UDC 632:615:89

eISSN 2406-1026

Pesticides & Phytomedicine

Pesticidi i fitomedicina

Scientific Journal of the Serbian Plant Protection Society

Vol. 37 * No. 1 * 2022

eISSN 2406-1026

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Scientific Journal of the Serbian Plant Protection Society

Vol. 37 * No. 1 * 2022

Pesticides & Phytomedicine

eISSN 2406-1026

Published trinnually

PUBLISHER

Institute of Pesticides and Environmental Protection, Belgrade, Serbia

Phone: (011) 3076-133, 3076-136

Fax: (011) 3076-136

CO-PUBLISHER

Serbian Plant Protection Society, Belgrade, Serbia

FOR PUBLISHER

Emil Rekanović

LAYOUT

Miodrag Panić

COPYEDITOR

Dušica Marinkov-Jovanović

Cited in: ***Chemical Abstracts; CAB International; DOAJ; EBSCO; AGRIS; Scindeks***
Full text articles available at: www.pesting.org.rs; www.doaj.org;
http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949

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Pestic. Phytomed. (Belgrade), 37(1), 2022, 1–7
DOI: https://doi.org/10.2298/PIF2201001J

UDC 632.38:634.711
Original scientific paper

Impact of raspberry leaf blotch emaravirus on red raspberry 'Willamette' fruits

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Received: 8 December 2021
Accepted: 27 December 2021

SUMMARY

Raspberry leaf blotch emaravirus (RLBV) has become established in many Serbian raspberry orchards as the most prevalent virus of raspberries in the country. The aim of this study was to evaluate the impact of RLBV on the red raspberry 'Willamette' variety. A trial was conducted in four raspberry orchards located in Western Serbia. Fruits from RLBV-infected and uninfected canes were analyzed for fruit size (fruit length, width, height, shape, and weight), soluble solids content, pH, titratable acidity, total sugars, and total phenolic and anthocyanin contents. The results of the study confirmed that RLBV significantly decreases fruit size and weight (9.15-27.49%) of 'Willamette' fruits. Soluble solids content was higher in infected fruits (1.55-7.39%), but the increase was not significant. RLBV did not cause significant changes in titratable acidity of raspberry juice, pH or total sugars content. Total phenolic and anthocyanin contents were higher in fruits of RLBV-infected plants in two out of four locations.

Keywords: raspberry, plant viruses, raspberry leaf blotch emaravirus, rasberry fruits

INTRODUCTION

Raspberries are hosts of more than 40 viruses and virus-like agents. The majority of viruses infecting raspberries do not cause any visible symptoms on leaves or fruits, but some induce different symptoms on infected plants. These symptoms may be easily confused with those caused by other pathogens (mainly fungi) and insects. Raspberry leaf blotch of raspberries has been known for decades and it is manifested by yellow and light green leaf blotches and patches, distortion of leaf margins and twisting of leaves. For a long time, these symptoms were mainly attributed to feeding damage caused by raspberry leaf and bud mite (*Phyllocoptes gracillis* Nalepa) (Dobrivojević & Petanović, 1985). In the past decade, raspberry leaf blotch disorder has become an important problem for raspberry growing in many countries (Bi et al., 2012; McGavin et al., 2012; Milenković & Marčić, 2011). The development and application of molecular techniques has led to the discovery of a new virus tentatively named raspberry leaf blotch emaravirus (RLBV) (McGavin et al., 2012). The virus was detected in raspberry plants originating from Great Britain and Serbia with leaf blotch symptoms, and in raspberry leaf and bud mites. The RLBV is a member of the genus *Emaravirus,* with segmented, linear, single-stranded negative-strand RNA genome (Elbeaino

et al., 2018). Raspberry leaf blotch emaravirus has been reported to infect numerous raspberry cultivars in several European countries: Bulgaria, Bosnia and Herzegovina, Finland, Great Britain, Montenegro, Poland, Serbia, Slovakia and Ukraine (Delić et al., 2020; Jevremović et al., 2019; Pozhylov et al., 2021). So far, RLBV has not been reported outside Europe. Raspberry leaf and bud mite is a suspected vector of the RLBV.

Red raspberry (*Rubus idaeus* L.) is the most important small fruit in the agricultural production of the Republic of Serbia. Frozen raspberries are one of the most important export products, ranking Serbia one of the world's leading exporters of this commodity (Petrović et al., 2017). Red raspberries are cultivated on more than 14,000 hectares and the main cultivated variety is 'Willamette' with a production share of about 90%. Red raspberry 'Willamette' variety had been bred in Oregon, the United States. It is a mid-summer floricane variety with medium-sized fruits. Since the mid-1970s, 'Willamette' has been predominant in raspberry production in Serbia due to its excellent fruit characteristics and suitability of fruits for deep freezing. Raspberries are grown in Serbia according to integrated pest management principles, and in small limited areas in organic farming. Over the last decade, one of the major concerns in raspberry orchard management has been the leaf blotch disorder. Recent studies have confirmed high incidence of RLBV in the country, causing more or less severe symptoms on infected plants (Jevremović et al., 2016, 2019). Also, the raspberry leaf and bud mite, which is a suspected RLBV vector, is considered to be the most important secondary pest of raspberry in Serbia (Milenković & Marčić, 2011).

Since information on the impact of RLBV on infected raspberry plants has been scarce, the aim of our study was to evaluate the influence of RLBV on physical properties and chemical composition of 'Willamette' raspberry fruits in Serbia.

MATERIAL AND METHODS

Experimental design

The trial was conducted during 2019 in four raspberry 'Willamette' orchards located in Western Serbia: Cerova (43° 44.662'N 20° 6.937'E, 336 m altitude), Tvrdići (43° 51.716'N, 19° 56.167'E, 473 m), Bedina Varoš (43° 33.776'N 20° 14.313'E, 686 m), and Devići (43° 25.667'N 20° 22.999'E, 942 m). Raspberries were trained in all orchards in the linear system with planting distance of 2.2 × 0.25 m. Raspberries were trained to wire trellis which is a common system in practice. In each orchard, 6-8 floricanes per row meter were selected and tied to the wire forming a trellis. All orchards were maintained according to an integrated pest and disease management system. The raspberry leaf and bud mite was controlled using acaricides according to an insect and disease spray schedule.

Twenty floricanes with leaf blotch symptoms and 20 asymptomatic floricanes were randomly selected from each orchard. In total, 160 canes were selected from 4 orchards. Symptomatic canes expressed typical leaf blotch symptoms: yellow blotches and patches, leaf twisting and distortion (Figure 1). To analyze the presence of RLBV in the selected canes, 5 leaves were sampled from each cane in May 2019. Leaves originating from the same cane represented one sample.

Figure 1. Symptoms of raspberry leaf blotch emaravirus on 'Willamette' leaves

Total nucleic acid extraction and reverse-transcription polymerase chain reaction (RT-PCR) analysis

All collected leaf samples (80 symptomatic and 80 asymptomatic) were analyzed by reverse-transcription polymerase chain reaction (RT-PCR).

A modified CTAB procedure was used for the extraction of TNA (Li et al., 2008). In brief, 200 mg of leaf tissue was ground in 2 ml of 2% cetyltrimethylammonium-bromide (CTAB) buffer in extraction bags (Flexo duga, Serbia). One ml of plant extract was incubated at 65 °C for 15 min and centrifuged at 10,400 rpm for 5 min. Upper aqueous phase (650 µl) was mixed with equal volume of 24:1 chloroform/isoamyl alcohol and centrifuged at 12,800 rpm for 10 min. The supernatant (500 µl) was mixed with 350 µl of ice-cold isopropanol and centrifuged at 12,800 rpm for 10 min. The obtained TNA pellet was washed with 1 ml ice-cold 70% ethanol by centrifugation at 12,800 rpm for 5 min. The pellet was then dried at room temperature and dissolved in 100 µl Tris-EDTA (TE) buffer. The extracted TNA was kept at -20 °C until further analysis.

Two-step reverse transcription (RT) was done with random hexamers [$d(N)_6$] and Maxima Reverse Transcriptase according to the manufacturer's instruction (Thermo Scientific, USA). PCR reactions were performed with primer pair 1287/1095, which amplifies the 567 base-pair (bp) fragment of the nucleocapsid of RLBV RNA3 (McGavin et al., 2012). Reactions were carried out in a T-personal thermal cycler (Biometra, Germany). Amplified products were analyzed by horizontal electrophoresis in 1.5% agarose gel. After electrophoresis, gel was stained with ethidium-bromide. Visualization of fragments was done in Gel Doc EZ System (Biorad, USA) using UV tray and the presence of the expected fragment of 567 bp was considered as a positive reaction.

To avoid any interaction with RLBV, all samples were also tested to other viruses that are present in Serbia and reported to infect raspberries. Samples were analyzed on the presence of raspberry bushy dwarf virus (RBDV) with ELISA test with reagents of BIOREBA AG, Switzerland. The presence of black raspberry necrosis virus (BRNV), raspberry leaf mottle virus (RLMV), raspberry vein chlorosis virus (RVCV), and rubus yellow net virus (RYNV) was assessed by RT-PCR and PCR, respectively.

Physical properties of fruits

In each orchard/location, fruits from the selected RLBV-infected and RLBV-free canes were hand-picked at the optimal maturity stage during mid-harvest. Fruits were hand-picked from 20 RLBV-infected and 20 RLBV-free canes (five fruits per cane, respectively) in each orchard, chilled in a field refrigerator and transported to the laboratory. In total, 800 fruits were picked and measured. Fruit dimensions (fruit length, width and height) were measured with digital calipers (Carl Roth, Germany). Fruit shape index was expressed as the ratio of mean height to mean width. Fruit weight was measured with a digital balance XL-1810 (Denver instruments, USA).

Chemical properties fruits

For these measurements fruits were picked from the same canes and on the same date as for the evaluation of fruit physical properties.

Soluble solids content (SSC %, °Brix) in fruits was determined in a small sample of fruit juice with a digital brix MA871 refractometer (Milwaukee, USA) at 20 °C. SCC was determined in 25 individual fruits in each variant. Distilled water was used to calibrate the refractometer.

Fruit pH was measured with a pH meter (Mettler Toledo, Switzerland). Titratable acidity (TA) was determined by neutralization of fruit extract with 0.1 N NaOH at pH 8.2, where phenolphthalein was used as an indicator. The results were expressed as grams (g) of citric acid equivalent per 100 g of fresh weight (FW).

The Luff-Schoorl method was used to evaluate total sugars (TS) (Tanner & Brunner, 1979). The results were expressed in percent per 100 g of FW.

Total anthocyanin content (TAC) was determined following a pH-differential method described by Liu et al. (2002). Absorption was measured at 520 and 700 nm in a spectrophotometer (Beckman, USA). The data are expressed as milligrams of cyanidin-3-glucoside equivalents (C3G) per 100 g FW using a molar extinction coefficient of 26,900.

Total phenolic content (TPC) was determined according to a modified Folin-Ciocalteu colorimetric method (Singleton et al., 1999; Liu et al., 2002). TPC was expressed in milligrams of gallic acid equivalents (GAE) in 100 g FW.

Statistical analysis

The obtained data was analyzed by a one-way analysis of variance (ANOVA) test followed by Duncan's test, where significant differences among the tested traits were assessed at $p < 0.01$ using the CoStat software, version 6.311 (CoHort Software, Monterey, CA, USA).

RESULTS AND DISCUSSION

RT-PCR analysis

All of the 160 collected raspberry leaf samples (40 samples per orchard) were tested for RLBV presence by RT-PCR using the recommended primer pair targeting RNA3 of the RLBV genome (Dong et al., 2016; Jevremović et al., 2019). RT-PCR analysis confirmed RLBV presence in all 80 analyzed samples showing leaf blotch symptoms (20 samples per orchard). RLBV was not detected in any of the 80 analyzed asymptomatic samples (20 samples per orchard). Other viruses (RBDV, RYNV, RLMV, RVCV and BRNV) were not detected in the analyzed samples.

RLBV is a common pathogen in red raspberry orchards in Serbia (Jevremović et al., 2016, 2019). The majority of cultivated raspberry varieties express typical and clear symptoms of RLBV infection on leaves. 'Willamette' has been the prevalent raspberry variety grown in Serbia for decades. Typical leaf blotch symptoms may be observed on infected 'Willamette' plants, as well as some other varieties ('Fertödi Zamatos', 'Glen Ample', 'Meeker', 'Polana', and 'Tulameen') reported as RLBV hosts in Serbia.

Physical properties of fruits

The results of our study clearly showed an influence of raspberry leaf blotch emaravirus on fruit dimensions (fruit length, width and height) and weight of infected plants, compared to those from RLBV-free plants (Table 1). The impact of the virus on these traits was confirmed in all four locations.

Depending on location, the decrease in fruit length was 4.01-9.8%, while fruit width decreased 4.06-9.47%, and fruit height 5.88-14.9%. The highest RLBV influence on all examined fruit dimensions was detected in the location Cerova. There were no differences between infected and uninfected fruits regarding the fruit shape index in any location. During this study we did not observe any fruit deformities of fruits from RLBV-infected canes.

The results of statistical evaluation of the tested parameters using variance analysis (ANOVA) showed that RLBV had significant effects on the fruit length, width, height and weight of 'Willamette' raspberry fruits from all four locations ($p < 0.01$) (Table 1). Statistical significance at this level was not achieved only for fruit weight in the location Bedina Varoš, but the difference between mean values of fruit height was found to be significant at $p < 0.05$.

Decrease in fruit weight due to RLBV infection ranged from 9.15-27.49%. As for fruit dimensions, the highest percentage of fruit weight decrease was noted on the location Cerova. Earlier visual field observations had indicated that the fruits on canes with blotch symptoms were generally smaller in size compared to those on canes without any leaf symptoms (Jevremović et al., 2016).

According to reports in literature, the RLBV causes significant production losses to raspberry growers, but no further details are available (Bi et al., 2012; Dong et al., 2016). Our results clearly demonstrated a decrease in fruit weight of the infected 'Willamette' raspberry fruit. The revealed decrease in yield from the test orchards is directly proportional to the percentage of infected canes. Differences between orchards regarding fruit weight decrease were evidenced. They can be attributed to factors such as the specific environmental conditions existing in different locations and the applied agro-technical measures.

There are no other literature data on the influence of RLBV on red raspberry fruit. The presence of the virus has been confirmed in several European countries with more or less significant raspberry fruit production. The variety structure differs from country to country and the influence of RLBV should be studied in other important cultivars.

Fruit chemical properties

The obtained values of SCC, TA, pH and TS are presented in Table 2. Soluble solids contents were higher in RLBV-infected canes on all four locations. The increase

Table 1. The effect of raspberry leaf blotch emaravirus (RLBV) infection on fruit length, width, height, fruit shape index and weight of 'Willamette' raspberry

Evaluated trait	Location							
	Tvrdići		Bedina Varoš		Devići		Cerova	
	RLBV-	RLBV+	RLBV-	RLBV+	RLBV-	RLBV+	RLBV-	RLBV+
Fruit length (mm)	19.43 ± 0.35 a	18.65 ± 0.23 b	19.12 ± 0.44 a	18.14 ± 0.48 b	20.62 ± 0.81 a	18.86 ± 0.44 b	19.79 ± 0.54 a	17.83 ± 0.48 b
Fruit width (mm)	18.19 ± 0.30 a	17.46 ± 0.21 b	18.09 ± 0.31 a	16.97 ± 0.50 b	18.38 ± 0.47 a	17.12 ± 0.52 b	18.19 ± 0.46 a	16.60 ± 0.39 b
Fruit height (mm)	21.45 ± 0.73 a	19.83 ± 0.42 b	19.54 ± 0.37 a	18.39 ± 0.67 a	22.26 ± 0.83 a	20.05 ± 0.73 b	21.43 ± 0.32 a	18.22 ± 0.52 b
Fruit shape index	1.07 ± 0.01 a	1.07 ± 0.01 a	1.06 ± 0.01 a	1.07 ± 0.01 a	1.12 ± 0.02 a	1.10 ± 0.02 a	1.09 ± 0.02 a	1.07 ± 0.03 a
Fruit weight (g)	3.28 ± 0.18 a	2.98 ± 0.18 b	2.95 ± 0.13 a	2.52 ± 0.19 b	3.85 ± 0.24 a	2.92 ± 0.24 b	3.31 ± 0.15 a	2.40 ± 0.14 b

Letters differing row-wise per location indicate significant difference ($p < 0.01$)

in SCC ranged from 1.55-7.39%. The highest SCC increase was evidenced on the location Cerova (7.39%). Statistical evaluation of SCC mean values showed that RLBV had no significant impact on this trait ($p < 0.01$) in fruits from any of the examined localities.

There was no statistically significant effect of RLBV on titratable acidity on three out of four locations. The obtained TA values of raspberry juice made from infected fruits were up to 11.04% higher compared to uninfected fruits within each locality.

The determined pH values of fruits from RLBV-infected plants were equal or up to 1.93% lower than those from uninfected plants. No statistically significant effect of RLBV was found at $p < 0.01$, except on the location Cerova.

Total sugars content in infected fruits was higher on locations Tvrdići and Cerova (by 4.05 and 15%, respectively), but lower in Bedina Varoš and Devići (by 4.84 and 8.21%, respectively). There were no significant differences between RLBV-infected and -uninfected plants regarding total sugars within each examined location.

The RLBV did not have a crucial role in changes of total sugars content in the infected fruits. Statistical analysis showed that RLBV had no effect on this trait at $p < 0.01$.

Total anthocyanin content in RLBV-infected and -uninfected raspberries from four locations is presented in Figure 2. A large variation was observed among values of total anthocyanin content for 'Willamette' raspberry from different locations, regardless of RLBV infection. In two locations (Bedina Varoš and Cerova) TAC values were higher in fruits from infected plants. On the other two localities (Devići and Tvrdići), TAC values were lower in fruits from infected plants. Total phenolic content was shown to correlate with total anthocyanin content (Figure 2).

Table 2. Soluble solids content, titratable acidity, pH value of the fruit juice, and total sugars content in fruits from RLBV-infected (RLBV+) and RLBV-uninfected (RLBV-) plants

Evaluated trait	Location							
	Tvrdići		Bedina Varoš		Devići		Cerova	
	RLBV-	RLBV+	RLBV-	RLBV+	RLBV-	RLBV+	RLBV-	RLBV+
Soluble solids content (%, °Brix)	9.48 ± 1.37 a	9.82 ± 1.54 a	8.39 ± 1.02 a	8.52 ± 0.57 a	8.67 ± 0.87 a	9.09 ± 1.02 a	10.28 ± 0.81 a	11.04 ± 0.82 a
Titritable acidity (%, w/v, citric acid)	1.63 ± 0.03 a	1.65 ± 0.04 a	1.33 ± 0.18 a	1.71 ± 0.05 b	1.66 ± 0.07 a	1.69 ± 0.05 a	1.83 ± 0.10 a	1.87 ± 0.19 a
pH	3.27 ± 0.05 a	3.27 ± 0.04 a	3.17 ± 0.07 a	3.15 ± 0.07 a	3.07 ± 0.05 a	3.05 ± 0.00 a	3.11 ± 0.01 a	3.05 ± 0.01 b
Total sugars	4.26 ± 0.18 a	4.44 ± 0.12 a	3.72 ± 0.36 a	3.54 ± 0.06 a	4.38 ± 0.30 a	4.02 ± 0.06 a	5.10 ± 0.18 a	6.00 ± 0.36 a

Letters differing row-wise per location indicate significant difference ($p < 0.01$)

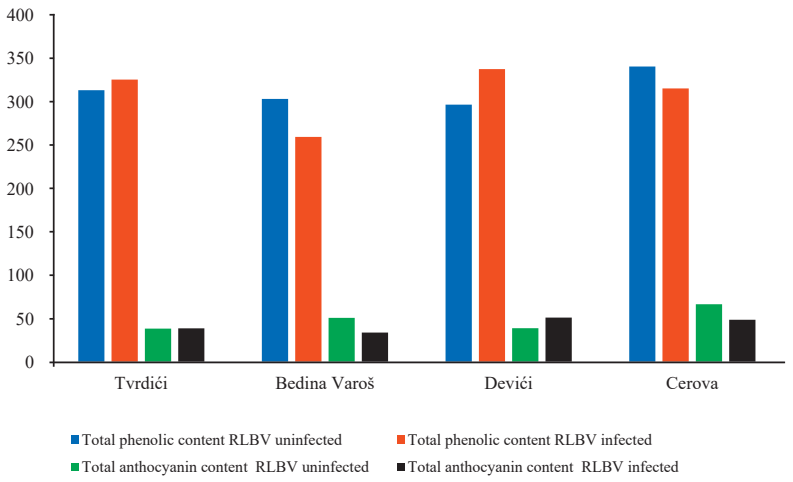

Figure 2. Total phenolic and total anthocyanin contents in raspberry fruits from RLBV-infected and RLBV-uninfected plants

Anthocyanins are an important class of flavonoids that represent a large group of plant secondary metabolites. Environmental growing conditions can affect the ability of fruit species to synthesize anthocyanin (Howard et al., 2003). Other important factors that influence total anthocyanins content are: fruit species, variety, maturity stage, soil substrate, and plant health status. Kalt et al. (2001) also reported that the synthesis of anthocyanins and phenolic compounds can be influenced by biotic and abiotic factors (irradiation, temperature, and pathogen attacks). The only factor that varied among plants in the examined locations was RLBV infection. RLBV infection increased total anthocyanin and total phenolic contents in some locations, while decreasing them in others.

CONCLUSIONS

The presented results clearly demonstrate that the RLBV significantly decreased the dimensions and weight of infected raspberry 'Willamette' fruits. Soluble solids content was higher in fruits collected from RLBV-infected plants, but with no significant effect. The analysis confirmed that RLBV did not have a statistically significant impact on pH, titratable acidity or total sugars content in fruits from infected plants. Total anthocyanin and total phenolic contents were higher in fruits from RLBV-infected plants on two examined locations, but lower on the other two localities.

ACKNOWLEDGEMENTS

This work was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia, contract 451-03-68/2022-14/200215.

The authors would also like to express gratitude to Ivan Radojičić, BSc in agronomy, and students of the Čačak Faculty of Agronomy Miodrag Gizdavić, Dragomir Marković and Stefan Popović for their assistance during field activities.

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Uticaj virusa mrljavosti lista maline na plodove maline sorte 'Willamette'

REZIME

Virus mrljavosti lista maline (raspberry leaf blotch emaravirus, RLBV) je prisutan u velikom broju zasada maline u Srbiji i najrasprostranjeniji je virus maline u zemlji. Cilj ovog rada je bio ispitivanje uticaja RLBV na plodove maline sorte 'Willamette'. Ogledi su sprovedeni u četiri zasada maline u lokalitetima zapadne Srbije. Analizirani su plodovi sa RLBV zaraženih i nezaraženih izdanaka: dužina, širina i visina ploda, indeks oblika ploda, težina ploda, sadržaj rastvorljive suve materije, pH vrednost voćnog soka, titraciona kiselost, ukupni šećeri, sadržaj ukupnih fenola i antocijana. Rezultati istraživanja su pokazali da je virus mrljavosti lista maline imao značajan uticaj na smanjenje veličine i težine (9,15-27,49%) ploda maline. Sadržaj rastvorljive suve materije je bio viši kod zaraženih plodova (1,55-7,39%), ali ovo povećanje nije bilo od statističkog značaja. RLBV nije imao uticaj na titracionu kiselost voćnog soka, pH vrednost i sadržaj ukupnih šećera. Sadržaj ukupnih fenola i antocijana je bio viši kod plodova sa zaraženih izdanaka u dva od četiri ispitivana lokaliteta.

Ključne reči: malina, biljni virusi, virus mrljavosti lista maline, plodovi maline

Pestic. Phytomed. (Belgrade), 37(1), 2022, 9–19
DOI: https://doi.org/10.2298/PIF2201009D

UDC 581.524.13:634.51+635.45:632.51
Original scientific paper

Antioxidant activity of *Juglans regia* L. and *Rumex obtusifolius* L. leaf extracts and screening for their allelopathic potential

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Received: 30 December 2021
Accepted: 10 February 2022

SUMMARY

Secondary plant metabolites with allelopathic activity or phytotoxicity could be biotechnologically important, serving as a source of allelochemicals, and thus contributing to the agro-industrial sector. The objective of this study was to use the obtained common walnut (*Juglans regia* L.) and bitter dock (*Rumex obtusifolius* L.) leaves extracts rich in phenolic compounds, i.e. with high antioxidant potential, and to identify their phytotoxicity to *Setaria glauca* (L.) P. Beauv. and *Sorghum halepense* (L.) Pers. weed seedlings grown *in vitro*. The obtained plant extracts had remarkably high affinity for scavenging free radicals, having DPPH IC_{50} values of 0.127 mg/ml for common walnut leaf extract and 0.194 mg/ml for bitter dock leaf extract. Ferric reducing antioxidant power of the extracts was also high, FRAP value of the common walnut leaf extract was 384.4 ± 8.1 μmol Fe^{2+}/g dry mass, and of the bitter dock leaf extract 321.6 ± 2.5 μmol Fe^{2+}/g dry mass. At the highest used concentration, common walnut leaf extract reduced germination of *S. glauca* by 67.3%, while bitter dock leaf extract reduced germination of that weed by 54.5%. Shoot length of *S. glauca* was inhibited 80.7% when subjected to common walnut leaf extract, and 78.2% under the influence of bitter dock leaf extract, and its root length was inhibited 96.4% and 93.1% respectively. Germination of *S. halepense* was inhibited 100% under the influence of the obtained common walnut leaf extract at its highest test concentration, and 79.2% when subjected to bitter dock leaf extract at the same concentration. Shoot length of this weed was reduced 100% after treatment with common walnut leaf extract, and 93.7% when subjected to bitter dock leaf extract. Root length was reduced 100% and 99.3%, respectively. Overall, the extracts demonstrated pronounced antioxidant activity and remarkable allelopathic potential.

Keywords: common walnut, bitter dock, weeds, antioxidant activity, allelopathy

INTRODUCTION

The fact of increasing world population has put some durable challenges before the food and agricultural sector through increased demand of total food availabilities coupled with higher quality standards (safety, environment, welfare and ethics). Along with increased crop production, pressure from weeds, insects and diseases has also risen. Among crop pests, weeds are regarded as a major one, responsible for 45% of crop yield

losses (Gnanavel & Natarajan, 2014). Weed infestation decreases crop productivity or reduces the quality of harvested products due to direct competition with crop plants for limited resources (Kaur et al., 2018).

For decreasing crop yield and product quality losses caused by weeds, control strategies are necessary and chemical control is still regarded as the most common and successful control method across the globe, despite problems and issues associated with the use of synthetic herbicides, (Hossen et al., 2021). However, extensive uses of herbicides in modern agriculture cause more and more problems, including development of weed resistance, increment of toxic residues in products, health concerns and environmental pollution. As a result, the tendency towards finding effective and safer alternatives for synthetic herbicides is rapidly increasing. Among non-chemical approaches for weed control, using the phenomenon of allelopathy to suppress weeds turns out to be one of the most effective weed management methods (Jabran & Farooq, 2013).

As allelopathy utilization in agro-ecosystems relies on allelochemicals, i.e., secondary metabolites produced and released by plants, and their effect on the germination, development, reproduction and survival of other nearby plants in the same population (Rice, 1984), it is precisely those phytochemicals that are at the focus of research in the field of bioherbicide development. Due to their destructive effects on plants (seed germination inhibition, shoot and root length restriction and photosynthesis and water/nutrient uptake disruption), various allelopathic compounds such as phenolic compounds (Arroyo et al., 2018), sterols and terpenes (Gaaliche et al., 2017), essential oils (Hazrati et al., 2018) and fatty acids (Qian et al., 2018) are already being tested for potential usage in weed control within organic agricultural systems. One of the advantages of using herbicides based on the mentioned natural compounds is that their half-lives are usually short, indicating that those bioherbicides will degrade quickly and leave no residues in the soil after harvest (Hazrati et al., 2018). Hence, literature data shows that secondary metabolites from plants could be a promising tool for weed control and their implementation in weed management would probably reduce chemical herbicide usage, which would further promote human health protection and environment preservation.

Among the identified chemicals with phytotoxic activity, phenolic compounds are shown to be one of the most important ones (Simões et al., 2008; Li et al, 2010; Mominul Islam & Kato-Noguchi, 2014). Thus a promising start for finding candidates for potential bioherbicides is to obtain plant extracts rich in phenolic compounds, i.e., with high antioxidant potential, and conduct quick testing of their phytotoxic activity by *in vitro* bioassays. Therefore, the main objective of this study was to test the antioxidant activity of extracts of common walnut (*Juglans regia* L.) and bitter dock (*Rumex obtusifolius* L.) leaves, and evaluate their impact on seed germination and seedling growth of *Setaria glauca* (L.) P. Beauv. (yellow foxtail) and *Sorghum halepense* (L.) Pers. (Johnson grass), two invasive weeds considered highly damaging to agricultural crops.

MATERIAL AND METHODS

Plant material and plant extraction

Fresh leaves of common walnut (*Juglans regia* L.) and bitter dock (*Rumex obtusifolius* L.) were collected in Vojvodina province, Serbia, in April 2019, then air dried under shade conditions, stored in paper bags in order to protect them from light and milled just before extraction.

A mix of methanol:acetone:distilled water (40:40:20 v/v) was used as extraction solvent. The powdery material was soaked in the solvent in 1:5 solid to volume ratio and sonicated for 15 min in an ultrasonic bath. Extraction was performed in triplicate and aliquots from three extractions were merged after centrifugation (3000 rpm, 10 min) and filtration (Whatman filter paper grade 1). Extract was evaporated to dryness at 50°C using a vacuum rotary evaporator. Residues were dissolved in distilled water, freeze dried, and kept at -20°C for future analysis.

In vitro evaluation of antioxidant activity

Antioxidant activity of the obtained extracts was tested by determining their radical scavenging activity (2,2-diphenyl-1-picrylhydrazyl [DPPH] scavenging capacity - DPPH assay) and by measuring their reducing potential (ferric ion reducing antioxidant power - FRAP assay).

DPPH assay: For evaluation of free radical scavenging activity, 0.25 g of dry leaf extract was dissolved in distilled water (25 ml), and series of dilutions were prepared from the stock solution in order to obtain concentrations within a range from 0.05 to 0.5 mg/ml. A volume of 0.5 ml of each dilution was mixed with 1 ml of DPPH solution (0.2 mM in methanol) and 3 ml of methanol in test tubes and vortexed well. Volume was adjusted up to 6 ml with methanol, and the solution was incubated for 30 minutes at 25 °C in the dark. The absorbance was measured by spectrophotometer at 517 nm. The solution containing only reagents, excepting the extract, was considered as a control, and antioxidant activity was calculated using the formula:

% inhibition = $[(Abs_{control} - Abs_{sample})/(Absc_{ontrol})] \times 100$. IC_{50} values (concentration of sample required to scavenge 50% of free radicals) were calculated. Ascorbic acid was used as a positive control.

FRAP assay: Acetate buffer (pH 3.6) (300 mM), TPTZ solution (10 mM in 40 mM HCl) and $FeCl_3 \times 6H_2O$ solution (20 mM) were mixed (10:1:1 v/v) into a FRAP solution. Dry leaf extract (150 µl, concentration 0.5 mg/ml in methanol) and 150 µl of distilled water were mixed with 3 ml of FRAP solution and incubated in the dark at 37 °C for 30 minutes. FRAP solution was used as a blank. Absorbance was measured at 593 nm by spectrophotometer. Ferrous sulphate heptahydrate ($FeSO_4 \times 7H_2O$) was used as equivalents for calibration curve preparation, and the results were expressed as µmol of ferric ion (Fe^{2+}) per g of dry extract. Ascorbic acid was used as a positive control.

Germination bioassay

A Petri-dish experiment was set up for *Setaria glauca* (L.) P. Beauv. (yellow foxtail) and *Sorghum halepense* (L.) Pers. (Johnson grass) under controlled conditions. Seeds of *S. glauca* were collected in fields around Batajnica (Belgrade, Central Serbia) in October 2017. Seeds of *S. halepense* were collected in fields around Zemun (Belgrade, Central Serbia) in October 2017. The seeds were cleaned and stored in paper bags in the laboratory at a temperature of 20-22 °C. Prior to the experiment the seeds were surface sterilized for 3 minutes in a 5% aqueous solution of sodium hypochlorite and washed several times with distilled water. Fifteen disinfected seeds were placed into each Petri dish (Ø = 9 cm) lined with sterilized filter paper disk. Common walnut and curly dock leaf extracts were diluted in distilled water to 0.5, 0.75 and 1% (w/v) concentrations and 5 ml was applied to each Petri dish. Distilled water served as a control. All dishes were sealed with parafilm to avoid evaporation. The dishes were placed in an incubator at 27±1 °C and kept in darkness. After a period of 7 days, the percentage of germination was calculated and early seedling growth (shoot and radical length) was measured. The experiment design was a randomized complete block with four replications, repeated twice, and data were combined for analysis.

Statistical analysis

All chemical measurements were performed in triplicates. The results of the FRAP assay were expressed as mean ± standard deviation. The IC_{50} values for DPPH assay were calculated using the GraphPad Prism statistical software package. Germination bioassay data were analyzed by a one-way analysis of variance (ANOVA), using the STATISTICA 8.0. software package. When F-values were statistically significant ($p<0.05$) treatments were compared using Fisher's Least Significant Difference (LSD) test.

RESULTS

Antioxidant activity of plant extracts

The scavenging effect of plant extracts on DPPH radical is shown in Figure 1. Both common walnut and bitter dock leaf extracts showed remarkably high DPPH reductions, compared with ascorbic acid at the same concentrations.

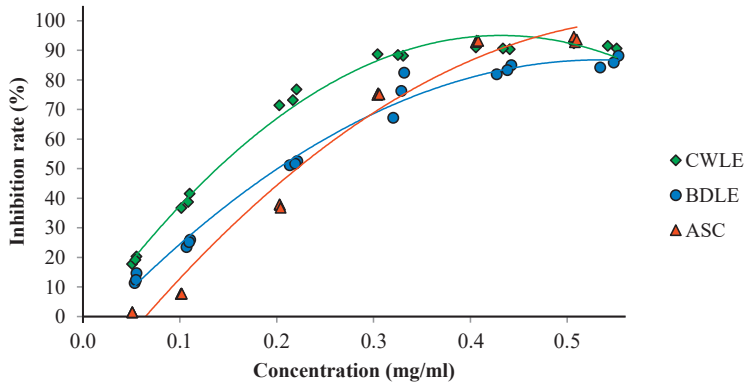

Figure 1. DPPH radical scavenging ability of common walnut (*J. regia*) and bitter dock (*R. obtusifolius*) leaf extracts (CWLE-common walnut leaf extract; BDLE-bitter dock leaf extract; ASC-ascorbic acid)

Common walnut leaf extract achieved approximately 20% inhibition already at its concentration of 0.05 mg/ml, and reached a little above 90% with the concentration of 0.5 mg/ml. Bitter dock leaf extract had somewhat lower scavenging ability as inhibition was approximately 13% at the concentration of 0.5 mg/ml, and reached around 85% at the concentration of 0.5 mg/ml. In comparison, the highest concentration (0.5 mg/ml) of ascorbic acid inhibited almost 94% of DPPH radicals, however, at lower concentrations this antioxidant had lower DPPH reduction activity than the obtained plant extracts. Powerful scavenging ability of the plant extracts is especially noticeable when comparing the calculated IC_{50} values. Thus, IC_{50} values were 0.127 mg/ml for common walnut leaf extract and 0.194 mg/ml for bitter dock leaf extract, both significantly lower compared with the IC_{50} value of ascorbic acid (0.228 mg/ml).

The ferric reducing antioxidant power (FRAP) of plant extract was also high. The results presented in Figure 2 demonstrate that the tested plant leaf extracts possess a significant ferric reducing capacity compared to the standard used (ascorbic acid), although pure antioxidant showed higher potency for this ability.

The FRAP value of common walnut leaf extract was 384.4 ± 8.1 μmol Fe^{2+}/g dry mass, the FRAP value of bitter dock leaf extract was 321.6 ± 2.5 μmol Fe^{2+}/g dry mass, while ascorbic acid had FRAP value of 2576.3 ± 21.5 μmol Fe^{2+}/g dry mass.

Germination bioassay

The effects of common walnut and bitter dock leaf extracts on *Setaria glauca* germination and seedling growth at different concentrations are presented in Figure 3.

Data show that over 90% of the seeds germinated in control petri dishes. Inhibition of *S. glauca* germination by both plant extracts was significant at all used concentrations, and especially high at the highest concentration. Common walnut leaf extract was overall more effective. Although this extract caused lower germination inhibition with its lowest concentration (0.5% w/v), compared to the same concentration (9.1% inhibition) of bitter dock leaf extract, inhibition of *S. glauca* germination caused by common walnut leaf extract at the concentration as low as 0.75% w/v was 34.5%, while it reached 67.3% at the highest test concentration (1% w/v). The lowest concentration of bitter dock leaf extract inhibited *S. glauca* germination by 18.2%, the 0.75% concentration achieved somewhat higher inhibition (23.6%), although not significant, while the highest used concentration reached 54.5% inhibition.

Considering seedling growth, both plant extracts significantly affected shoot and radical elongation of *S. glauca*. Reduction in shoot length was similarly high after treatments with common walnut and bitter dock leaf extracts. At the lowest and mid concentrations, inhibition of shoot length caused by walnut was 63.0 and 69.3%, while 59.4 and 68.3% was caused by dock, and the difference

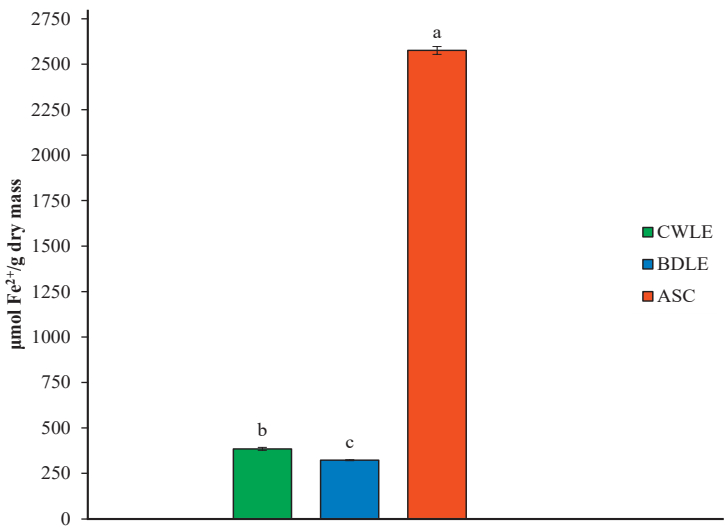

Figure 2. Ferric reducing antioxidant power (FRAP) of common walnut (*J. regia*) and bitter dock (*R. obtusifolius*) leaf extracts (CWLE-common walnut leaf extract; BDLE-bitter dock leaf extract; ASC-ascorbic acid). Data represent the mean values of three experiments (±SD). Values marked with different letters differ significantly ($p \leq 0.05$))

between these results was without statistical significance. As expected, the highest inhibition of shoot length occurred under the highest concentration of common walnut and bitter dock leaf extracts, and without statistical significance between the plant extracts – 80.7% and 78.2%, respectively. The extracts of test plants had even higher negative effect on radical elongation of *S. glauca*, and, although at lower concentrations the reduction in radical length caused by both plants was more or less similar (70.5-89.7% for common walnut; 86.1-89.9% for bitter dock), a significant difference was noted for the highest concentration, where common walnut leaf extract inhibited radical length by 96.4%, and bitter dock leaf extract by 93.1%.

The effects of common walnut and bitter dock leaf extracts on *Sorghum halepense* germination and seedling growth at different concentrations are presented in Figure 4.

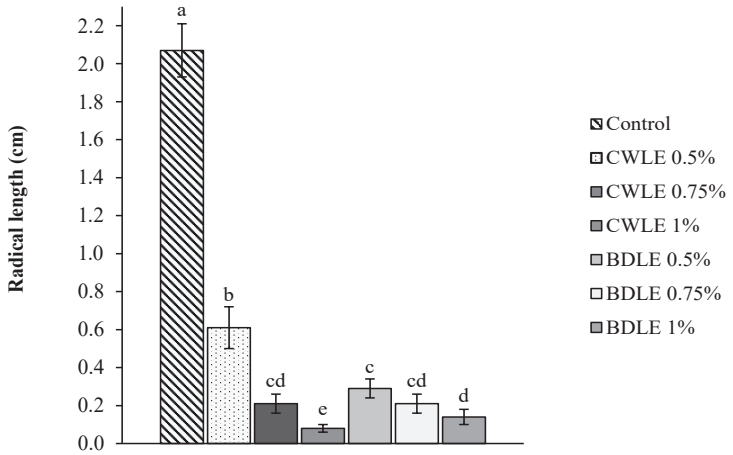

Figure 3. Effects of common walnut (*J. regia*) and bitter dock (*R. obtusifolius*) leaf extracts on germination (a), shoot elongation (b) and radical elongation (c) of *Setaria glauca* seed at different concentrations (CWLE-common walnut leaf extract; BDLE-bitter dock leaf extract). Data represent the mean values of experiments (±SD). Values marked with different letters differ significantly ($p \leq 0.05$)

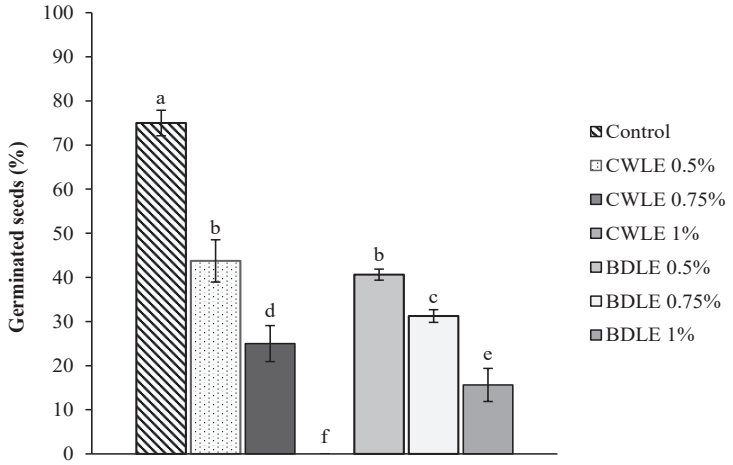

Figure 4. Effects of common walnut (*J. regia*) and bitter dock (*R. obtusifolius*) leaf extracts on germination (a), shoot elongation (b) and radical elongation (c) of *Sorghum halepense* seed at different concentrations (CWLE-common walnut leaf extract; BDLE-bitter dock leaf extract). Data represent the mean values of experiments (±SD). Values marked with different letters differ significantly ($p \leq 0.05$)

The results showed that seeds of *S. halepense* germinated also at high rate in the control - 75%. Both plant extracts highly inhibited the seed germination of this weed at all used concentrations. Inhibition by the lowest concentration, although high, was still under 50% and without a significant difference between the plants (41.7% for walnut and 45.8% for dock). However, the mid and highest concentrations of common walnut leaf extract showed extremely high effects on germination rate. Inhibition caused by the concentration of 0.75% w/v was 66.7%, while 1% w/v concentration of this extract prevented germination of *S. halepense* (100% inhibition). Bitter dock leaf extract did not cause total inhibition of germination of this weed, although the effect was still very high. Germination inhibition was 58.3% at the mid concentration and it reached 79.2% at the highest test concentration.

Seedling growth of *S. halepense* was significantly affected by both plant extracts. Common walnut leaf extract was overall more effective in reducing shoot length, as inhibition of shoot elongation was 90.4 and 92.0% already at the lowest and mid concentrations, respectively (without statistical differences). As mentioned before, germination inhibition at the highest concentration, and consequently shoot length inhibition, was 100%. Inhibition of shoot length caused by the three concentrations of bitter dock leaf extract from the lowest to the highest, was 72.0, 86.2 and 93.7%, respectively, and differences between those results were statistically significant.

As for *S. glauca*, negative effects of plant extracts on its radical length were even more pronounced in comparison with shoot length. At lower concentrations the reduction of radical elongation caused by both plants was without statistical differences and extremely high: 94.0-95.4% for common walnut and 92.9-96.1% for bitter dock. At the highest used concentration, as mentioned earlier, common walnut leaf extract inhibited germination, and consequently radical length, by 100%, while inhibition was 99.3% after treatment with bitter dock leaf extract.

DISCUSSION

Antioxidant activity of plant extracts

Walnut and dock leaves have been intensively used in medical practice, but also as a source of valuable compounds for various industrial applications based on their antioxidant properties. The varied biological activities of *Juglans* and *Rumex* species is due to the presence of various groups of biologically active substances in them, most belonging to phenolic compounds: tannins (galotannins and ellagitannins), a naphthoquinone derivative (juglone), anthraquinones, flavonoids (quercetin, kaempferol, etc.), phenolic acids (caffeic acid, p-coumaric acid, etc.) (Amaral et al., 2008; Zhou et al., 2015; Rusu et al., 2018; Jimoh et al., 2008; Wegiera et al., 2007; Litvinenko & Muzychina, 2008). Phenolic profiles of extracts obtained from these plants may differ quite significantly depending on the polarity of solvents used for extraction. In this study a mix of three solvents (acetone, methanol and water) was used in order to extract antioxidative phenolic compounds with a wide range of polarity. As antioxidants from plants respond in a different manner to different radical or oxidant sources (Prior et al., 2005), two methods based on different reaction mechanisms were used to determine the antioxidant activity of the obtained extracts.

Both common walnut and bitter dock leaf extracts obtained in this experiment showed to possess high antioxidant activities. Their affinity for scavenging free radicals was remarkably high, compared with ascorbic acid, with DPPH IC_{50} values being lower (higher antioxidant activity) than the IC_{50} value of ascorbic acid. Ferric reducing antioxidant power (FRAP) of plant extract was also significantly high. However, compared to the standard used (ascorbic acid), plant extracts showed lower ability to reduce transition metal ions.

The DPPH scavenging ability of the obtained common walnut leaf extract was higher than it was in bitter dock. Its IC_{50} value (0,127 mg/ml) was lower than those Pereira et al. (2007) found for walnut leaves aqueous extracts (0.151-0.202 mg/ml) or Carvalho et al. (2010) for walnut leaves methanol (0.199 mg/ml) and petroleum ether (2.921 mg/ml) extracts. On the other side, Santos et al. (2013) recorded even lower IC_{50} values (0.066 mg/ml) for walnut leaves methanol extracts from certain cultivars. Bitter dock leaf extract obtained in our present study had somewhat lower DPPH scavenging ability, compared to walnut, although still significantly higher than ascorbic acid. Its IC_{50} value (0.194 mg/ml) was significantly lower than those found for dock leaf extracts obtained by ultrasonic extraction, using methanol:water 80:20 solvent mix (approximately 40 mg/ml) (Wegiera et al., 2011), but higher than those obtained by Soxhlet extraction with methanol (0.078 mg/ml) (Harshaw et al., 2010). Inconsistencies in results are most likely due to the extraction methodology, which is important in any antioxidant assay as the yield of antioxidative phenolic compounds depends on the

solubility of natural products and choice of solvent. However, the importance of plant cultivar itself, as well as the growth environment and phase of plant ontogenetic development should not be underestimated. Extracts of common walnut and bitter dock leaves obtained in this experiment could be considered to have high radical scavenging ability.

Considering the ferric reducing antioxidant power, common walnut leaf extract showed a moderate ability to reduce metal ions. FRAP value obtained for this plant (384.4 μmol Fe^{2+}/g dry mass) was somewhat higher compared with bitter dock leaf extract. However, its potency regarding this ability was notably lower compared to ascorbic acid. A similar FRAP value for walnut leaves extract was obtained by Shah et al. (2018) (418.92 μmol Fe^{2+}/g dry mass), although this group of authors reported even higher values (up to 1067.94 μmol Fe^{2+}/g dry mass), depending on walnut genotype tested. The ferric reducing antioxidant power of bitter dock leaf extract was moderate, somewhat lower than the power of walnut, with a FRAP value 321.6 μmol Fe^{2+}/g dry mass. Antioxidant activity has been examined in a number of *Rumex* species, thus Jimoh et al. (2008) reported a significant ferric reducing antioxidant power of *Rumex ecklonianus* with FRAP value of 384.64 μmol Fe^{2+}/g dry mass for acetone leaf extract, 707.26 μmol Fe^{2+}/g dry mass for methanol leaf extract and 47.88 μmol Fe^{2+}/g dry mass for water leaf extract, while Chelly et al. (2021) tested bioproperties of methanol extracts of different parts of *Rumex roseus* and obtained a FRAP value for leaf of 700 μmol Fe^{2+}/g dry mass. Generally, regarding FRAP assay, it is difficult to make comparison with literature data primarily due to different interpretations of results, but also differences in plant cultivars, their phenological phase and plant growth conditions, as well as extraction solvents used and sample preparation procedures, especially considering that the extraction method has proved to have significant influence on FRAP.

Overall, bioactive compounds present in the obtained common walnut and bitter dock leaf extracts could be considered as interesting and economical sources of antioxidants with strong antioxidant capacity for various applications, including potential weed control.

Allelopathic potential of plant extracts

The results of our research indicate that common walnut and bitter dock leaf extracts were phytotoxic to both tested weeds - *S. glauca* and *S. halepense*.

For *S. glauca,* the allelopathic effect expressed through the inhibition of seed germination was more pronounced only after treatments with the highest used concentrations of extracts, reaching over 50% inhibition, and common walnut leaf extract was more efficient regarding this bioassay parameter. Regarding *S. halepense,* both extracts used already at their mid concentrations caused over 50% seed germination inhibition. At the highest concentration, bitter dock leaf extract extremely reduced the germination rate of *S. halepense*, while common walnut leaf extract fully stopped its germination (100% inhibition). Inhibition of seed germination by the tested extracts can be attributed to the presence of phenolic compounds and effects may be due to their synergistic effect rather than a single constituent. Based on the strong antioxidant capacity of the extracts it could be assumed that phenolic compounds (flavonoids, phenolic acids, tannins, quinines, etc.) are most probably present in large quantities in common walnut and bitter dock leaves extracts. As those phenolic compounds could interfere with the activities of respiratory enzymes in seed germination (Muscolo et al., 2001) or alter the activities of the growth hormone gibberellic acid, thereby causing inhibitory effect on its germination (Olofsdotter, 2001), their presence in the extracts could probably be the reason for the obtained inhibitions.

Considering seedling growth, both plant extracts significantly reduced shoot and radical length of both tested weeds. Regarding *S. glauca,* shoot length was similarly affected by the same concentrations of common walnut and bitter dock leaf extracts, and both tested extracts inhibited shoot elongation at almost the same rate without statistically significant difference. Radical elongation of this weed was more sensitive to bitter dock leaf extract at the lowest tested concentration but the highest concentration of common walnut leaf extract achieved higher inhibition of this parameter. *S. halepense* was even more sensitive to the tested plant extracts, and overall the common walnut leaf extract had a higher impact on its shoot and radical length than the bitter dock leaf extract. Radical elongation was for this weed also more intensively inhibited than shoot elongation. Generally, roots often show higher sensibility compared with shoots, as roots of plants are in fact the first plant organ to absorb allelopathic compounds from extracts (Nishida et al., 2005). Besides, root tissue is more permeable than shoot tissue (Mominul Mominul Islam & Kato-Noguchi, 2013). Phenolic compounds, namely flavonoids, have been shown to influence the expression of specific genes

associated with root tissue differentiation, decreasing root development (Franco et al., 2015), and generally many studies report suppressions of root growth due to decrease in mitotic cell division in root apex when exposed to different plant extracts (Levizou et al., 2002; Piyatida & Kato-Noguchi, 2010; Morikawa et al., 2012, Rob et al., 2021).

Overall, common walnut leaf extract showed greater efficacy in inhibiting germination and seedling elongation of the test weeds, compared to bitter dock leaf extract, and there is a consistency regarding antioxidant activities of the extracts and their phytotoxicity as the walnut leaf extract also had a higher antioxidant potential. Walnut is one of the most famous allelopathic plants (Ercisli et al., 2005) and its toxicity is associated mostly with the powerful napthoquinone juglone (Strugstad & Despotovski, 2012). However, juglone is not expected to be the only allelochemical present in *Juglans* species. Studies regarding the allelopathic potential of walnut are generally focused on the effect of juglone itself, while some studies have also investigated the effect of walnut leaf extracts with or without quantification of contents of juglone and/or other phenolic compounds in them, and all studies reported high inhibitory effects on germination and seedling growth of various cultivated or native plants (Babula et al., 2014; Zubay et al., 2021; Ercisli et al., 2005; Kocacë Aliskan & Terzi, 2001; Ercisli & Turkkal, 2005; Medic et al., 2021). Regarding *Rumex* species, less literature data is available dealing with its allelopathic potential. Zaller (2006) tested the effect of an aqueous extract of bitter dock leaves on seed germination of 14 plant species belonging to graminoids, non-leguminous forbs and leguminous forbs, and revealed that all tested grasses were heavily inhibited by the extracts, while herbs and legumes varied from unaffected to heavily inhibited. Another group of authors also pointed out strong inhibiting effects of *R. crispus* and *R. obtusifolius* leaf extracts on tested grassland grass species (Dragomir et al., 2017), while allelopathic effects of those extracts were severe for some grassland perennial legume seeds (*Medicago sativa*, *Trifolium pratense* and *Lotus corniculatus*) and moderate for others (*Trifolium repens*) (Camen et al., 2017). As indicated in numerous previous studies, the effects of identical extracts are not necessarily the same across various plants, i.e. each plant species has its own response to allelochemicals (Medic et al., 2021). Our research showed that *S. halepense* seeds were more sensitive to the tested common walnut and bitter dock leaf extracts than *S. glauca* seeds.

CONCLUSION

The results of the present study demonstrated that the obtained common walnut (*Juglans regia* L.) and bitter dock (*Rumex obtusifolius* L.) leaves extracts have pronounced antioxidant activity, and it could be inferred from the presented preliminary investigation that they possess remarkable allelopathic potential as both had significant negative impact on the germination and seedling growth of the tested weeds *Setaria glauca* (L.) P. Beauv. and *Sorghum halepense* (L.) Pers. The level of growth suppression varied with extract concentration and examined plant species. An association was noted between the antioxidative potential of extracts and their phytotoxicity, suggesting that phenolic compounds, the dominant antioxidant components in plants, could be responsible for their allelopathic potential. The result may be useful for future research in the field of bioherbicide development, but additional studies are required to validate the present results under field conditions and to test extract phytotoxicity to cultivated plants before proceeding towards development of herbicides based on natural products.

ACKNOWLEDGEMENT

This investigation was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grants No. 451-03-9/2021-14/200214 and 451-03-9/2021-14/200125).

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Antioksidativna aktivnost i skrining alelopatskog potencijala ekstrakata lista *Juglans regia* L. i *Rumex obtusifolius* L.

REZIME

Alelopatski potencijal i fitotoksičnost sekundarnih metabolita biljaka značajni su sa aspekta biotehnologije imajući u vidu da ove fitohemikalije u svojstvu alelohemikalija mogu značajno da doprinesu razvoju agroindustrijskog sektora. Cilj rada bio je da se dobiju ekstrakti lista oraha (*Juglans regia* L.) i štavelja (*Rumex obtusifolius* L.) bogati fenolnim jedinjenjima odnosno sa visokim antioksidativnim potencijalom i da se ispita njihova fitotoksičnost prema korovskim vrstama *Setaria glauca* i *Sorghum halepense* kroz inhibiciju klijanja i rasta klijanaca. Dobijeni biljni ekstrakti pokazali su izražen potencijal u neutralisanju DPPH radikala, sa IC_{50} vrednostima od 0,127 mg/ml za ekstrakt lista oraha i 0,194 mg/ml za ekstrakt lista štavelja. Redukciona sposobnost jona metala dobijenih biljnih ekstrakata takođe je bila veoma visoka, FRAP vrednost za ekstrakt lista oraha iznosila je 384,4 ± 8.1 μmol Fe^{2+}/g suvog ekstrakta, dok je za ekstrakt lista štavelje iznosila 321,6 ± 2.5 μmol Fe^{2+}/g suvog ekstrakta. Pri najvećoj korištenoj koncentraciji ekstrakt lista oraha inhibirao je klijanje semena *S. glauca* za 67,3%, dok je ekstrakt lista štavelja inhibirao klijanje ovog korova za 54,5%. Porast stabaoceta *S. glauca* inhibiran je 80,7% nakon tretmana ekstraktom lista oraha, a 78,2% pod uticajem ekstrakta lista štavelja, dok je porast korenka inhibiran 96,4%, odnosno 93,1%. Klijanje semena *S. halepense* potpuno je inhibirano (100%) pod uticajem ekstrakta lista oraha, dok je ekstrakt lista štavelja pri najvećoj korišćenoj koncentraciji prouzrokovao 79,2% inhibicije. Porast stabaoceta ovog korova potpuno je redukovan (100%) nakon tretmana ekstraktom lista oraha, a ekstrakt lista štavelja prouzrokovao je 93,7% inhibicije ovog parametra. Porast korenka inhibiran je 100% odnosno 99.3% nakon tretmana ovim ekstraktima. Dobijeni rezultati ukazuju na postojanje značajne antioksidativne aktivnosti i izraženog alelopatskog potencijala dobijenih ekstrakata lista oraha i lista štavelja.

Ključne reči: orah, štavelj, korovi, antioksidativna aktivnost, alelopatija

Pestic. Phytomed. (Belgrade), 37(1), 2022, 21–27
DOI: https://doi.org/10.2298/PIF2201021J

UDC 632.35:632.982.6:631.41:582.583(63)
Original scientific paper

Management of ginger bacterial wilt (*Ralstonia solanacearum*) epidemics by biofumigation at Tepi, southwestern Ethiopia

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Received: 15 November 2021
Accepted: 22 February 2022

SUMMARY

Bacterial wilt of ginger, caused by *Ralstonia solanacearum*, is the most damaging disease, which brings rapid and serious wilting, and reduces the quality and yield of ginger rhizome in Ethiopia. Thus, an experiment was carried out to evaluate the effect of different biofumigants on bacterial wilt in Ethiopia during the 2019 and 2020 main cropping seasons. The experiments were conducted at the Tepi Agricultural Research Center. Different biofumigation soil amendments (citronella, palmarosa, mint, lemongrass and Chinese chive) were applied before planting. The trials were arranged in a randomized complete block design with three replications. Examination of variance showed that soil amendments with biofumigants strongly decreased bacterial wilt severity and improved rhizome yield and components. Rhizome yield gains of about 90.2% were achieved by soil biofumigation with lemongrass, as compared to untreated control. The relative mean rhizome yield damage due to bacterial wilt in the control plot was 47.4%. Wilt severity was inversely and very significantly ($p \leq 0.01$) proportional ($r = -0.90^{**}$) to rhizome yield. The overall results of the study show that soil amendments with botanicals, particularly lemongrass, before planting should be used to manage ginger bacterial wilt in experimental areas and further similar agro-ecologies.

Keywords: ginger, bacterial wilt, biofumigants, yield, Ethiopia

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.), which belongs to the family Zingiberaceae, is a herbaceous plant with rhizomes which are used as spice. For small scale farmers in southwestern Ethiopia, ginger is one the most important cultivated spices. In southwestern parts of the country ginger has a major share in cropping systems. According to Geta & Kifle, A, (2011), of the total arable land owned by farmers in the Southern Nations, Nationalities and Peoples Regional State, 85% of the land and 35% of the growers are associated with ginger production. It has been one of the leading spice export commodities in Ethiopia over the past almost 10 years, and it earned the country 22.6 million $USD (Wubshet, 2018). However, ginger production is largely affected by diseases caused by bacteria, fungi, viruses, mycoplasma and nematodes (Paret et al., 2010;

Sharma et al., 2010). Bacterial wilt of ginger (*Ralstonia solanacearum*) imposes serious economic losses and it is widely disseminated in most tropical and subtropical areas of the world (Yabuuchi et al., 1995; Kumar & Sarma, 2004). Bacterial wilt is a very significant disease in major ginger growing areas of southwestern Ethiopia, where ginger is extensively and mainly produced for marketable purposes (Habetewold et al., 2015).

The use of essential oils to kill or suppress a pathogen causing disease is called biofumigation. Those oils are constituents of some green compost plants, viz. mint, palmarosa, citronella and lemon-grass. After these florae are mixed or cultivated into soil for 2 or 3 months prior to planting, they decay and release vital oils that are poisonous to the virulent pathogen present in that soil. According to a report by Paret et al., (2010), palmarosa and lemongrass oils highly reduced the incidence of bacterial wilt both in the laboratory and under greenhouse conditions.

Also biofumigation is a cultural practice that uses explosive chemicals released from decaying *Brassica* cells to overcome soil-borne pathogens and pests. The main volatile oils released during decomposition of *Brassica* tissues are isothiocyanates. Isothiocyanates are associated with the active ingredient of the marketable fumigants dazomet and metham sodium, and are very toxic to different pathogens and pests. They are produced subsequent to tissue injury, after myrosinase enzyme hydrolyses glucosinolates at neutral pH. Glucosinolates containing sulfur (thioglucosides) are released as secondary metabolites by *Brassica* species and most investigators have confidence in their initial role as deliverers of resistance against pathogens and pests. Bactericidal effect of different isothiocyanates produced by *Brassica* tissues is known (Brown & Morra, 2005). Thus it is very important to have a certain level of bacterial wilt management through soil amendments with different biofumigants as disease management schemes. Therefore, the present study was designed to determine the magnitude of bacterial wilt disease epidemics and ginger rhizome yield losses through soil amendments with biofumigants.

MATERIAL AND METHODS

Description of experimental area

Field trials were conducted at the Tepi Agricultural Research Centre during the 2019 and 2020 main growing periods. It is located at 35°08' longitude and 7°08' latitude and 1200 m asl. The lowest and highest mean temperatures are 15 and 30 °C, respectively. Its annual mean rainfall is 1630 mm (Jibat et al., 2018).

Soil amendment with biofumigant plants, viz. citronella, lemongrass, palmarosa, mint and chinese chive, applied at 10 tones/ha one month before planting and mulching after planting, was implemented as a cultural management practice to reduce pathogen inoculum and prevent disease epidemics, and an unamended plot was used as a control or check plot. The experiment relied entirely on a natural epidemic of bacterial wilt because the sites had been confirmed as hot spots of disease in previous field history.

A total of six treatments, including controls, were laid out in a randomized complete block design with three replications. Planting was carried out on a gross plot size of 18 m^2 (3 m width and 6 m length) with ten 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 1.5 and 2 m, respectively.

Data collected

Growth and yield parameters

Data about ginger growth and yield parameters were recorded from the central four rows of each plot. The number of tillers per plant (NTPP) was recorded as the number of tiller shoots produced by 12 sample plants and their mean was considered as the number of tillers per plant per plot at the time of physiological maturity. The number of fingers per plant (NFPP) was determined as the number of rhizome fingers that arose from mother rhizomes of 12 plants and their mean was taken to represent the number of fingers per rhizome per plot at the time of harvesting. Total rhizome yield (kg ha^{-1}) was also calculated as the rhizome yield per kilogram harvested from four central rows and converted to per hectare using the following formula:

$$\text{Yield (kg ha}^{-1}) = \frac{\text{Yield (kg) of four central rows} \times 10{,}000 \times \text{m}^2}{\text{Net area (m}^2\text{) of four central rows/plot}}$$

Relative yield loss (%) from each plot was calculated using the formula suggested by Sharma et al. (2008):

$$\text{RYL (\%)} = \frac{(Y1 - Y2) \times 100}{Y1}$$

where RYL = relative yield loss in rhizome yield (reduction in rhizome yield); Y_1 = maximum mean

rhizome yield of the best treatment in the experiment; and Y_2 = mean yield of the other treatment/control plots.

Wilt incidence assessment

Ginger bacterial wilt occurrence (number of plants wilted) was calculated visually starting from the observance of symptoms in the field. Ginger plants that showed either complete or partial wilting were all considered wilted and staked to avoid double counting in subsequent assessments. Wilt incidence was then calculated for each treatment as the percentage of total number of plants emerged, and the result on the final assessment date was presented in Table 1.

Data Analysis

Analysis of variance (ANOVA) was run for growth, yield and wilt incidence data to compare the effects of treatments. The least significant difference at 5% level of significance was used for mean separation. Analysis of variance was done using the SAS GLM procedure version 9.3 (SAS, 2014). Correlation coefficients of ginger growth, yield and yield components with the last date of disease incidence assessment were computed to establish their associations.

RESULTS AND DISCUSSION

Disease incidence

Two years data were pooled because of homogeneity of variances as tested using Bartlett's test (Gomez & Gomez, 1984) and the F-test was non-significant for most of the parameters studied in each year. Therefore, data were combined for analysis. The analysis of variance shows significant differences among different biofumigant plants evaluated and control treatments at the final date of wilt assessments and area under disease progress curve (AUDPC). The minimum mean disease incidence was found in the plot amended with lemongrass (49.43%), while the maximum mean wilt disease incidence was recorded in control plots (61.13%) at the final date of assessment. Similar trends were also observed for AUDPC (Table 1).

To date there is no single and effective control measure against the bacterial wilt-causing pathogen (Jibat et al., 2018). In this study, however, lower wilt incidence and higher reductions in mean wilt incidence were possible through the application of lemongrass alone, compared to control plots. This might be because lemongrass suppresses certain microorganisms in soil and also it could have stimulated development and growth of other microorganisms as biofumigation might have augmented the soil organic matter, which is the energy basis for microbial activities (Wang et al., 2012).

A related study also revealed that tomato yield from a biofumigated field was higher than in a control field as biofumigation decomposition increased soil fertility, thus encouraging further growth and yield of the crop (Katan et al., 1980). Similarly, it was found that biofumigation of soil maximizes nitrogen, calcium and magnesium accessibility (Stapleton et al., 2000).

Table 1. Effects of biofumigation on bacterial wilt (*Ralstonia solanacearum*) disease at the final date of disease assessment (%) and AUDPC (%-days) at Tepi, Ethiopia, during the 2019 and 2020 main cropping seasons

Treatments	PSI (%)[1]	AUDPC (%-days)[2]
Citronella	55.4^{abc}	112.29^{a}
Palmarosa	49.66^{c}	101.79^{ab}
Mint	56.43^{ab}	111.53^{a}
Lemongrass	42.43^{d}	84.84^{b}
Chinese chive	53.23^{bc}	107.23^{a}
Control	61.13^{a}	113.25^{a}
LSD (5%)	6.63	17.51
CV (%)	6.87	9.15

[1]Percent severity index 120 days after planting (DAP), [2]Area under progress of bacterial wilt disease of ginger. Values in each column followed by the same letters are not significantly different at 5% probability level

Effects of biofumigation on ginger growth parameters

The management practices of biofumigation soil amendment produced highly and significantly ($P < 0.01$) different effects in both growth and yield parameters, except for rhizome length and width, which showed non-significant difference. The maximum number of tillers per plant (4.8) was recorded in plots treated with lemongrass but it was statistically on par with the plots treated with citronella and palmarosa. In contrast, the lowest number of tillers per plant was recorded in the untreated control plots (2.83) (Table 2).

A marked difference was also observed between treated and untreated control plots in rhizome length, rhizome width and number of fingers per rhizome. Higher rhizome length (11.21 cm) and width (4.11 cm) were measured in plants from the plots amended with

lemongrass. The highest number of fingers per rhizome was also counted in lemongrass plots (6.3), compared to the rest of the treatments. The lowest number of tillers per plant, rhizome length, rhizome width and number of fingers per rhizome were recorded in the control plots, i.e. 2.83, 7.66, 2.88 and 2.53, respectively.

The advantage in comparative growth resulting from the application of soil amendment could be due to increased soil health and its improved physical and chemical status, which in turn reduced infection by soil pathogens. In this context, Bailey et al. (2003) indicated that organic amendments to soil have direct effect on plant health and crop productivity by improving the physical, chemical and biological properties of soil, which then have positive effects on plant growth. On the other hand, degradation of organic matter in soil directly affects the viability and existence of pathogens in soil by limiting available nutrients and releasing natural chemical substances with variable inhibitory properties, and stimulates the activities of microorganisms that are antagonistic to those pathogens (Akhtar & Malik, 2000), and increases soil microbial activities thereby leading to intense competition (Bailey et al., 2003).

Table 2. Effects of biofumigation on growth, yield and yield components at Tepi, Ethiopia during the 2019/2020 main cropping seasons

Treatments	NTPP*	RL*	RW*	NFPR*	Yield (t ha^{-1})
Citronella	4.26^{ab}	8.96^{bc}	3.76^{ab}	3.3^{c}	9.63^{bc}
Palmarosa	4.23^{ab}	9.93^{ab}	4.18^{a}	5.3^{b}	10.4^{b}
Mint	3.63^{b}	7.96^{c}	3.41^{ab}	2.7^{c}	8.96^{c}
Lemongrass	4.8^{a}	11.21^{a}	4.11^{ab}	6.3^{a}	13.43^{a}
Chinese chive	3.6^{b}	8.6b^{c}	3.30^{ab}	5.2^{b}	12.36^{a}
Control	2.83^{c}	7.66^{c}	2.88^{b}	2.53^{c}	7.06^{d}
LSD (5%)	0.72	1.86	1.23	0.78	1.31
CV (%)	10.28	11.29	18.78	10.19	7.01

*NTPP: number of tillers per plant; RL: rhizome length (cm); RW: rhizome width (cm); NFPR: number of fingers per rhizome; Means followed by the same letter(s) columnwise and in adjacent columns are not significantly different at 5% level of significance.

It is well documented that soil amendments that enhance host plant growth and resistance have significant effects in reducing disease incidence (Datnoff et al., 2007), improving tolerance to environmental and pest stress, and enhancing crop growth, yield and quality parameters (Sahebi et al., 2016). For instance, application of potassium fertilizer with lemongrass soil supplement increased ginger growth and yield (Jibat et al., 2018), and the same was shown in this study. Analysis of variance also revealed highly significant ($P < 0.001$) variation between different botanical soil amendments in rhizome yield. The highest (13.43 t ha^{-1}) rhizome yield was obtained from plots treated with lemongrass, followed by plots treated with Chinese chive (12.36). The lowest rhizome yield was harvested from untreated plots, which was about 7.06 t ha^{-1}.

Cultural practices that include soil biofumigation are the most popular approach to manage bacterial wilt by reducing the incidence and severity of wilt, and consequently sustaining productivity of crops. Several studies have also described the effects of cultural practices in reducing bacterial wilt incidence and increasing yield of various crops (Anith et al., 2000; Ji et al., 2005; Yadessa et al., 2010; Ayana et al., 2011; Lee et al., 2012; Sahebi et al., 2016). The mechanisms of disease suppression and increase in rhizome yield was supposed to be based on increase in soil nutrients, changes in physical and chemical properties of the experimental soil due to fertilizer and lemongrass incorporation. In this regard, lemongrass might release essential oils into soil and so reduce bacterial population. Moreover, soil fumigation with botanicals could enhance the capabilities of beneficial microbes against target pathogens.

In line with this finding, Yadessa et al. (2010) found that soil amendments with cocoa peat, farmyard manure, compost and green manure significantly reduced bacterial wilt incidence by 81% and enhanced tomato yield in amended over unamended soil. Some other studies have also noted that lemongrass oil provided protection from tomato wilt by reducing pathogen population and increasing yield under controlled conditions; and disease suppression reached 45-60% under field conditions (Ji et al., 2005). However, the efficacy of lemongrass excelled when integrated with other soil amendment tactics under field conditions (Hong et al., 2011). Furthermore, other related findings demonstrated that soil solarization, combined with fumigation (Yamada et al., 1997) and biological control agents (Kumar & Sood, 2002), reduced the incidence of tomato bacterial wilt and increased fruit yield.

The relative yield loss due to bacterial wilt, calculated against untreated plots, was 47.4% (Table 3). Relative yield loss was reduced by soil amendments with lemongrass. The lemongrass-amended plots recorded 8.97-33.29% higher yields in comparison with other treatments, excluding the control. Soil amendments with Chinese chive caused the next lowest (8.97%) mean relative yield loss. Yield gains of 90.2% were obtained due to soil biofumigation with lemongrass. Theoretically, integrated disease management is intended to eliminate

or reduce initial inocula, reduce the effectiveness of initial inocula, increase resistance of hosts, delay disease onset and slow down secondary cycles of infections (Agrios, 2005). This might imply that lemongrass biofumigation along with soil solarization and fertilization highly reduced *R. solanacearum* population and subsequent damage of ginger by mechanisms discribed above. Previous research results have also shown that the application of *Brassica* spp. as green manure is effective in reducing soilborne pathogens through the release of toxic and volatile chemicals after decomposition (Brown & Morra, 2005). Application of plastic mulch, followed by green manure incorporation, would enhance the decomposition process, minimize the escape of volatile gases into the atmosphere and raise soil temperature to kill soilborne pathogen propagules and, as a result, reduce plant yield losses due to disease (Katan et al., 1980).

Association of disease, growth and yield parameters

Computing correlation between final mean disease incidence, and growth and yield parameters was important since change of wilt incidence influenced the response of growth and yield parameters during the experiment. The average bacterial wilt incidence (120 DAP) had a negative and highly significantly ($P \leq 0.01$) association ($r = -0.90^{**}$) to rhizome yield (Table 4). Moreover, the final mean disease incidence (120 DAP) and number of fingers per rhizome were observed to correlate ($r = -0.73^{**}$) negatively and highly significantly ($P \leq 0.01$) over the cropping season. More or less similar phenomena were noted for the correlation between mean wilt incidence and growth and yield parameters. Such findings confirm negative effects of bacterial wilt on the growth, rhizome yield and components of ginger. This complies with the findings of Bekele and Gebremedhin (2000) who found that late blight disease parameters strongly and negatively correlated with final tuber yields. Research reports of Fekede (2011) also confirmed that disease parameter is associated with yield components. Inverse relations were found between chocolate spot disease and grain yield and components of faba bean in sole and mixed cropping systems (Sahile et al., 2010).

Table 3. Effects of biofumigation soil amendment on ginger rhizome yield (t ha^{-1}) and rhizome yield losses at Tepi, Ethiopia, during the 2019 and 2020 main cropping seasons

	Yield (t ha^{-1})	Relative yield (%)	Relative yield loss (%)
Citronella	9.63	71.70	–28.3
Palmarosa	10.4	77.43	–22.57
Mint	8.96	66.71	–33.29
Lemongrass	13.43	100	0.00
Chinese chive	12.36	92.03	–8.97
Control	7.06	52.6	–47.4

Table 4. Coefficients of correlation (r) between growth, yield and disease incidence in ginger at Tepi during the 2019 and 2020 main cropping seasons

Parameter	Yield (t ha^{-1})	Finger per rhizome	Rhizome length height	Tiller per plant	FDI (120 DAP)	AUDPC (% days)
Yield (t ha^{-1})	1					
Finger per rhizome	0.71**	1				
Rhizome length	0.94**	0.75**	1			
Tiller per plant	0.78**	0.65**	0.89**	1		
FDI (120 DAP)	–0.90**	–0.73**	–0.84**	–0.61**	1	
AUDPC (% days)	–0.91**	–0.75**	–0.86**	–0.64**	0.98**	1

** Describes level of statistical significance at $P \leq 0.01$

CONCLUSIONS

The present field data provided empirical evidence that soil amendment with plant biofumigants as green manure before planting reduced yield loss due to ginger bacterial wilt. Soil amendment with plant biofumigants improved rhizome yield, yield components and growth of ginger, compared to untreated control plots. The results demonstrated that, considering all biofumigants used, soil amendment with lemongrass as green manure before planting ginger enhanced ginger rhizome yield, yield components and growth parameters, and highly reduced associated damage due to bacterial wilt incidence, compared to the untreated control and other treatments. These present findings can benefit farmers through increased productivity and income by way of reducing inputs into non-chemical means of control of bacterial wilt epidemics. Additionally, much more research work can be done with biofumigant effects on soil physico-chemical properties and mechanisms by which green manure soil amendment reduces the incidence of ginger bacterial wilt.

ACKNOWLEDGEMENTS

We are very grateful to the Ethiopian Institute of Agricultural Research for financing the study.

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Suzbijaje epidemija bakterijskog uvenuća đumbira (*Ralstonia solanacearum*) biofumigacijom u Tepi, jugozapadna Etiopija

REZIME

Bakterijsko uvenuće đumbira, izazvano bakterijom *Ralstonia solanacearum*, je bolest koja prouzrokuje najveće štete u Etiopiji, dovodeći do ubrzanog i raširenog uvenuća, odnosno umanjenja prinosa i kvaliteta rizoma đumbira. Eksperiment je izveden kako bi se procenio uticaj različitih biofumiganata na bakterijsko uvenuće u Etiopiji tokom perioda vegetacije u godinama 2019-2020. Eksperiment je izveden u Agricultural Research Center u Tepi. Pre sadnje, primenjeni su različiti biofumiganti za obogaćivanje zemljišta (citronela, palmarosa, nana, limunova trava i kineski vlašac). Ogledi su izvedeni u potpuno slučajnom blok sistemu sa tri ponavljanja. Ocena varijanse pokazala je da je dodavanje biofumiganata zemljištu u velikoj meri umanjilo delovanje bakterijskog uvenuća i poboljšalo razvoj rizoma i njegovih komponenti. Poboljšanje prinosa rizoma od oko 90.2%, u poređenju sa netretiranom kontrolom, postugnuto je upotrebom biofumiganta sa limunskom travom. Relativna šteta u prinosu rizoma kao posledica bakterijskog uvenuća na kontrolnoj parceli bila je 47.4%. Smanjenje prinosa bilo je manje na parcelama gde je primenjena limunska trava. Delovanje uvenuća bilo je značajno ($p \leq 0.01$) i obrnuto proporcionalno ($r = -0.90^{**}$) prinosu rizoma. Rezultati istraživanja pokazuju da se limunska trava može koristiti za tretman zemljišta pre sadnje u eksperimentalnim zonama u budućim agro-ekološkim istraživanjima.

Ključne reči: đumbir, bakterijsko uvenuće, biofumiganti, prinos, Etiopija

Pestic. Phytomed. (Belgrade), 37(1), 2022, 29–39
DOI: https://doi.org/10.2298/PIF2201029T

UDC 632.951:504.054:504.42:591.16:593.95
Original scientific paper

Investigation of spermiotoxic, embryotoxic and cytotoxic effects of copper pyrithione on *Paracentrotus lividus* (Lamarck, 1816)

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Received: 22 September 2021
Accepted: 3 January 2022

SUMMARY

Spermiotoxic, embryotoxic and cytotoxic effects of the widely used biofouling biocide copper pyrithione (CuPt) were evaluated in bioassays to examine the inhibition of fertilization rate, offspring quality and effects on early development of the sea urchin *Paracentrotus lividus*. CuPt was non-spermiotoxic for fertilization rates but the frequency of embryonic malformations increased in a concentration-dependent manner when eggs were fertilized with CuPt-exposed sperm. CuPt EC_{50} was calculated to be 13.58 µg/l for embryotoxicity. While the frequency of normally developed plutei decreased, the number of larvae with skeletal deformations increased. The IC_{25} and IC_{50} values in cytotoxicity assays were calculated to be 12.79 and 47.85 µg/l, respectively. The study revealed statistically significant decrease in the number of mitotically dividing cells, increase in the percentage of interphase cells and increased chromosomal abnormalities in the exposed cells. According to these results, CuPt can be said to have a highly toxic effect on sea urchin embryos at the applied concentrations. This situation suggests that there may be a potential risk of marine contamination with CuPt for this species.

Keywords: biocides, copper pyrithione, developmental biology, sea urchin

INTRODUCTION

Biofouling is the growth of unwanted organisms on submerged/semi-submerged structures, adding friction to ships leading to increased fuel consumption and economic loss (Egardt et al., 2017). Anti-fouling biocides are used to impede the growth of aquatic organisms, such as mussels, crustacea, barnacles and algae, on the submerged parts of marine vessels. They interact with organisms and have variable toxic impacts, and high level of toxicity in some cases. Sublethal effects on aquatic organisms include decreased growth rates and lower reproducibility (Fernández-Alba et al. 2002). Common anti-fouling agents, such as tributyltin (TBT), were therefore banned in 2008. Alternative biocides which cause less toxicity to non-target organisms, broad

antimicrobial activity, low water solubility and high degradability have been used in marine antifouling paints as replacements for TBT. Pyrithione salts, such as zinc pyrithione (ZnPt) and copper pyrithione (CuPt), were introduced on the market in the 1990s as alternatives to TBT. They are both lipophilic metal complexes, which can interact with free metal ions in seawater by exchanging their metal ions (Maraldo & Dahllof, 2004; Martins et al. 2018).

The widespread use of CuPt has become a focal concern of recent studies and more research has been conducted on its ecotoxicity to non-target aquatic organisms, such as fish and invertebrates (Koutsaftis & Aoyama, 2007; Onduka et al., 2010; Bao et al., 2011; Wang et al., 2011). High CuPt toxicity to non-target species of different trophic levels, such as the algae *Skeletonemacostatum* (72-h EC_{50} = 1.5 μg/l), crustacean *Tigriopus japonicus* (24-h EC_{50} = 23 μg/L), and fish *Pagrus major* (96-h LC_{50} = 9.3 μg/L), was reported (Onduka et al., 2010). In a study where toxic effects of CuPt, zinc pyrithione (ZnPt), Sea Nine-211, diuron, Irgarol 1051 (also termed co-biocide) and KH101 to *Oncorhynchus tshawytscha* were studied, the highest toxicity was found to result from CuPt (Okamura et al., 2002). Embryotoxicity of CuPt to the sea urchin *Strongylocentrotus intermedius* was reported as EC_{50} = 32.93 nM (Wang et al., 2011). In another study using the same species, the EC_{50} value in the first 6 h after fertilization was 4000 nM (Xue et al., 2011).

In different studies with aquatic organisms, such as *Perinereis nuntia* (Mochida et al., 2011), *Artemia salina* (Koutsaftis & Aoyama, 2007) and sea urchins (Kobayashi & Okamura, 2002), the toxicity of various biocides was investigated, and the CuPt biocide was shown to be highly toxic to these organisms.

Toxicity bioassays are biological tools complementing analytical chemistry techniques for assessing biological effects of pollution in complex matrices, such as sediment, and they usually involve the use of test organisms in the laboratory to predict ecosystem-level effects. Their additional advantage is that they detect new/emerging contaminants for which no analytical techniques have yet been developed or validated, providing an insight into the bioavailability of pollutants or integrating the toxic effects of different substances in the environment. The sea urchin and bivalve embryo development test is a standardized, economic, rapid chronic toxicity tool successfully used in screening the toxicity of known, emerging contaminants and other pollutants and their mixtures in natural matrices, such as water and sediments (Carballeira et al., 2012; Soares & Junior, 2016; Pagano et al., 2017; Rial et al., 2017). *Paracentrotus lividus* is an indicator marine species, widely found in the Mediterranean and Aegean Seas, and its occurrence is not restricted and therefore is representative for a variety of ecosystems (Martins et al. 2018).

The ecotoxicology studies cited above have drawn attention to CuPt as an emerging marine pollutant with a potential to adversely affect marine life. A literature survey showed that no research had been conducted on the effects of CuPt on *P. lividus*. Therefore, the present study aims to reveal the spermiotoxic, embryotoxic and cytotoxic effects of CuPt on sea urchins as an important model organism.

MATERIAL AND METHODS

Adult samples *of Paracentrotus lividu*s, a test organism used in embryotoxicity and spermiotoxicity bioassays, were hand collected in unpolluted areas of the Dardanelles Strait, Güzelyalı, Çanakkale province (Turkey) and transferred to the laboratory in portable boxes. Salinity, temperature, and pH of the seawater were measured to be ‰ 31, 20.3°C, and 8.14, respectively. CuPt (purity: 95-100%; CAS: 14915-37-8; molecular weight: 315.86 g/mol) was obtained from Arch UK Biocides. Stock solution of CuPt was prepared in dimethyl sulfoxide (DMSO; purity: >99%, Amresco), at 400 μg/ml. Exposure solutions were prepared from stock solutions at concentrations of 1, 10, 20, 40, 60, 80 and 100 μg/l. According to the relevant literature, concentration range was made between concentrations with the highest mortality range and the lowest inhibitory effect. A positive control group was exposed to $3x10^{-4}$ M cadmium chloride ($CdCl_2$, CAS no:10108-64-2, purity: 99.9%; Fischer Scientific, Waltham, MA).

Gametes were obtained and embryos were allowed to develop according to Pagano's bioassay protocol (1986; 2001). The gametes were harvested by injecting 1-3 ml of 0.5 M KCl into the ceolomic cavity along the perioral membrane. The eggs were placed in sterilized beakers containing 25 ml of filtered seawater (FSW) and sperm was retrieved in dry media (without gametes being put into FSW). Bioassay protocols for fertilization, embryo development and quality control at all stages of the procedures were conducted as reported by Pagano et al. (1986) and Chapman et al. (1995). The spermiotoxicity experiment was carried out in quadruplicate, while the embryotoxicity experiment was conducted in triplicate to use less chemical and save time during counting. In the control group, the fertilization rate was expected to

be at least 70-90% and normal plutei to be at least 80% (Chapman et al. 1995).

Spermiotoxicity experiment

The spermiotoxicity experiment was carried out in two stages. In the first stage, the egg fertilization rate of CuPt-exposed sperms was evaluated: 20 µl aliquot of sperm harvested from 3 males was exposed to each consentration of CuPt diluted with 10 ml of FSW. The experiments were conducted in amber colored glass containers to avoid sunlight-induced degradation of copper pyrithione and to prevent its adhesion to plastic culture plates (Bao et al., 2011). Sperm was exposed to different concentrations at room temperature for 30 min, then 40 µl aliquot of sperm suspension was added to FSW containing 4 ml of egg suspension (about 50 eggs/ml) (Pagano et al., 1986). The eggs were left to incubate at 18 ± 1°C for 30 min. At the end of exposure time, fertilization was terminated with 1% formaldehyde (Novelli et al. 2003). Randomly selected 100 embryos were taken from each sample and evaluated blind under a light microscope and the percentage of fertilization was determined. Only embryos with fertilization membranes which were observed clearly were considered as fertilized.

In the second stage of the spermiotoxicity bioassay, embryos were allowed to develop from CuPt exposed sperms, and morphologically assessed. Eggs were fertilized with the sperm exposed to CuPt for 30 min and left to incubate at 18 ± 1°C for 48-72 h. After that developmental abnormalities were determined under the light microscope (Pagano et al. 2001).

Embryotoxicity experiment

The zygote solution was prepared by adding sperm suspension onto the egg solution prepared for the embryotoxicity experiment and left to undergo fertilization for 20 min. One ml of the fertilized egg solution was sampled and added into the control group and concentration series of test solutions (1, 10, 20, 40, 60, 80 and 100 µg/l CuPt concentrations) and 10 ml of aliquot was obtained. The embryos were left for incubation at 18 ± 1°C for 48-72 h until they reached the pluteus larva stage. At the end of the experiment, embryo development was terminated with 1% formaldehyde and 100 embryos were randomly enumerated under the light microscope for the frequency of malformations detected in embryos. Then the percentage of developmental anomalies were evaluated in the plutei: (1) normal pluteus size and symmetry, (2) malformed larvae P1+P2 (P1: malformed skeletal system; P2: missing skeletal system); (3) development retardation (R) (sizes ≤ 1/2 normal larvae and embryos having failed to reach pluteus stage (blastula and gastrula) (as modified from Pagano et al., 1986, 2001; Cairns, 1986).

Cytogenetic analysis

Fertilization and domestication of cells in cytotoxicity experiments were carried out as in the embryotoxicity bioassay. However, after 5-6 h fertilization exposure, the embryos were filtered and put into 10 ml of Carnoy's fluid (absolute ethanol:chloroform:acetic acid, 6:3:1) and fixed in this fixative for 30 min. Thirty min after fixation, the fixative was discharged and 10 ml of absolute ethanol was added. The samples were kept in absolute ethanol for 24 h and the solvent was replaced at the end of 24 h, and then stained with 2% of acetocarmine dye and examined under the light microscope (1000x). Enumeration and morphological measurements of mitotic degradation and chromosome anomalies, used to assess cytogenetic effects, were carried out in line with Pagano et al (1986) and Ferreira et al. (2009).

Statistical analysis

Dose-response relationships for CuPt were established to calculate the EC_{50} defined as the toxicant concentration causing 50% reduction in embryogenesis success. Data showing the percentages of abnormal larvae (identified for each exposure concentration and each control group: negative, solvent and positive controls) were analysed and are given as mean ± standard error or with 95% confidence interval (CI). The median effect concentration (EC_{50}) is the calculated value for 50% of the estimated toxic effect associated with CuPt exposure concentrations. The toxic endpoint in the embryotoxicity assay was observed as the percentage of abnormal larvae (25%, EC_{25}, and 50%, EC_{50}). In the spermiotoxicity and cytotoxicity experiments, IC_{25} and IC_{50} values were calculated using: i) a linear interpolation method (ICPIN Version 2.0, USEPA), and ii) a Toxicity Relationship Analysis Program (TRAP, USEPA, Duluth, MN, USA). The spermiotoxicity data (fertilization rate) were transformed into arc-sin square root and exposure concentrations log-transformed for analysis. Differences between mean deformities in embryotoxicity and cytotoxicity bioassays were analyzed using the non-parametric Kruskal-Wallis H test, since the data did not meet normal distribution assumptions. The significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

The sea urchin spermiotoxicity, embryo development and cytotoxicity bioassays were used in the present study to evaluate the toxicity of CuPt using the indicator species *Paracentrotus lividus*.

In the first stage of the spermiotoxicity test, control and DMSO control fertilization rates were very similar and the average was 90.87±4.84, therefore the tests were considered valid. However, spermiotoxicity of CuPt calculated as fertilization rate was minimum 75%, therefore the confidence intervals for IC_{25} and IC_{50} (95%) could not be calculated, since at least one dose (concentration) has to be under 50%. It is possible to conclude that CuPt did not significantly affect the fertilization rate of *P. lividus* sperms.

In the second stage of the spermiotoxicity test, malformations of embryos developed from the CuPt-exposed sperms increased depending on concentration. A decrease in the frequencies of normal (N) ($P < 0.05$, Figure 1) developed plutei from the CuPt-exposed sperm was significantly different from controls. Malformations of embryos (P1+P2 combinations) developed from CuPt-exposed sperm increased in a concentration-dependent way, and mean differences were significant between normal and malformed embryos (P1+P2) ($P<0.05$, Figure 2). There were no significant differences between the development retarded (R) embryos exposed to the given concentrations (1, 10, 20, 40, 60, 80 and 100 µg/l CuPt concentrations) ($P>0.05$, Fig. 3).

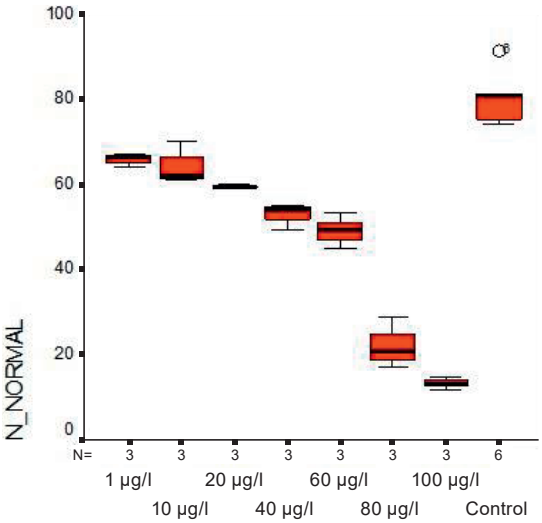

Figure 1. Alteration (expressed as percentage) in normal plutei (N) developed from eggs fertilized with sperm exposed to different CuPt concentrations

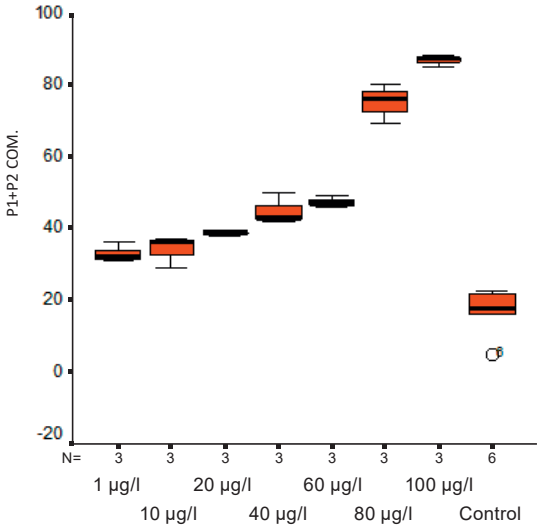

Figure 2. Alteration (expressed as percentage) in P1+P2 combination developed from eggs fertilized with sperm exposed to different CuPt concentrations

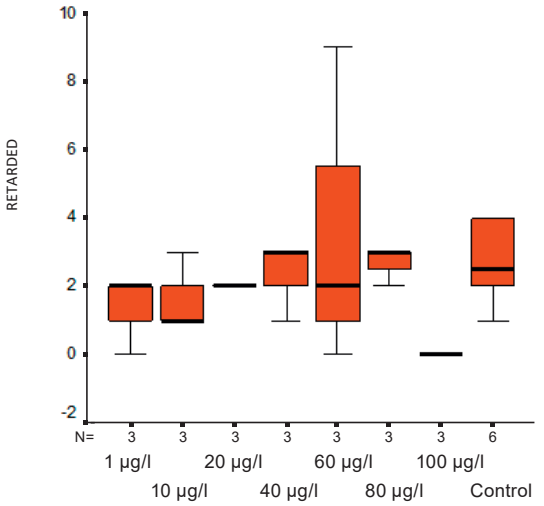

Figure 3. Alteration (expressed as percentage) in retarded embryos (R) developed from eggs fertilized with sperm exposed to different CuPt concentrations.

Embryotoxicity test

In embryotoxicity experiments, solvent control was considered safe. The percentage of abnormal embryos increased significantly after applying CuPt at all of its seven exposure concentrations (i.e., 1, 10, 20, 40, 60, 80, 100 µg/l). Decrease in the frequencies of normal (N) developed plutei in exposure groups was significantly different from controls ($P < 0.05$, Figure 4). A positive control group was exposed to $3x10^{-4}$ M cadmium chloride and 100% of the embryos showed development

retardation (R) malformation. The EC_{50} value of CuPt was calculated to be 13.58 µg/l for embryotoxicity to *P. lividus*. While the average value of normal pluteus was 61.33 ± 1.75 in the group exposed to 1 µg/l CuPt, it was 3.83 ± 7.52 in the group exposed to 100 µg/l ($P < 0.05$). As CuPt concentration increased, the mean percent of normally developing specimens decreased, whereas skeletal deformities increased with P_1+P_2 combinations (Figures 5-6).

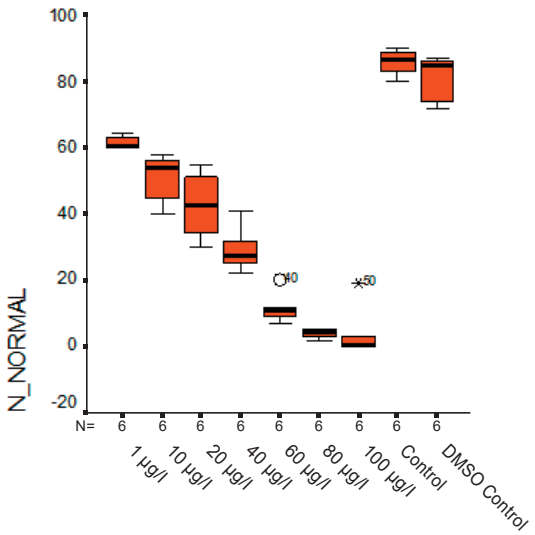

Figure 4. Embryotoxicity results for normal plutei (N, expressed as percentage) developed from normal eggs and sperm showing larval development effects due to different CuPt concentrations

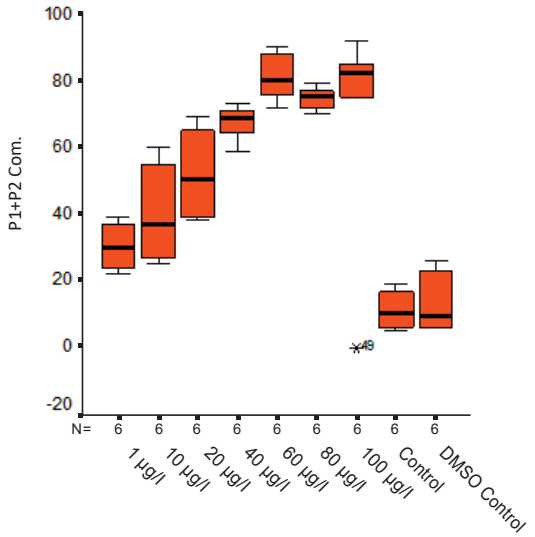

Figure 5. Variations in embryotoxicity expressed as P1 + P2 combination of developmental defects (%) depending on different CuPt concentrations

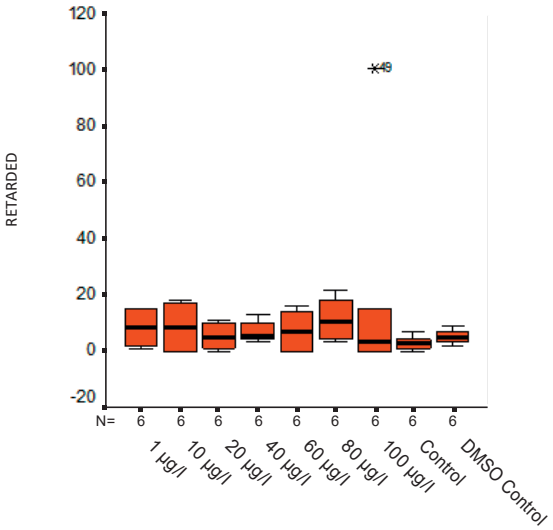

Figure 6. Variations in embryotoxicity expressed as retarded (R) developmental defect (%) depending on different CuPt concentrations

Cytotoxicity test

In cytotoxicity experiments, the IC_{25} and IC_{50} values for percentage mitotic index were calculated to be 12.79 µg/l and 47.85 µg/l, respectively. Our results showed the percentage mitotic index of the groups as: 28% ± 0.007 (control); 28% ± 0.005 (1 µg/l); 24%± 0.008 (10 µg/l); the percentages decreased from 16 to 7% under doses increasing from 20 to 100 µg/l (Figure 7). DMSO solvent control was not cytotoxic, based on the mitotic index. The positive control cadmium chloride was 3% ± 0.001. Mitotic index decreased in a concentration dependent manner. Interphase percentage per embryo decreased inversely with decreased mitotic index (Figure 8).

The metaphase and anaphase ratio (M/A) was 4.4 in the 60 µg/l exposure group and close to 2 in all other concentration groups, suggesting that cell transition from metaphase to anaphase was inhibited by the concentration of 60 µg/l (Figure 9). The number of lagged chromosomes, a chromosome abnormality, exhibited no significant difference between the 10 and 20 µg/l concentrations but tended to increase with concentration. The number of lagged chromosomes was 0.47 ± 0.06 and 2.9 ± 0.24 under 1 and 60 µg/l concentrations, respectively. The control group was 9.6 ± 0.23. Under 100 µg/l, the number was found to be 6 ± 0.19 due to much fewer cells undergoing mitosis. The number

of free and broken chromosomes also tended to increase depending on concentration. The numbers of free chromosomes were 0.35 ± 0.07, 0.68 ± 0.01 and 1.1 ± 1.1 in the control group and under 1 µg/l and 60 µg/l concentrations, respectively. In addition, the number of broken chromosomes was 1.6 ± 0.16, 3.2 ± 0.17 and 6.8 ± 0.21 in the control group and under 1 µg/l and in 100 µg/l, respectively. No increase in the number of sticky chromosomes was observed under concentrations up to 60 µg/l but they increased under 80 and 100 µg/l concentrations. No prominent differences in the formation of acentric fragment and anaphase bridge were observed between concentrations.

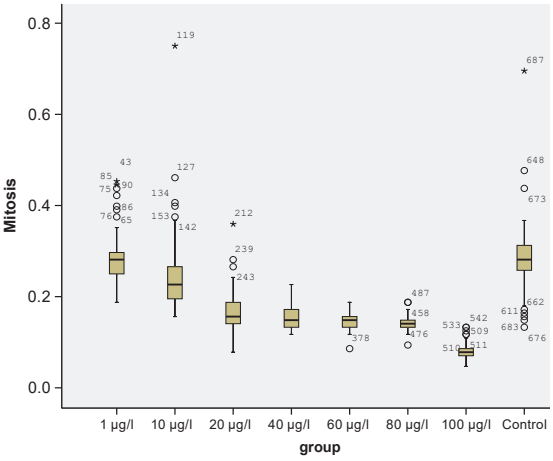

Figure 7. Variation in mitotic index counts per embryo due to CuPt exposure concentrations

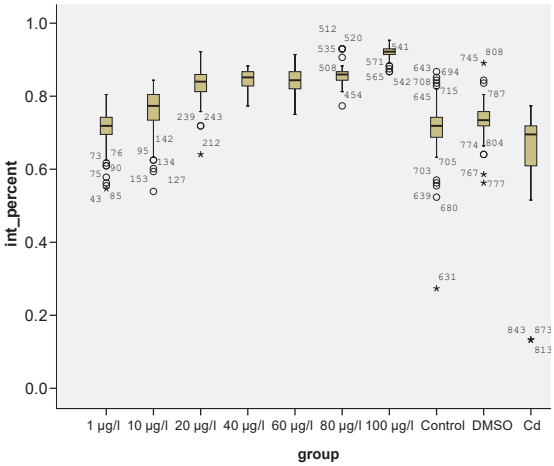

Figure 8. Variation in interphase percentages per embryo due to CuPt exposure concentrations

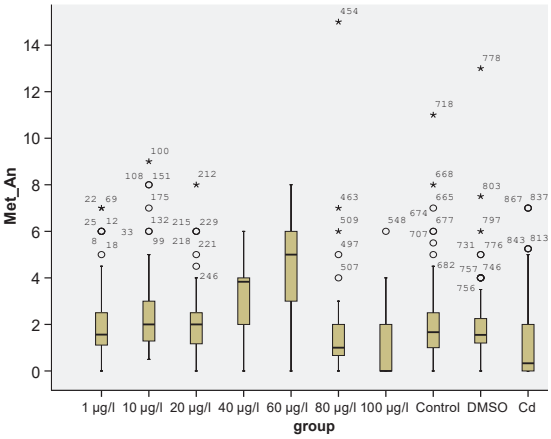

Figure 9. Changes in M/A ratio per embryo due to CuPt exposure concentrations

In toxicology, hormetic dose response occurs associated with exposures of biological organisms to environmental stressors. Some adaptive pathways extend the region of cellular homeostasis and are protective against toxicity. Hormesis denotes that cells can positively maintain their metabolic activities and adapt to lower concentrations of toxic agents (Calabrese, 2008; Tang et al., 2019). DMSO used as a solvent is known to protect cells from various lesions, to modify membrane permeability and to act as an antioxidant in biological systems (Grigoryan & Shiladzhyan, 2009). In the spermiotoxicity test of the present study, an increase of 9% in the number of fertilized embryos in 1 µg/l, compared with the control group, is considered to have stemmed from a probable hormetic effect of lower doses of CuPt. It is also thought that DMSO might have suppressed the toxic effect of CuPt on sperm motility most probably due to antioxidant effects. Onduka et al. (2010) estimated CuPt 24 h toxicity to the crustacean *Tigriopus japonicus* as EC_{50} = 23 µg/l, and our results agree with them. Xu et al. (2011) reported individual and joint-action toxicity of binary or multiple mixtures of heavy metal compounds for embryonic toxicity and spermiotoxicity in the sea urchin *Strongylocentyotus intermedius*. Among four metals, Cu was the most toxic with EC_{50} = 1.32 µM, while toxicities to larval development in sea urchin embryos decreased in the order: Cu > Pb > Zn > Cd. Cu was still very toxic (EC_{50} = 6.40 µM) regarding sea urchin fertilization success, spermiotoxicity, and the ranking was: Zn > Cu > Pb > Cd based on the EC_{50} values. Fernandez & Beiras (2001) found very comparable data for Cu embryotoxicity to *P. lividus*.

Exposure of sea urchin *Evechinus chloroticus* adults to moderately high levels of waterborne copper led to a significant increase in copper burden in gonads of both sexes; however spawning success was not impaired. Larval size was also significantly affected by the copper burden of mothers, and many larvae were severely abnormal at this early stage. The study concluded that in a polluted environment there is likely to be an overall reduced reproductive output across the population (Phillips & Rouchon, 2018).

The toxicity of CuPt to *Artemia salina*, the brine shrimp, was significantly influenced by the organic matter content, salinity and proportions of constituent salts in water. Upon combining with cupric ions in the medium, the non-hazardous degradation product 2-mercaptopyridine-N-oxide (HPT) exhibited increased toxicity due to its rapid transformation to the parent biocide. The 48 h CuPt acute survival effect to *A. salina* was estimated as EC_{50} = 250 μg/l (Lavtizar et al. 2018). The ranges agree with data in the present study.

Basallote et al. (2018) demonstrated effects of pH and pCO_2 on sediment metal toxicity to *Paracentrotus lividus* embryotoxicity and spermiotoxicity, and metal toxicity was significant at pH 7.0 and 6.5. The antifouling biocide CuPt easily photolyses (Lavtizar et al. 2018), similar to zinc pyrithione (ZnPt), and can interact with free metal ions by releasing the metal ion (zinc-zinc pyrithione or copper) and take up new free metal ions, e.g., manganese, iron or copper from seawater. The degradation half-life of ZnPt and CuPt was estimated to be between 7 and 9 min. when exposed to light under experimental conditions. Marinas and harbours have a high-risk potential for ZnPt and CuPt accumulation due to high concentration of boats in them that are sources of these biocides as paint particles continue to release them and cause persistent contamination of the sediment (Thomas et al., 2000; Maraldo & Dahllof, 2004).

Egardt et al. (2017) studied antifouling paints in sediments in a national park on the Swedish west coast and determined several banned antifouling compounds in surface sediments. Furthermore, the reported Cd and Cu levels were significantly higher than background values, and copper was significantly correlated with Cd, Cr and Pb, suggesting similar sources and similar correlation between Cd and Zn. Although Cu is an essential metal, it may pose a potential risk of being toxic in high concentrations, and may have sub-lethal effects on the benthic environment, raising concern for all sources of Cu whether organic, organometallic or metallic. Metal contamination of marine waters is not limited to known abundant pollutants, heavy rare earth elements (HREEs) are of concern too due to growing applications in several advanced technologies, necessitating in-depth, ad hoc investigations for potential environmental toxicology. Oral et al. (2017) studied HREE-associated toxicities to *Arbacia lixula* and *Paracentrotus lividus* and found different toxicities of the tested HREEs in terms of effects on embryogenesis, fertilization, cytogenetic and redox endpoints at 10^{-5} M. In agreement with our results, sperm exposure to HREEs (10^{-5}-10^{-4} M) resulted in a concentration-related decrease in fertilization success along with increase in offspring damage. The same research group extended their research to other sea urchin species for comparing species sensitivities, and found similar impacts. Gravina et al. (2018) reported effects of broadly unexplored HREEs effects to early life stages of the sea urchin *Sphaerechinus granularis* and observed significant developmental defect (DD) increases, i.e. significant decrease in mitotic activity with increased mitotic aberrations in embryos even at 10^{-5} M. *S. granularis* lives further offshore and deeper than the other species, *P. lividus* and *A. lixula,* and it is historically more sensitive to pollutants.

Toxic effects (spermiotoxicity) of three forms of selenium to *P. lividus* were also reported for the larval stage, and hypothesized to be associated with sperm since selenium was prominent in the early reproductive stages (Oral 1997). As in that study, it is considered in the present study that CuPt toxicity might have been conveyed to the embryo through the genetic material of the sperm.

Incresed skeletal deformities were induced by CuPt in the fish species *Fundulus heteroclitus*, where metabolites originating from CuPt degradation in sea water inhibited the AChE esterase enzyme (Mochida et al., 2009). Moreover, CuPt is likely to degrade cell membrane and pH gradient, and to bind such complex agents as metals and proteins (Ermolayeva & Sanders, 1995; Wang et al., 2011). It can be derived from these results that skeletal malformations observed in the embryotoxicity test on *P. lividus* may also be due to mechanisms similar to those cited in earlier studies. Bellas et al. (2005) tested four pesticides (chlorpyrifos, diuron, lindane and tributyltin) for potential threat to non-target marine species. These substances are also introduced to the marine environment and coastal areas by spray drift, surface runoff or accidental spills. Although they are not in the same chemical group with CuPt, their toxicities to early development of embryos and larvae of four marine invertebrates, chosen for their abundance, ecological importance and commercial relevance, namely: an echinoid (*Paracentrotus lividus*), an ascidian (*Ciona intestinalis*), and two crustacean species (*Maja squinado* and *Palaemon serratus*), were studied and

the presented results are in agreement with our work. Tributyltin embryotoxicity EC_{50} was 0.309 μg/l,, i.e. it was highly toxic to *P. lividus* embryogenesis, as our results also show. Another form of Cu is copper oxide nanoparticles (CuO NPs), extensively used in industrial and commercial applications. The effects of CuO NPs on the spermatozoa of the sea urchin *Paracentrotus lividus* were assessed using physiological and biochemical markers, and oxidative stress was found as the main driver of CuO NP spermiotoxic effects (Gallo et al., 2018). Male reproductive success depends on several abiotic and biotic factors. The authors emphasized sperm quality to be crucial for predicting male reproductive biology and since CuO NP exposure was associated with a reduction in sperm quality and fertilization failure, attention was drawn to such effects having potential implications for species fitness and survival.

In cytotoxicity studies on the effects of three forms of selenium on *P. lividus*, as the concentration increases the toxic agents are found to cause decrease in mitotic index by inhibiting mitotic activity, and increase in the percentage of interphase cells. Moreover, they are also thought to break chromosomes and inhibit chromosomes from binding to spindle fibers and to enable the formation of free chromosomes by hindering cell division shuttles in their varying concentrations. Inferring from all these findings on chromosome abnormalities, it is thought that these toxic agents are lethal to some embryos in early stages (blastula or gastrula) before differentiation, while causing skeletal malformations in the developed embryos (Oral 1997). Our results are in agreement with those of other studies regarding the cytogenetic toxicity analysis of CuPt (Oral, 1997; Oral et al., 2017; Gravina et al., 2018). CuPt also inhibits mitotic activity more as concentrations increase, and causes increased population of interphase cells and also affects chromosomal activities. Previous research had evidenced that CuPt used in anti-fouling paints was highly toxic to marine organisms (Mochida et al., 2009). When CuPt is rapidly disintegrated in seawater, its environmental concentrations change and therefore further research on CuPt should be carried out (Harino et al., 2007).

The present research revealed that the non-persistent antifouling biocide CuPt in the administered concentrations was highly toxic to *Paracentrotus lividus* embryos. CuPt was not toxic to sperm but to embryos developing from eggs fertilized by that sperm. Moreover, this study is significant in reporting cytogenetic effects of CuPt. Bioassays provide invaluable information on the bioavailability of pollutants, reveal complex interactions between emerging contaminants not previously considered as risks, and are rapid, reliable and simple. Limited work has been done on comparing the sensitivity of different species to pollutants, relying for hazard assessment of pollutants on single-species tests that cannot detect the full range of pollutants entering the marine environment, and the present work is a contribution to the field in that sense. There is a need to standardize these bioassays, especially for climate change impact and mixtures of pollutants, and more significantly for emerging contaminants (Pagano et al., 2017). Comparative toxicity data on pollutants across taxonomically distant echinoid species characterized by different habitats and by different sensitivities to xenobiotics will be most beneficial for standardizing and validating bioassays, especially for variable sensitivities of test species used. *Patella* spp. (Mollusca, Gastropoda) has also been evaluated for use as a standardized protocol for embryo-larval bioassay (Perez et al., 2016). Recently Morroni and co-workers (Morroni et al., 2016, 2018) developed a new Integrative Toxicity Index (ITI) for overall comparison of evaluation procedures and of results obtained from different experimental treatments. The ITI is expected to provide new insights into the capability of each metal to induce anomalies in the embryogenesis of echinoid embryos and their recovery to normal development after metal exposure, thus adding further ecological value to the sea urchin bioassay. The possibility to understand and weigh the reversibility of toxic effects improves the ecological relevance of embryo toxicity bioassays. These studies provide comparative toxicity data on single and mixture of emerging contaminants and should be considered a priority for monitoring and regulatory programs. Programs aimed at minimizing adverse health outcomes from single or mixed pollutant exposures will be used for regional and international level mitigation measures. As CuPt is already approved in the European Union, while ZnPt is pending approval (EU Commission, 2015), and Japan, Hong Kong, China, Australia and New Zealand have authorised the use of both in antifouling paint formulations (NZEPA, 2013; APVMA, 2017 as sited by Martins et al., 2018), comprehensive risk assessment studies should be launched soon.

ACKNOWLEDGEMENT

This study partly fulfilled the requirements for one of the authors' M.Sc. thesis (E.T.) and was supported by the Çanakkale Onsekiz Mart University Scientific Research Projects Commission (BAP 2015/645).

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Ispitivanje spermotoksičnog, embriotoksičnog i citotoksičnog delovanja bakar piritiona na *Paracentrotus lividus* (Lamarck, 1816)

REZIME

Ispitivano je spermotoksično, embriotoksično i citotoksično delovanje biocida bakar piritiona (CuPt), koji se koristi protiv biotaloženja, kako bi se u biotestu procenila inhibicija oplodnje, kvalitet potomstva i delovanje na rani razvoj morskog ježa *Paracentrotus lividus*. U pogledu nivoa oplodnje, CuPt je pokazao odsustvo spermotoksičnosti, ali se učestalost deformacija embriona povećala kada su jaja oplođena spermom izloženom delovanju CuPt, i to u zavisnosti od koncentracije. Dobijena je CuPt EC_{50} od 13.58 µg/l za embriotoksičnost. Dok je učestalost normalno razvijenih pluteusa opadala, broj larvi sa skeletnim deformacijama se povećavao. Odgovarajuće vrednosti IC_{25} i IC_{50} u biotestovima citotoksičnosti su bile 12.79 i 47.85 µg/l. Istraživanje je otkrilo statistički značajno smanjenje učestalosti deobe ćelija mitozom, povećanje procenta interfaznih ćelija i povećanje hromozomskih abnormalnosti kod izloženih ćelija. Prema ovim rezultatima, može se reći da primenjene koncentracije CuPt imaju visoko toksično delovanje na embrione morskog ježa. Ovakva situacija ukazuje na mogući rizik od kontaminacije ove vrste bakar piritionom.

Ključne reči: biocidi, bakar pirition, razvojna biologija, morski jež

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.

Manuscript submission

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.

Manuscript preparation

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).

Title should be concise and refer to the subject. Full names and surnames of all authors, details of their respective affiliations and emails should be indicated below the title. If discrepancy in such data occurs between the textual document and submission metadata in Assistant, the former will be given precedence.

Abstract (not exceeding 300 words) should briefly state the main results and conclusions.

Articles should contain the following sections: Introduction, Material and Methods, Results, Discussion, Acknowledgement and References.

Introduction should present the state-of-the-art in a particular research field, as well as research intent.

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.

Discussion should emphasize the importance of the results, as well as their place within the context of previous research. Wherever possible, Results and Discussion should be separate sections.

Acknowledgement should be collated at the end of the manuscript before References.

References cited in the text need to include the author's/ authors' surname(s) and year of publication:

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References mentioned in the manuscript must be listed in the References section at its end, in alphabetic order and using the **APA citation style** (see description at e.g. https://owl.english.purdue.edu/owl/resource/560/01/).

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.

Dedić, B. (2012). Testing sunflower inbred lines for tolerance to phoma black stem. *Pesticides* & *Phytomedicine*, *27*(4), 299-303. doi:10.2298/PIF1204299D

Abbaspoor, M. & Streibig, J.C. (2005). Clodinafop changes the chlorophyll fluorescence induction curve. *Weed Science, 53*(1), 1-9. doi:10.1614/WS-04-131R

Abbaspoor, M., Teicher, H.B. & Streibig, J.C. (2006). The effect of root-absorbed PSII inhibitors on Kautsky curve parameters in sugar beet. *Weed Research, 46*(3), 226-235. doi:10.1111/j.1365-3180.2006.00498.x

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

Timbrell, J. (2000). *Principles of biochemical toxicology* (3rd ed). London, UK: Taylor and Francis Ltd.

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.

Book chapters and articles in conference proceedings: author(s), year of publication, title of chapter/article/abstract, source title (with editors names), pages, place of publication and publisher.

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.

Internet references: author(s), year of publication, title, source title, link.

Graora, D., & Spasić, R. (2008). Prirodni neprijatelji *Pseudaulacaspis pentagona* Targioni-Tozzetti u Srbiji. *Pesticidi i fitomedicina*, *23*(1) 11-16. Retrieved from http://www.pesting.org.rs/media/casopis/2008/no.1/23_1_11-16.pdf

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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.

Diagrams should be processed in Corel Draw (version 9 or later) or in Adobe Illustrator (version 9 or later) and all data written in Times New Roman font. Diagrams should be submitted as supplementary files and their approximate locations in the text marked.

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.

Authors are expected to use the accepted International System of Units (SI). Abbreviations should be defined in brackets at their first in-text mention. Provide full Latin names along with common names of organisms, and italicize only Latin names of genera and species, e.g. Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). After first mention, the Latin name can be abbreviated (e.g. *L. decemlineata*).

Review articles need to contain an introduction, appropriate subtitles and a reference list.

Publishing in *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* is free of charge.

The editorial staff practice a policy of plagiarism prevention.

Uputstvo autorima

O časopisu

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.

Časopis *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* predstavlja nastavak publikacije *Pesticidi*, koja je pod tim imenom izlazila u periodu 1986-2003.

Časopis *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* objavljuje originalne i pregledne, prethodno neobjavljene radove.

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Radovi koji se prilažu moraju biti napisani na engleskom jeziku, sa rezimeom na engleskom i srpskom jeziku.

Od 2020. godine, časopis izlazi četvoromesečno (tri broja godišnje).

Od 2021. godine, časopis Pesticidi i fitomedicina (Pesticides and Phytomedicine) objavljuje sveske samo u elektronskom obliku, bez štampane verzije. Osnovne platforme na kojima se postavljaju sadržaji časopisa su: Scindeks (http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949) i zvanični veb sajt izdavača (http://www.pesting.org.rs/).

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Tokom 2011. godine, časopis je prešao na sistem onlajn uređivanja (Elektronsko uređivanje – e-Ur) na portalu SCIndeks Asistent **http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949**. Ovaj sistem uređivanja omogućava funkcionalniju prijavu radova i komunikaciju uredništva sa recenzentima i autorima, a obuhvata i servise za kontrolu kvaliteta radova: *CrossRef* za dodelu DOI, *CrossChek* za prevenciju plagijarizma i *KWASS* za opremanje radova ključnim rečima ekstrahovanim iz rečnika/tezaurusa. Elektronsko uređivanje je usaglašeno sa Aktom o uređivanju časopisa Ministarstva prosvete, nauke i tehnološkog razvoja RS i obezbeđuje vođenje evidencije koje ovaj akt nalaže.

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Publikovanje u časopisu *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* podrazumeva da rad sadrži rezultate originalnih istraživanja koji nisu objavljeni, odnosno nisu dostavljeni nekom drugom časopisu za objavljivanje. Pregledni radovi treba da sadrže sveobuhvatan prikaz određene teme zasnovan na referentnoj literaturi i publikovanim rezultatima sopstvenih istraživanja. Svi radovi se recenziraju, a recenzija je obostrano anonimna.

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Autori u radu NE NAVODE ključne reči. Njih će glavni urednik ekstrahovati iz *KWASS* tezaurusa (rečnika), što će značajno poboljšati vidljivost rada. Autori imaju pravo da dodeljene ključne reči prihvate ili da neke od njih zamene.

Priprema rada

Rad treba pripremiti u programu za obradu teksta Word (format A4, margine 25 mm, font Times New Roman 12 pt). Radovi treba da budu isključivo na engleskom jeziku sa naslovom i rezimeom na oba jezika (engleskom i srpskom).

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.

Zahvalnica se navodi na kraju teksta rada, pre literature.

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

Disertacije: autor, godina odbrane, naslov, i puni naziv institucije u kojoj je disertacija odbranjena.

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, UK: Cambridge University 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

Tabele se obeležavaju arapskim brojevima prema predviđenom redosledu. Tabele se izrađuju isključivo u programu Word for Windows, kroz meni Table-Insert-Table, koristeći font Times New Roman, 12 pt i osnovni prored. Fusnotama neposredno ispod tabela treba dati prednost nad drugim objašnjenima u zaglavlju tabela ili u samim tabelama, a tekst se daje u fontu Times New Roman, 10 pt. Svaka tabela mora imati zaglavlje. Tabele se prilažu kao dopunske (zasebne) datoteke, a u samom tekstu se obeležava njihovo približno mesto.

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.

Dijagrami treba da budu urađeni i dostavljeni u programu Corel Draw (verzija 9 ili novija), ili u programu Adobe Illustrator (verzija 9 ili novija). Za unos podataka treba koristiti font Times New Roman. Grafikoni se prilažu kao zasebne (dopunske) datoteke, a u samom tekstu se obeležava njihovo približno mesto.

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.

Od autora se očekuje da koriste preporučene jedinice međunarodnog sistema (SI). Skraćenice je potrebno definisati u zagradama nakon prvog pominjanja u tekstu. Narodni nazivi organizama se daju uz pun latinski naziv, a kurzivom se obeležavaju samo latinski nazivi rodova i vrsta, npr. krompirova zlatica, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). Nakon prvog pojavljivanja, latinsko ime dalje treba pisati skraćeno (npr. *L. decemlineata*).

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

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

Uredništvo preduzima mere protiv grubog kršenja etičkih normi u vezi sa plagijarizmom.

Funded by the European Union

Harmonization and Innovation in PhD Study Programs for Plant Health in Sustainable Agriculture (HarISA) – State of the Art

The HarISA project, which is in its third year of implementation, follows the overall objectives of Erasmus+KA2 call. It improves the quality of higher education and increases its relevance for the labor market and society. At the same time, it improves the level of competences and skills in higher education by developing new and innovative educational programs.

The project is implementing through five activities divided in eight Work packages (WP1-WP8). In summary, the activities of the groups included the following:

WP1: An overview of existing partners PhD study programs was done first. Than, PhD programs and courses that involve tasks related to plant health were analysed and the compliance of those programs with Bologna process and with national and EU qualification framework have been established. The learning outcomes of PhD study programs and subjects related to plant health were compared.

WP2: Through the work of this group, development of the joint framework for harmonization of the PhD study programs and a curriculum draft has been enabled. This is based on the exchange of the best practices among partner institutions (PIs) related to PhD study program management and mentoring, as well as research needs for plant health in sustainable agriculture. The descriptions of the PhD study programs management and tutoring system were prepared by PIs and the best practices were identified. The needs related to plant health knowledge for sustainable agriculture were identified. This WP2 was developed draft of the curriculum, created in order to address all important issues related to plant health, of the International Joint PhD study program.

WP3: In this work package seven sub-groups (SG) were formed. Members of these sub-groups were analyzed existing subjects taught at PIs, compared the methods, tools, human capacities and learning outcomes, identified teaching needs and proposed the new research topics for PhD students. The common teaching material were developed for the courses that are similar and taught at different PIs. Within these seven sub-groups, a total of 22 new courses were developed, as follows:

SG1 - Diagnosis in plant health and IPM:

1. Advanced diagnostic methods and techniques for detection of prejudicial and beneficial organisms
2. Integrated approach to surveillance of prejudicial organisms affecting plant health
3. Control of quarantine prejudicial organisms, managing of non-native beneficial organisms and evaluation of risk assessment based on EU protocols

SG2 - Sustainable use of pesticides:

4. Plant protection products in sustainable agriculture
5. Environmental fate of pesticides
6. Toxicology and ecotoxicology of pesticides

SG3 - Plant feeders:

7. Advanced techniques in plant feeders
8. Frontiers in invertebrate pest and resistance management
9. Advanced invertebrate pathology

10. Invasive alien pests
11. Vectors of plant pathogens
12. Integrated Management of urban pests

SG4 - Plant pathology:

13. Molecular plant microbe interactions

SG5 - Weed science:

14. Weed management in precision agriculture
15. Modelling in weed science
16. Invasive plant species

SG6 - Mycotoxins and food safety:

17. Mycotoxins and food safety

SG7 - General contents of transversal interest:

18. Principles of scientific work in bio-science
19. Bio-diversity and bio-indicators in sustainable agriculture
20. GIS & Spatial data analysis
21. Bio-informatics
22. Knowledge and management of research funding systems

WP4: Establishment of diagnostic and training hubs (DTH) at partner universities aims to develop the criteria and identify the excellence within the partner universities and to upgrade existing facilities in service of PhD students', staff and professionals' needs. The selection procedure for DTHs has been established. A list of procurment equipment has been prepared and procurement is largerly complete.

WP5: The task of this group was to organize the PhD students and staff mobility and training. This WP aims to organise mobility for research purposes and training for project application and for mentoring in order to ensure the sustainability of the PhD study programs in plant health by increasing the number of scientific project application (more research and PhD candidates in future) and by improving the quality of mentoring. The mobility plan is based on the achievements of WP2-WP4. The research topics and mentorship together with the need for exchange of teachers have been identified and were the base for the creation of mobility plan. Two types of trainings were organized; one for teachers withouth or with poor mentoring experience and the second one for PhD students and staff for writing of scientific project application.

WP6: Quality assurance aims to monitor and evaluate the project progress and management, and also to evaluate the developed curricula and PhD student satisfaction with all elements of PhD study program (management, tutoring, scientific content). Evaluation Board (EB) is formed from members of program countries PIs. They prepared quality control plan and monitored implementation of the project. They also surveyed PhD students' and staff after the mobility period.

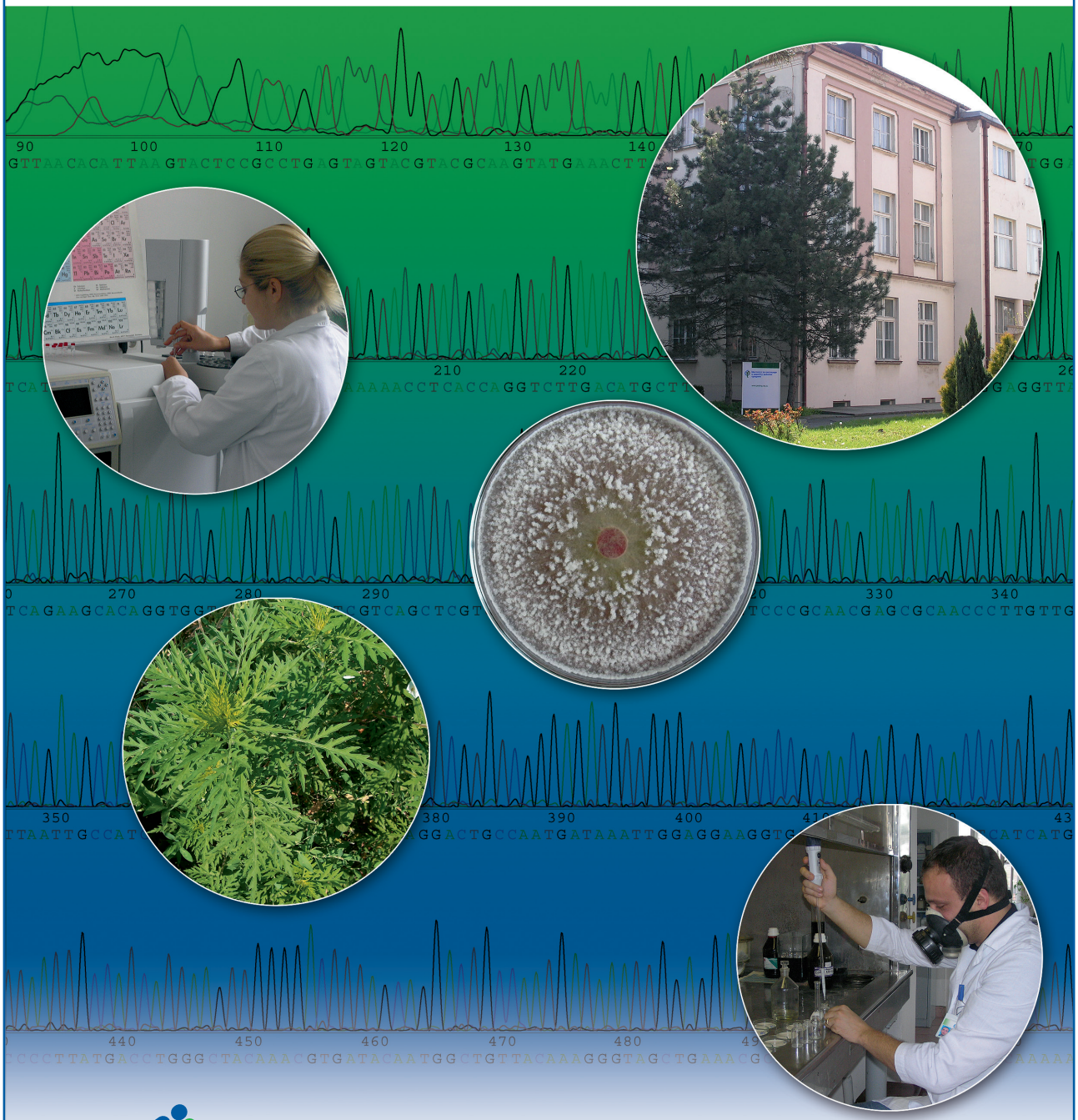
WP7: This group deals with the project dissemination, ie ensures project visibility and sustainability. A communication and visibility action plan have been created. The basic way of disseminating the project results is realized through designing and maintaining of the project website. The projects' Facebook profile has also been created and regularly updated. All relevant HarISA associated partners web sites have been updated with the partnership's outcomes and results. Documents were released and distributed within the framework of the Project conferences, meetings, workshops, etc. Project activities as well as research results were presented and promoted at conferences and meetings organized by associated partners.

WP8: Project management

This WP aims to ensure a structured, timely, cost efficient and successful project start, implementation, reporting, controlling, project internal communication and project finalization. The MB has been established during the Kick off meeting consisting of project coordinators from all partner institutions. The project coordinator has the main role and the principal responsibility in organizing project activities and ensuring their successful implementation. Also, the coordinator is responsible for preparing Interim and Final reports.

Although the HarISA project was supposed to be completed in January this year, considering the global COVID-19 pandemic, the project is expected to be expanded to October 15, 2022.

Nauka u službi zdravlja bilja, čoveka i prirode



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