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Influence of *Xanthomonas euvesicatoria* on quality parameters of pepper seed from Serbia

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SUMMARY

The present study focused on detecting bacteria of the *Xanthomonas* spp. complex (*X. euvesicatoria*, *X. vesicatoria, X. perforans* and *X. gardneri*) and examining their influence on certain quality parameters of pepper seed collected from the territory of Smederevska Palanka (Serbia). The analysis included 27 non-commercial pepper seed genotypes (including chili and sweet pepper) collected in 2021. Several parameters of the quality of analyzed pepper seed (germination energy, total germination, moisture and seed health) were determined. The results showed that out of a total of 27 analyzed samples of pepper seed, the presence of *X. euvesicatoria* was detected in 13 of them. The presence of *X. vesicatoria, X. gardneri* and *X. perforans* was not confirmed. Germination energy of infected seed was 52-84%, and of bacteria-free seed 63-90%; total germination of infected seed was 66-91%, and of bacteria-free seed 80-95%. Seed moisture of infected seed samples was 6.1-12%, and of bacteria-free seed 6.2-8.1%. These parameters did not show significant statistical difference ($p>0.05$). The presence of seed-borne fungi *Fusarium* sp. accounted for up to 3% in 25 samples, while it was up to 6% in the remaining two; *Alternaria* sp. ranked from 1-4% in 25 samples, and up to 5% in only two samples. The results led to a conclusion that the bacterium *X. euvesicatoria* is the predominant pathogen of the *Xanthomonas* spp. complex, but it did not affect the quality parameters of the tested pepper seed.

Keywords: pepper, bacterial spot, germination, moisture, seed health

INTRODUCTION

Pepper (*Capsicum annuum* L.) as a commercial species is cultivated worldwide. The annual production of pepper (dried chillies and peppers) has reached approximately 3.9 million tons (Li et al., 2018). In Serbia, the area under pepper production was assessed at 10.278 ha in 2021, and overall production at 147.663 tons (Statistical Office of the Republic of Serbia, 2022).

Production of *C. annuum* is greatly hampered by many biotic factors, especially fungal, bacterial and viral diseases. Bacterial spot, caused by four distinct plant pathogenic *Xanthomonas* species (*X. euvesicatoria*, *X. vesicatoria, X. perforans* and *X. gardneri*) is one of the most destructive diseases affecting sweet and chili pepper (Potnis et al., 2015; Schwartz et al., 2015; Horuz, 2019). Major losses occur under conditions of high humidity, intense rainfall and temperatures between 20-30 °C

(Kurozawa & Pavan, 2005). Rain and wind facilitate *Xanthomonas* spp. spreading from infected to healthy plants (Osdaghi et al., 2021). *Xanthomonas* spp. bacteria have been recognized as a serious disease accompanied by significant damage in pepper production in Serbia (Ignjatov et al., 2010; Vlajić et al., 2017). These pathogens cause lesions on pepper leaves, irregular shape, and haloes of concentric necrotic and surrounding chlorotic tissue. When a severe infection occurs, leaves may fall off. Symptoms on fruit are scab-like, raised, whitish lesions, which leads to their decreased market value (EPPO, 2013).

One of the most important management strategies and preventive control measures for plant pathogens is the testing and certification of seed and its production in areas where pathogens are not present or under unfavorable conditions for their development. In addition, seed is very important for crop production as more than 80% of crops are propagated from seed worldwide. Some studies have reported that even a low level of infection in the seed is enough to cause epidemics in the field (Kolb et al., 2007). According to the ISTA (2020) and the Official Gazette of the Socialist Federal Republic of Yugoslavia (1987), the most common methods for selecting high-quality seed are based on their physical properties, such as weight and germination, biochemical and other physical tests. *Xanthomonas* spp. bacteria are able to survive in seed (externally or internally), and can be spread by infected seed as a primary source of inoculum, leading to infection of subsequent crops (Bashan et al., 1982, Ritchie, 2000; Dutta et al., 2014; Utami et al., 2022). Seed is the major source of long distance dissemination. According to van der Wolf and Duriat (2006), *X. campestris* pv. *vesicatoria* survives in seed over long periods, even more than 16 years. Seeds may also be externally or internally infected with seed-borne pathogenic fungi (Martín et al., 2022). Pepper seed-borne fungi have been reported by several authors, indicating the presence of *Fusarium* sp., *Alternaria* sp., *Colletotrichum capsici*, *Phytophthora capsici, Rhizoctonia solani*, *Phoma capsici*, *Macrophomina phaseolina* and *Verticillium* sp. (Mushtaq & Hashmi 1997; Ali, 2007; Agarwal et al., 2007; Chigoziri & Ekefan, 2013). The most common fungi in *C. annuum* are *Fusarium* spp., which alone cause seed rot, seedling rot and root rot leading to significant damage in pepper production (Hasan et al., 2012).

The aim of this work was to determine the presence of plant pathogenic bacteria of the genus *Xanthomonas* (*X. euvesicatoria, X. vesicatoria, X. gardneri* and *X. perforans*) in pepper seed, and their influence on certain parameters (germination energy, total germination, moisture and seed health) of the quality of pepper seed collected in the territory of Smederevska Palanka in Serbia.

MATERIAL AND METHODS

Seed material

A total of 27 pepper seed samples originated from the locality Smederevska Palanka in 2021 (coded as P1-P27). The samples were retrieved from a collection of non-commercial genotypes of pepper seeds (sweet and chili pepper) of the Institute for Vegetable Crops (Smederevska Palanka). All samples for the analysis were stored in paper bags at a temperature of 20-22 °C in the laboratory.

Detection of *Xanthomonas* spp. in pepper seed

Extraction of bacteria from pepper seed

Each pepper seed sample (consisting of 20 g) was soaked in 3 ml g^{-1} seed of sterile 10 mM phosphate buffered saline - PBS (containing: Na_2HPO_4 x $12H_2O$ 2.7 g; NaH_2PO_4 x $2H_2O$ 0.4 g; NaCl 8.0 g; distilled water 1000 ml) for a minimum of 14 h at 4 °C. The samples were then shaken for 2 h at room temperature (24 °C) and 115 rpm, then filtered and centrifuged at 11 000 *g* for 20 min at 10 °C. The supernatant was discarded, and the pellet was resuspended in 1 ml of sterile distilled water.

DNA extraction

Genomic DNA was isolated from the extracts obtained from seed using the DNeasy Plant Mini Kit (QIAGEN, Diagnostics GmbH, Qiagen AG) according to a protocol given by the manufacturer.

Polymerase chain reaction (PCR)

Two conventional duplex-PCR tests were used for detection of 4 species of the *Xanthomonas* complex (*X. euvesicatoria*, *X. vesicatoria*, *X. gardneri* and *X. perforans*). Amplification was performed in two separate reactions, each including two primer combinations: Bs-XeF/Bs-XeR and Bs-XvF/Bs-XvR; Bs-XgF/Bs-XgR and Bs-XpF/Bs-XpR, according to a protocol proposed by Koenraadt et al. (2009) (Table 1). PCR was programmed as follows: initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 10 min. As a positive control, a strain coded as X22 from pepper isolated in Irig locality in 2016 and identified as *X. euvesicatoria* was used.

Table 1. Primers used in the study

Primer	Primer sequence (5'-3')	Species
Bs-XeF	CATGAAGAACTCGGCGTATCG	*Xanthomonas euvesicatoria*
Bs-XeR	GTCGGACATAGTGGACACATAC	
Bs-XvF	CCATGTGCCGTTGAAATACTTG	*Xanthomonas vesicatoria*
Bs-XvR	ACAAGAGATGTTGCTATGATTTGC	
Bs-XgF	TCAGTGCTTAGTTCCTCATTGTC	*Xanthomonas gardneri*
Bs-XgR	TGACCGATAAAGACTGCGAAAG	
Bs-XpF	GTCGTGTTGATGGAGCGTTC	*Xanthomonas perforans*
Bs-XpR	GTGCGAGTCAATTATCAGAATGTGG	

Amplified PCR products were visualized by gel electrophoresis on 1.5% agarose gel stained with ethidium bromide under UV light. The expected amplicon sizes in base pairs were as follows: Bs-XeF/R primers 173 bp; Bs-XvF/R primers 138 bp; Bs-XpF/R primers 197 bp and Bs-XgF/R primers 154 bp. The DNA molecular weight marker GeneRuler Low Range DNA Ladder, ready-to-use, was used for fragment size estimation.

Analysis of pepper seed quality parameters

Seed testing of 27 genotypes of pepper was performed using standard methods for assessing seed quality and health (ISTA, 2020). Pepper seed quality was evaluated based on germination parameters (germination energy and total germination), moisture and seed health.

Germination

Germination energy and total seed germination were tested using the standard filter paper method (ISTA, 2020). Samples of different pepper genotypes consisting of a total of 400 seeds (100 per replicate) were placed in Petri dishes with filter paper moistened with 0.2% KNO_3. Quality analyses, especially germination, revealed abnormal seed unable to develop by the end of the test period, which will not eventually develop into healthy seedlings. Seeds were incubated for 7 and 14 days at 23 °C. The final seedling count was made after 14 days for all pepper genotypes.

Moisture

For moisture determination, pepper seed samples were measured thermogravimetrically to their constant weight. To measure moisture content in seed samples, consisting of 5 g, they were kept at a temperature of 105 °C ± 2 °C for 17 h ± 1 h. Seed moisture (SW) is defined as the water in seed, and calculation was performed according to the following formula:

$$SW(\%) = \frac{(m3-m1)}{(m2-m1)} \times 100$$

where:
m1 (g) = the mass of a container and lid;
m2 (g) = the mass of a container, lid, and content before drying;
m3 (g) = the mass of a container, lid, and content after drying.

Seed health

The seed health of pepper genotypes was tested for the presence of *Alternaria* sp. and *Fusarium* sp. Seed testing was performed using the standard method on filter paper (ISTA, 2020). According to the Official Gazette of the Socialist Federal Republic of Yugoslavia (1987), the percentage limit for seed infected with either plant pathogenic fungus is under 5%.

After incubation, the results were rated according to the following formula:

$$\text{Seed health (\%)} = \frac{\text{number of infected seeds}}{\text{total number of seeds}} \times 100$$

Statistical analysis

Statistical analysis was performed using the SPSS software (version 23, IBM, USA). The effects of factors were evaluated by ANOVA (F-test) and Tukey's Multiple Range Test ($p \leq 0.05$) to determine the effects of their means. The coefficients of correlation (r) were calculated for the interrelationships between the observed traits. Differences of $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

The presence of a plant pathogenic bacterial population of *Xanthomonas* spp. in seed of different pepper genotypes, and bacterial influence on seed quality parameters (germination energy, total germination, moisture, seed health) were determined in this study. The analysis included 27 genotypes of pepper (sweet and chili pepper) seed obtained in the season of 2021.

Detection of *Xanthomonas* spp. in pepper seed

The results showed that out of 27 analyzed pepper seed samples, 13 samples were confirmed for the presence *X. euvesicatoria* after amplification of product size of 173 bp using the primers Bs-XeF/Bs-XeR and Bs-XvF/Bs-XvR in duplex-PCR (Figure 1). *X. vesicatoria*, *X. gardneri* and *X. perforans* were not detected in any of the analyzed pepper seed samples.

In this study *X. euvesicatoria* was identified as the only species present in pepper seed samples collected in 2021. It is not surprising since this species is found as the most common in Serbian pepper production (Ignjatov et al., 2010; Vlajić et al., 2017; Giovanardi et al., 2018). Moreover, the presence of *X. euvesicatoria* has been identified as prevalent on pepper in the American continent (Potnis et al., 2015; Hernández-Huerta et al., 2021; Areas et al., 2015), and in European countries (Bogatzevska et al., 2007; Vancheva et al., 2021). It suggests an expansion of this species as an emerging threat to pepper production (Potnis et al., 2015).

As a *Xanthomonas* sp. can be disseminated via contaminated seed or plant material, its detection in tomato or pepper seed and seedlings is critical for the reduction of potential inoculum sources (Potnis et al., 2015). The pathogen can be detected using several techniques, but largely via a combination of classical and molecular approaches. Conventional pathogen detection is based on cells and culture characterization, while PCR and Loop-Mediated Isothermal Amplification (LAMP) are available among molecular methods (Koenraadt et al., 2009; Araújo et al., 2012; Utami et al., 2022). Considering that *Xanthomonas* species remain inside the seed, the use of disease-free seed and seedlings is one of the key management tools. Diagnostic testing of seed health is typically used to detect the level of seed infection (Utami et al., 2022). In that context, our further efforts will be focused on the use of appropriate seed-pathogen isolation methods in order to detect viable *Xanthomonas* cells, and to demonstrate their role in further development and spreading of bacterial spot disease under field conditions.

Pepper seed quality parameters

Germination energy in infected pepper seed ranged from 52 to 84%, and in bacteria-free seed from 63-90%. Total germination of infected seed was found to range from 66 to 91% and of bacteria-free seed between 80 and 95% (Table 2). For all tested pepper genotypes, total germination was above the proposed minimum of 55%; however, some genotypes had total germination of more than 90% (P15, P16, P219 and P23).

The results revealed no statistically significant difference among most of the pepper genotypes ($p>0.05$) (Table 2) relating to germination energy and total germination; only the genotypes coded as P1, P2, P7, P10, P13, P15, P16, P19, P23 differed at the statistical level of $p<0.05$, but with no evidence of an influence of *X. euvesicatoria* infection.

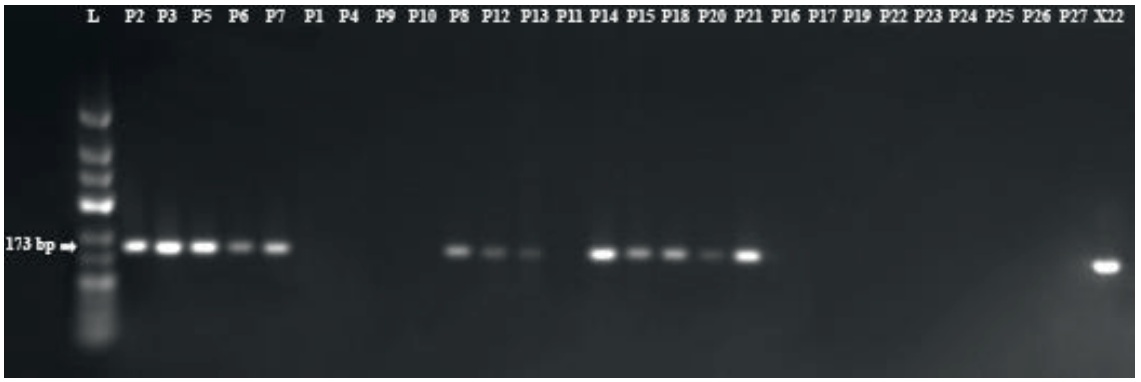

Figure 1. Identification of *X. euvesicatoria* in seed samples using primers Bs-XeF/Bs-XeR and Bs-XvF/Bs-XvR in duplex-PCR. Legend: L–Ladder (GeneRuler Low Range DNA Ladder, ready-to- use); P–tested pepper seed samples; X22– reference strain.

Germination is the most important parameter for high classification of seed quality. In a study conducted by Kurtulmus et al. (2016), 101 different pepper varieties had germination rates of c. 85%, so that the authors suggested that such seeds can be selected for producing high-quality pepper plants.

In all analysed pepper genotypes total seed germination was high and *X. euvesicatoria* contamination did not influence the quality of seed ($p>0.05$). The lowest total germination was observed in the samples P2 and P13, which could be associated with higher moisture percent and detected fungi (*Fusarium* sp. and *Alternaria* sp.). Seed moisture ranged from 6.1-7.9% in the infected and 6.2-8.1% in bacteria-free pepper seed (Table 2). Only in the samples P2 and P13 moisture was higher than 11.5% and 12%, respectively, and with statistical significance compared to the other tested seed ($p>0.05$). According to Gebeyehu (2020), moisture content in seeds gradually increases during storage, reducing seed quality depending on the reduction in germination percentage. Low seed moisture and naturally-occurring antifungal substances have been suggested to be the main barriers to their development (Costa et al., 2019).

The presence of *Fusarium* sp. seed-borne fungi was in the range of 2-3% for both infected and bacteria-free tested seed, and *Alternaria* sp. presence ranged from 1-3% in infected seed, while it was estimated at 4% in bacteria-free seed. Only the genotypes P2 and P13 had higher percentages of *Fusarium* sp. (5% and 6%, respectively) and *Alternaria* sp. (4% and 5%, respectively) infection, compared to the other pepper genotypes. In general, the obtained results revealed that the seed-borne fungi were present in all pepper seed samples but they did not significantly affect total germination or reduce overall germination.

Table 2. Parameters of quality (energy, total germination and moisture) of *Xanthomonas euvesicatoria*-infected (13 genotypes) and bacteria-free (14 genotypes) pepper seed samples

	Pepper genotype	Germination energy (%)	Total germination (%)	Moisture (%)	*Fusarium* sp. (%)	*Alternaria* sp. (%)
Infected seed	P2	$52^{\pm 0.5b}$	$66^{\pm 0.4b}$	$12^{\pm 0.2b}$	$5^{\pm 0.5}$	$4^{\pm 0.5}$
	P3	$67^{\pm 0.4b}$	$88^{\pm 0.2a}$	$6.2^{\pm 0.5ab}$	$1^{\pm 0.2}$	$1^{\pm 0.1}$
	P5	$76^{\pm 0.2a}$	$85^{\pm 0.1a}$	$7.3^{\pm 0a}$	$2^{\pm 05}$	$1^{\pm 0.5}$
	P6	$75^{\pm 0.5a}$	$82^{\pm 1.0a}$	$7.2^{\pm 0.1a}$	$3^{\pm 0.1}$	$1^{\pm 0.3}$
	P7	$65^{\pm 0.7b}$	$75^{\pm 0.5b}$	$6.4^{\pm 0ab}$	$1^{\pm 0.1}$	$2^{\pm 0.1}$
	P8	$77^{\pm 0.2a}$	$87^{\pm 0.8a}$	$7.5^{\pm 0.1a}$	$1^{\pm 0.5}$	$3^{\pm 0.3}$
	P12	$78^{\pm 0.8a}$	$86^{\pm 0.2a}$	$7.5^{\pm 0.1a}$	$2^{\pm 0.6}$	$1^{\pm 0.2}$
	P13	$53^{\pm 0.4b}$	$63\pm^{0.4b}$	$11.5^{\pm 0.1b}$	$6^{\pm 0.1}$	$5^{\pm 0.1}$
	P14	$78^{\pm 0.8a}$	$87^{\pm 0.9a}$	$6.9^{\pm 0.1a}$	$2^{\pm 0}$	$1^{\pm 0.4}$
	P15	$84^{\pm 0b}$	$91^{\pm 0.7b}$	$6.9^{\pm 0.2a}$	$1^{\pm 0.4}$	$2^{\pm 0.2}$
	P18	$79^{\pm 0.5ab}$	$88^{\pm 0.7a}$	$7\pm^{0.1a}$	$1^{\pm 0.3}$	$2^{\pm 0}$
	P20	$82^{\pm 1.0ab}$	$89^{\pm 0.6a}$	$7.9^{\pm 0.1b}$	$2^{\pm 0.2}$	$1^{\pm 0}$
	P21	$77\pm^{0.2a}$	$89^{\pm 0.7a}$	$6.1^{\pm 0ab}$	$2^{\pm 0.1}$	$1^{\pm 0.1}$
Bacteria-free seed	P1	$63^{\pm 0.4b}$	$80^{\pm 0.6ab}$	$6.9^{\pm 0a}$	$3^{\pm 0.2}$	$4^{\pm 0}$
	P4	$77^{\pm 0.5a}$	$85^{\pm 0.6ab}$	$6.8^{\pm 0.1a}$	$1^{\pm 0.1}$	$1^{\pm 0.1}$
	P9	$75^{\pm 0.2a}$	$84^{\pm 0.2ab}$	$6.9^{\pm 0.3a}$	$2^{\pm 0.1}$	$2^{\pm 0}$
	P10	$68^{\pm 0.4b}$	$81^{\pm 0.2ab}$	$6.2^{\pm 0.5ab}$	$1^{\pm 0.4}$	$2^{\pm 0}$
	P11	$75^{\pm 0.4a}$	$81^{\pm 0.2ab}$	$7.1^{\pm 0.3a}$	$1^{\pm 0.2}$	$3^{\pm 0}$
	P16	$79^{\pm 1.0ab}$	$92^{\pm 0.2b}$	$7.3^{\pm 0.5a}$	$2^{\pm 0.3}$	$1^{\pm 0.2}$
	P17	$77^{\pm 0.4a}$	$87^{\pm 1.0a}$	$7.5^{\pm 0.1a}$	$3^{\pm 0.1}$	$1^{\pm 0.4}$
	P19	$90^{\pm 0.6b}$	$95^{\pm 0.6b}$	$6.9^{\pm 0.1a}$	$2^{\pm 0.4}$	$1^{\pm 0.7}$
	P22	$72^{\pm 0.6b}$	$82^{\pm 0.7ab}$	$7.1^{\pm 0.1a}$	$1^{\pm 0.2}$	$2^{\pm 0.5}$
	P23	$82^{\pm 0.5b}$	$95^{\pm 0.4b}$	$8.1^{\pm 0.1b}$	$2^{\pm 0.5}$	$1^{\pm 0.1}$
	P24	$77^{\pm 0.5a}$	$85^{\pm 0.5a}$	$7.2^{\pm 0.1a}$	$3^{\pm 0.3}$	$3^{\pm 0.5}$
	P25	$75^{\pm 0.8a}$	$86^{\pm 0.5a}$	$6.8^{\pm 0.3a}$	$1^{\pm 0.2}$	$4^{\pm 0.2}$
	P26	$74^{\pm 0.7a}$	$80^{\pm 0.7ab}$	$6.9^{\pm 0.4a}$	$1^{\pm 0.1}$	$2^{\pm 0.1}$
	P27	$75^{\pm 0.8a}$	$85^{\pm 0.8a}$	$7.1^{\pm 0.1a}$	$2^{\pm 0.1}$	$1^{\pm 0}$

Different lowercase letters mean significant effect: a-no statistical significance between genotypes; b- statistical significance between genotypes; ab-difference between genotypes ($p \leq 0.05$); Tukey's Multiple Range test for the column. Values are means ±standard deviation of the mean.

Table 3. The correlation coefficient (r) for the observed traits in 27 pepper genotypes (including seed infected with *Xanthomonas euvesicatoria*)

Traits	Total germination	Germination energy	Moisture	*Fusarium* spp.	*Alternaria* spp.	Infected pepper seed
Total germination		0.958***	-0.694	-0.649	-0.418	
Germination energy			-0.422	-0.450	-0.436	
Moisture				0.580	0.582	
Fusarium sp.					0.462	
Alternaria sp.						
Bacteria-free pepper seed						0.341

Pearson's correlation coefficient: ***p< 0.001

The presence of *Fusarium* sp. fungi can result in significant reductions in crop production. Study results on the effects of phytopathogenic fungi on pepper seed germination showed that *F. oxysporum* and *F. solani* reduced seed germination more than the other species. These seed-borne pathogens can reduce pepper seed germination by more than 50% (Liang, 1990; Ali, 2007). Moreover, *Alternaria* spp. is a common pathogen of *Capsicum* spp. worldwide (Nasehi et al., 2014). Soomro et al. (2020) found significant differences in seed germination, number of abnormal seedlings and root length of rapeseed (*Brassica napus*) infected with *Alternaria* spp., compared to fungi-free seed.

In the analysis of parametars of seed quality, the strongest correlation (r =0.958, p<0.001) was found between total germination and germination energy, which indicates a positive correlation between them (Table 3). Pearson's correlation coefficient for these traits indicates that an increase in germination energy will lead to an increase in total germination. Similar results were obtained in a study by Poštić et al. (2020) performed on tomato seeds (r = 0.8711, p <0.001). A negative correlation coefficient indicates the possibility of declining energy and total germination, allowing positive growth of *Fusarium* sp. and *Alternaria* sp. at a statistically significant level (p<0.05). The positive correlation coefficient for the traits moisture, *Fusarium* sp. and *Alternaria* sp. indicates that the percentage of seed infected with those fungal species will grow with increasing moisture.

CONCLUSION

The presented research showed that the plant pathogenic bacterium *X. euvesicatoria* is the predominant pathogen of the *Xanthomonas* spp. complex in seed of several pepper genotypes collected in Smederevska Palanka in Serbia during 2021. The parameters of quality (germination energy, total germination, moisture and seed health) of all 27 tested samples were above the threshold minimum, indicating no influence on the tested quality parameters of pepper seed.

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Uticaj prisustva *Xanthomonas euvesicatoria* na parametre kvaliteta semena paprike u Srbiji

REZIME

U ovom radu izvršena je detekcija bakterija iz kompleksa *Xanthomonas* (*X. euvesicatoria*, *X. vesicatoria, X. perforans* i *X. gardneri*) i ispitan uticaj prisustva bakterija na određene parametre kvaliteta semena paprike poreklom sa teritorije Smederevske Palanke (Srbija). Analiza je obuhvatila 27 nekomercijalnih genotipova semena paprike (uključujući čili i slatku papriku) prikupljenih u sezoni 2021. godine. U radu su ocenjeni parametri kvaliteta semena paprike i to energija klijanja, ukupna klijavost, vlaga i zdravstvena ispravnost. Rezultati su pokazali da je od ukupno 27 analiziranih uzoraka semena paprike, prisustvo *X. euvesicatoria* detektovano kod ukupno 13 uzoraka. Prisustvo *X. vesicatoria, X. gardneri* i *X. perforans* nije utvrđeno ni u jednom uzorku semena paprike. Energija klijanja zaraženog semena je iznosila od 52-84%, a kod semena bez prisustva bakterija od 63-90%; ukupna klijavost u zaraženom semenu je bila od 66-91%, a u semenu bez prisustva bakterija 80-95%. Vlažnost semena u zaraženim uzorcima je iznosila 6,1-12%, a u uzorcima bez prisustva bakterija između 6,2-8,1%. Utvrđivani parametri kvaliteta se nisu značajno razlikovali na statističkom nivou ($p>0,05$). Prisustvo fitopatogenih gljiva koje se prenose semenom je utvrđeno u svim uzorcima semena paprike, i to *Fusarium* sp. do 3% kod ukupno 25 uzoraka, dok je kod dva uzorka zaraza bila i do 6%; prisustvo *Alternaria* sp. je bilo od 1-4% kod ukupno 25, a kod dva uzorka više, do 5%. Dobijeni rezultati ukazuju da je bakterija *X. euvesicatoria* dominantan patogen iz kompleksa *Xanthomonas* vrsta, ali da utvrđeno prisustvo nije značajno uticalo na parametre kvaliteta semena paprike.

Ključne reči: paprika, bakteriozna pegavost, klijavost, vlaga, zdravstvena ispravnost

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Original scientific paper

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

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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.

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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

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Original scientific paper

Management of ginger bacterial wilt (*Ralstonia solanacearum*) epidemics by soil solarization and botanical mulching at Tepi, southwestern Ethiopia

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SUMMARY

Ginger is one of the most valuable cash crops for farmers in different parts of Ethiopia. Bacterial wilt disease, caused by *Ralstonia solanacearum*, creates major limitation to production of ginger in Ethiopia. Heavy losses due to the disease occur regularly, causing an additional impediment for production in infected areas. Field tests were conducted at Tepi, South-Western Ethiopia, during the 2019 and 2020 main cropping seasons to assess the effects of soil solarization and botanical mulch on epidemics of bacterial wilt of ginger. Four soil solarization periods, lasting two, four, six or eight weeks before planting were integrated with four different botanical mulch treatments after planting: vetivar grass, lemon grass, Chinese chive and *Lantana camara*. Unsolarized and unmulched plots were used as the control for comparison. Treatments were arranged in factorial arrangements with randomized complete block design with three replications. The outcomes indicated that soil solarization integrated with lemon grass mulch treatments significantly reduced bacterial wilt mean incidence by 22.1% up to 42.2%, compared to control plots. These treatments also dramatically reduced AUDPC and disease progress rates. Soil solarization for eight weeks integrated with lemon grass mulch resulted in the lowest (42.2%) final mean disease severity and AUDPC (33.8%) in comparison to the control. Typical results of this study indicated that soil solarization integrated with botanical mulch treatments were effective in slowing dawn the epidemics of bacterial wilt and in recovering ginger production and productivity, and they are consequently recommended for application in the study areas along with other crop management schemes.

Keywords: ginger, bacterial wilt, mulching, soil solarization, plant disease management

INTRODUCTION

Ginger has a significant contributing role to the local economy of Ethiopia. It has an export potential, adds value to economic growth, creates a lot of job opportunity locally, and impacts gender empowerment, accessibility and government priorities regarding small farmers in Ethiopia (Vijayalaxmi & Sreepada, 2012). Bacterial wilt of ginger caused by the soil-borne bacteria *Ralstonia solanacearum* is a problem demanding careful consideration in ginger-growing areas of Ethiopia. This disease regularly occurs late in the rainy period. Diseased plants show inward curling, yellowing and browning of the entire shoot, and near-death signs. The basal portion of the yellow stem (shoot) is water-soaked and easily broken off from the underground rhizome and there is milky bacterial ooze exuding from cut stems or rhizomes (Habetewold et al., 2015, Jibat et al ., 2018).

Bacterial wilt is triggered by *R. solanacearum* and its management is challenging once it has established in the field. Owing to its extensive host range, long persistence in soil, propagation in many ways (planting material, irrigation water, farm implements and vectors), it lives in vegetation as dormant infection and hereditarily assorted strains (Allen et al., 2005). However, knowing these features of the disease, it is quite valuable to examine the conditions that regulate disease development and plan a sound disease management approach.

Soil solarization is a nonchemical technique for monitoring soil-borne pests using high temperatures produced by captivating radiant energy from the sun as a 5% rise in soil temperature reduces bacterial diseases. Soil solarization during the hot seasonal months raises soil temperature to levels that kill many disease-producing organisms (Elmore et.al., 1997) and speeds up the breakdown of organic material in soil and so add the benefit of release of soluble nutrients, such as nitrogen ($N0_3^-$, NH_4^+), calcium (Ca^{++}), magnesium (Mg^{++}), potassium (K^+), and fulvic acid, making them more available to plants (Wilen & Elmore, 2007). Studies done by Stapleton et al. (2008) show that plants often grow faster and produce both higher and better quality yields when grown in solarized soil. When correctly done, the top 15 cm of soil will heat up to as high as 60 °C, depending on conditions on the site. Plastic sheets shelter the soil for 4 to 6 weeks allowing solar radiation to be confined in the soil, heating the top 30 to 45 cm and killing a wide variety of soil-borne pests, such as weeds, pathogens, nematodes and insects (Wilen & Elmore, 2007).

Mulching the plant beds with green botanical leaves/organic wastes is crucial to avoid soil splashing and erosion of soil due to heavy rain which decreases bacterial disease feasts. It also complements organic matter to the soil, checks weed emergence and preserves moisture during the latter part of the cropping period. It can recover soil structure by cumulative availability of nitrogen and other essential nutrients for growing healthy plants, as well as controlling a range of diseases. Typically, soil solarization and mulching leave no chemical residues and thus provide a simple method, suitable to reduce the inoculum of the bacterial wilt-causing pathogen in the soil. Therefore, the aim of this study was to reduce ginger bacterial wilt epidemic development and enhance ginger rhizome yield through soil solarization and botanical mulching of the soil.

MATERIALS AND METHODS

Experimental site

The trial was undertaken at Tepi Agricultural Research Centre (TARC), Ethiopia during the 2019 and 2020 main cropping seasons. TARC is located in Yeki district, Southern Nations Nationalities and Peoples' Regional State, which is 600 km south-west of the capital, Addis Ababa. It is situated between 35°08' longitude and 7°08' latitude and at an altitude of 1200 m.a.s.l. The average lowest and highest temperatures are 15 and 30 °C, respectively. It obtains an average annual rainfall of 1630 mm (Guji et al., 2019).

Experimental materials and treatments

Soil solarization for 2, 4, 6 and 8 weeks before ginger planting was evaluated in relation to bacterial wilt either alone or in integration with botanical mulch consisting of vetiver grass, lemon grass, Chinese chive and *Lantana camara* at 10 tons/ha. Soil solarization over different time periods before planting and mulch treatment after planting with different botanicals were applied as cultural management practices to reduce pathogen inocula and prevent disease epidemics.

The plot to be solarized was systematically cultivated and smoothed so as to prevent ripping of the sheet. Later, all plots were watered and covered with transparent polyethylene sheets of 1.5 cm thickness for 2, 4, 6 and 8 weeks under high and direct solar radiation, and an unsolarized plot was used as the control (Stapleton et al., 2008). All free ends were buried and then the soil

around them compressed so as to prevent leakage of heated air or moisture from solarized plots. The first mulching was done at the time of planting with green leaves of vetiver grass, lemon grass, Chines chive and *Lantana camara* at 10 tons/ha, and the unmulched plot was used as the control. The experiment depended on repetitive epidemics of bacterial wilt since the site is a hot spot area of disease with a former history.

Experimental design and trial management

A total of 17 treatments including controls were laid out in a randomized complete block design in a factorial arrangement with three replications. Planting was done on a total plot size of 4 m^2 (2 m width and 2 m length) with six rows of ginger and four harvestable central rows. A recommended spacing of 0.15 m between plants and 0.3 m between rows were used. Spacing between plots and blocks were 0.5 and 1 m, respectively. Total area allocated for the experiment was 44.5 m × 8 m (356 m^2). The four central rows were considered for data collections. All other cultural practices for growing ginger under field conditions were applied uniformly following recommended practices.

Disease assessment

Ginger bacterial wilt incidence (number of plants wilted) was visually evaluated at 15-days interval starting from 60 days after planting (DAP). Plants that displayed either whole or partial wilting were all deliberately wilted and staked to avoid double counting in succeeding assessments. Wilt incidence for each treatment was then considered as a percentage in the total number of plants grown. Disease progress was plotted by considering the disease incidence against time. The area under disease progress curve (AUDPC) based on disease incidence was calculated using the formula recommended by Campbell and Madden (1990):

$$\mathrm{AUDPC} = \sum_{i=1}^{n-1} \left(\frac{X_i + X_{i+1}}{2} \right) \left(t_{i+1} - t_i \right)$$

where n is the total number of assessments, t_i is time of the i^{th} assessment in days from the first assessment date, x_i is the percentage of disease incidence at i^{th} assessment. AUDPC was expressed in %-days since incidence (x) was expressed as percentage and time (t) in days (Campbell & Madden, 1990). AUDPC values were standardized by dividing the values by the epidemic periods (Campbell & Madden, 1990).

Data analysis

Data on bacterial wilt incidence was scrutinized. Analysis of variance (ANOVA) was done for disease incidence and rAUDPC to see the outcome of treatments and their interactions. A logistic ln (Y/1-Y), (Van der Plank, 1963) model was used for estimation of disease progression parameters from each treatment. The converted disease incidence data were regressed over time (DAP) to decide the rate. The fitness of the models was established based on degrees of the coefficient of determination (R^2) and residuals (SE) reached using the model (Campbell & Madden, 1990). The slope of the regression line predicted the disease progress rate.

Regression was considered using Minitab (Release 15.0 for windows®, 2007). The least significant difference (LSD) was used for mean separation at 5% level of significance. ANOVA was executed using the General Linear Model (GLM) of SAS procedure version 9.3 (SAS, 2014). The association of final disease incidence and rAUDPC with yield and yield constituents was examined using correlation analysis. The two years were considered as the same because of the homogeneity of variances confirmed using Bartlett's test (Gomez & Gomez, 1984) and the F-test was nonsignificant for most of the parameters calculated in each year. Thus, data were pooled for analysis.

RESULTS AND DISCUSSION

Disease incidence

Pooled analysis of bacterial wilt incidence data revealed non-significant ($P>0.05$) variation between the 2019 and 2020 main cropping seasons. Therefore, data for both years were pooled and analyzed. Interaction effects of soil solarization with botanical mulch on disease incidence showed highly significant ($P<0.001$) difference at the last date of assessment. Standardized AUDPC values similarly showed significant difference and revealed likely patterns in wilt incidence for both soil solarization and botanical mulch.

The maximum (50.02%) disease incidence was documented from the unsolarized and unmulched control plot, followed by plots solarized for six weeks and mulched with *Lantana camara*, which scored 40.14% at the final date of assessment (120 DAP). The lowest (29.0%) level of disease incidence was obtained in plots with soil solarized for eight weeks,

and mulched with lemon grass, at the final assessment 120 DAP. An analogous trend was observed for the rAUDPC (Table 1). This might be attributed to soil solarization as it increases the tilth and nutrient status of soil. It had been previously shown that microorganisms beneficial to plant growth were either stimulated (*Rhizobium* spp. and *Trichoderma* spp.) or less affected (*Bacillus* spp. and Actinomycetes) by soil solarization than pathogenic organisms (Stapleton & Devay, 1982).

Lower wilt incidence and rAUDPC values found in plots treated with soil solarization for eight weeks and mulched with lemon grass might be credited with the accessibility of nutrients, release of essential oils and boosted population of beneficial soil microorganisms. Available nutrients may increase crop potency and essential oils may incorporate lethal chemicals that could consequently reduce wilt epidemics. In line with this result, a study conducted by Guji et al. (2019) reported that an addition of potassium fertilizer (100 kg ha^{-1}) in combination with soil solarization and lemon grass abridged bacterial wilt incidence by 42.5% over the control. Another earlier study integrated biofumigation with *Brassica* spp., palmarosa and lemon grass with mulching, which released volatiles of essential oils into pathogen-infected fields and reduced bacterial wilt incidence (Arthy et al., 2005). Such plants have high glucosinolates and upon mulching they hydrolyse to antimicrobial isothiocyanates, nitriles or thiocyanates, thereby reducing *R. solanacearum* populations in the soil and wilt incidence in crops (Blok et al., 2000).

Disease progress rate

The disease development rates and parameter estimates of bacterial wilt displayed variations regarding soil solarization periods and mulch types. Disease progress rates in the vetivar grass mulched plots ranged from 0.024 to 0.028 units/day (Table 2), whereas the rates in the lemon grass mulch treatment ranged between 0.011 and 0.025 units/day. In Chinese chive mulched plots, the rates were in between 0.012 and 0.025 units/day, and 0.015 to 0.027 unit/day in *Lantana camara* mulch treatment. It was also obvious that the disease progressed at relatively quicker rates on unsolarized and unmulched plots (0.032 units/day) than on solarized and mulched plots over the years. The results showed that the rate at which bacterial wilt progressed was slower when soil solarization for four weeks was applied along with the lemon grass mulch. This technique could help to explore the synergetic ability of each treatment to obstruct wilt epidemic development. Previous studies had also shown that combinations of soil solarization with other cultural practices decreased disease progress rates by strengthening the resistance of plants to the pathogen. In a study, for instance, soil solarization combined with potassium fertilizer and lemon grass increased plant resistance and decreased disease progress rate (Guji et al., 2019).

Table 1. Interaction effects of soil solarization and botanical mulch on bacterial wilt (*R. solanacearum*) final disease incidence (%) and standardized area under disease progress curve (%-days) at Tepi, Ethiopia, during 2019/2020 main cropping seasons

Treatment combination[1]	Disease incidence (%) and rAUDPC (%-days)				Disease incidence (%) and rAUDPC (%-days)			
	Week 2		Week 4		Week 6		Week 8	
	PDI_f[2]	rAUDPC[3]	PDI_f[2]	rAUDPC[3]	PDI_f[2]	rAUDPC[3]	PDI_f[2]	rAUDPC[3]
VG	34.68^{defg}	23.05^{fgh}	38.26^{bcd}	25.46^{cde}	39.41^{bc}	24.08^{efg}	33.76^{efgh}	24.7^{6def}
LG	39.1^{bc}	24.17^{efg}	33.27^{fghi}	22.54^{gh}	30.33^{hi}	21.63hi	29.0^{i}	20.3^{i}
Ch	37.57^{bcde}	25.18^{cde}	36.06^{cdef}	28.95^{b}	$31.56g^{hi}$	24.38^{ef}	29.53^{i}	22.10^{h}
LC	39.85^{bc}	26.42^{cd}	39.08^{bc}	26.63^{c}	40.14^{b}	25.6^{cde}	38.13^{bcd}	24.86^{cde}
Control	50.2^{a}	33.43^{a}	50.2^{a}	33.43^{a}	50.2^{a}	33.43^{a}	50.2^{a}	33.43^{a}
LSD (0.05)	3.87	1.8						
CV (%)	6.37	4.34						

[1] VG = vetivar grass; LG = lemon grass; Ch = Chinese chive; LC = *Lantana camara*. [2] Percent disease incidence 120 days after planting (DAP). [3] rAUDPC = standardized area under disease progress curve of ginger bacterial wilt. Means followed by the same letter(s) within a column are not significantly different at 5% level of significance.

Table 2. Effects of soil solarization and botanical mulch on disease progress rate (r) and parameter estimates of bacterial wilt (*R. solanacearum*) on ginger at Tepi, Ethiopia, during 2019/2020 main cropping seasons

Botanical mulch	Soil solarization period [1]	Disease progress rate (r) at Tepi			
		Disease progress rate (unit day^{-1}) [2]	SE of rate [3]	SE of intercept [4]	R^2 (%) [5]
Vetivar grass	Control	0.032	0.278	0.278	90.7
	W2	0.025	0.138	0.138	96.2
	W4	0.028	0.078	0.078	98.7
	W6	0.024	0.124	0.124	96.0
	W8	0.024	0.140	0.140	94.6
Lemon grass	Control	0.032	0.278	0.278	90.7
	W2	0.025	0.063	0.063	98.9
	W4	0.020	0.107	0.107	95.5
	W6	0.018	0.118	0.118	93.4
	W8	0.011	0.073	0.073	93.8
Chinese chive	Control	0.032	0.278	0.278	90.7
	W2	0.012	0.066	0.066	95.4
	W4	0.022	0.057	0.057	98.9
	W6	0.025	0.042	0.042	99.5
	W8	0.022	0.202	0.202	89.5
Lantana camara	Control	0.032	0.278	0.278	90.7
	W2	0.015	0.155	0.155	88.0
	W4	0.027	0.134	0.134	96.6
	W6	0.026	0.125	0.125	97.0
	W8	0.024	0.117	0.117	96.8

[1] W2 = soil solarization for two weeks; W4 = soil solarization for four weeks; W6 = soil solarization for six weeks; W8 = soil solarization for eight weeks; [2] Disease progress rate obtained from regression line of disease incidence with time of assessment (days). [3] Standard error of rate. [4] Standard error of parameter estimates. [5] Coefficient of determination of the Logistic model.

Disease progress curve

The disease development curves of bacterial wilt (incidence versus DAP) were drawn separately for each mulch type with different weeks of soil solarization before planting (Figure 1). Each curve for soil solarization period and mulch type revealed that disease severity developed increasingly starting from the onset to the final severity examination during the study periods. The four disease progress curves for each solarization period also indicated that disease progress was not analogous for each mulch type used. Disease severity in control plots followed relatively high progressive curves and exhibited the peak levels of bacterial wilt severity. Soil solarization for two weeks alone and in combination with *Lantana camara* treatment showed similar curves as control plots. However, disease progress curves for plots treated with soil solarization for eight weeks and mulching with lemon grass rose gradually and displayed the lowest bacterial wilt severity at different days after planting. Consistently,

a previous study showed that, during solarisation of soil, positive changes occur in the structure of soil, in solubility of mineral substances available to plants and microbial growth, and in populations of soil-borne microorganisms (Katan et al., 1980). These fluctuations affect the inoculum concentration of plant pathogens, as well as their aggressiveness and survival. Changes in the populations of other soil-borne microorganisms that occur during and after soil solarization may influence disease suppression in soil and enhance plant growth.

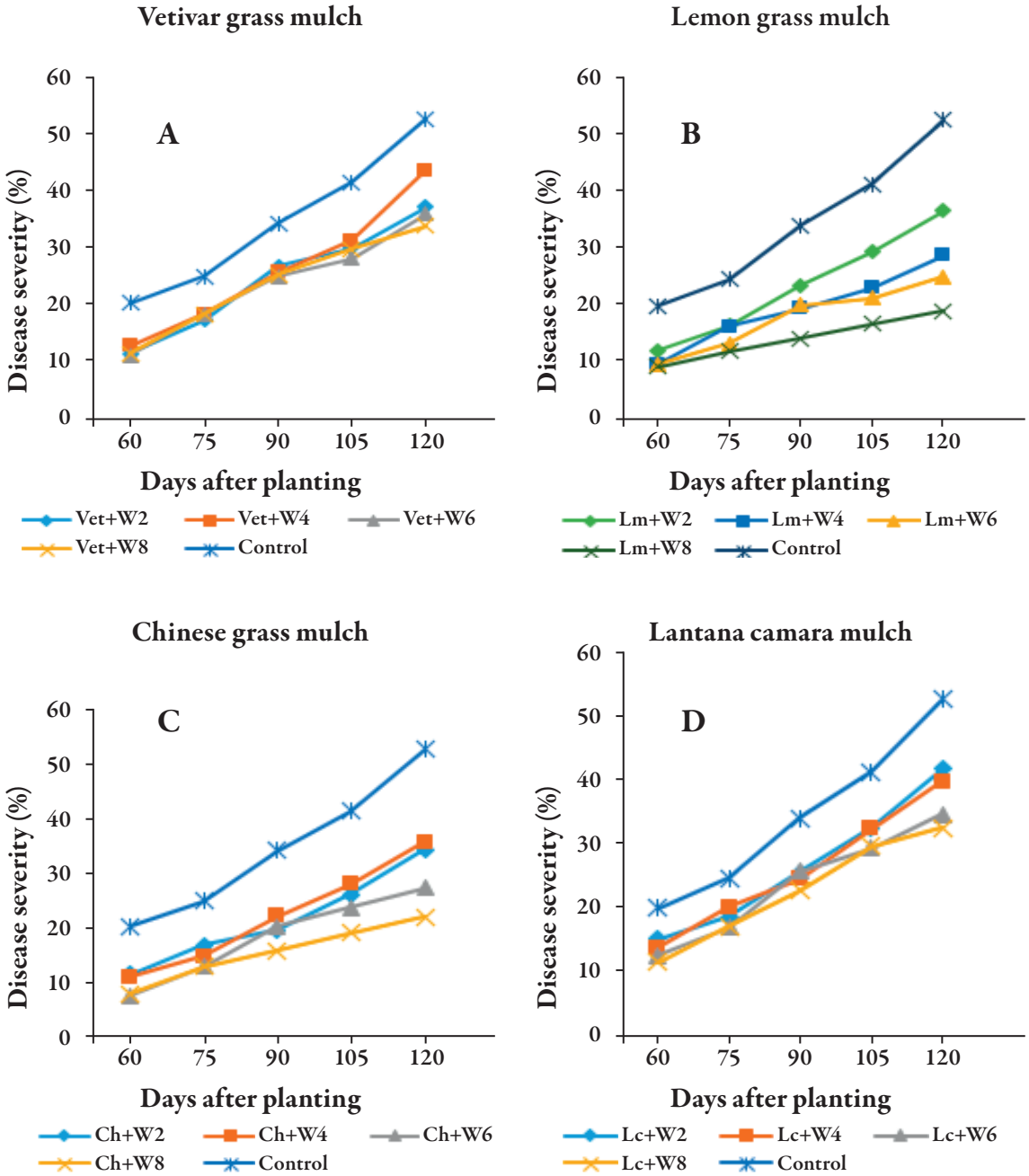

Figure 1. Ginger bacterial wilt (*Ralstonia solanacearum*) disease progress curves as affected by soil solarization for two weeks (W2), four weeks (W4), six weeks (W6) and eight weeks (W8), and botanical mulch with Vet (vetivar grass), Lm (lemon grass), Ch (Chinese chive), and Lc (*Lantana camara*) at Tepi in 2019 and 2020 main cropping seasons.

Table 3. Coefficients of correlation (r) between ginger yield and disease parameters at Tepi, Ethiopia, during 2019 and 2020 main cropping seasons

Parameter	RL (cm)[1]	NFPR[1]	Yield (t ha^{-1})	PSI f (%)[1]	rAUDPC[1]	Dpr (units day^{-1})
RL (cm)	1					
NFPR	0.069^{ns}	1				
Yield (t ha^{-1})	0.423**	0.334**	1			
PSI f (%)	-0.366**	-0.538**	-0.522**	1		
rAUDPC[1]	-0.549**	-0.516**	-0.663**	0.792	1	
Dpr (units day^{-1})	0.026^{ns}	-0.065 ^{ns}	0.184^{ns}	-0.078^{ns}	-0.081^{ns}	1

[1] RL= rhizome length, NFPR= no. of fingers per rhizome, PSI f= final disease severity index, rAUDPC = standardized area under disease progress curve of bacterial wilt incidence of ginger. ** Level of statistical significance at P ≤ 0.01. ^{ns} non-significant at P > 0.05.

Association of yield and disease parameters

Calculating the relationships between and among the final disease incidence, rAUDPC, disease progress rate, yield and yield-related components was vital since modification of each parameter influenced the reaction of another during the trial. For studying the relationship between disease and yield parameters, a simple correlation analysis was used. Diverse levels of association were observed among disease incidence, rAUDPC, disease progress rate and yield and yield-related components and the data are offered in Table 3.

Standardized area under disease progress curve and final disease incidence were absolutely and highly significantly (P≤0.01) correlated (r = 0.792**). This is consistent with Guji et al. (2019), whose study showed an exceptional correlation between the epidemiological parameters PSI and AUDPC. Conversely, negative correlation of rhizome yield with bacterial wilt progress was found to be stronger with rAUDPC than with the final disease incidence. Yield and rAUDPC were seriously and highly significantly (P≤ 0.01) correlated (r =-0.663**). Such result indicates the presence of strong negative effects of bacterial wilt on rhizome yield of ginger. In addition, similar findings appeared for the correlation between disease parameters and yield-associated components of ginger. This result complies with the findings of Guji et al. (2019), who confirmed that bacterial wilt severity, AUDPC and infection rates were powerfully and negatively associated with ginger rhizome yields.

CONCLUSION

Based on the outcomes found in this study, it can be decided that bacterial wilt incidence, rAUDPC, progress rates and curves were intensely influenced by soil solarization and botanical mulch. Soil solarization for eight weeks before planting and botanical mulch with lemon grass after planting greatly reduced the bacterial wilt of ginger. It is therefore suggested to solarize the soil for several weeks before planting ginger and to apply lemon grass mulch after planting along with other crop management strategies to manage bacterial wilt of ginger in the face of the present and upcoming climate dynamics in southwestern Ethiopia. Additional studies on integrated management of ginger bacterial wilt would continue.

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Suzbijanje epidemija bakterijskog uvenuća (*Ralstonia solanacearum*) đumbira primenom solarizacije zemljišta i botaničkog malčiranja u Tepi, jugozapadna Etiopija

REZIME

Đumbir je jedna od najprofitabilnijih kultura za uzgajivače u različitim delovima Etiopije. Bakterijsko uvenuće, koje prouzrokuje *Ralstonia solanacearum*, ograničava proizvodnju đumbira u Etiopiji. Značajni gubici usled ove bolesti javljaju se redovno, stvarajući dodatne prepreke u područjima zaraze. Ogledi su izvedeni u Tepi, jugozapadna Etiopija, tokom proizvodnih sezona 2019. i 2020. kako bi se procenili efekti solarizacije zemljišta i botaničkog malčiranja na epidemiju bakterioznog uvenuća đumbira. Četiri perioda solarizacije u trajanju od dve, četiri, šest i osam nedelja pre sadnje integrisana su sa četiri tretmana malčiranjem nakon sadnje i to biljkama: vetiver, limunova trava, kineski vlašac i lantana (*Lantana camara*). Kontrolne parcele nisu solarizovane, niti malčirane. Tretmani su uređeni faktorijalno u nasumičnom kompletnom blok sistemu sa tri ponavljanja. Rezultati su pokazali da je integrisana primena solarizacije zemljišta i malčiranja limunovom travom značajno smanjila srednje vrednosti za učestalost bakterijskog uvenuća i to od 22.1% do 42.2%, u odnosu na kontrolne parcele. Ovi tretmani su drastično smanjili AUDPC i stopu napredovanja bolesti. Solarizacija u trajanju od osam nedelja integrisana sa malčiranjem limunovom travom dala je najnižu (42.2%) konačnu srednju vrednost stepena oboljevanja, kao i AUDPC (33.8%), u poređenju sa kontrolom. Rezultati istraživanja pokazuju da integrisana primena solarizacije i botaničkog malčiranja efikasno usporava epidemije bakterijskog uvenuća i omogućava oporavak proizvodnje đumbira i njegove produktivnosti, pa se stoga preporučuju za primenu u području istraživanja zajedno sa ostalim metodama zaštite useva.

Ključne reči: đumbir, bakterijsko uvenuće, malčiranje, solarizacija zemljišta, zaštita biljaka od bolesti

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Original scientific paper

Sensitivity of *Cuscuta* species and their hosts to *Anethum graveolens* essential oil

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SUMMARY

The purpose of this study was to examine *in vitro* the herbicidal effect of an essential oil of dill (*Anethum graveolens*) on germination and early seedling growth of two parasitic flowering plants in the genus *Cuscuta* (*Cuscuta campestris* and *C. epithymum*), as well as its phytotoxic impact on germination and early seedling growth of two host plants (alfalfa and red clover). Chemical analysis of the essential oil extracted from dill leaves and flowers showed that carvone (51.69%) and limonene (39.88%) predominated. The results of a seed bioassay showed inhibitory effects of different concentrations (1%, 0.5%, 0.1%, and 0.01% v v^{-1}) of the essential oil of dill leaves on germination and early seedling growth of both tested species of the genus *Cuscuta*. Germination of *C. campestris* seeds was inhibited between 67% and 94%, while seed germination of *C. epithymum* was inhibited between 67% and 100%. A similar inhibitory effect was observed regarding the seedling length parameter. Moreover, the bioassay results indicated significant phytotoxic effects of dill essential oil on the seed germination and early seedling growth of host plants. Red clover proved more sensitive as even the lowest concentration inhibited germination by 35%, while higher concentrations caused 70-100% inhibition. On the other hand, lower concentations inhibited germination of alfalfa seeds by 5%, while higher concentrations caused greater inhibition (36-100%). Future research should include both pot experiments and field micro-trials to determine herbicidal, i.e. phytotoxic, effects of dill essential oil on parasitic flowering plants and cultivated species.

Keywords: dodder, alfalfa, red clover, dill, essential oils, phytotoxicity, bioherbicide

INTRODUCTION

The widespread use of synthetic chemicals may result in the accumulation of toxic residues in agricultural products and cause soil and groundwater contamination, the development of weed resistance, and adverse effects on human and animal health (Hatcher & Melander, 2003). In addition, synthetic chemicals can be immobilized in soil through adsorption or binding to colloids (Hossard et al., 2017; Kanissery et al., 2019), affecting both soil organic matter turnover and microbial community composition (Haney et al., 2000; Lancaster et al., 2010; Ntalli et al., 2019). Therefore, one of the main challenges of agriculture in the 21st century is to minimize the use of pesticides in crop production (Villa et al., 2017). One potential solution is to find alternative natural and safe products,

and exploit renewable resources, such as medicinal and aromatic plants known for their allelopathic properties (Benvenuti et al., 2017; Della Pepa et al., 2019). Due to their structural diversity, natural compounds are a good source of new bioherbicides owing to their new modes of action.

Anethum graveolens L. (dill) is one of the useful essential oil-providing spices, and medicinal plants, because it contains essential oil in its leaves, stems, flowers, fruits, and seeds, which is used in the food and pharmaceutical industries. Dill essential oil has demonstrated various biological activities, such as antimicrobial, antifungal, antioxidant, insecticidal, and anti-inflammatory, due to the presence of biologically active compounds, such as carvone, p-cymene, and α-phellandrene (Chahal et al., 2017).

Plants belonging to the genus *Cuscuta* (common name: dodder) are the most important group of obligate parasitic weeds in the world, inhabiting virtually all continents and causing sweeping damage to both crop and non-crop species (Press & Phoenix, 2005). From an agricultural aspect, the most important *Cuscuta* species in Serbia are *C. campestris* and *C. epithymum*, and both have a wide host range.

Effective control of dodders is extremely difficult because of the nature of attachment and close association between the host and the parasite (Dawson et al., 1994). In the past, control of dodders was also difficult due to a limited selection of registered herbicides for its control in legumes, as well as in sugar beet. So, the need for alternative solutions to control this parasitic flower is increasing. To our best knowledge, there are no reports of the allelopathic effects of essential oils of *A. graveolens* on species in the genus *Cuscuta* and phytotoxic effects on their hosts. This study focused on identifying the chemical composition of an essential oil isolated from leaves of *A. graveolens*. It also aimed to investigate its allelopathic effect on seed germination and seedling growth of two species in the genus *Cuscuta* (*C. campestris* and *C. epithymum*) and its phytotoxic effect on two host species (alfalfa and red clover).

MATERIAL AND METHODS

Plant material

Leaves and flowers of *A. graveolens* were collected in Kosjerić on July 2019. Seeds of *C. campestris* were collected in fields around Odžaci in September 2021, and seeds of *C. epithymum* in fields around Požega in September 2020. The seeds were cleaned and stored in paper bags in the laboratory at a temperature of 20-22 °C.

Preparation and analysis of essential oil

All plant material was air-dried in the shade at room temperature for 20 days and then hydrodistilled in a Clevenger-type apparatus for 2.5 h. The obtained essential oil was dried over anhydrous sodium sulphate and preserved in sealed vials at 4°C until further analysis. Chemical characterization of the essential oil under study was performed by gas chromatography (GC), using two types of detector. Quantitative analysis was performed using an Agilent GC (model 7890A) equipped with a split/splitless injector, flame ionization detector (FID), and HP-5 capillary column (30 m, 0.32 mm i.d., 0.25 μm film thickness). Injector and detector temperatures were set to 250 and 300°C, respectively, while nitrogen flow rate was 1 ml/min. Column temperature was programmed linearly to increase from 50 to 250°C at 4°C/min before being held for 10 min. Qualitative chemical analyses were performed using a Varian CP-3800 GC equipped with Saturn 2200 mass spectrometer (MS) as a detection device. The injector temperature and column temperature were the same as for the GC-FID analysis, while separation was performed using an Agilent DB-5MS column (30 m, 0.25 mm i.d., 0.25 μm film thickness). Helium was used as the carrier gas (1 ml/min), and the ion trap and transfer line temperatures were set to 250°C and 280°C, respectively. The mass detector was operated in the electron impact (EI) mode (70 eV; 40–600 m/z range). In both cases, the solutions of essential oils in n-hexane (1%) were injected in the split mode (1:20). To determine the retention indices (RI), a mixture of n-alkanes (C6-C28) was analyzed using both GC-FID and GC-MS under the same conditions as the essential oils. Identification of essential oil components was performed using both the Wiley 7.0 mass spectral library and the RI data obtained, while quantitative data were expressed as area percent obtained by the GC-FID analysis. The RI data obtained were compared with those from the available literature (Adams, 2007). These data are used as an additional tool to confirm the MS results.

Petri dish germination bioassay

The effect of *A. graveolens* essential oil was tested on two parasitic species, *C. campestris* and *C. epithymum*, and their host plants alfalfa and red clover. The experiment was conducted under controlled conditions in an incubator in darkness (Velpro, Serbia) at 27±1°C. The solutions were prepared with 0.5 ml of essential oil emulsified with Tween 20 (v/v 0.1%) (REANAL Finomvegyszergyar Rt., Hungary, No.805383) at a 1:1 ratio and dissolved in distilled water to obtain a stock solution of 1% concentration. The other concentrations (0.5%, 0.1%, and 0.01% v v^{-1}) were

prepared by dilution. Water and a solution of Tween 20 at a concentration of 1.0% were used for the control. Seed surface was sterilized with 5% sodium hypochlorite solution (NaOCl) for 3 minutes and then rinsed three times with distilled water. Twenty disinfected seeds were placed into each petri dish and than 5 ml of each solution was added. All dishes were sealed with parafilm to avoid evaporation. After 8 days, the percentage of germination was calculated and early seedling growth (seedling length) was measured.

The inhibition percentage, reflected through germination and seedling length, was calculated using the formula:

$$\% \text{ inhibition} = [(Xc - Xt)/Xc] \times 100 \qquad [1]$$

where Xc is the % of germination and seedling length in control, and Xt is the % of germination and seedling length in treatment with essential oil.

Germination rate (GR, sum of germination data per day) was calculated using a formula described by Maguire (1962):

$$GR = n1/t1 + n2/t2 + ... + n_x/t_x, \qquad [2]$$

where n1, n2, ...nx are the numbers of germinated seeds at times t1, t2, ...tx in days.

The experiment design was a randomized complete block with four replications, repeated twice, and data were combined for analysis.

Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA) using STATISTICA 8.0. software package. Normality distribution and homogeneity of variances were checked for all data using the Kolmogorov-Smirnov and Levene tests. When F-values were statistically significant ($p<0.05$) treatments were compared using Fisher's least significant difference (LSD) test.

RESULTS AND DISCUSSION

Chemical analysis of essential oil

Chemical composition of the obtained dill essential oil is presented in Table 1. It shows that 18 identified compounds accounted for 99.61% (v/w) of total oil mass with carvone (51.69%) and limonene (39.88%) being the predominant components. With an exception of

Table 1. Chemical composition of *A. graveolens* essential oil

Chemical class	Components	RI_{EXP}[a]	RI_{LIT}[b]	Content (%)
MH[c]	α-thujene	923	924	0.11
MH	α-pinene	932	932	0.35
MH	sabinene	970	969	0.08
MH	β-pinene	975	974	0.14
MH	α-phellandrene	1001	1002	4.25
MH	p-cymene	1018	1020	0.21
MH	limonene	1023	1024	39.88
MH	terpinolene	1085	1086	0.11
MH	allo-ocimene	1127	1128	0.08
OM[d]	cis-limonene oxide	1132	1132	0.09
OM	trans-limonene oxide	1136	1137	0.22
OM	dill ether	1182	1184	0.55
OM	cis-dihydrocarvone	1191	1191	0.26
OM	trans-dihydrocarvone	1200	1200	1.21
OM	trans-carveol	1214	1215	0.09
OM	cis-carveol	1228	1226	0.18
OM	carvone	1241	1239	51.69
FAD[e]	(2E)-decenal	1262	1260	0.11
	Total			99.61

[a]RI_E – Experimentally determined Retention Indexes
[b]RI_{LIT} – Retention Indexes – literature data (Adams, 2007)
[c]Monoterpene hydrocarbon
[d]Oxygenated monoterpene
[e] Fatty acid and fatty acid derived compound (aliphatic aldehyde)

α-phellandrene (4.25%) and trans-dihydrocarvone (1.21%), all other compounds were present at much lower level. In general, monoterpene hydrocarbons accounted for 45.21%, oxygenated monoterpenes for 4.29%, while (2E)-decenal as aliphatic aldehyde accounted for 0.11% of total oil mass. Carvone and limonene are the predominant components of essential oils produced from dill cultivated in Serbia (Aćimović et al. 2014). Analyzing three essential oils obtained from dill grown at different locations in Vojvodina Province the authors found that the contents of carvone and limonene varied in a range from 51.7-54.5 and 40.6-43.1, respectively, which is consistent with our results.

Seed bioassay

The results of seed bioassay showed that different concentrations of essential oil extracted from dill leaves and flowers inhibited germination and growth of seedlings of both tested *Cuscuta* species. Germination of *C. campestris* seeds was inhibited between 67% (solution concentration 0.01%) and 94% (solution concentrations 0.5% and 1%), while inhibition of *C. epithymum* seeds by the two higher concentrations (0.5% and 1%) was 100%, and 80%, respectively, and 67% by lower concentrations (0.1% and 0.01%) (Figure 1). The inhibitory effect on *C. campestris* was confirmed by significant statistical differences between all test concentrations and the control, and between the applied concentrations, except for the two lower ones which caused no statistically significant difference (Figure 1a). In contrast, *C. epithymum* showed a significant difference ($p<0.05$) even at the lowest essential oil concentration (Figure 1b). The data suggest a greater susceptibility of *C. epithymum* than *C. campestris*, which was confirmed by measurements of the growth rate parameters, which were higher for *C. campestris* than for the other species tested at all concentrations (Table 2).

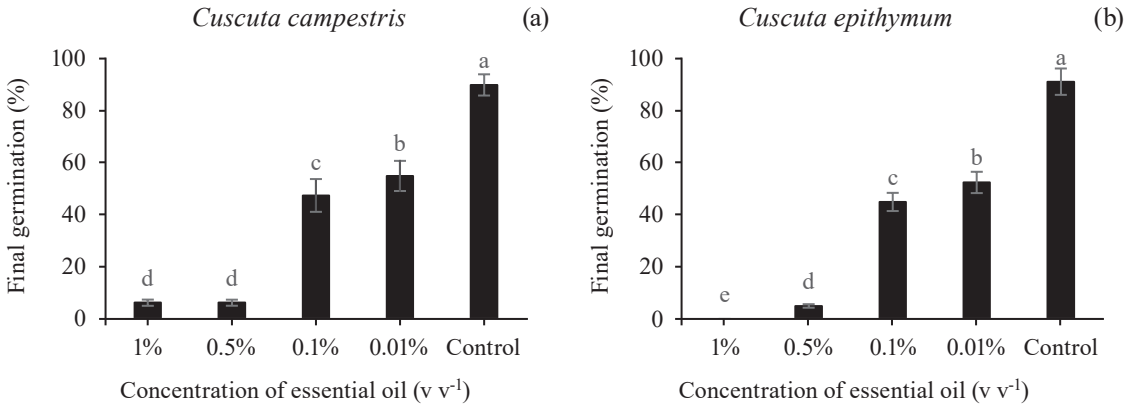

Figure 1. Effects of different concentrations of *A. graveolens* essential oil on seed germination of *C. campestris* (a) and *C. epithymum* (b). Differences were evaluated by one-way analysis of variance (ANOVA) completed with Fisher's least significant difference (LSD) test, $p<0.05$. Means marked by different letters (a, b, c, d) differ significantly ($p<0.05$) for final germination at different concentrations.

Table 2. Effects of different concentrations of *A. graveolens* essential oil on germination rate (%) and seedling growth (cm) of *C. campestris* and *C. epithymum*

Concentration of essential oil	*C. campestris*		*C. epithymum*	
	Germination rate	Seedling growth	Germination rate	Seedling growth
1%	0.52 ± 0.10 c	0.23 ± 0.08 c	0.00 ± 0.00 d	0.00 ± 0.00 c
0.5%	0.58 ± 0.12 c	0.46 ± 0.12 c	0.43 ± 0.10 c	0.24 ± 0.17 c
0.1%	6.46 ± 0.89 b	1.08 ± 0.18 b	6.15 ± 0.98 b	1.98 ± 0.77 b
0.01%	6.98 ± 0.96 b	5.23 ± 0.87 a	6.06 ± 1.01 b	5.21 ± 0.89 a
Control	17.6 ± 1.40 a	5.53 ± 0.93 a	35.21 ± 4.83 a	5.40 ± 0.85 a

Data are reported as means ± standard deviation. Differences were evaluated by one-way analysis of variance (ANOVA) completed with Fisher's least significant difference (LSD) test, $p<0.05$. Means in the same column marked by different letters (a, b, c, d) differ significantly ($p<0.05$).

Seedling length and germination rate were also examined as parameters in the present study for both *Cuscuta* species. Similar to the former parameters, seedling length sustained an inhibitory effect in both species, ranging from 5% (0.01%) to 96% (1%) for *C. campestris,* and from 4% (0.01%) to 100% (1%) in *C. epithymum* (Table 2). Statistically significant differences ($p<0.05$) in seedling length were noted in both species between the control and treatments with concentrations from 0.1% to 1%, while no significant differences were found between the control and the lowest concentration (0.01%), and between the two higher concentrations (0.5% and 1%).

Bioassay results show significant phytotoxic effects of the tested dill essential oil on seed germination and early seedling growth of two hosts (alfalfa and red clover). Red clover was found more susceptible as its germination was inhibited 35% by the lowest concentration, while higher concentrations caused inhibition ranging from 70% to 100% (Figure 2b). Germination of alfalfa seed was inhibited 5% by lower concentrations (0.1% and 0.01%), while higher concentrations caused higher inhibition (36-100%) (Figure 2a). A similar inhibitory trend was noted for the two other parameters (seedling length and germination rate) (Table 3). No significant differences were found between the control data and treatments of alfalfa with lower concentrations, while a significant difference was shown between the control and both higher concentrations ($p<0.05$) for all tested parameters. Conversely, red clover seed showed significant differences ($p<0.05$) between the control and all concentrations, which further confirms a greater susceptibility of red clover seedlings than alfalfa seedlings to the tested dill essential oil (Figure 2).

Control of parasitic weeds is a challenge in agriculture. In conventional agriculture, chemical agents are widely used for weed control, and the overuse of agrochemicals has led to environmental pollution (Muzell Trezzi et al., 2016). Allelopathic compounds (essential oils, phenols), plant residues or extracts, plant mulches and cover crops rich

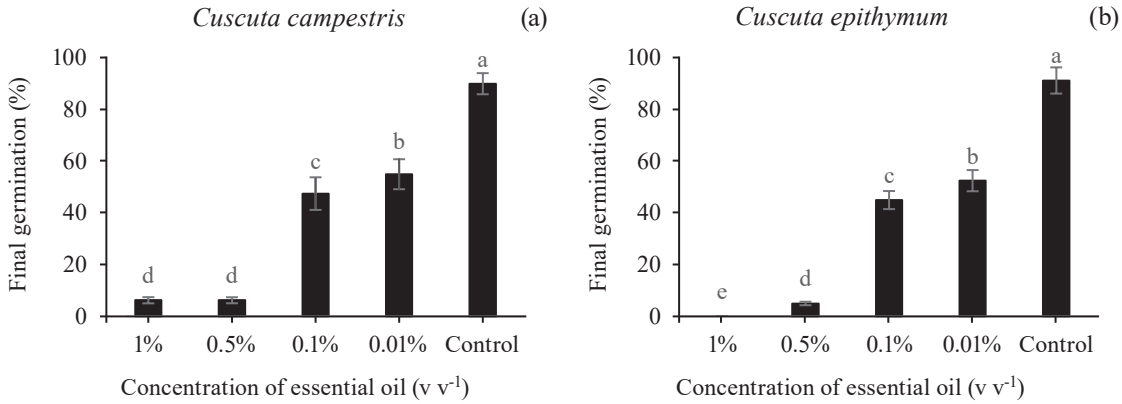

Figure 2. Effects of different concentrations of *A. graveolens* essential oil on seed germination of alfalfa (a) and red clover (b). Differences were evaluated by one-way analysis of variance (ANOVA) completed with Fisher's least significant difference (LSD) test, $p< 0.05$. Means marked by different letters (a, b, c, d) differ significantly ($p< 0.05$) for final germination at different concentrations.

Table 3. Effects of different concentrations of *A. graveolens* essential oil on germination rate (%) and seedling growth (cm) of alfalfa and red clover

Concentration of essential oil	Alfalfa		Red clover	
	Germination rate	Seedling growth	Germination rate	Seedling growth
1.0%	0.00 ± 0.00 d	0.00 ± 0.00 c	0.00 ± 0.00 d	0.00 ± 0.00 c
0.5%	6.59 ± 1.16 c	0.25 ± 0.03 c	0.00 ± 0.00 d	0.00 ± 0.00 c
0.1%	20.00 ± 2.68 b	2.86 ± 0.88 b	5.87 ± 0.69 c	2.16 ± 0.71 b
0.01%	32.13 ± 4.10 a	5.56 ± 1.06 a	16.95 ± 3.95 b	4.80 ± 0.49 a
Control	35.21 ± 5.51 a	6.10 ± 0.08 a	23.96 ± 2.04 a	4.87 ± 0.54 a

Data are reported as means ± standard deviation. Differences were evaluated by one-way analysis of variance (ANOVA) completed with Fisher's least significant difference (LSD) test, $p<0.05$. Means in the same column marked by different letters (a, b, c, d) differ significantly ($p<0.05$).

in allelochemicals can be used for natural weed control. Allelopathy is a field that has been developing more and more over the last few decades but research in the field of parasitic weeds is not as advanced. In fact, several studies have investigated the effects of various allelopathic plant residues on the control of field dodder (Seyyedi et al., 2013; Abbasvand et al., 2020). The effects of residues of three plants, *Zygophyllum fabago* L., *Calendula officinalis* L. and *Datura stramonium* L., on two cultivars of sweet basil infested with field dodder (*C. campsetris*) were studied (Abbasvand et al., 2020). The results suggested that the residues of *Z. fabago* were preferable to other residues used in that study for suppressing field dodder in basil production. In addition, flower and leaf extracts from *Nepeta meyeri* inhibited the germination and growth of *C. campestris* seedlings (Shekari et al. 2022).

Our *in vitro* study confirmed the inhibitory effect of different concentrations of dill essential oil on seed germination and early seedling growth of *C. campestris* and *C. epithymum*. Although the bioassay results showed significant inhibitory effects on the measured parameters of both species in the genus *Cuscuta*, they also revealed phytotoxic effects on the germination and early seedling growth of their host plants (alfalfa and red clover). Future research will need to include both pot trials and field micro-trials to determine the herbicidal, i.e. phytotoxic effects of dill essential oil on the tested parasitic flowering plants and their hosts.

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Osetljivost vrsta roda *Cuscuta* i njihovih domaćina na etarsko ulje mirođije *Anethum graveolens*

REZIME

Cilj ovog rada je bio da se *in vitro* ispita herbicidni uticaj etarskog ulja mirođije (*Anethum graveolens*) na klijanje i rani porast klijanaca dve parazitne cvetnice iz roda *Cuscuta* (*Cuscuta campestris* i *Cuscuta epithymum*), kao i fitotoksični uticaj na klijanje semena i rani porast klijanaca semena domaćina (lucerka i crvena detelina). Hemijskom analizom etarskog ulja izolovanog iz listova i cvetova mirođije je dobijeno da su karvon (51.69%) i limonen (39.88%) dominantne komponente. Rezultati biotesta sa semenima su pokazali inhibitorni uticaj etarskog ulja izolovanog iz listova mirođije pri različitim koncentracijama na klijavost i rani porast klijanaca obe vrste roda *Cuscuta*. Naime, inhibicija klijanja semena *C. campestris* se kretala od 67% do 94%, dok je kod semena *C. epithymum* inhibicija bila od 67% do 100%. Sličan inhibitorni uticaj je zabeležen i za parametar dužina klijanaca. Pored ovoga, u rezultatima biotesta su zabeleženi značajni fitotoksični efekti etarskog ulja mirođije na klijanje semena i rani porast klijanaca domaćina. Crvena detelina se pokazala kao osetljivija, jer je i pri najnižoj koncentarciji inhibicija klijanja bila 35%, a pri višim se kretala od 70% do 100%. Nasuprot ovome inhibicija klijanja semena lucerke pri nižim koncentracijama je bila 5%, dok je na višim koncentracijama zabeležena veća inhibicija (36-100%). Buduća istraživanja moraju obuhvatiti oglede u saksijama, kao i poljske mikrooglede da bi se utvrdili herbicidni, odnosno fitotoksični efekti etarskog ulja mirođije na testirane parazitne cvetnice i gajene vrste.

Ključne reči: vilina kosica, lucerka, crvena detelina, mirođija, etarska ulja, fitotoksičnost, bioherbicid

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Corrigendum to "The dark-red spider mite, *Tetranychus ludeni* Zacher (Acari: Tetranychidae) – a new pest in Serbian acarofauna"

Ivana Marić*, Irena Međo and Dejan Marčić
(Volume 37, Issue 3, pp. 85-93; doi: https://doi.org/10.2298/PIF2203085M)
**Corresponding author: ivamar24@gmail.com*

The original published version of the article contained two errors:

(1) In Table 1B: tomato was not indicated as a host plant for *T. evansi*

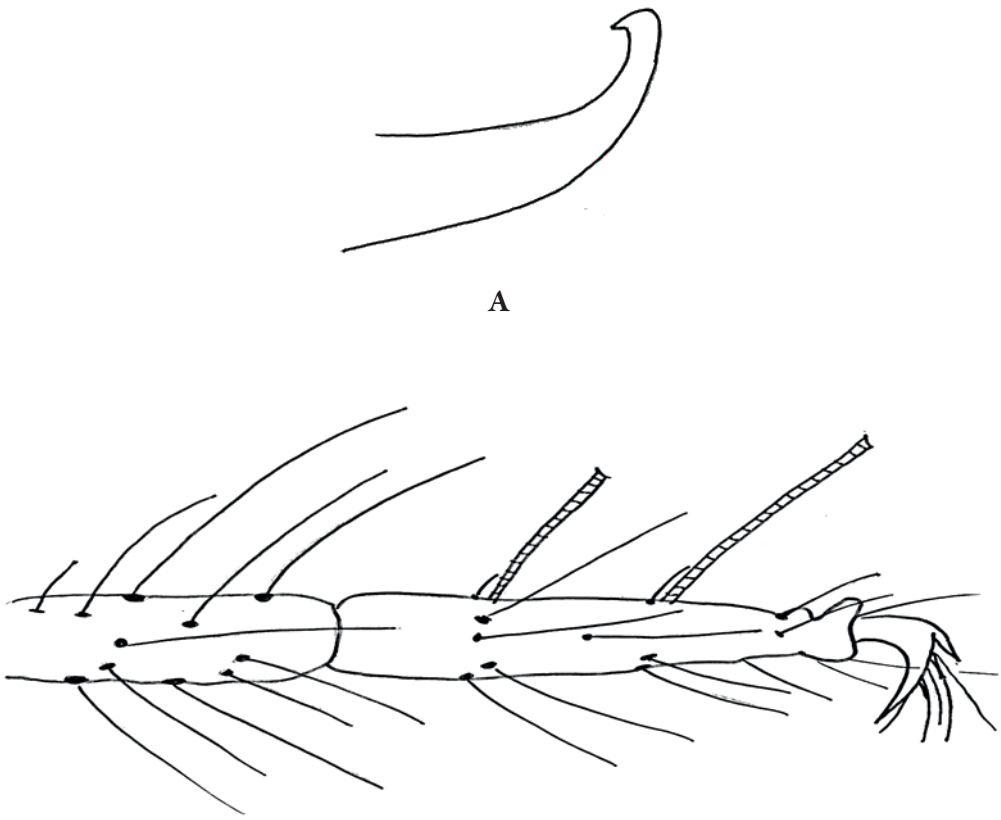
(2) In Figure 1: After carefully looking at the previous drawings of *T. ludeni* body parts, we concluded that it is necessary to provide new and more precise drawings of the male aedeagus and female tibia/tarsus I

The corrigendum presents the corrected version of Table 1B and Figure 1.

Table 1B. Distribution records of *Tetranychus ludeni* in Serbia in 2020

No.	Sampling locations (see Fig. 1)	Host plants (see Tab. 2)	Habitat codes	Population size	Other spider mite species
25.	Kruševac - Velika Drenova	9	I1.2	+++	*T. urticae*
26.	Trstenik - Počekovina	9	I1.2	+++	*T. urticae, T. turkestani*
27.	Vrnjačka Banja - Štulac	9, 16	I1.1	+++	*T. turkestani, T. evansi*
28.	Trstenik – Medveđa	9	I1.1	+	
29.	Trstenik - Čairi	10	I1.1	+++	
30.	Paraćin - Čepure	7	I1.1	+	
31.	Paraćin - Ratare	9	I1.3	+	
32.	Batočina - Badnjevac	8, 16	I2.3	+	*T. urticae, T. evansi*
33.	Šabac - Štitar	16	J2.4	+	*T. evansi*
34.	Šabac - Dobrić	9	J2.4	++	*T. urticae, T. turkestani*
35.	Bogatić	9	I1.2	++	*T. turkestani*
36.	Vladimirci - Jalovnik	5, 9	I1.2	+	*T. urticae*
37.	Prnjavor - Petlovača	11	I1.2	+	
38.	Barajevo - Guncati	19	I2.3	+	
39.	Barajevo - Beljina	9	J2.4	+	*T. urticae*
40.	Mladenovac - Međulužije	9, 11	J2.4	+++	*T. urticae*
41.	Velika Plana - Krnjevo	11	I1.1	+++	
42.	Sm. Palanka - Selevac	7	I1.1	+	
43.	Sremska Mitrovica - Manđelos	10	I1.1	+	
44.	Bačka Topola - Krivaja	10	I2.3	+	
45.	Zrenjanin - Orlovat	9, 10	I1.3	+	*T. urticae*
46.	Niš - Donje Međurovo	9	I1.1	+++	
47.	Leskovac - Donje Krajince	9	I1.1	+	
48.	Leskovac - Gornji Bunibrod	9, 10	I1.1	+++	*T. urticae*
49.	Predejane - Gravovo	11	I1.2	+	*T. turkestani*

I **Regularly or recently cultivated agricultural, horticultural and domestic habitats:** I1.1 = Intensive unmixed crops; I1.2 = Mixed crops of market gardens and horticulture; I1.3 = Arable land with unmixed crops grown by low-intensity agricultural methods; I2.3 = Recently abandoned garden areas. **J = Constructed, industrial and other artificial habitats:** J2.4 = Agricultural constructions



A

B

Figure 1. - *T. ludeni*; A-male aedeagus; B-tibia/tarsus, female

Instructions for Authors

About Journal

Pesticidi i fitomedicina (Pesticides and Phytomedicine) is dedicated to the following research fields: toxicology and ecotoxicology of pesticides; phytopathology; applied entomology and zoology; weed science; plant and food products protection; use of pesticides in agriculture, sanitation and public health.

The journal continues the title *Pesticidi*, which was published over the period 1986-2003.

Pesticidi i fitomedicina (Pesticides and Phytomedicine) publishes original scientific papers and review papers that have not been published previously.

Pesticidi i fitomedicina (Pesticides and Phytomedicine) is an Open Access journal.

Contributions to the journal must be submitted in English, with summaries in English and Serbian (Serbian-speaking authors only).

As of 2020, *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* is issued triannually (three issues annually).

As of 2021, Pesticides and Phytomedicine (Pesticidi i fitomedicina) will be published **online only,** and paper copies of future issues will no longer be available. The primary platforms for journal publication will continue to be: Scindeks (http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949) and the publisher's official web site (http://www.pesting.org.rs/).

The journal is indexed in: Chemical Abstracts, CAB International; DOAJ, EBSCO, AGRIS, Scindeks.

In 2011, the journal converted to an electronic online journal management system on the SCIndeks Assistant portal at **http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949.** The system enables easy article submission and communication among the editorial staff, reviewers and authors. It also includes several quality control services: *CrossRef* for DOI assignment, *CrossChek* for plagiarism prevention and *KWASS* for equipping articles with keywords extracted from a dictionary/thesaurus. Electronic editing is in compliance with the Journal Editing Act of the Ministry of Education, Science and Technological Development of the Republic of Serbia, and provides record-keeping stipulated in the Act.

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To be published in *Pesticidi i fitomedicina (Pesticides and Phytomedicine),* an article must be based on original scientific results that have not been previously published and are not under consideration for publication elsewhere. Review articles should contain a comprehensive survey of a particular subject based on referenced literature and published results of the author(s) own research. All contributions are peer reviewed in a double blind process.

A click on "submit a manuscript" on the left-hand side of the journal home page in SCIndeks Assistant will lead users to a registration page and further on into a guided process of electronic manuscript submission. Serbian authors are requested to fill out the application form in both English and Serbian. Each visual or graphic item (table, chart, diagram or photo) should be submitted as a separate (supplementary) file.

Authors need NOT specify keywords in their articles. They will be extracted and selected by the Editor-in-Chief from the *KWASS* thesaurus (dictionary), which will significantly improve article visibility. Authors are entitled to accept or change some of the keywords.

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The manuscript should be prepared in Microsoft Word (A4 format, all margins 25 mm, font Times New Roman 12 pt). Articles have to be written in the English language, and only the title and abstract in both English and Serbian (Serbian summary will be furnished by the copyeditor for foreign authors'manuscripts).

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Material and Methods should provide sufficient detail to allow the work to be reproduced. Conventional methods should only be referenced.

Results should be presented in a logical order, clearly and concisely, using adequate tables and graphics. Avoid repetition of the results in tables and graphics, or in the text.

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Journal references are required to contain the following information: name(s) of author(s), year of publication, title of article, title of journal, volume, issue number (unless pagination is continuous), pages (from-to) and DOI if available.

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Books: name(s) of author(s) or editor(s), year of publication, title, place of publication and name of publisher.

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Frank, R. H. & Bernanke, B. (2007). *Principles of macroeconomics* (3rd ed.). Boston, MA: McGraw-Hill/Irwin.

Saari L.L. & Thill, D.C. (Eds.). (1994). *Resistance to acetolactate synthase inhibiting herbicides: Herbicide resistance in plants*. Boca Raton, FL, USA: CRC Press.

Dissertations: author's name, year of presentation, title, full name of the institution at which dissertation was defended.

Stepanović, M. (2012). *Osetljivost izolata Alternaria solani (Sorauer) iz različitih krajeva Srbije na fungicide i rizik rezistentnosti*. (Doktorska disertacija). Univerzitet u Beogradu, Poljoprivredni fakultet, Beograd.

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Hammond, K. R. & Adelman, L. (1986). Science, values, and human judgment. In H. R. Arkes & K. R. Hammond (Eds.), *Judgement and decision making: An interdisciplinary reader* (pp. 127-143). Cambridge, England: Cambridge University Press.

Edwards, J.P., Fitches, E.C., Audsley, N. & Gatehouse, J.A. (2002). Insect neuropeptide fusion proteins – A new generation of orally active insect control agents. In T. Margini (Ed.), *Proceedings of the BCPC – Pests and diseases* (pp. 237-242). Brighton, UK: University of Brighton Press.

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Kerruish, R.M. & Unger, P.W. (2010). *Plant protection 1 – Pests, diseases and weeds*. Retrieved from APPS at http://www.appsnet.org/Publications/Kerruish/PP1.pdf

Tables need to be numbered in Arabic numerals consecutively as they appear in text. Tables should be made exclusively in Word for Windows using the toolbar menu Table-Insert-Table, Times New Roman font, 12 pt, and single line spacing. Footnotes immediately below the table body should be given priority over other explanation in table header or in table cells, and text should be in Times New Roman font, 10 pt. Each table must have a header. Tables should be submitted as supplementary (separate) files, and their approximate location in the text marked.

Graphs should be processed in Microsoft Excel and all data in Times New Roman font. Explanations should be provided in captions, consecutively and marked with Arabic numerals. Graphs should be submitted as supplementary files, and their approximate location in the text marked.

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Photos need to be taken by digital camera (resolution at least 150 dpi, photo dimension A4, file format JPG or TIFF). If authors are unable to submit original photos, those should be scanned in RGB mode (colour) or as Grayscale (black and white), with 300 dpi resolution in original size. Photos need to be marked with Arabic numerals in consecutive order. Provide each photo with a caption, mark its approximate location in the text and submit it as a supplementary file.

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Časopis *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* objavljuje naučne radove iz oblasti: toksikologije i ekotoksikologije pesticida; fitopatologije; primenjene entomologije i zoologije; herbologije; zaštite bilja i prehrambenih proizvoda; primene pesticida u poljoprivredi, komunalnoj higijeni i javnom zdravstvu.

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Naslov treba da bude kratak i da upućuje na temu. Puna imena i prezimena svih autora, puni nazivi i adrese institucija svih autora i njihove email adrese treba navesti ispod naslova rada. U slučaju neslaganja ovih podataka u samom tekstu rada i u prijavi na platformi za uređivanje, prioritet će se dati podacima u samom tekstu rada.

Rezime (obima do 300 reči) treba da predstavi ono što je za rad najznačajnije.

Rad treba, po pravilu, da sadrži sledeća poglavlja: Uvod, Materijal i metode, Rezultati, Diskusija, Zahvalnica i Literatura.

Uvod treba da sadrži najnužniji pregled istraživanja u datoj oblasti i ciljeve istraživanja.

Materijal i metode treba opisati dovoljno detaljno da omoguće ponavljanje ispitivanja. Poznate metode i tehnike označiti samo odrednicom iz literature.

Rezultate predstaviti logičnim redosledom, jasno i precizno, koristeći prigodne tabele i grafičke prikaze. Izbegavati ponavljanje rezultata u tabelama i grafikonima, ali i u tekstu rada.

Diskusija treba da istakne značaj dobijenih rezultata, kao i njihovo mesto u kontekstu prethodnih istraživanja. Kad god je to moguće, diskusiju treba odvojiti od rezultata.

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Stepanović, M. (2012). *Osetljivost izolata Alternaria solani (Sorauer) iz različitih krajeva Srbije na fungicide i rizik rezistentnosti*. (Doktorska disertacija). Univerzitet u Beogradu, Poljoprivredni fakultet, Beograd.

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Kerruish, R.M. & Unger, P.W. (2010). *Plant protection 1 – Pests, diseases and weeds*. Retrieved from APPS at http://www.appsnet.org/Publications/Kerruish/PP1.pdf

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Grafikoni treba da budu urađeni i dostavljeni u programu Excel, sa podacima u fontu Times New Roman. Potrebna objašnjenja daju se u legendama obeleženim arapskim brojevima prema redosledu. Grafikoni se prilažu kao zasebne (dopunske) datoteke, a u samom tekstu se obeležava njihovo približno mesto.

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Fotografije treba da budu snimljene digitalnim fotoaparatom (rezolucija najmanje 150 dpi, dimenzija fotografije A4, a format zapisa JPG ili TIFF). Ukoliko autori nisu u mogućnosti da dostave originalne fotografije, treba ih skenirati u RGB modelu (ukoliko su u boji), odnosno kao Grayscale (ukoliko su crno-bele), sa rezolucijom 300 dpi u originalnoj veličini. Fotografije je potrebno obeležiti arapskim brojevima prema predviđenom redosledu. Za svaku fotografiju se daje legenda i obeležava njeno približno mesto pojavljivanja u tekstu. Svaka fotografija se prilaže kao zasebna (dopunska) datoteka.

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Pregledni radovi treba da sadrže uvod, odgovarajuće podnaslove i spisak literature.

Objavljivanje radova u časopisu *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* je besplatno.

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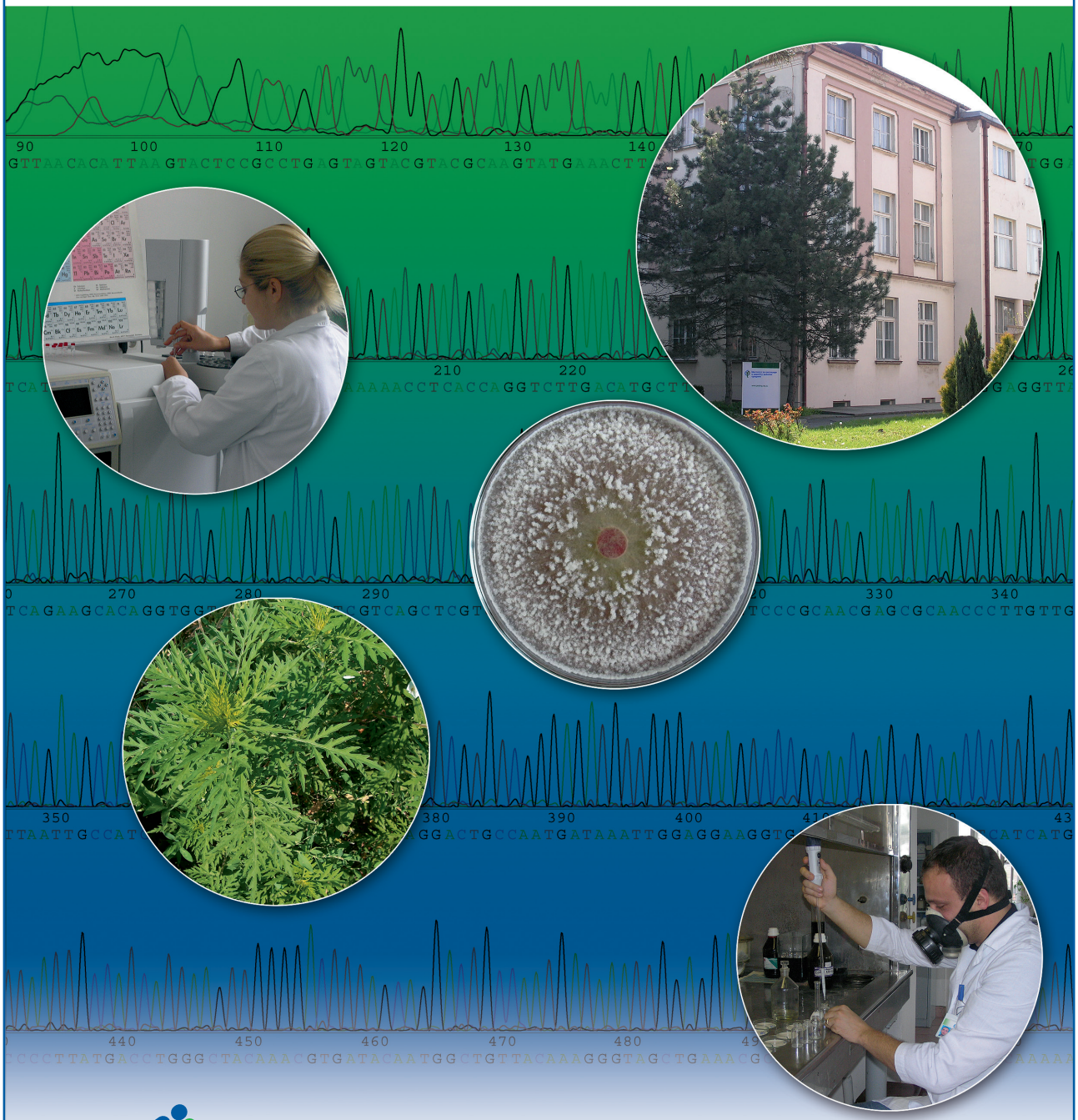

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