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Original scientific paper

In vitro sensitivity of *Alternaria solani* to conventional fungicides and a biofungicide based on tea tree essential oil

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SUMMARY

A study of *in vitro* sensitivity of five *Alternaria solani* isolates to cooper-oxychloride, chlorothalonil, difenoconazole, pyraclostrobin and a biofungicide based on tea tree essential oil was carried out. The isolates were obtained from infected tomato leaves collected from five different locations in Serbia. The tested isolates showed the highest sensitivity to pyraclostrobin with EC_{50} values ranging from 0.0014 to 0.0041 μg ml^{-1}. The EC_{50} values of difenoconazole were 0.018-0.037 μg ml^{-1}, chlorothalonil 2.99-4.54 μg ml^{-1}, and cooper-oxychloride 13.27-15.63 μg ml^{-1}. All tested *A. solani* isolates were the least sensitive to tea tree oil (1323.97-3307.08 μg l^{-1}).

Keywords: *Alternaria solani*; Fungicides; Sensitivity

INTRODUCTION

Early blight of tomato, caused by the necrotrophic fungus *Alternaria solani* Sorauer, is one of the most common foliar diseases of tomato (*Solanum lycopersicum* L.). The disease can occur over a wide range of climatic conditions but is most intense in areas with heavy dew, rainfall and high relative humidity. It causes damping-off of tomato seedlings, as well as collar rot, leaf spots, stem lesions and fruit rot in later stages. Plant infection may result in a complete loss of yield through foliage destruction and direct fruit damage by the pathogen, as well as sun blotch on defoliated plants (Rotem, 1998). Significant yield losses (35-78%) have been reported all over the world (Basu, 1974; Datar & Mayee, 1982; Jones et al., 1997).

Typical symptoms of tomato early blight are dark spots with concentric rings of spores surrounded by a halo of chlorotic area on leaves. Early blight lesions first occur on older senescing leaves, which become chlorotic and abscise prematurely, eventually spreading to other foliage in plant canopy under conditions conducive for disease development (Franc & Christ, 2001; Ganie et al., 2013). Periods of profuse moisture from rain, dew or overhead irrigation are required for spore germination and infection, while temperatures ranging from 5 to

30°C are favourable for pathogen sporulation and disease development (Gudmestad et al., 2013).

Most currently grown tomato cultivars are susceptible to early blight to varying degrees, so that foliar fungicides are frequently used to manage the disease. The most effective early blight control measure consists of frequent applications of fungicides starting early in the growing season before the first symptoms appear (Pscheidt & Stevenson, 1988).

A number of foliar fungicides can be used to manage early blight in tomato. Copper-oxychloride, mancozeb and chlorothalonil are the most frequently used protectant fungicides for early blight management but they provide insufficient control under high disease pressure (Holm et al., 2003; Pasche & Gudmestad, 2008). Quinol-oxidizing inhibitor (QoI) fungicides have been used successfully since their introduction in 1999, and provided a very high level of disease control (Stevenson & James, 1999; Pasche & Gudmestad, 2008). Unfortunately, QoI resistance due to the F129L mutation in the cytochrome b gene was first detected in North Dakota and Nebraska in 2001 (Pasche et al., 2004; Pasche et al., 2005). The F129L mutation conveys a moderate level of resistance to QoI fungicides such as azoxystrobin and pyraclostrobin. Development of resistance of pathogenic fungi towards synthetic fungicides is a great problem that may affect significantly the efficacy of chemical fungicides.

As an alternative to synthetic fungicides, and in congruence with improvements in integrated pest management, a search for natural products has become a very important task. Many researchers have examined the influence of essential oils on fungi that are plant pathogens, and fungi important in food industry, and proved that such plant compounds could provide a solution (Sitara et al., 2008; Soković et al., 2009; Tanović et al., 2009; Parveen et al., 2010; Mihajlović et al., 2013). Essential oils are plant volatiles containing monoterpenes, sesquiterpenes and phenyl propionoids. The essential oil of *Melaleuca alternifolia*, commonly known as tea tree oil, has a long history of use as a topical antiseptic (Markham, 1999). Tea tree oil is produced by steam distillation of leaves and terminal branches of *M. alternifolia* and consists largely of cyclic monoterpenes (Brophy et al., 1989), of which about 50% are oxygenated and about 50% are hydrocarbons. Tea tree oil exhibits a broad-spectrum antimicrobial activity which may be principally attributed to terpinen-4-ol (Markham, 1999, Carson et al., 2006).

The aim of this study was to evaluate *in vitro* sensitivity of *Alternaria solani* isolates originating from Serbia to several fungicides and a tea tree oil-based product.

MATERIAL AND METHODS

Isolation and identification of *Alternaria solani*

The isolates of *A. solani* were derived from symptomatic tomato leaves collected from five locations in Serbia. Fragments of infected leaf tissue were placed on potato dextrose agar (PDA) medium and incubated at 25°C for seven days to allow mycelial growth. Fragments excised from the mycelia developed were transferred onto PDA medium and purified by monospore isolation. *A. solani* cultures were identified on the basis of colony morphology and microscopic observation of conidia, and the identity of isolates was confirmed by polymerase chain reaction (PCR). The obtained isolates were stored on PDA medium at 4°C in the culture collection of the Institute of Pesticides and Environmental Protection, Belgrade.

Table 1. *Alternaria solani* isolates, origin and year of isolation

Code of isolate	Location	Year of isolation
AS-1	Kraljevo	2006
AS-2	Mladenovac	2006
AS-3	Trstenik	2007
AS-4	Gložan	2008
AS-5	Bela Crkva	2008

Fungicides

Commercial fungicide formulations were used as active ingredients (a.i.): copper-oxychloride (Cuprozin 35 WP, 350 g kg^{-1}, Spiess Urania Chemical), chlorothalonil (Bravo 720 SC, 720 g l^{-1}, Syngenta Agro), difenoconazole (Score 250 SC, 250 g l^{-1}, Syngenta Agro), pyraclostrobin (as 99.9% technical grade, BASF), tea tree oil (Timorex Gold, 23.8%, Stockton Chemical Corporation).

Pyraclostrobin was first dissolved in dimethylsulfoxide (DMSO), and then a set of stock solutions for each tested fungicide and tea tree essential oil were made using sterile distilled water. Freshly-made stock solutions were prepared to give specific concentrations of each active ingredient in μg ml^{-1}. Volumes of stock solution were added to molten (50°C) PDA medium prior to pouring, thereby producing active ingredient concentrations ranging from 0.0001 to 6000 μg ml^{-1} (Löcher & Lorenz, 1991).

In vitro fungicide sensitivity tests

A. solani isolates were grown on PDA medium amended with the fungicides: copper-oxychloride, chlorothalonil, difenoconazole, pyraclostrobin and

tea tree oil, and used in sensitivity tests. Based on preliminary data, the following concentrations were selected for further study: 3.125, 6.25, 12.5, and 25 μg ml^{-1} of copper oxychloride; 0.01, 0.1, 1, 10 and 100 μg ml^{-1} of chlorothalonil; 0.001, 0.01, 0.1, 1 and 10 μg ml^{-1} of difenoconazole; 0.0001, 0.001, 0.01, 0.1, 1, and 10 μg ml^{-1}of pyraclostrobin; and 750, 1500, 2500, 3000, 4000, 5000 and 6000 μg ml^{-1} of tea tree oil. Control plates were not amended with fungicides. Tests for each isolate were replicated three times per concentration of each fungicide. Mycelial plugs of 7-days old culture (10 mm in diameter) were removed from the edges of colonies grown on PDA medium, placed upside down on fungicide-amended and fungicide-free PDA media in Petri dishes, and incubated at 25°C in the dark. After 7 days, colony diameter of each isolate was measured in three directions (minus the diameter of inoculation plug) and the percent inhibition (PI) values per each fungicide rate were calculated using the formula below:

$$PI = \frac{a - b}{a} x\ 100$$

where a = colony diameter of control plates and b = colony diameter of fungicide-amended plates. PI values were subjected to regression analysis against the logarithmic values of fungicide rates. The EC_{50} (fungicide concentration which inhibits mycelial growth by 50%) was determined for each isolate and data on fungicide concentration and relative inhibition were analysed using probit analysis, according to Finney (1971).

RESULTS

The sensitivity of *A. solani* isolates to fungicides and tea tree oil is shown in Tables 2, 3, and 4. Of all tested fungicides, pyraclostrobin exibited the highest toxicity. The growth of all tested isolates was significantly reduced (approximately 43%) even by the lowest concentration of pyraclostrobin (0.0001 μg ml^{-1}). Growth inhibition of over 82% was achieved by pyraclostrobin concentration of 10 μg ml^{-1}. Concentrations which inhibited mycelial growth by 50% (EC_{50}) ranged from 0.0014 to 0.0041 μg ml^{-1}.

The tested *A. solani* isolates were also highly sensitive to difenoconazole. All isolates were able to grow well at 0.001 μg ml^{-1} difenoconazole concentration (<10% growth inhibition), but they were partially inhibited by the next two higher concentrations (0.01 and 0.1 μg ml^{-1}) and significantly inhibited by the highest concentration of 10 μg ml^{-1} (>95% growth inhibition). The EC_{50} values for difenoconazole ranged between 0.018 and 0.037 μg ml^{-1}.

Chlorothalonil was less inhibitory than either pyraclostrobin or difenoconazole at all investigated concentrations. Mycelial growth was reduced less than 10% by the lowest chlorothalonil concentration (0.01 μg ml^{-1}), while the highest concentration (100 μg ml^{-1}) caused between 65.3 and 82.2% inhibition. Chlorothalonil EC_{50} values ranged from 2.99 to 4.54 μg ml^{-1}.

Among all tested conventional fungicides, copper-oxychloride exhibited the lowest toxicity to *A. solani* isolates. All isolates were partially inhibited by the two lowest concentrations (3.125 and 6.25 μg ml^{-1}), while inhibition by the highest concentration of copper-oxychloride (25 μg ml^{-1}) was more than 55%. The obtained EC_{50} values for copper-oxychloride were higher than those of the other investigated fungicides, ranging between 13.27 and 15.63 μg ml^{-1}.

All *A. solani* isolates showed the lowest sensitivity to tea tree oil, in contrast to the conventional fungicides. Mycelial growth of the isolate AS-3 was significantly inhibited by the concentration of 3000 μg ml^{-1}, while AS-1 showed a corresponding value of over 5000 μg ml^{-1}. The EC_{50} values for tea tree essential oil ranged from 1323.97 to 3307.08 μg ml^{-1}.

Table 2. *In vitro* sensitivity of *A. solani* isolates to copper-oxychloride and chlorothalonil

Isolate code	Copper-oxychloride		Chlorothalonil	
	EC_{50} (μg ml^{-1}) value/range*	Slope (b) value/range	EC_{50} (μg ml^{-1}) value/range	Slope (b) value/range
AS-1	14.74 (12.16–18.85)	1.65 (1.44–1.86)	3.00 (1.81–5.17)	0.56 (0.61–0.61)
AS-2	13.27 (10.63–17.66)	1.35 (1.15–1.55)	2.99 (1.82–5.07)	0.58 (0.53–0.63)
AS-3	13.98 (11.56–17.74)	1.64 (1.43–1.85)	4.18 (2.36–7.94)	0.49 (0.44–0.54)
AS-4	15.41 (12.26–21.14)	1.37 (1.17–1.57)	4.54 (2.91–7.34)	0.68 (0.63–0.73)
AS-5	15.63 (12.05–22.86)	1.19 (0.99–1.39)	3.21 (0.46–69.14)	0.41 (0.37–0.45)

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

Table 3. *In vitro* sensitivity of *A. solani* isolates to difenoconazole and pyraclostrobin

Isolate code	Difenoconazole		Pyraclostrobin	
	EC_{50} (μg ml^{-1}) value/range	Slope (b) value/range	EC_{50} (μg ml^{-1}) value/range	Slope (b) value/range
AS-1	0.030 (0.019–0.045)	0.74 (0.68–0.80)	0.0030 (0.0008–0.007)	0.24 (0.19–0.27)
AS-2	0.022 (0.013–0.034)	0.72 (0.66–0.78)	0.0014 (0.0004–0.008)	0.24 (0.19–0.27)
AS-3	0.037 (0.024–0.056)	0.72 (0.66–0.78)	0.0020 (0.0005–0.006)	0.24 (0.21–0.27)
AS-4	0.034 (0.008–0.120)	0.74 (0.68–0.80)	0.0030 (0.0008–0.007)	0.24 (0.19–0.27)
AS-5	0.018 (0.009–0.032)	0.51 (0.46–0.56)	0.0041 (0.0007–0.013)	0.26 (0.22–0.30)

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

Table 4. *In vitro* sensitivity of *A. solani* isolates to tea tree oil

Isolate code	Tea tree oil	
	EC_{50} (μg ml^{-1}) value/range	Slope (b) value/range
AS-1	3307.08 (798.74–13692.59)	1.35 (0.19–2.51)
AS-2	1968.89 (1572.01–2465.98)	4.07 (2.97–5.17)
AS-3	1323.97 (763.39–2295.97)	3.25 (2.19–4.31)
AS-4	1329.87 (675.02–2620.02)	2.19 (1.08–3.30)
AS-5	1509.14 (1205.64–1889.04)	3.70 (2.57–4.83)

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

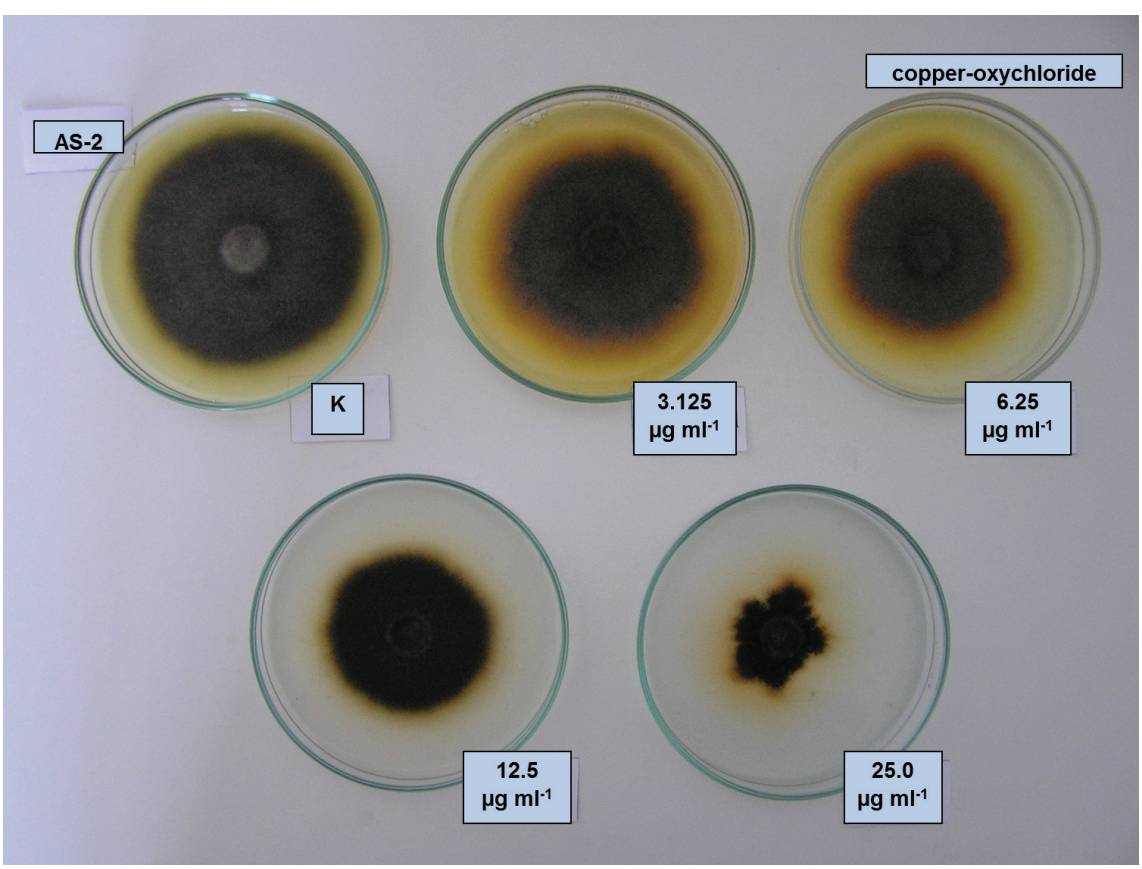

Figure 1. Growth of *A. solani* isolate AS-2 on PDA amended with copper-oxychloride

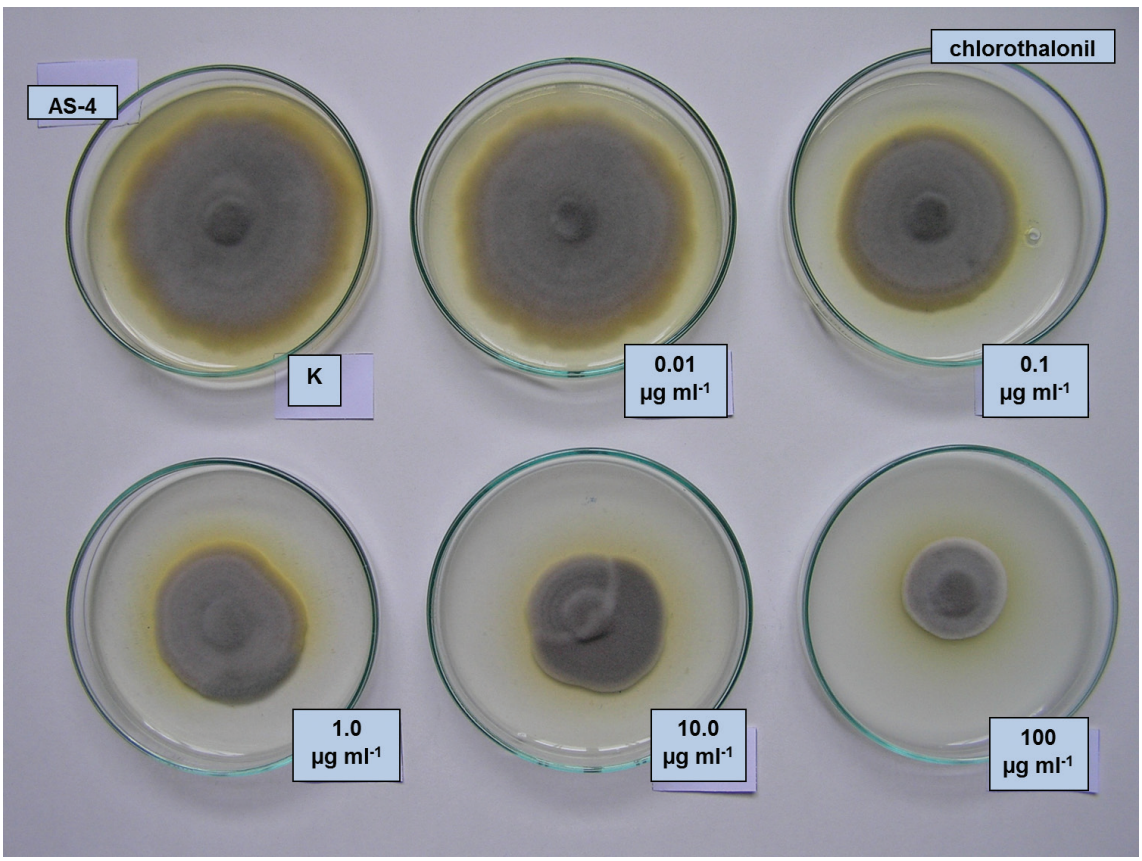

Figure 2. Growth of *A. solani* isolate AS-4 on PDA amended with chlorothalonil

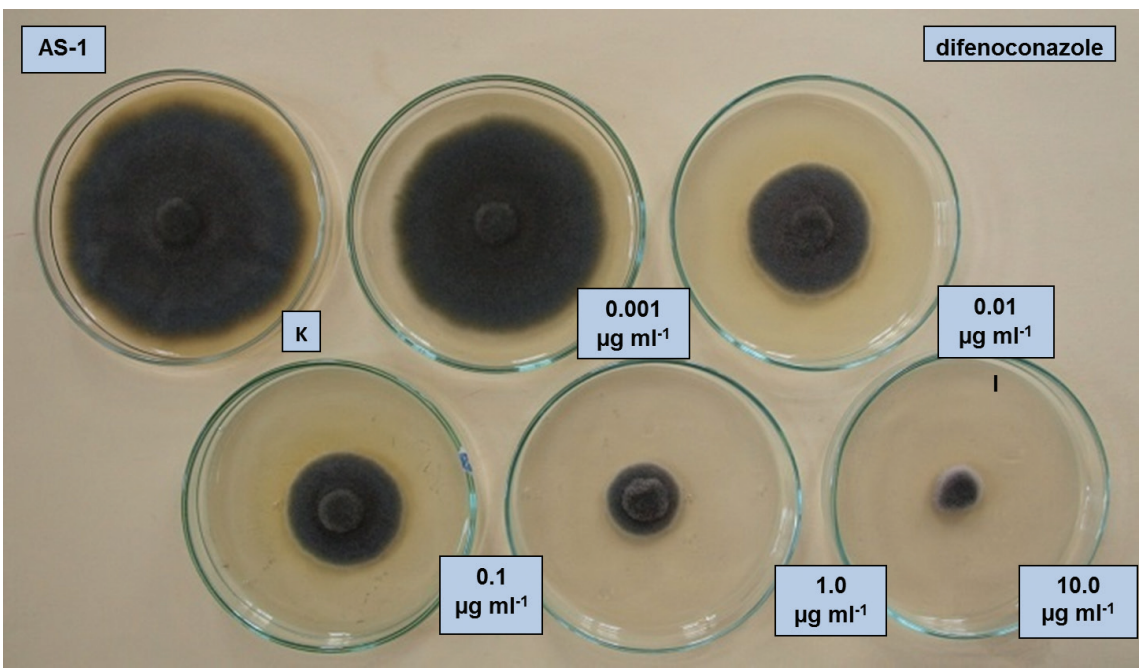

Figure 3. Growth of *A. solani* isolate AS-1 on PDA amended with difenocolazole

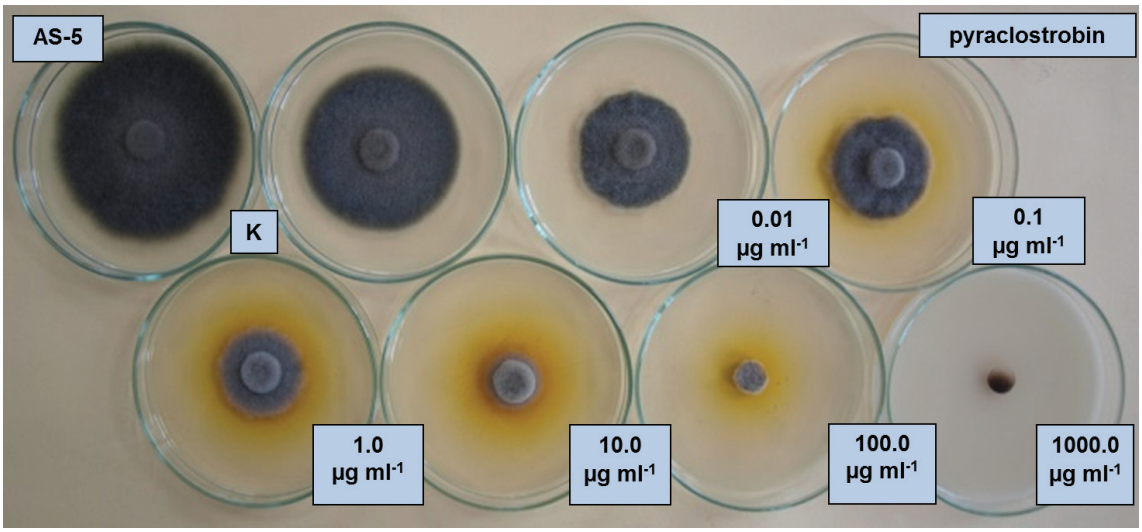

Figure 4. Growth of *A. solani* isolate AS-5 on PDA amended with pyraclostrobin

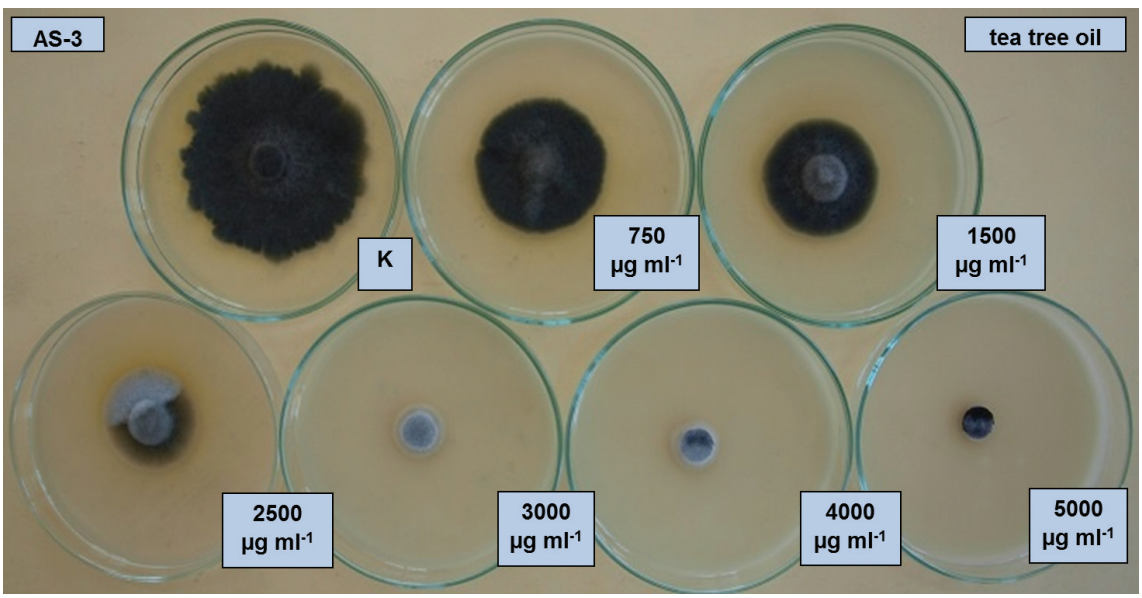

Figure 5. Growth of *A. solani* isolate AS-3 on PDA amended with tea tree oil

DISCUSSION

The results of this study showed that the tested fungicides exhibited different levels of toxicity to *A. solani* isolates. Pyraclostrobin and difenoconazole demonstrated the highest toxicity to all tested *A. solani* isolates. Töfoli et al. (2003) had reported that tebuconazole and difenoconazole provided elevated inhibition of mycelial growth and partial inhibition of conidium germination. In that study, azoxystrobin and pyraclostrobin+methiram showed intermediary inhibitory effect on mycelial growth and complete inhibition of conidium germination starting from 1 µg ml^{-1}, while chlorothalonil and mancozeb demonstrated minor inhibitory levels but superior nevertheless to the control. Pasche et al. (2004) evaluated azoxystrobin sensitivity in an *in vitro* spore germination assay. They detected no significant differences in mean EC_{50} values between baseline isolates and all other isolates collected in 1999. Mean azoxystrobin EC_{50} values of *A. solani* isolates collected in 2000 and 2001 were significantly higher than means from previous years, and mean azoxystrobin EC_{50}

values in 2001 were significantly higher than those of isolates collected in 2000. Nearly all isolates examined in that azoxystrobin assessment were classified into two groups: one that included 76 isolates with EC_{50}s below 0.10 μg ml^{-1}, which was designated as clearly "azoxystrobin sensitive", and another one with EC_{50} values above 1.450 μg ml^{-1}, designated as "azoxystrobin reduced-sensitive". Wang et al. (2008) had tested sixty field *A. solani* isolates for their sensitivity to difenoconazole employing the mycelial lineal growth method. The results showed that the isolates were all sensitive to difenoconazole, no strain was found with sharply decreasing sensitivity, and the averaged EC_{50} value (0.3050±0.1361 μg ml^{-1}) could therefore be used as the baseline-sensitivity of *A. solani* to difenoconazole. Isolates from Serbia were even more sensitive to difenoconazole than those from China, having EC_{50}s below 0.037 μg ml^{-1}.

Antimicrobial activity of the essential oils of various aromatic and medicinal plants has been recognized for a long time. However, strong antimicrobial effects of some other essential oils have also been reported (Tanović et al., 2005; Soylu et al., 2006; Lee et al., 2007; Tanović et al., 2009; Đorđević et al., 2011). In our present study, tea tree oil, which is a biofungicide, had higher EC_{50} values than the synthetic fungicides. Regarding EC_{50} values, the most sensitive was isolate AS-3 (EC_{50}=1323.97 μg ml^{-1}), while AS-1 had the highest EC_{50} value (3307.08 μg ml^{-1}). In another study, Abbo et al. (2009) had reported that tea tree essential oil had a highly suppressive effect against *Alternaria* sp. and could be used as a successful inhibitory agent against tomato early blight pathogen. These results are similar to the findings that Gustafson et al. (1998) and Carson et al. (2006) had reported on inhibitory effects of that oil against Gram-positive and Gram-negative bacteria, yeast and fungi.

A study conducted by El-Mougy (2009) showed that carnation, caraway and thyme oils had inhibitory effects on *A. solani in vitro*. Mycelial growth of *A. solani* was completely inhibited by 1% concentration of carnation oil, while inhibition by the same concentration of caraway and thyme oils was 85.4% and 79.3%, respectively. These results showed that carnation, caraway and thyme oils had greater toxicity to *A. solani* than tea tree oil.

Babagoli and Behdad (2012) also examined the effect of essential oils from *Carum copticum*, *Zataria multiflora* and *Satureja hortensis* on the mycelial growth of *A. solani*. They concluded that, since *Carum copticum* was able to inhibit 99.5% of fungus growth by a concentration of 200 μg ml^{-1}, and 100% by 400 μg ml^{-1}, it could potentially replace chemical fungicides currently used against early blight of tomato caused by this fungus.

Among the five fungicides analyzed in our present study, pyraclostrobin and difenoconazole demonstrated the highest toxicity to *A. solani*. The tested isolates were medium sensitive to chlorothanlonil. Copper-oxychloride showed low toxicity to the pathogen. Tea tree oil did not exhibit a significant antifungal activity *in vitro*, but its efficacy in the field will nevertheless be the subject of a detailed study in our future research.

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Osetljivost izolata *Alternaria solani* na konvencionalne fungicide i biofungicid na bazi etarskog ulja čajnog drveta *in vitro*

REZIME

Ispitivana je osetljivost pet izolata *Alternaria solani* u *in vitro* uslovima na bakar-oksihlorid, hlorotalonil, difenokonazol, piraklostrobin, kao i etarsko ulje čajnog drveta. Izolati su dobijeni iz zaraženih listova paradajza sakupljenih sa pet lokaliteta na teritoriji Republike Srbije. Ispitivani izolati su pokazali najveću osetljivost na piraklostrobin, sa EC_{50} vrednostima u intervalu od 0,0014 do 0,0041 µg/ml. Vrednosti EC_{50} za difenokonazol su bile od 0,018 do 0,037 µg/ml, hlorotalonil 2,99-4,54 µg/ml i bakar-oksihlorid 13,27-15,63 µg/ml. Svi ispitivani izolati *A. solani* su ispoljili najmanju osetljivost na etarsko ulje čajnog drveta (1323,97-3307,08 µg/ml).

Ključne reči: *Alternaria solani*; fungicidi; osetljivost