based on tea tree oil that was tested under laboratory conditions in the current study exhibited low toxicity to the tested isolates of *R. solani* with EC_{50} values of 496.79 mg/l. On the other hand, it exhibited very high efficacy in the greenhouse experiment, 90.50%, compared to control plants.

R. solani is an important pathogen of pepper in all its growth stages from seedlings to harvest. Knowledge of the sensitivity of this pathogen to commercial fungicides and alternative natural compounds is an important tool for the success of Rhizoctonia root rot management in fields with detected presence of *R. solani*. The present study revealed high efficacy of the tea tree oil product tested (90.50%), suggesting that it could be used as a safe solution for application after the disease has been detected and the pathogen identified.

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Efekte fungicida i biofungicida na *Rhizoctonia solani* patogena paprike

REZIME

U radu je ispitivana *in vitro* i *in vivo* osetljivost *Rhizoctonia solani* na nekoliko komercijalnih fungicida i biofungicida. Izolat *R. solani* dobijen je iz obolelih biljaka paprike iz plastenika (Knjaževca, Srbija). U uslovima staklenika, najveća efikasnost utvđena je za iprodion (95,80% u poređenju sa biljkama iz kontrole), iako razlika u efikasnosti između tretmana iprodionom, etarskim uljem čajnog drveta, azoksistrobinom i tiofanat-metilom nije bila statistički značajna. Ispitivani izolat ispoljio je osetljivost na sve ispitivane fungicide i biofungicide *in vitro*. Dobijene su sledeće EC₅₀ vrednosti: 0,43 mg/l za iprodion, 1,84 mg/l za tiofanat-metil, 13,84 mg/l za prochloraz, 430,37 mg/l za fluopiram, 596,60 mg/l za azoksistrobin i 496,79 mg/l za ulje čajnog drveta.

Ključne reči: *Rhizoctonia solani*, paprika, fungicidi, biofungicidi, osetljivost, efikasnost