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Original scientific paper

# *In vitro* and *in vivo* toxicity of fungicides and biofungicides for the control of Verticillium and Fusarium wilt of pepper

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**SUMMARY**

A survey of *in vitro* and *in vivo* sensitivity of *Verticillium dahliae* and *Fusarium oxysporum* to several commercial fungicides and biofungicides was undertaken. In *in vitro* assays, the tested isolate of *V. dahliae* proved to be very sensitive to difenoconazole ($EC_{50}$ = 0.02 mg/l). However, under greenhouse conditions, the highest efficacy in *V. dahliae* control on inoculated pepper plants was recorded for a product based on thiophanate-methyl (83.10% compared to control). Among the tested fungicides, the lowest efficacy was recorded for a product based on azoxystrobin (23.10 %) with no significant difference compared to control ($p > 0.05$). In *in vitro* assays, the tested *F. oxysporum* isolate was the most sensitive to prochloraz ($EC_{50}$ = 0.07 mg/l) and the least sensitive to fluopyram ($EC_{50}$ = 1075.01 mg/l). In *in vivo* assay, the highest efficacy was achieved by products based on captan (95.60%), and the lowest by a product based on thiophanate-methyl (54.40%). Antagonistic activity of the bacterium *B. subtilis* under laboratory conditions was not satisfying. Also, the antifungal activity and spectrum of a tested product based on tee tree oil was not efficient in suppressing pepper wilting caused by *V. dahliae* and *F. oxysporum*.

**Keywords**: soil-borne plant pathogens, wilt disease, pepper, fungicides, tea tree oil, *Bacillus subtilis*

## INTRODUCTION

Pepper production is severely affected by soil-borne plant pathogens worldwide. *Verticillium* spp. and *Fusarium* spp., the causal agents of wilt disease, are among the most important pathogens in economic terms (Mijatovic et al., 2005; Fradin & Thomma, 2006). They can infect pepper plants at any stage of development, causing yellowing, wilting and shriveling of leaves, followed by stunting, bark cracking and twig or branch dieback. Eventually, diseased plants may die, leading to significant yield losses. Due to the endophytic

growth of these pathogens, as well as long persistence of their resting survival structures (microsclerotia, chlamydospores) in soil, wilt disease is very difficult to control (Alström, 2001).

Over the past several decades, soil fumigation with methyl bromide has been the primary method of controlling soil-borne diseases. However, in 1992 methyl bromide was listed as a Class I ozone-depleting substance that should be removed from the market by 2015 (Bell, 2000; UNEP, 2006). Without proper control, soil-borne diseases could increase crop losses to unpredictable levels. Although crop rotation slowly reduces inoculum density, it is not always profitable to practice it in intensive cropping. In addition, resistant pepper cultivars are not commercially available, while grafting of plants on resistant rootstock would not be cost-effective under practical conditions (Gilreath et al., 2004).

It is believed that the application of naturally-occurring and widespread substances as crop protectants could give a convenient solution. Thus, essential oils from many plants, as well as secondary metabolites of many microorganisms are well-known for their strong antimicrobial activity (Daferera et al., 2003). Tea tree essential oil has a long history of use as a topical microbicide in human pharmacology (Carson et al., 2006). Its suppressive activity against many phytopathogenic fungi, including *Aspergillus fumigatus*, *Fusarium solani*, *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus oryzae*, has also been documented (Bishop & Reagan, 1998; Inouye et al., 1998; Bowers & Locke, 2000; Inouye et al., 2000; Angelini et al., 2006). In addition, the tea tree oil-based formulated product Timorex Gold has already been registered in more than 25 countries for the control of foliar and fruit diseases in both conventional and organic farming. However, its possible use against soil-borne plant pathogens has not been tested before. On the other side, *Bacillus* species, including *Bacillus subtilis,* are successfully used for the control of numerous plant and animal diseases (Fravel, 1988; Weller, 1988; Pandey et al., 1997; Mihajlović, 2014). Not only that they produce toxins (Mukry et al., 2010) and other metabolites that reduce pathogenic organisms, but they also increase plant growth (Awais et al., 2007) and significantly improve the uptake of fertilizing elements by plants. Due to its very high plant stimulating activity, a strain known as Ch-13 of *B. subtilis* has been registered in several countries as a biofertiliser under the trade name Extrasol. Further studies, however, have shown that it is also effective against some important plant pathogens (*Fusarium*, *Helminthosporium, Alternaria, Puccinia, Phythophtora,* etc.). Nevertheless, its effects on soil-borne pathogens and particularly wilt disease causal agents remains unknown (Pertot et al., 2015).

Therefore, the aim of this study was to determine whether tea tree oil and *B. subtilis*-based products could be effectively used for suppressing wilt diseases in pepper. As no standardized method for such evaluation is available, a reference fungicide, as well as the best plant quantitative parameter(s) for disease assessment, were applied based on *in vitro* and *in vivo* experiments. Fungicides with different modes of action were chosen based on literuture data and professional experience.

## MATERIALS AND METHODS

### Isolates

*V. dahliae* and *F. oxysporum* were isolated from infected pepper plants sampled from two locations in Serbia: Padinska Skela and Smederevska Palanka, respectively, using a method described by Dhingra and Sinclair (1995). Small fragments of diseased xylem tissue were washed under running tap water for 30 minutes, surface disinfected by 2% NaClO, placed aseptically on potato dextrose agar (PDA) and incubated at 25±1°C for 7-10 days. The developed mycelia were transferred to fresh PDA medium to obtain pure cultures. The isolates were maintained on PDA slants at 5°C in the culture collection of the Institute of Pesticides and Environmental Protection, Belgrade, Serbia. Preliminary identification of the isolates was based on morphological and pathogenic characteristics, according to Waterhouse and Waterston (1964). The identity of the isolates was confirmed by amplification and sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using the primers ITS1 and ITS4 (White et al., 1990).

### Crop protection products

Crop protection products for *in vitro* and *in vivo* studies were selected based on available literature data on their modes of action and spectra of activity, on pathogens' biology, as well as the authors' practical experience. Tables 1 and 2 summarize the products used against *V. dahliae* and *F. oxysporum*, respectively.

**Table 1.** Active ingredients, trade names, producers and *in vivo* application rates of products tested in this study against *Verticillium dahliae*

| Active ingredient | Product | Producer | Concentration (%) |
|---|---|---|---|
| *B. subtilis* | Ekstrasol | Bisolbi Inter | $1\times10^7$ cfu/ml |
| Tea tree oil | Timorex Gold | Stockton | 1 |
| Thiophanate-methyl | Funomil | Agromarket | 0.1 |
| Difenoconazole | Score 250-EC | Syngenta | 0.05 |
| Fluopyram | Luna Privilege | Bayer CropScience | 0.1 |
| Azoxystrobin | Quadris | Syngenta | 0.075 |
| Prochloraz | Spartak 450-EC | Sinochem Ningbo | 0.08 |

**Table 2.** Active ingredients, trade names, producers and *in vivo* application rates of products tested in this study against *Fusarium oxysporum*

| Active ingredient | Product | Producer | Concentration (%) |
|---|---|---|---|
| *B. subtilis* | Ekstrasol | Bisolbi Inter | $1\times10^7$ cfu/ml |
| Tea tree oil | Timorex Gold | Stockton | 1 |
| Fluopyram | Luna Privilege | Bayer CropScience | 0.1 |
| Captan | Agrokaptan | Agromarket | 0.3 |
| Prochloraz | Spartak 450-EC | Sinochem Ningbo | 0.08 |
| Propiconazole | Bumper 25-EC | Magan Agrochemicals | 0.03 |
| Thiophanate-methyl | Funomil | Agromarket | 0.1 |

### *In vitro* sensitivity of the isolates studied

Sensitivity of *V. dahliae* and *F. oxysporum* isolates to fungicides and tea-tree-oil *in vitro* was determined in a radial growth assay described by Leroux and Gredt (1972). Mycelial plugs (10 mm in diameter), cut from the edge of actively growing colonies of *V. dahliae* and *F. oxysporum* isolates on PDA, were used for inoculation of fungicide-amended and fungicide-free media. Preliminary concentrations of all investigated fungicides ranged from 0.0001 to 100 mg a.i./l, while tea tree oil was applied at 100 to 5000 mg a.i./l (Löcher & Lorenz, 1991).

***V. dahlia*** – Based on preliminary studies, the following concentrations of fungicides were used in the medium: thiophanate-methyl 1.25, 2.5, 3.5, 5, and 7.5 mg/l; difenoconazole - 0.09, 0.19, 0.37, 0.75, and 1.5 mg/l; fluopyram 5000 mg/l; azoxystrobine 25, 50, 75, and 100 mg/l; prochloraz 0.01, 0.1, and 1 mg/l. The tea tree oil concentration range was 1500, 2500, 3000, and 5000 mg/l.

***F. oxysporum*** – The selected concentrations of thiophanate-methyl were 25, 50, 75, and 100 mg/l; captan 50, 75, 100, and 250 mg/l; fluopyram 250, 500, 1000, 1500, and 2000 mg/l; propiconazole 1.56, 3.12, 6.25, 12.5, and 25 mg/l; prochloraz 0.06, 0.125, 0.25, 0.5, 1 mg/l, and tea tree oil 125. 250, 500, and 1000 mg/l.

Instead of fungicide dispersion, control plates were amended with the same amount of sterile distilled water. Three replicates per each fungicide concentration and each isolate were used. After 7-15 days of incubation at 25°C in the dark, the mean colony diameter of each isolate was measured and growth inhibition (PI [%]) was calculated using the formula below:

$$PI\ (\%) = [(a - b)/a] \times 100$$

where a = the mean colony diameter of control plates, and b = the mean colony diameter of fungicide-amended plates. The median effective concentration value ($EC_{50}$, fungicide concentration which inhibited mycelial growth by 50%) was determined for each isolate by probit analysis (Finney, 1971).

Azoxystrobin sensitivity of the *V. dahlia* isolate was determined as described above with a slight modification. In order to inhibit an alternative respiratory pathway, salicylhydroxamic acid (SHAM) (Sigma-Aldrich, Saint Louis, MO) was added at the concentration of 0.1 mg/l into both fungicide-amended and fungicide-free media (Ziogas et al., 1997).

### *In vitro* antagonistic activity assay

Fungal pathogens, *V. dahliae* and *F. oxysporum* isolates grown in potato-dextrose broth (PDB) at 24°C for 48 h and homogenized on magnetic stirrer, were used as an inoculum source.

The antagonistic microorganism *B. subtilis* strain Ch-13 was isolated from the commercial product Ekstrasol (Bisolbi Inter, Russia) by plating and grown in submersed culture in Erlenmeyer flasks on the shaker (200 rpm) at 28°C for four days on Meynell media containing: 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). After cultivation, a sample of the cultivation medium was centrifuged at 10 000 g for 10 min and the supernatant was used for *in vitro* antagonistic activity assay.

Two-layer-PDA medium in 90-mm petri dishes was used in the antagonistic assay. The first layer was 2% PDA, while the second layer consisted of 1.2% PDA containing previously prepared suspension of each fungal pathogen. One 10-mm well per plate was made and 100 µl of prepared antagonistic supernatant was added to each well. Antagonistic activity was tested in four replicates against each isolate. As reference treatments, 100 µl of conventional fungicide dispersions (thiophanate-methyl for *V. dahliae* and prochloraz for *F. oxysporum*) were used at the rate of 1% a.i., while 100 µl of sterile, distilled water was added to the wells in control plates. The whole experiment was repeated twice.

The assessment of antagonistic activity was performed after 48 h incubation at 25°C by visual observation of the presence of clear inhibition zones around the wells, as well as by measuring of the diameter of the whole activity zone that consisted of clear inhibition zone + a partial mycelial growth inhibition zone (mm). Since experimental conditions were identical in all replications, the obtained data were pulled together and the average values were presented.

### *In vivo* studies

***Inoculum preparation:*** A pure culture of *V. dahliae* grown on PDA at 25°C for 2 weeks was used as a source of inoculum. The medium from 10 petri dishes, each containing 15-day-old fungal cultures, was blended with 1000 ml of distilled water until complete homogenization. Pepper plants were previously dipped in the inoculum suspension for two to three minutes. The prepared amount of inoculum was used for inoculation of 20 pepper plants (D'Ercole et al., 2000). After transplanting, the plants were first watered with the remaining inoculum suspension (5 ml per plant) and then with water.

Inoculum of *F. oxysporum* was prepared by growing the isolate in glass bottles containing 150 g double sterilized barley grains at 25°C for 21 days. Then, the inoculum was mixed thoroughly with sterilized clay soil at the rate of 3% and added into pots (Hashem et al., 2010).

***Greenhouse experiment:*** Five-week-old pepper plants (cv. Novosadska babura), grown in 60-celled polystyrol trays, were transplanted into 10 cm × 5 cm pots filled with 400 ml sterile growth substrate (Floragard®, Germany). Sixty ml of each fungicide, at the rates given in Table 1 and Table 2, were added to each pot prior to inoculation, while inoculated plants (methods described previously in the chapter *Inoculum preparation*), watered with 60 ml of sterile distilled water, served as a positive control (C). Uninoculated pepper plants, watered with 60 ml sterile distilled water, served as a negative control (AC). The pots were kept in a greenhouse (24±2°C). The degree of wilting was recorded daily, while final evaluation was performed 25 (*F. oxysporum*) and 45 days (*V. dahliae*) after inoculation. Disease severity was estimated by visual observation based on a scale 0-5, 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. 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 evaluated using Townsend-Heuberger's formula (Puntner, 1981):

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

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 determined using Abbott's formula. The data were analyzed separately for each trial using ANOVA and the means were separated by Duncan's multiple range test.

In addition to visual assessment of both Verticillium and Fusarium wilt incidence, measurements of height and fresh weight of plants from the soil level to the uppermost leaf tip was performed. In case of Verticillium wilt, the length of the vascular necrotic zone on longitudinal section was also recorded. The data were processed by ANOVA and the means separated by Duncan's multiple range test.

## RESULTS

### Morphological characteristics of isolates

***V. dahliae:*** The isolates grown on PDA formed white mycelium, which later became black when microsclerotia formed (50-100 µm). Conidiophores were abundant, hyaline, verticilliately branched. Conidia were hyaline, ellipsoidal to irregularly sub-cylindrical, with an average size of 4.9 µm (2.7-7.5 µm) × 2.6 µm (2.0-3.2 µm). Based on macroscopic and microscopic characters of the isolate, it was established that they apparently belong to the species *V. dahlia*.

***F. oxysporum***: The isolate produced delicate, white to pink mycelium on PDA media. After seven days of incubation at 25°C in the absence of light, the fungus formed a colony of 7 cm in diameter. The presence of conidia on the PDA substrate was not observed. However, in the 5-day-old culture on synthetic nutrient-poor agar (SNA) media, a large number of unicellular, elyptical, oval-shaped microconidia and straight to slightly curved macroconidia with 3 septates formed. Based on the studied characteristics, it was determined that the test isolate belongs to *F. oxysporum*.

### Molecular identification of isolates

Using ITS1/ITS4 primers, PCR products of approximately 450 bp and 600 bp were noted. No amplicons occurred in negative control. BLAST analysis of the nucleotide sequence of the amplified product of 450 bp in size showed identity (was identical) with the KC156634.1 sequence of *V. dahliae*, while an analysis of nucleotide sequence of the amplified product 600 bp in size showed it was identical with the sequence of *F. oxysporum* EF495230.1.

### *In vitro* sensitivity of the studied *V. dahliae* isolate

Sensitivity of the studied isolate to test fungicides was determined based on $EC_{50}$ values (Table 3). Of all tested products, difenoconazole exhibited the greatest toxicity, severely inhibiting hyphal growth at the concentration of 0.09 mg/l. The calculated $EC_{50}$ value for mycelial growth inhibition was 0.02 mg/l. The isolate also showed high susceptibility to difenoconazole (0.002 mg/l) and prochloraz ($EC_{50}$=0.03 mg/l). On the other hand, azoxystrobin exhibited a significantly lower toxicity than the mentioned fungicides ($EC_{50}$=71.95 mg/l), whereas fluopyram was completely ineffective even at the concentration of 5000 mg/l. It was also found that all conventional fungicides were more toxic than the studied formulated tea tree oil product. The tested *V. dahlia* isolate demonstrated an ability to tolerate tee tree oil at concentrations higher than 1500 mg/l. The calculated $EC_{50}$ value for mycelial growth inhibition was 1507.65 mg/l.

### *In vitro* sensitivity of the studied *F. oxysporum* isolate

Sensitivity of the *F. oxysporum* isolate to the tested fungicides and tea tree oil is presented in Table 4. The isolate expressed very high sensitivity to prochloraz with an $EC_{50}$ value of 0.07 mg/l. The isolate was capable to grow well on the medium containing 0.06 mg/l of prochloraz, while its growth was severely inhibited at 0.25 mg/l and higher concentrations. Propiconazole was also highly toxic ($EC_{50}$=4.69 mg/l), while the toxicity of captan was moderate ($EC_{50}$= 19.14 mg/l). Tea tree oil showed the lowest toxicity to *F. oxysporum* with an $EC_{50}$ value of 1205.77 mg/l.

**Table 3.** *In vitro* sensitivity of *Verticillium dahliae* to fungicides and tea tree oil

| Fungicide | $EC_{50}$ (mg/l) | | b | |
|---|---|---|---|---|
| | Value | Range | Value | Range |
| Tee tree oil | 1507.65 | 1351.73-1681.93 | 3.46 | 3.10-3.82 |
| Prochloraz | 0.03 | 0.02-0.06 | 0.66 ±0.98 | |
| Fluopyram | >5000 | *NS | *NS | |
| Difenoconazole | 0.02 | 0.0004-0.05 | 0.51 | 0.37-0.65 |
| Azoxystrobine | 71.95 | 61.05-89.56 | 2.19 | 1.86-2.52 |
| Thiophanate-methyl | 3.48 | 3.03-3.87 | 2.72 | 2.42-3.02 |

$EC_{50}$ – fungicide concentration which inhibits mycelial growth by 50%; *95% confidence interval (p=0.05)
*NS – not specified

**Table 4.** *In vitro* sensitivity of *Fusarium oxysporum* to fungicides and tea tree oil

| Fungicide | $EC_{50}$ (mg/l) | | b | |
|---|---|---|---|---|
| | Value | Range | Value | Range |
| Tea tree oil | 1205.77 | 711.74-3470.31 | 0.71 | 0.57-0.85 |
| Prochloraz | 0.07 | 0.04-0.10 | 0.87 | 0.68-1.06 |
| Fluopyram | 1075.01 | 670.50-2321.21 | 0.56 | 0.39-0.73 |
| Propiconazole | 4.69 | 3.27-6.35 | 0.86 | 0.72-1.00 |
| Captan | 19.14 | 2.79-35.75 | 1.10 | 0.80-1.40 |
| Thiophanate-methyl | 71.95 | 61.05-89.56 | 2.19 | 1.86-2.52 |

$EC_{50}$ – Fungicide concentration which inhibits mycelial growth by 50%; *95% confidence interval (p=0.05)

**Table 5.** Fungal growth inhibition zones in treatments (%) with *Bacillus subtilis* and conventional fungicide, compared with control

| Pathogen | Inhibition zone (%) | |
|---|---|---|
| | *Bacillus subtilis* strain Ch-13 | Conventional fungicide |
| *Verticillium dahliae* | + | +++ |
| *Fusarium oxysporum* | – | +++ |

– no inhibion zone;
+ partia l inhibition of mycelial growth only;
++ clear inhibition zone > 25 mm;
+++ clear inhibition zone > 50 mm;

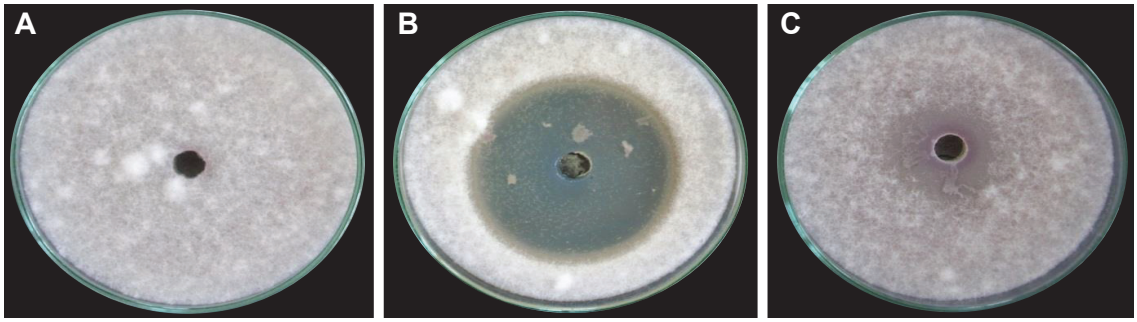


**Figure 1.** *In vitro* antagonistic activity assay of *B. subtilis* against *Fusarium oxysporum* A) Total activity zone; B) Clear inhibition zone C) Partial mycelial growth inhibition zone

### Assessment of antagonistic activity of *B. subtilis* strain Ch13 *in vitro*

Assessment of th e antagonistic activity of the bacterium *B. subtilis* strain Ch-13 was conducted after 48 h incubation at 25°C by measuring inhibition zone diameter (mm) (Table 5).

The *B. subtilis* strain Ch-13 showed higher antagonistic activity against *V. dahliae* than against *F. oxysporum* isolate. Mycelial growth of *V. dahliae* in the activity zone of *B. subtilis* was not completely inhibited, yet a zone of partial inhibition of mycelial growth with a diameter of 16 mm was observed. Low level of antagonistic activity of the *B. subtilis* strain Ch-13, again with a zone of partial inhibition of mycelial growth, was recorded for the isolate of *F. oxysporum* (70 mm). Inhibition zone in the prochloraz treatment of *F. oxysporum* was 61.7 mm, while the radius of inhibition zone in the treatment with thiophanate-methyl was 52.75 mm (Figure 1).

**Table 6.** Verticillim wilt severity and treatment efficacy on pepper plants 45 days after fungicide and biocontrol agent application

| Fungicide | Rate (%) | Disease index (%) | | Efficacy (%) |
|---|---|---|---|---|
| | | Ms[1] | Sd[2] | E[3] |
| *B. subtilis* | 1.00 | 42.00 c* | 25.10 | 35.40 |
| Tee tree oil | 1.00 | 35.00 bc | 13.70 | 46.20 |
| Thiophanate-methyl | 0.10 | 11.00 a | 8.90 | 83.10 |
| Difenoconazole | 0.05 | 19.00 ab | 8.20 | 70.80 |
| Fluopyram | 0.10 | 16.00 ab | 8.20 | 75.40 |
| Azoxystrobin | 0.075 | 50.00 cd | 25.00 | 23.10 |
| Prochloraz | 0.08 | 17.00 ab | 21.10 | 73.80 |
| Control | – | 65.00 d | 13.70 | 0.00 |
| AC[3] | – | 0.00 a | 0.00 | 100.00 |

[1]Average number of diseased plants; *Mean values in columns followed by different letters are significantly ($p<0.05$) different according to Duncan's test; [2]Standard deviation; [3]Efficacy (%). [3]AC - non-inoculated control plants

**Table 7.** Height (in cm), fresh weight (in g) and necrosis zone (in cm) of pepper plants inoculated with *Verticillium dahliae* 45 days after fungicide and biocontrol agent treatments

| Fungicide | Rate (%) | Height (cm) | | Fresh weight (g) | | Necrosis (cm) | |
|---|---|---|---|---|---|---|---|
| | | Ms[1] | Sd[2] | Ms[1] | Sd[2] | Ms[1] | Sd[2] |
| *B. subtilis* | 1.00 | 10.40 ab* | 7.52 | 2.63 a | 1.33 | 1.30 c | 0.84 |
| Tee tree oil | 1.00 | 9.00 a | 2.24 | 1.72 a | 0.68 | 2.25 d | 0.75 |
| Thiophanate-methyl | 0.10 | 24.60 de | 2.07 | 7.37 cd | 2.76 | 0.45 ab | 0.32 |
| Difenoconazole | 0.05 | 26.60 ef | 2.97 | 7.97 d | 1.46 | 0.06 ab | 0.13 |
| Fluopyram | 0.10 | 17.30 bc | 7.51 | 3.94 ab | 1.51 | 0.75 bc | 0.43 |
| Azoxystrobin | 0.075 | 11.50 b | 5.09 | 1.98 a | 0.58 | 1.25 c | 0.56 |
| Prochloraz | 0.08 | 19.00 cd | 6.16 | 5.32 bc | 3.46 | 0.45 ab | 0.32 |
| Control | – | 12.20 abc | 6.73 | 1.72 a | 1.06 | 2.30 d | 0.57 |
| AC[3] | – | 31.80 f | 1.30 | 11.34 e | 1.65 | 0.00 a | 0.00 |

[1]Average number of diseased plants; *Mean values in columns followed by different letters are significantly ($p<0.05$) different according to Duncan's test; [2]Standard deviation; [3]AC - non-inoculated control plants

## Greenhouse experiment

***V. dahliae*:** Table 6 summarizes the results of Verticillium wilt severity and the efficacy of substances applied prior to inoculation. Under greenhouse conditions, the lowest disease index was found in pepper plants treated with thiophanate-methyl (11%), corresponding to the efficacy of 83.10%, compared to the inoculated untreated control. However, the observed differences in disease index among treatments with thiophanate-methyl (11%), fluopyram (16%), prochloraz (17%), and difenoconazole (19%) was not statistically significant. In this experiment, tea tree oil- and *B. subtilis*-based products were moderately effective (46.20 and 35.40%, respectively). Among the tested products, the highest disease incidence was recorded in plants treated with azoxystrobin (23.10%). Moreover, the difference between azoxystrobin treatment and untreated control was not significant ($p > 0.05$).

Plant height, fresh weight and vascular necrotic zone length of plants treated with the investigated products are presented in Table 7. Maximum plant height was recorded in treatments with difenoconazole (26.60 cm) and thiophanate-methyl (24.60 cm), while the height of plants treated with tea tree oil and *B. subtilis* was not significantly different from the untreated control. Similarly, the highest fresh weight was recorded in treatments with difenoconazole (7.97 g) and thiophanate-methyl (7.37 g), whereas tee tree oil and azoxystrobin treatments resulted in the lowest fresh weight (1.72 g and 1.98 g, respectively).

The average length of vascular necrotic zone in inoculated untreated plants was 2.30 cm. The lowest

values of this parameter were found in treatments with difenoconazole (0.06 cm), thiophanate-methyl (0.45 cm) and prochloraz (0.45 cm), respectively. In the tea tree oil treatment, the length of necrosis was not significantly different from that in inoculated untreated plants, while a significantly lower value was found in the treatment with *B. subtilis*.

High negative correlations between disease severity and plant height (r = 0.81) and between disease severity and fresh weight of pepper plants (r = 0.84) were found. On the other hand, the length of vascular necrotic zone was in positive correlation with disease severity (r = - 0.84).

***F. oxysporum:*** Table 8 summarizes the results of Fusarium wilt severity and efficacy of the products applied prior to inoculation. Based on the disease severity observation, captan (95.60%) and prochloraz (92.20%) showed the highest efficacy. High efficacy of *B. subtilis* was also noted.

The height and fresh weight of pepper plants inoculated with *F. oxysporum* and treated with the investigated substances are presented in Table 9. As compared to control pepper plants (0.50 cm), all inoculated treatments other than propiconazole (0.84 cm) and prochloraz (1.10 cm) exhibited significantly higher values. Maximum plant height was recorded in treatments with captan (4.84 cm) and *B. subtilis* (4.46 cm). Treatments with thiophanate-methyl (0.64 g) and fluopyram (0.91 g) showed the lowest plant fresh weight, while maximum values were noted in treatments with prochloraz (2.04 g) and captan (2.03 g). Statistically significant differences were not observed in the weight of plants in all inoculated treatments, when compared with untreated uninoculated control pepper plants (14.24 g).

Correlation between disease incidence and plant height was moderate (r = 0.56), while correlation between efficacy and fresh weight of plants was weak (r = 0.43).

**Table 8.** Fusarium wilt severity and treatment efficacy on pepper plants 25 days after fungicide and biocontrol agent application

| Fungicide | Rate (%) | Disease index (%) | | Efficacy (%) |
|---|---|---|---|---|
| | | Ms [1] | Sd[2] | E[3] |
| *B. subtilis* | 1.00 | 20.00 ab* | 32.60 | 77.80 |
| Tea tree oil | 1.00 | 85.00 c | 22.40 | 5.60 |
| Fluopyram | 0.10 | 30.00 ab | 27.40 | 66.70 |
| Captan | 0.30 | 4.00 ab | 5.50 | 95.60 |
| Prochloraz | 0.08 | 7.00 ab | 11.00 | 92.20 |
| Propiconazole | 0.03 | 22.00 ab | 43.80 | 75.60 |
| Thiophanate-methyl | 0.10 | 41.00 b | 43.40 | 54.40 |
| Control | – | 90.00 c | 22.40 | 0.00 |
| AC[4] | – | 0.00 a | 0.00 | 100.00 |

[1]Average number of diseased plants; *Mean values in columns followed by different letters are significantly (p<0.05) different according to Duncan's test; [2]Standard deviation; [3]Efficacy (%); [4]AC - non-inoculated control plants

**Table 9.** Height (in cm) and fresh weight (in g) of pepper plants inoculated with *Fusarium oxysporum* 25 days after fungicide and biocontrol agent application

| Fungicide | Rate (%) | Height (cm) | | Fresh weight (g) | |
|---|---|---|---|---|---|
| | | Ms[1] | Sd[2] | Ms[1] | Sd[2] |
| *B. subtilis* | 1.00 | 4.46 cd* | 1.70 | 1.98 a | 1.18 |
| Tea tree oil | 1.00 | 1.90 ab | 1.60 | 1.06 a | 0.89 |
| Fluopyram | 0.10 | 3.74 cd | 1.30 | 0.91 a | 0.58 |
| Captan | 0.30 | 4.84 d | 1.40 | 2.03 a | 1.09 |
| Prochloraz | 0.08 | 1.10 a | 0.70 | 2.04 a | 0.76 |
| Propiconazole | 0.03 | 0.84 a | 0.70 | 1.82 a | 1.27 |
| Thiophanate-methyl | 0.10 | 2.94 bc | 1.50 | 0.64 a | 0.39 |
| Control | – | 0.50 a | 0.30 | 1.50 a | 0.91 |
| AC[3] | – | 10.50 e | 1.90 | 14.24 b | 1.28 |

[1]Average number of diseased plants; *Mean values in columns followed by different letters are significantly (p<0.05) different according to Duncan's test; [2]Standard deviation; [3]AC - non-inoculated control plants

## DISCUSSION

Data in the present study showed that the tested *V. dahliae* and *F. oxysporum* isolates were sensitive to the tested fungicides. The fungicides exhibited different levels of toxicity, and different $EC_{50}$ values indicate heterogeneity in responses to fungicides with different modes of action.

Our results provide novel information on the efficacy of the succinate dehydrogenase inhibitor (SDHI) fungicide fluopyram in controlling Verticillium and Fusarium wilt of pepper and efficacy of difenoconazole in suppressing *V. dahliae*.

Fluopyram provided moderate control against these two diseases under greenhouse conditions, causing 75.40% and 66.70% reduction, respectively. However, in the laboratory test, the isolate of *V. dahliae* did not show sensitivity to fluopyram, and it was ineffective even at the concentration of 5000 mg/l. Of all *in vitro* tested fungicides, fluopyram also exhibited the lowest toxicity to the studied *F. oxysporum* isolate with its relatively high $EC_{50}$ value of 1075.01 mg/l. Fluopyram ineffectiveness in *in vitro* studies could be attributed to its mode of action. Fluopyram is an SDHI fungicide that specifically inhibits fungal respiration by blocking the ubiquinone-binding sites in the mitochondrial complex II and plays an important role in integrated management programs for many plant diseases (Avenot & Michailides, 2010).

Difenoconazole was effective against *V. dahliae* in our experiments. This demethylation inhibitor (DMI) fungicide caused a 70.80% reduction in Verticillium wilt severity. Again, another DMI fungicide, prochloraz, showed a high toxicity to propiconazole-tested isolates of *V. dahliae* ($EC_{50}$=0.03 mg/l) and *F. oxysporum* ($EC_{50}$=0.07 mg/l) under laboratory conditions. These results appear to be in partial agreement with results published by Kurt et al. (2003) for *V. dahliae*. They showed that the mean effective concentration ($EC_{50}$) for *V. dahliae* isolates from Turkey ranged from 0.52 to 0.84 mg/l. The $EC_{50}$ values recorded in our experiments with *F. oxysporum* were similar to those reported by Amini and Sidovich (2010). They found that isolates of *F. oxysporum* f. sp. *lycopersici* were most sensitive to prochloraz in a group of fungicides used in that test (benomyl, carbendazim, fludioxonil, bromuconazole and azoxystrobine) ($EC_{50}$ = 0.005 mg/l).

In our experiment, antagonistic activity of the bacterium *B. subtilis* was generally not satisfying under laboratory conditions. The strain Ch-13 showed no inhibitory effects *in vitro* against the tested isolates. However, the efficacy of *B. subtilis* strain Ch-13 (77.80%) against *F. oxysporum* was relatively high. It could be related to the mechanisms of biological control. Besides direct antagonism to plant pathogen growth, *B. subtilis,* as a plant growth promoting rhizobacterium, can promote plant growth. It could be indirectly by reducing mobilizing nutrients in soils, producing numerous plant growth regulators that protect plants from phytopathogens by controlling or inhibiting them. Also, *B. subtilis* affects them by improving soil structure and bioremediating polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds (like pesticides) (Rajkumar et al., 2010; Braud et al., 2009). Therefore, our results show that the local strain Ch-13 of *B. subtilis* is an efficient tool to control Fusarium wilt but it was not as efficacious as the conventional chemical pesticides (captan and prochloraz >90%).

Numerous studies have demonstrated that vascular pathogens are able to activate all factors that affect plant growth rate (Yadeta & Thomma, 2013; Rekanovic et al., 2007; Tanovic et al., 2004). The present study also indicates high negative correlations between Verticillium wilt disease severity and plant height, and between disease severity and fresh weight of pepper plants. However, our experiments showed that plant height and fresh weight are not reliable parameters for evaluating fungicide efficacy in controlling *F. oxysporum*. Correlation between disease incidence and plant height was moderate, while it was weak between the efficacy and fresh weight of plants.

Also, the antifungal activity and spectrum of the tested product based on tee tree oil were not efficient in suppressing pepper wilting caused by *V. dahliae* and *F. oxysporum*. Under laboratory conditions, this biofungicide exhibited low toxicity to the tested isolates with $EC_{50}$ values of 1507 mg/l and 1205.77, respectively. In the present study, the results obtained in *in vitro* tests partially confirmed those obtained under greenhouse conditions. Considering all products tested against the *F. oxysporum* isolate, the lowest control efficacy was recorded in the treatment with tee tree oil (5.60%), while a slightly higher value was found for *V. dahliae*. The same observation was reported by Tanovic et al. (2004). They confirmed a very low antimicrobial activity of this oil against *V. dahliae* isolated from pepper and *Fusarium oxysporum* f. sp. *lycopersici*.

## CONCLUSION

A product based on thiophanate-methyl showed the best result in controlling Verticillium wilt, while azoxystrobine was the most effective against *F. oxysporum* on pepper. The activity spectrum of the

tested biofungicides was not satisfying. The results therefore reveal a risk associated with the application of these products in contemporary conventional model of pepper production. However, the high efficacy of *B. subtilis* strain Ch-13 found against *F. oxysporum* (77.80%) could provide a basis for using these substances after pathogens have been detected and identified in soil on fields intended for pepper production.

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# Efekti fungicida i biofungicida u uslovima *in vitro* i *in vivo* u suzbijanju verticilioznog i fuzarioznog uvenuća

**REZIME**

U radu je ispitivana *in vitro* i *in vivo* osetljivost izolata *Verticillium dahliae* i *Fusarium oxysporum* na nekoliko komercijalnih fungicida i biofungicida. U *in vitro* testovima izolat *V. dahliae* je ispoljio visoku osetljivost na difenokonazol ($EC_{50}$ = 0,02 mg/l). Međutim, u uslovima staklenika, najveća efikasnost na inokulisanim biljkama paprike utvrđena je kod preparata na bazi tiofanat-metila (83,10%). Među testiranim fungicidima, najniža efikasnost koja se nije statistički značajno razlikovala u poređenju sa inokulisanom i netretiranom kontrolom ($p > 0,05$), utvrđena je u tretmanu azoksistrobinom (23,10%). Prohloraz je bio najtoksičniji fungicid u laboratorijskim uslovima ispitivanja za izolat vrste *F. oxysporum*, sa vrednošću $EC_{50}$ = 0,07 mg/l. U *in vivo* ispitivanjima najveća efikasnost utvrđena je kod preparata na bazi kaptana (95,60%), a najmanja u tretmanu tiofanat-metilom (54,40%). Antagonistička aktivnost biološkog preparata na bazi bakterije *Bacillus subtilis* u laboratorijskim uslovima nije bila zadovoljavajuća. Takođe je utvrđeno da biopreparat na bazi ulja čajnog drveta nije bio efikasan u suzbijanju uvenuća paprike čiji su prouzrokovači vrste *V. dahliae* i *F. oxysporum*.

**Ključne reči**: zemljišni patogeni biljaka, uvenuće, paprika, fungicidi, ulje čajnog drveta, *Bacillus subtilis*