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AFLATOXIGENIC AND OCHRATOXIGENIC MOULDS AND THEIR TOXINS IN TREE NUTS ON SERBIAN MARKET

ABSTRACT: In this study, moulds and mycotoxins presence in different tree nuts were investigated. The results showed that all of the 25 samples were contaminated with moulds. Mean values of total mould count varied from 1–4.9 cfu per grain. The most frequent species in hazelnut samples were *Rhizopus oryzae* (32.2%) and *Aspergillus niger* (28.9%). In walnuts *A. niger* (75.6%), in cashews also *A. niger* (42.4%) while in pistachio samples *Alternaria alternata* (20.7%), and *Cladosporium cladosporioides* (20.7%) were the most dominant. *Rhizopus oligosporus* was the only identified species in all almond samples (100%). Using Enzyme Linked Immunosorbent Assay (ELISA), the presence of total aflatoxins and ochratoxin A was examinated. In all analyzed samples, levels of ochratoxin A were below the limit of detection. Total aflatoxins were detected only in walnut samples with average concentration of 7.1 μg/kg.

KEYWORDS: contamination, moulds, mycotoxins, tree nuts

INTRODUCTION

Tree nuts are valuable crops which are widely used in human consumption around the world. They include walnuts, almonds, hazelnuts, cashews and pistachios. The world largest producers of hazelnuts are Turkey, Italy, United States and China. Also, USA are the biggest producers and exporters of almonds. Walnuts are mainly produced in China, cashews in India and Nigeria, while the biggest producers of pistachios are Iran and USA (FAO, 2012).

The low water activity of tree nuts (a_w <0.70) is not suitable for growth of bacteria and yeasts, which need higher water content (minimal a_w for the most bacteria growth is 0.90 and for the most of the yeasts 0.88) (Beuchat, 1981).

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Tree nuts are more susceptible to moulds contamination, because these microorganisms can grow on substrates with lower a_w. Some xerophyllic moulds can grow even on substrates with a_w 0.65 (Ray, 2003; Pitt and Hocking, 2009). If insects damage the grain or if nuts are in contact with the ground, the possibility of moulds contamination increases. Grain damage and moulds contamination may occur during growth, harvesting, processing or storaging. It also depends on usage of fungicide, storage time and storage conditions (Schatzki and Ong, 2001; Campbell et al., 2003; Baquião et al., 2012).

There are many reports about mould contamination of tree nuts (Abdel- Hafez and Saber, 1993; Gürses, 2006; Ozay et al., 2008; Xu et al., 2011; Baquião et al., 2012; Tournas et al., 2015; Alsuhaibani, 2018). The most frequent contaminants are *Aspergillus*, *Rhizopus* and *Penicillium* species. It is of great concern because some *Aspergillus* and *Penicillium* species have potential to produce toxic secondary metabolites- mycotoxins (Bayman et al., 2002; Gürses, 2006; Rodrigues et al., 2012). Since most of the mycotoxins are thermostable at high temperatures, they can be found in processed food, and thus enter food chain. These toxins can cause diverse acute or chronic toxic effects in humans and animals. Today it is known over 300 different mycotoxins but the most common and the most toxigenic is aflatoxin B1 (AFB1) (Moreu, 1979; Heperkan, 2006). AFB1 is a potent carcinogenic and teratogenic mycotoxin which can lead to serious health problems, including liver cancer, chronic hepatitis, jaundice, hepatomegaly and cirrhosis (Ozay et al., 2006). International Agency for Research on Cancer (IARC) clasiffied AFB1 in group 1 (agents carcinogenic to humans). Beside AFB1, common mycotoxin which can be found in tree nuts is ochratoxin A (OTA). It is nephrotoxic mycotoxin, classified by IARC in group 2B (possible human carcinogen) (Castegnaro and Wild, 1995; Nielsen et al., 2009).

European Union (EU) set the maximum levels for AFB1 in almonds and pistachios intended for direct human consumption at 8 μg/kg and 10 μg/kg for total aflatoxins (AFB1, AFB2, AFG1, AFG2), for hazelnuts at 5 μg/kg for AFB1; 10 μg/kg fot total aflatoxins and in walnuts and cashews at 2 μg/kg for AFB1; 4 μg/kg fot total aflatoxins. Maximum level for ochratoxin A in tree nuts intended for direct human consumption is limited at 3 μg/kg (Maurice, 2002; EC, 1881/2006; Campagnollo et al., 2016). Serbia also set maximum levels for AFB1 and total aflatoxin (AFB1, AFB2, AFG1, AFG2) presence in tree nuts, wich is in agreement with European Union. However, in Serbian Regulation there is no data about maximum levels for ochratoxin A presence in tree nuts (*Službeni glasnik RS* / Official Gazette RS, 2019). Also, in Serbian Regulation there is no information about microbiological safety of tree nuts (*Službeni glasnik RS* / Official Gazette RS, 2010).

Considering that tree nuts are widely used in human consumption, and bearing in mind potentially toxigenic and carcinogenic effects of mycotoxin on human health, the aim of the present work was to identify and evaluate mould and mycotoxin contamination in different tree nuts from Serbian market.

MATERIAL AND METHODS

Samples

Twenty-five raw tree-nut samples were analyzed for mould contamination and mycotoxin production. Analyzed hazelnuts (*Corylus avellana* L.), almonds (*Prunus dulcis* L.), walnuts (*Juglans regia* L.), cashews (*Anacardium occidentale* L.) and pistachios (*Pistacia vera* L.) were collected from different local markets and "healthy food" stores from Novi Sad, Serbia. All of the samples were sampling randomly, in bulk, without original packaging. In each group of tree-nuts there was five samples (100 g of each). Walnuts were originally from Serbia while other samples were imported. Hazelnuts were imported from Turkey, almonds from California and Dalmatia, cashews from Vietnam and Indonesia and pistachios from Turkey and Iran.

Mycological analysis

Using direct-plating method, five grains of hazelnuts, almonds, cashews and pistachios and four grains of walnuts were plated on Dichloran 18% Glycerol agar (DG18) plates. All of the samples were done in two replicates, without surface disinfection. The plates were incubated at 25 ºC for 5 days, after which moulds were counted. Total mould count was expressed as the number of colony-forming units (cfu) per grain.

Identification of moulds

Alternaria, *Cladosporium*, *Monascus*, *Monilia*, *Moniliella*, *Rhizomucor*, *Rhizopus, Syncephalastrum*, *Pteroconium* and *Ulocladium* species were recultured on Sabouraud Maltose Agar (SMA), and *Aspergillus*, *Penicillium,* and *Eurotium* on Czapek's agar (CZA). The plates were incubated at 25 ºC for 7 days. *Fusarium* species were reculted on Potato Dextrose Agar (PDA) and Carnation Leaf Agar (CLA). In order to stimulate growth of conidiogenic structures, plates were incubated 10–14 days in combined light conditions (12 h of light and 12 h of darkness) at 25 ºC. After incubation period, identification of moulds was done based on their microscopic characteristics and macroscopic characteristics of the colonies. The color of colony and reverse, diameter, texture and edges of colony, the presence or absence of exudate etc. were observed. Their morphological characteristics, the length and shape of metules and phialides, the size and the shape of conidia, hyphaes, etc. were observed under microscope. Identification was done according to Samson et al. (2004), Samson and Frisvad (2004), Lević (2008), and Leslie and Summerell (2006) using "Leitz Aristoplan Trinocular Phase Contrast Microscope", Wild Leitz, Germany.

Mycotoxicological analysis

Determination of mycotoxin presence in analyzed samples was performed using Enzyme-Linked Immunosorbent Assay (ELISA).

For ochratoxin A determination was used I' screen Ochratoxin ELISA kit, Tecna srl, Trieste, Italy, with limit of detection of 1 μg/kg. Extracts for ochratoxin test were prepared using 5 g of finely grinded sample mixed with 15 ml of HCl 1M and 30 ml of dichlormethane. The solution was shaking for 15 minutes with a low speed shaker after which 5 ml of lower dichlormethane phase was transfered in a tube. Sodium bicarbonate in the amount of 5 ml was added and shaken for 15 minutes with a low speed shaker, which was followed by centrifugation at 2200 × g for 15 minutes. Upper acqueous phase in the amount of 150 μl was taken and diluted with 350 μl of bicarbonate solution. The analyses were performed according to the test kit instructions. The absorbances were read using a microplate reader (Multiskan EX, Thermo Electron Corporation) at 450 nm.

Veratox Aflatox (Total) Quantitative Test kit, Neogen, Lansing, USA, with detection limit of 2.5 μg/kg was used for total aflatoxins determination. Extracts for total aflatoxin test were prepared using 5 g of finely grinded sample mixed with 25 ml of 70% methanol. Prepared solution was shaken for 3 minutes and filtered through Whatman No.1 filter paper. The analyses were performed according to the test kit instructions. The absorbances were read using a microplate reader (Multiskan EX, Thermo Electron Corporation) at 650 nm.

RESULTS AND DISCUSSION

In Figure 1 are shown results of mycological analysis expressed as mean values of total moulds count for each group of analyzed samples.

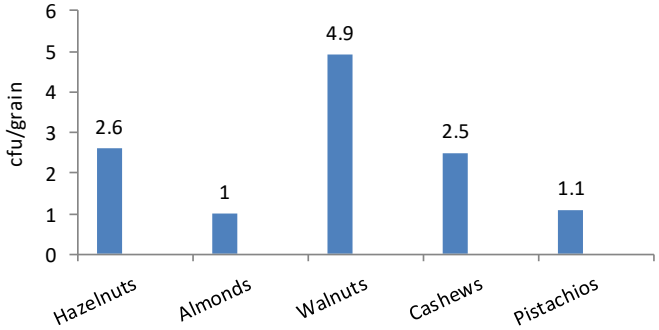

Figure 1. Mean values of total moulds count (cfu/grain) in analysed samples

As it can be seen, almond samples had the lowest mean value of total mould count (1 cfu/grain), and the highest mean value of total mould count had walnut samples (4.9 cfu/grain).

Tournas et al. (2015) also reported that among other analysed tree nuts, walnuts were the most contaminated with moulds (5.34 log10 cfu/g). Moulds were detected in every walnut sample, in 91% of pine nuts and in 76% of almond samples. Ozay et al. (2008) found that total mould count in Turkish hazelnuts varied from $1.8 \times 10^1 – 3.8 \times 10^6$ cfu/g. Using direct plating method, Xu et al. (2011) reported that compared to ground meal (1.2 × 103 cfu/g) and the kernel (7.0 × 102 cfu/g), whole nuts were the most contaminated with moulds (3.4×10^4 cfu/g), because of the greater mold contamination on the surface of the shell. Alsuhaibani (2018) reported that total mould count of analysed samples ranged from 3.0×10^3 cfu/g in the pistachios and cashews to 4.0×10^3 cfu/g in the walnuts, almonds and hazelnuts.

The list and the frequency of identified mould species in each group of samples are given in Table 1.

Table 1. The list and frequency of identified mould species (%)

Samples	Species	Frequency of species (%)
Hazelnuts	*Aspergillus flavus*	17.8
	A. niger	28.9
	Penicillium expansum	1.1
	Rhizomucor pusillus	1.1
	Rhizopus oligosporus	18.9
	R.oryzae	32.2
Almonds	*R. oligosporus*	100
Walnuts	*A. flavus*	5.1
	A. niger	75.6
	A. parasiticus	1.9
	Fusarium oxysporum	4.5
	Monascus ruber	1.9
	R. oligosporus	10.9
Cashews	*Alternaria alternata*	1.5
	A. flavus	3.0
	A. niger	42.4
	Eurotium chevalieri	24.2
	E. herbariorum	4.5
	M. ruber	1.5
	Monilia sitophila	7.6
	Penicillium glabrum	1.5
	R. oligosporus	12.1
	Syncephalastrum racemosum	1.5
Pistachios	*A. alternata*	20.7
	A. niger	10.3
	Aspergillus versicolor	3.4
	Cladosporium cladosporioides	20.7
	C. sphaerospermum	6.9
	Moniliella acetoabutens	3.4
	P. nalgiovense	6.9
	P. polonicum	6.9
	P. solitum	3.4
	Pteroconium intermedium	3.4
	R. oligosporus	6.9
	R. oryzae	3.4
	Ulocladium atrum	3.4

In hazelnuts the most frequent species were *R. oryzae* (32.2%) and *A. niger* (28.9%) while in almond samples *R. oligosporus* was the only identified species. In walnuts *A. niger* (75.6%) was the most dominant. In cashews prevalent species was *A. niger* (42.4%) while in pistachio samples the most frequent species were *A. alternata* (20.7%) and *C. cladosporioides* (20.7%).

Many authors reported similar results about identified moulds in tree nuts. Xu et al. (2011) reported that *Penicillium*, *Alternaria* and *Cladosporium* were the most frequent species in analyzed tree nut samples. *Aspergillus* was not identified. On the other hand, Abdel-Hafez and Saber (1993) showed that *Aspergillus* represent 57.35% of all identified species in analyzed hazelnuts and walnuts. The most frequent species were *A. flavus*, *A. fumigatus*, *A. niger*, *C. cladosporioides*, *C. herbarum*, *P. chrysogenum*, *P. citrinum* and *P. oxalicum*. Ozay et al. (2008) reported that *A. flavus* (89%) and *A. parasiticus* (11%) were the only identified moulds in all analyzed hazelnut samples. Also, Tournas et al. (2015) found that *A. niger* was the most frequent mould representing 35% of all identified species in almonds, 36% in pine nuts and 30% in walnut samples. In Serbia, mould contamination of tree nuts was also reported previously. Dimić et al. (2005) reported that 17% of analyzed almond samples were contaminated with moulds, while contamination of hazelnuts was negligible. In almond and hazelnut samples were identified *Aspergillus*, *Penicillium* and *Eurotium* species as well as *Paecilomyces variotii*. Also, Radojevic (2016) found that 12 of 15 peanut samples from Serbian market were contaminated with moulds. The most frequent were *Penicillium* (33.9%) and *Aspergillus* (32.9%) species.

These results indicate that *Aspergillus* species are very common contaminants of tree nuts. It is of great concern because many *Aspergillus* species are known for mycotoxins production. In Table 2 is given the list of mould species identified in this examination which may produce aflatoxins and ochratoxins.

Table 2. The list of potentially aflatoxigenic and ochratoxigenic moulds identified in this study and their mycotoxins (according to Frisvad and Thrane, 2004)

Moulds	Toxins
A. flavus	aflatoxin B1, aspergillic acid, kojic acid, 3-nitropropionic acid, cyclopiazonic acid
A. niger	ochratoxin A, naphtho-4-pyrones, malformins
A. parasiticus	aflatoxin B1, B2, G1,G2, kojic acid, aspergillic acid

The frequency of potentially aflatoxigenic and ochratoxigenic species in each group of analyzed samples is given in Figure 2 (a, b).

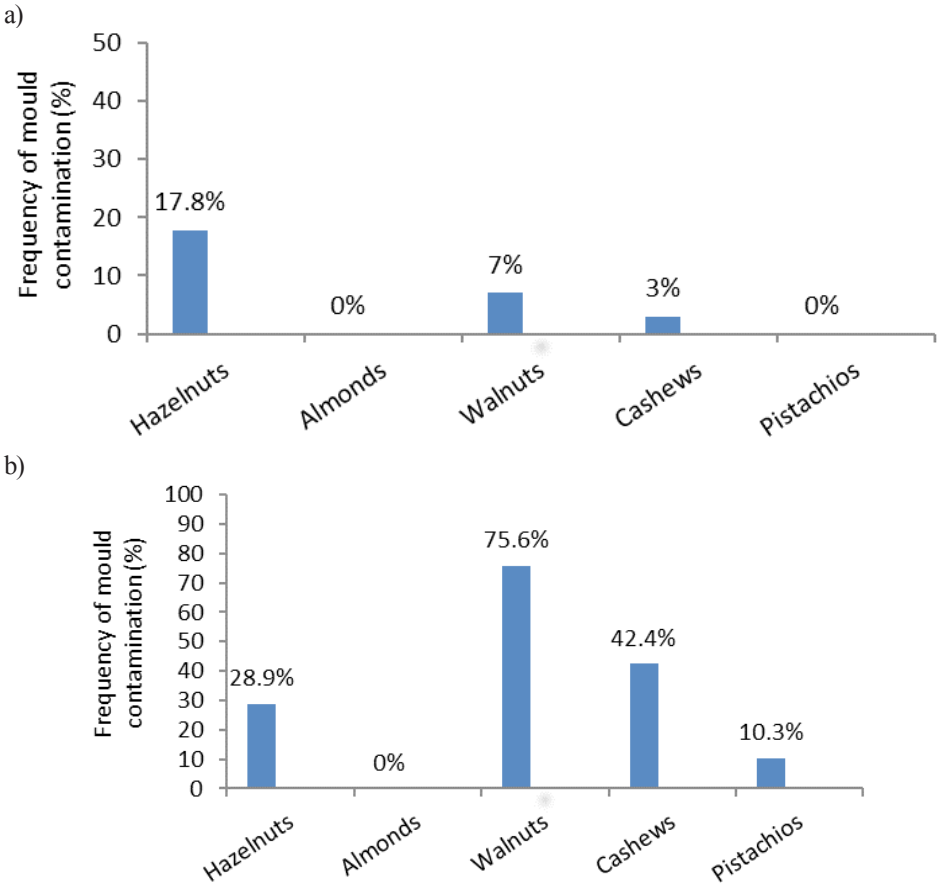

Figure 2. The frequency (%) of potentially aflatoxigenic (a) and ochratoxigenic (b) moulds in tree nuts

Hazelnut samples had the highest percentage (17.8%) of potentially aflatoxigenic species, while in almonds and pistachios was not identified none of the potentially aflatoxigenic species. In walnut samples potentially ochratoxigenic species represented 75.6% of all identified moulds. Among almond samples there was identified none of the ochratoxigenic species.

There are many studies which have reported mycotoxin presence in tree nut samples. Back in 1993, Abdel-Hafez and Saber detected aflatoxins in 90% of hazelnut samples and in 75% of walnut samples (Abdel-Hafez and Saber, 1993). Also, Gürses (2006) reported aflatoxin presence in 27.66% tested tree nut samples. Essawet et al. (2017) analyzed different tree nut samples for aflatoxin presence. The percentages of mycotoxin positive samples were 33.3% (almonds), 40.0% (Brazilian nuts), 20.0% (hazelnuts), 13.3% (cashews), 26.6% (walnuts) and 53.3% (peanuts). Also, there are earlier reports about mycotoxin presence in tree nuts in Serbia. Dimić et al. (2005) reported that aflatoxin G1

was detected in one almond sample (of total 3) in concentration of 0.14 μg/kg while ochratoxin A was detected in two almond samples in concentration of 8.00 μg/kg and 16.00 μg/kg. In hazelnut samples mycotoxin presence was not detected. Also, Radojević (2016) detected aflatoxin presence in 20% of analyzed peanut samples in concentrations of 1.538 μg/kg, 1.935 μg/kg and 2.372 μg/kg. Investigating mycotoxicological food safety in Serbia, Milićević and Nedeljković (2004) found aflatoxin presence in 9.09% of hazelnut and walnut samples with average concentration of 0.27 μg/kg. Also, in 5.45% of these samples ochratoxin A with average concentration of 0.54 μg/kg was detected.

Although ochratoxigenic moulds are found in almost every sample, all of the samples were ochratoxin A free, while total aflatoxins were detected in all walnut samples with average concentration of 7.1 μg/kg. This concentration is higher than maximum approved level of European Union and Serbian Regulation, which indicates that these walnuts are not safe for human consumption. It is important to stress that presence of mycotoxigenic species does not indicate the must presence of mycotoxins. Mycotoxin production depends on genetic ability of mould and different factors such as temperature, relative humidity, insect infestation, usage of fungicides and fertilizers, type of substrate etc. Therefore, they only indicate potential risk for toxin production. Also, the absence of mycotoxigenic species does not exclude the presence of mycotoxin (Bullermall, 1986; D'Mello and Macdonald, 1997). Our results also showed no correlation between presence of mycotoxigenic species and mycotoxin presence.

As it was mentioned, walnut samples were the ones most contaminated with moulds, where mean value of total moulds count was 4.9 cfu/grain. Also, in these samples two potentially aflatoxigenic species were identified – *A. flavus* and *A. parasiticus* – which explains aflatoxin presence in these samples. Although hazelnuts had the highest percentage of potentially aflatoxigenic species (17.8%), in these samples aflatoxin presence was not detected.

CONCLUSION

The present work showed that all of the tested samples were contaminated with moulds, mostly walnuts. Almonds had the lowest mean value of total mould.

The most frequent species in hazelnut samples were *R. oryzae* and *A. niger*. In walnuts *A. niger* was predominant, in cashews *A. niger* and *E. chevalieri*, while in pistachios *A. alternata* and *C. cladosporioides* were the most common species. *R. oligosporus* was the only identified species in almond samples.

In spite the presence of several mycotoxigenic species, all tested samples were ochratoxin A free. Total aflatoxins were detected only in walnuts, but in concentrations above maximum prescribed. Therefore, it can be concluded that monitoring of mycotoxin level in tree nuts is necessary. Prevention of toxigenic moulds contamination and continuous monitoring of mycotoxins presence will ensure food safety and human health.

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REFERENCES

Abdel-Hafez AII, Saber SM (1993): Mycoflora and mycotoxin of hazelnut (*Corylus avellana* L.) and walnut (*Juglans regia* L.) seeds in Egypt. *Zentralbl. Mikrobiol.* 148: 137–147.

Alsuhaibani AMA (2018): Effects of storage periods and temperature on mold prevalence and aflatoxin contamination in nuts. *Pak. J. Nutr.* 17: 219–227.

Baquião AC, Zorzete P, Reis TA, Assunção E, Vergueiro S, Correa B (2012): Mycoflora and mycotoxins in field samples of Brazil nuts. *Food Control* 28: 224–229.

Bayman P, Baker JL, Mahoney NE (2002): *Aspergillus* on tree nuts: incidence and associations. *Mycopathologia* 155: 161–169.

Beuchat LR (1981): Microbial stability as affected by water activity. *Cereal Foods World* 26: 345–349.

Bullermall LB (1986): Mycotoxins and food safety. *Food Technol.* 40: 59-66.

Campagnollo FB, Ganev KC, Khaneghah AM, Portela JB, Cruz AG, Granato D, Corassin CH, Oliveira CAF, Sant'Ana AS (2016): The occurrence and effect of unit operations for dairy products processing on the fate of aflatoxin M1: A review. *Food Control* 68: 310–329.

Campbell BC, Molyneux RJ, Schatzki TF (2003): Current research on reducing pre- and post-harvest aflatoxin contamination of US almond, pistachio, and walnut. *Toxin. Rev.* 22: 225–266.

Castegnaro M, Wild CP (1995): IARC activities in mycotoxin research. *Nat. Toxins* 3: 327–331.

Dimić G, Maletić Z, Kocić-Tanackov S (2005): Xerotolerant mycopopulations and mycotoxins in muesli components. *Matica Srpska Proc. Nat. Sci.* 109: 81–87.

D'Mello JPF, Macdonald AMC (1997): Mycotoxins. *Anim. Feed Sci. Tech.* 69: 155–166.

EC 1881 (2006): European Commission, Commission Regulation on 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, *Official J. EU*, L 364: 5–18.

Essawet N, Abushahma H, Inbaia S, Najii A, Amra HA (2017): Natural incidence of aflatoxins and ochratoxin a nuts collected from local market in Tripoli. *Int. J. Curr. Microbiol. App. Sci.* 6: 1479–1486.

FAO (2012): FAO Statistical Yearbook. Agricultural production. Food and Agriculture Organization of the United Nations.

Frisvad JC, Thrane U (2004): Mycotoxin production by common filamentous fungi. In: Samson RA, Hoekstra ES, Frisvad JC (eds.), *Introduction to Food- and Airborne fungi*, Centraalbureau voor Schimmelcultures, Utrecht.

Gürses M (2006): Mycoflora and aflatoxin content of hazelnuts, walnuts, peanuts, almonds and roasted chickpeas (Leblebi) sold in Turkey. *Int. J. Food. Prop.* 9: 395–399.

Heperkan D (2006): The importance of mycotoxin and a brief history of mycotoxin studies in Turkey. *Bull. Tech. Univ. Instanb.* 54: 18–27.

Leslie FJ, Summerell AB (2006): *The Fusarium Laboratory Manual*. Blackwell Publishing, USA.

Lević TJ (2008): *Vrste roda Fusarium*. Institut za kukuruz „Zemun Polje" i društvo genetičara Srbije. Beograd: Cicero.

Maurice OM (2002): Risk assessment for aflatoxins in foodstuffs. *Int. Biodeterior. Biodegrad.* 50: 137–142.

Milićević D, Nedeljković Lj (2004): Mikotoksikološka ispravnost životnih namirnica u periodu od 2001–2004. III Međunarodna Eko-konferencija – Zdravstveno bezbedna hrana, Novi Sad, pp. 339.

Moreu C (1979): *Moulds, Toxins and Food*. 2nd ed., London: Wiley.

Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC (2009): Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Anal. Bioanal. Chem.* 395: 1225–1242.

Ozay G, Seyhan F, Yilmaz A, Whitaker TB, Slate AB (2006): Sampling hazelnuts for aflatoxin: uncertainty associated with sampling, sample preparation and analysis. *J. AOAC Int.* 89: 1004–1011.

Ozay G, Seyhan F, Pembeci C, Saklar S, Yilmaz A (2008): Factor influencing fungal and aflatoxin levels in Turkish hazelnuts (*Corylus avellana* L.) during growth, harvest, drying and storages: A 3-year study. *Food Addit Contam. Part A* 25: 209–218.
Pitt JI, Hocking AD (2009): *Fungi and Food Spoilage*. London: Blackie Academic.
Radojević J (2016): *Toksigene plesni i aflatoksini u kikirikiju i suvom grožđu na tržištu Republike Srbije*. Specijalistički rad, Tehnološki fakultet, Univerzitet u Novom Sadu, Srbija.
Ray B (2003): *Fundamental Food Microbiology*. Third Edition, CRC Press.
Rodrigues P, Venancio A, Lima N (2012): Aflatoxigenic fungi and aflatoxins in Portuguese almonds. *Sci. World J*. 47: 1–9.
Samson RA, Frisvad JC (2004): *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. *Stud. Mycol*. 49: 1–260.
Samson RA, Hoekstra ES, Frisvad JC (2004): *Introduction to food and airborne fungi*. 7th Edition, Utrecht: Centraalbureau voor Schimmelcultures.
Schatzki TF, Ong MS (2001): Dependence of aflatoxin in almonds on the type and amount of insect damage. *J. Agric. Food Chem*. 49: 4513–4519.
Službeni glasnik RS (Official Gazette RS) (2010): Pravilnik o opštim i posebnim uslovima higijene hrane u bilo kojoj fazi proizvodnje, prerade i prometa, 72/10.
Službeni glasnik RS (Official Gazette RS) (2019): Pravilnik o maksimalnim koncentracijama određenih kontaminenata u hrani, 41/09; 17/19.
Tournas VH, Niazi NS, Kohn JS (2015): Fungal presence in selected tree nuts and dried fruits. *Microbiol. Insights* 8: 1–6.
Xu Y, Bianchini A, Hanna MA (2011): Evaluation of mold and mycotoxin contaminations in hybrid hazelnuts grown in Nebraska. *J. Food Process. Technol*. 2: 119.

АФЛАТОКСИГЕНЕ И ОХРАТОКСИГЕНЕ ПЛЕСНИ И ЊИХОВИ ТОКСИНИ У ОРАШАСТИМ ПЛОДОВИМА НА ТРЖИШТУ СРБИЈЕ

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РЕЗИМЕ: У овом раду испитано је присуство плесни и микотоксина у узорцима орашастих плодова. Резултати истраживања показали су присуство плесни у свих 25 узорака. Средња вредност укупног броја плесни кретала се од 1 до 4,9 cfu по зрну. Најзаступљеније врсте у узорцима лешника биле су *Rhizopus oryzae* (32,2%) и *Aspergillus niger* (28,9%), у узорцима ораха *A. niger* (75,6%), у индијском ораху такође *A. niger* (42,4%), док су у узорцима пистаћа *Alternaria alternata* (20,7%) и *Cladosporium cladosporioides* (20,7%) биле најдоминантније врсте. *Rhizopus oligosporus* је једина идентификована врста у узорцима бадема (100%). Присуство укупних афлатоксина и охратоксина А у узорцима орашастих плодова одређено је ELISA методом. У свима анализираним узорцима концентрација охратоксина А била је испод границе детекције. Укупни афлатоксини су детектовани једино у узорцима ораха са просечном концентрацијом од 7,1 μg/kg.

КЉУЧНЕ РЕЧИ: контаминација, микотоксини, орашасти плодови, плесни

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PRESENCE OF AFLATOXINS AND DEOXYNIVALENOL IN SEEDS OF MAIZE, SPELT WHEAT AND SOYA BEAN – PRELIMINARY RESEARCH

ABSTRACT: Mycotoxins naturally contaminate plant – based food. Since organic production does not allow the use of synthetic pesticides in plant protection, many researchers state that organically produced foods are more contaminated with mycotoxins than conventional ones. In this regard, the aim of this study was to observe the content of aflatoxins B1, B2, G1 and G2 and deoxynivalenol, as the most common mycotoxins, in organically and conventionally produced seeds of maize, spelt wheat and soya been during the 2015–2017 period. The HPLC/FLD and HPLC/DAD were used to determine the presence of aflatoxins and deoxynivalenol, respectively. The highest number of samples was not positive to the presence of these mycotoxins. Aflatoxin B1 (1.16 μg/kg) and deoxynivalenol (101.53 μg/kg) were detected only in the sample of organic maize harvested in 2015. Based on obtained results, no conclusion can be drawn on the effects of organic and conventional production on contents of mycotoxins in seeds of maize, spelt wheat and soya bean, and further long-term studies are required.

KEYWORDS: aflatoxins, deoxynivalenol, maize, spelt wheat, soya bean

INTRODUCTION

Mycotoxins are secondary metabolites of fungi. They naturally contaminate food of plant origin that is the source of nutrition for the population of the majority of countries. These are cereals, oil plants, dry fruits and vegetables,

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hazelnuts, peanuts, sesame, almonds, coffee, etc. (Bryden, 2012). Fungi contaminate food in fields and/or during food storage. Foodstuffs can be directly contaminated by the development of fungi in them, or indirectly by consuming foods containing the mycotoxin residues or by the use of contaminated ingredients in their processing (Marriot and Gravani, 2006). Indirect contamination with mycotoxins through the food chain via animal – based products is a particularly high risk to human health (Soriano, 2007). It is estimated that 25–40% of cereals in the world are contaminated with mycotoxins (Pittet, 1998), the most common of which are aflatoxins, deoxynivalenol, zearalenone, fumonisin and T-2 toxins (Sokolović, 2005).

Aflatoxins are heterocyclic metabolites, products of secondary metabolism of fungi *Aspergillus flavus* Link, *A. parasiticus* Speare, *A. nominus, A. pseudotamarii* and *A. bombzcis* and belong to most studied types of mycotoxins (Peterson et al., 2001). Aflatoxins B1, B2, G1 and G2 can be found in a wide range of foodstuffs, such as cereals, dry fruits, figs, nuts and spices (EFSA, 2007). Deoxynivalenol is a secondary metabolite of the *Fusarium*, *Trichoderma, Trichothecium, Myrothecium* and *Stachybotrys* species (WHO, 1990). It belongs to the type B trichothecenes. It most often contaminates wheat, barley and maize (EFSA, 2004), and to a lesser extent rye, oats and rice (Kuiper-Goodman, 2002).

Since the use of synthetic pesticides in control of pathogenic microorganisms is not permitted in organic farming, the question arises whether there is a higher amount of mycotoxins in organic food than in conventional ones. This issue has been the objective of many studies worldwide (Frank Hansen, 1990; Jörgensen et al., 1996; Kuhn, 1999; Birzele et al., 2000; Usleber et al., 2000; Malmauret et al., 2002; Schneweis et al., 2005; Ghidini et al., 2005; Winter and Davis, 2006; Herrera et al., 2009; Lairon, 2010).

The aim of the present study was to observe the presence and potential differences in the content of mycotoxins aflatoxins B_1, B_2, G_1 and G_2 and deoxynivalenol in seeds of maize, spelt wheat and soya bean conventionally and organically produced.

MATERIALS AND METHODS

Samples

Fourteen seed samples of maize, soya bean and spelt wheat organically and conventionally produced were drawn and analysed during the three – year period (2015–2017). Seed samples were grown and collected at three different locations: 1) Maize Research Institute, Zemun Polje, Belgrade (organic and conventional maize – variety Rumenka; organic spelt wheat – Nirvana variety), 2) Institute of Field and Vegetable Crops, Novi Sad, location Bački Petrovac (organic and conventional soya bean – cultivar Kaća) and 3) Nova Varoš

(conventional wheat – Nirvana variety). Damaged seeds and weed seeds were removed from drawn seed samples (100 g of each plant species). Such cleansed seed samples were ground into fine powder.

The distribution of aflatoxins B1, B2, G1 and G2, as well as deoxynivalenol, was determined in fourteen samples of maize, spelt wheat and soya bean (in three replicates).

Determination of aflatoxins

The liquid chromatograph Agilent 1260 HPLC system (Agilent Technologies, USA) with the degasser G1379B, binary pump G1312C, autosampler G1329B, thermostat column and the fluorescence detector was used to determine aflatoxins. The Zorbax Eclipse XDB C18 column (150 × 4.6 mm, 5μm) (Agilent, USA) thermostatted at 30 °C (SRPS EN 14123:2012) was used for the chromatographic development. Water was used as a mobile phase A, while the mixture of acetonitrile and water (50/50, V/V) at the ratio of 60% : 40%, and the isocratic regime and the flow rate of 1.2 mL/min was used as a mobile phase B. The sample volume entering the system amounted to 50 μl. The detection was performed at λex=365 nm and λem=455 nm, after photochemical derivatisation using the LCTech photochemical reactor. Data were processed using Agilent ChemStatin software (version B 04.02, Agilent Technologies 2001–2010), using the external standard.

Twenty g of samples were weighed and extracted with a mixture of methanol and water (70/30, V/V) in a blender at the maximum speed of 60 sec / 2 min. The extract was filtered through the Whatman blue ribbon filter paper (No. 42). A 10-ml aliquot was diluted with 40ml PBS buffer (Ph 7.4). The complete volume of the prepared extract was filtered through the immunoaffinity column (Vicam Aflatest, 3 ml wide bore). The column was rinsed with 10 ml distilled water and air – dried. Eluation of mycotoxins into a 5-ml normal vessel was done with 2 ml methanol, and then steamed in the nitrogen current and dissolved in the mobile phase.

The method linearity was confirmed in the range of 0.4–20 μg/kg, with the coefficient of correlation of $r2 > 0.99$ for all four aflatoxins. Within the validation parameters, the detection limit (AB1 and AB2 0.16 μg/kg; AG1 0.08 μg/kg and AG2 0.07 μg/kg) and the quantification limit set at 0.4 μg/kg for all aflatoxins were experimentally confirmed. The accuracy and precision of the method were established by spiking two samples on two concentration levels (0.4 and 4.0 μg/kg). The mean extraction yield for the level of 0.4 μg/kg was AB1 – 118.3% (RSDr 22.75%), AB2 – 119.6%, (RSDr 28.82%), AG1 – 116.9% (RSDr 23.28%) and AG2 – 53.5% (RSDr 18.01%), and for the level 4 μg/kg AB1 – 79.0% (RSDr 4.55%), AB2 – 76.5% (RSDr 2.30%), AG1 – 83.7% (RSDr 7.24%) and AG2 – 57.2% (RSDr 9.70%). Obtained values meet the requirements set by the Commission Regulation (EC) No 401/2006.

Determination of deoxynivalenol (DON)

The liquid chromatograph Agilent 1260 HPLC system (Agilent Technologies, USA) with the quaternary pump G1311B, autosampler G1316A, thermostat column G1316A and the VWD detector was used to determine deoxynivalenol. The Zorbax SB-Aq column (250 × 4.6 mm, 5µm) (Agilent, USA, Part No 880975-914) thermostatted at 30 °C (SRPS EN 15791:2009) was used for the chromatographic development. Water was used as a mobile phase A, while the mixture of acetonitrile and water (50/50, V/V) at the ratio of 90% : 10%, at the isocratic regime and the flow rate of 0.6 mL/min was used as a mobile phase B. The sample volume entering the system amounted to 100 µl. The detection was performed at λ=218 nm for 20 minutes. Data were processed using Agilent ChemStatin software (version B 01.07 (27), Agilent Technologies 2001–2014), using the external standard.

A total of 25 g of samples were weighed in a beaker and 5 g NaCl were added. After that, 200 ml de-ionised water was added and extracted in a blender at the maximum speed for 2 min. The aliquot was centrifuged at 4,000 rpm for 10 min. The extract was filtered through the Whatman black ribbon filter paper (No. 4), and then 2 ml of a filtered sample was transferred to the immunoaffinity column. The aliquot was rinsed with 2 × 5 ml de-ionised water and air-dried with the application of weak vacuum. DON was eluated with 1.0 ml methanol and 1.0 ml de-ionised water. The sample was then transferred into the autosampler and analysed by using HPLC/VWD.

The method linearity was confirmed in the range of 25–1000 µg/kg, with the coefficient of correlation of r2 > 0.99 (0.99974). Within the validation parameters, the detection limit (9 µg/kg) and the quantification limit (25 µg/kg) were tested. The accuracy and precision of the method were established by spiking two samples on two concentration levels (25 and 750 µg/kg). The mean extraction yield for the level of 25 µg/kg was 85.1% (RSDr 11.08%), and for the level 750 µg/kg was 99.0% (RSDr 5.32%). Obtained values meet the requirements set by the Commission Regulation (EC) No 401/2006.

RESULTS AND DISCUSSION

The analyses of seed samples of maize, spelt wheat and soya bean for the presence of deoxynivalenol and aflatoxins B1, B2, G1 and G2 revealed that the majority of samples were not positive for the presence of these mycotoxins (Table 1; mycotoxin quantification limits are presented in Table 1). Deoxynivalenol and aflatoxin B1 were detected only in the sample (Figure 1 and 2) at concentrations lower than the maximum levels prescribed by the Regulation on the maximum allowed quantities of residues of plant protection products in food and feed and on food and feed for which the maximum permitted quantities of residues of plant protection products is determined, *Official Gazette of the Republic of Serbia*, No. 22/2018 and 90/2019.

Table 1. Mycotoxin contents in seed samples of maize, spelt wheat and soya bean

	MYCOTOXIN (µg/kg)				
SAMPLE	DON	AB1	AB2	AG1	AG2
OM15	101.53	1.16	< LOQ	< LOQ	< LOQ
OM16	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
OM17	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
CM16	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
CM17	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
OS15	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
OS16	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
OS17	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
CS16	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
CS17	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
OSo16	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
OSo17	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
CSo16	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
CSo17	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
LOQ (µg/kg)	25	0.4	0.4	0.4	0.4
MDK (µg/kg)	1250*	2	/	/	/

O – organic; C – conventional; S – spelt wheat; M – maize; So – soya bean; DON – deoxynivalenol; AB1, AB2, AG1, AG2 – aflatoxins
*raw cereals except durum wheat, oats and maize.

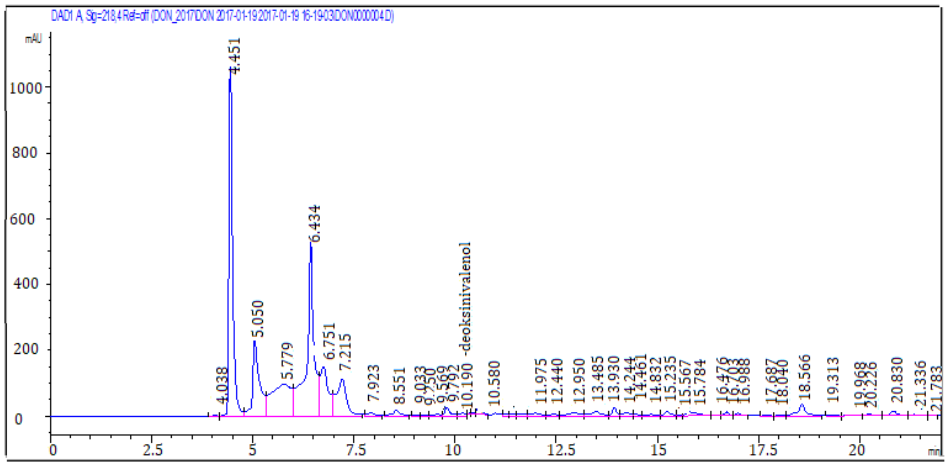

Figure 1. Chromatogram of the sample OK15 in which the presence of deoxynivalenol (101.53 µg/kg) was detected

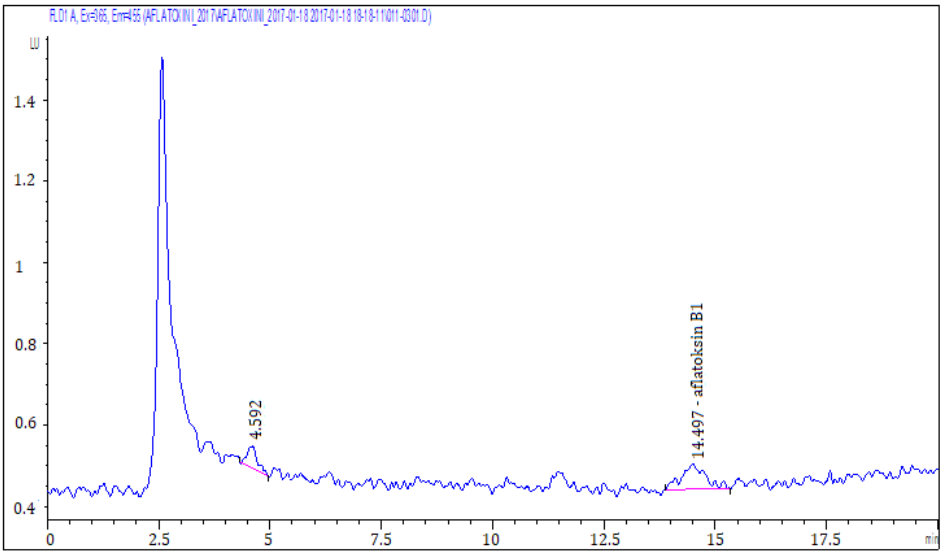

Figure 2. Chromatogram of the sample OK15 in which the presence of aflatoxin B1 (1.16 µg/kg) was detected

According to the report presented by Kuhn (1999), a lower level of deoxynivalenol was detected in organic wheat (74 ppb), and a slightly higher level was established in conventionally produced wheat (109 ppb). The latter was more frequently contaminated with *Fusarium* and contained more zearalenone and deoxynivalenol than organic wheat (Schneweis et al., 2005). According to Usleber et al. (2000), the following concentrations of deoxynivalenol in wheat and rye samples acquired in health food stores were determined: 200 µg/kg in wheat flour type 405; 410 µg/kg in wheat flour type 550; 370 µg/kg in wheat flour type 1050; 210 µg/kg in wheat bread premix; 300 µg/kg in whole grain flour; 280 µg/kg in whole grain wheat, 830 µg/kg in wheat bran; 120 µg/kg in rye flour and grits. Almost half of the organic products were contaminated with deoxynivalenol, with lower levels than conventionally produced products. Based on results obtained in analyses of mycotoxins presence in organically and conventionally produced foodstuffs (beef, pork, poultry, eggs, milk, lettuce, tomatoes, carrots, apples, spinach, French beans, buckwheat, barley and wheat), Malmauret et al. (2002) stated that these results did not provide clear evidence of whether conventional products were safer than organic ones. Herrera et al. (2009) have observed the occurrence of mycotoxins in organic and conventional durum semolina – obtained from organically and conventionally grown wheat in Spain. The percentage of ochratoxin A in organic and conventional semolina amounted to 20% and 8.3%, respectively, while the mean levels were lower in conventional samples. Deoxynivalenol was found in 16.7% and 20% of conventional and organic samples, respectively. Mean levels of deoxynivalenol were lower in conventional semolina samples (77 $\mu g/kg^{-1}$) than in organic ones (89 $\mu g/kg^{-1}$). Jestoi et al. (2004) observed the presence of mycotoxins and cy-

toxicity in organic and conventional products in the form flour, muesli and infant food produced from maize, wheat, spelt, oat, barley, rye, etc., purchased from the Finish and Italian markets. Concentrations of observed mycotoxins were low in all samples and the lowest in cereal – based food for infants. Enniatins B (6–124 μg/kg) and B1 (90–184 μg/kg) and deoxynivalenol (55–118 μg/kg) were the most dominant mycotoxins present in 97%, 97% and 90% samples, respectively. The zearalenone levels ranged from 80 to 127 μg/kg, while levels of aflatoxin B1 were significantly lower (16–40 μg/kg). According to these authors, the effects of the organic and conventional production method did not have statistical significance on the total levels of mycotoxins, although the mean concentration of total mycotoxins was somewhat higher in organic products. According to the study carried out by Gourama (2015) in south-eastern Pennsylvania on 100 samples (50 organic and 50 conventional) of popcorn, rice, maize, walnuts, almonds, peanuts, pumpkin seeds, green peas, flaxseeds, soya bean and cashew nuts, the most common fungal genera were *Aspergillus* and *Penicillium*. On this occasion, organically and conventionally produced walnuts, peanuts, soya bean and maize had the highest levels of contamination. The total level of aflatoxins (B1, B2, G1, G2) in three conventional samples of walnuts, almonds and peanuts amounted to 564, 306 and 538 μg/kg, respectively, while the corresponding levels in organic peanuts and maize were 524 μg/kg and 465 μg/kg, respectively. Cirillo et al. (2003) analysed the following organic (101) and conventional (101) products for three *Fusarium* toxins – deoxynivalenol and fumonisins B1 and B2: maize (popcorn, flour, polenta, etc.), wheat (flour, bran, biscuits, bread, pasta, etc.) and rice (biscuits, rice flakes), as well as mixed-based foodstuff consisted of rye, barley, spelt, millet, oats, milled cereals, wholemeal bread. The authors found out that organic foods were more contaminated with deoxynivalenol (80%). Fumonisin B1 was determined in 20% organic foods and 31% conventional ones and fumonisin B2 in more than 32% of food samples from both production methods. The highest medium concentration of deoxynivalenol was determined in conventional rice – based foodstuffs (207 μg/kg). According to results gained by Arino et al. (2006), the organic maize crop was more contaminated with *Fusarium* fungi (63.20%) than conventional one (40.27%). However, the distribution of the genera *Fusarium* was higher in conventional (34.93%) than in organic (18.15%) maize. Mankevičienė et al. (2014) studied the distribution of mycotoxins in organically grown certified common wheat (*Triticum aestivum* L., winter and spring) and spelt (*Triticum spelta* L.) during the two – year period and found out that concentrations of deoxynivalenol, zearalenone and T-2/HT-2 toxin, detected in spelt grain with glumes, spelt glumes, as well as in spring wheat, were as follows: concentrations of T-2/HT-2 toxins in common wheat were up to 115.0 μg/kg, in bran 120,6–286.8 μg/kg, zearalenone and deoxynivalenol were higher than 20 and 200 μg/kg, respectively. The contents of mycotoxins varied over different production years. The authors pointed out to significantly higher concentrations of *Fusarium* toxins in spelt grain glumes than in dehulled grain, which proves the protection role of glumes in grain of this type of cereal.

CONCLUSION

In order to determine the presence of aflatoxins B1, B2, G1 and G2 and deoxynivalenol, 14 seed samples of maize, spelt wheat and soya bean organically and conventionally produced in the 2015–2017 period were analysed. The presence of deoxynivalenol and aflatoxin B1 was determined only in one sample – 2015 organic maize.

Furthermore, the presence of deoxynivalenol (101.53 μg/kg) and aflatoxin B1 (1.16 μg/kg) was detected at concentrations lower than the maximum values prescribed by the Regulation on the maximum allowed quantities of residues of plant protection products in food and feed and on food and feed for which the maximum permitted quantities of residues of plant protection products is determined, Official Gazette of the Republic of Serbia, No. 22/2018 and 90/2019.

According to gained results, it cannot be clearly concluded on the influence of the production method on the mycotoxin content in seeds. Namely, although organic maize was contaminated, it does not mean that organically produced crops conditionally contain greater amounts of mycotoxins than conventionally produced crops. In order to elucidate the effect of the production method on the mycotoxin contents in a plant material, long-term studies with a greater number of species are necessary.

REFERENCES

Arino A, Juan T, Estopanan G, Gonzalez/Cabo JF (2006): Natural occurrence of *Fusarium* species, Fumonisin production by toxigenic Strains, and concentrations of Fumonisins B1 and B2 in conventional and organic maize grown in Spain. *J. Food Prot.* 70: 151–156.

Birzele B, Prange A, Krämer J (2000): Deoxynivalenol and ochratoxin A in German wheat and changes of level in relation to storage parameters. *Food Addit. Contam.* 17: 1027–1035.

Bryden WL (2012): Mycotoxin contamination of the feed supply chain: Implications for animal productivity and feed security. *Anim. Feed Sci. Tech.* 173: 134–158.

Cirillo T, Ritieni A, Visone M, Amodio Cocchieri A (2003): Evaluation of conventional and organic italian foodstuffs for deoxynivalenol and fumonisins B1 and B2. *J. Agric. Food Chem*. 51: 8128–8131.

EFSA (2004): Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to Deoxynivalenol (DON) as undesirable substancein animal feed. *EFSA J.* 73: 1–42.

EFSA (2007): Opinion of the scientific panel on contaminants in the foodchain on a request from the commission related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. *EFSA J.* 446: 127.

Frank Hansen L (1990): Characterization of organic milk. *Proceedings of the Ecological Agriculture NJF* – seminar 166 – Miljövard.

Ghidini S, Zanardi E, Battaglia A, Varisco G, Ferretti E, Campanini G, Chizzolini R (2005): Comparison of contaminant and residue levels in organic and conventional milk and meat products from Northern Italy. *Food Addit. Contam.* 22: 9–14.

Gourama H (2015): A preliminary mycological evaluation of organic and conventional foods. *Food Prot. Trends* 35: 385–391.

Herrera M, Juan T, Estopanan G, Arino A (2009): Comparison of deoxynivalenol, ochratoxin A and aflatoxin B1 levels in conventional and organic durum semolina and the effect of milling. *J. Food Nutr. Res.* 48: 92–99.

Jestoi M, Carmela SM, Kouva M, Veijalainen P, Rizzo A, Ritieni A, Peltonen K (2004): Levels of mycotoxins and sample cytotoxicity of selected organic and conventional grain – based products purchased from Finnish and Italian markets. *Mol. Nutr. Food Res.* 48: 299–307.
Jörgensen K, Rasmussen G, Thorup I (1996): Ochratoxin A in Danish cereals 1986-92 and daily uptake by Danish population. *Food Addit. Contam.* 13: 95–104.
Kuhn F (1999): *Bestimmung von Trichothecenen in Weizen aus verschiedenen Anbausystemen mittels HPLC-MS*, Diplomarbeit, Universität Basel, 60 p.
Kuiper-Goodman T (2002): Recent developments in the risk assessment of deoxynivalenol. Toxicology, quality and impact on industry. *Second Canadian workshopon Fusarium head blight*, Ottawa.
Lairon D (2010): Nutritional quality and safety of organic food. A review. *Agron. Sustain. Dev.* 30: 33–41.
Malmauret L, Parent-Massin D, Hardy J-L, Verger P (2002): Contaminants in organic and conventional foodstuffs in France. *Food Addit. Contam.* 19: 524–532.
Mankevičienė A, Jablonskytė-Raščė D, Maikštėnienė S (2014): Occurrence of mycotoxins in spelt and common wheat grain and their products. *Food Addit. Contam.* Part A 31: 132–138.
Marriot NG, Gravani RB (2006): *Principles of Food Sanitation*, Springer, USA.
Peterson SW, Ito Y, Horn BW, Goto T (2001): *Aspergillus bombycis*, a new aflatoxigenic species and genetic variation in its sibling species, *A. nomius*. *Mycologia* 93: 689–703.
Pittet A (1998): Natural occurrence of mycotoxins in foods and feeds – an updated review. *RevMedVet* 149: 479–492.
Schneweis I, Meyer K, Ritzmann M, Hoffmann P, Dempfle L, Bauer J (2005): Influence of organically or conventionally produced wheat on health, performance and mycotoxin residues in tissues and bile of growing pigs. *Arch. Anim. Nutr.* 59: 155–163.
Sokolović M (2005): Značaj trikotecenskih mikotoksina u hrani za perad. *Zbornik / Proceedings. VI. simpozij peradarski dani 2005, s međunarodnim sudjelovanjem*, Hrvatska, Poreč, 11–14. svibnja 2005. Zagreb: Centar za peradarstvo.
Soriano MJ (2007): *Micotoxinas en Alimentos* (1st ed.). España: Ediciones Díaz de Santos (Chapter 12).
SRPS EN 15791:2009: Određivanje deoksinivalenola u hrani za životinje – HPLC metoda sa UV detekcijom i prečišćavanjem na imuno afinitetnoj koloni.
SRPS EN 14123:2012: Određivanje aflatoksina B1 i ukupnih aflatoksina B1, B2, G1 i G2 u lešnicima, kikirikiju, pistaćima, smokvama i mlevenoj začinskoj paprici — Metoda tečne hromatografije visoke performanse sa postkolonskom derivatizacijom uz prečišćavanje na imunoafinitetnoj koloni.
Usleber E, Lepschy J, Mätlbauer E (2000): Deoxynivaleol in Mehlproben des Jahres 1999 ausdem Einzelhandel, *Mycotoxin Res.* 16: 30–33.
WHO (1990): Environmental health criteria 105, Selected mycotoxins: Ochratoxins, trichothecenes, ergot. Available from: http://www.inchem.org/documents/ehc/ehc/ehc105.htm
Winter CK, Davis SF (2006): Organic Foods. *J. Food Sci.* 71: 117–124.

ПРИСУСТВО АФЛАТОКСИНА И ДЕОКСИНИВАЛЕНОЛА У СЕМЕНУ КУКУРУЗА, СПЕЛТЕ И СОЈЕ – ПРЕЛИМИНАРНА ИСТРАЖИВАЊА

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РЕЗИМЕ: Микотоксини природно контаминирају намирнице биљног порекла. Пошто органски начин производње искључује употребу синтетичких пестицида за заштиту биља, бројни истраживачи наводе да су органски произведене намирнице више контаминиране микотоксинима у поређењу са конвенционалним. У том смислу, циљ овог рада био је да испита садржај афлатоксина B1, B2, G1 и G2 и деоксиниваленола, као најчешћих микотоксина, у органски и конвенционално произведеном семену кукуруза, спелте и соје током 2015, 2016 и 2017. године. Присуство афлатоксина одређено је методом HPLC/FLD, а деоксиниваленола методом HPLC/DAD. Највећи број узорака није садржавао микотоксине. Афлатоксин B1 (1,16 μg/kg) и деоксиниваленол (101,53 μg/kg) били су детектовани само у узорку органског кукуруза из 2015. године. Према добијеним резултатима, не може се извести јасан закључак о утицају органског и конвенционалног начина производње на садржај микотоксина у семену кукуруза, спелте и соје те су неопходна даља вишегодишња испитивања.

КЉУЧНЕ РЕЧИ: афлатоксини, деоксиниваленол, кукуруз, спелта, соја

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Leucotelium cerasi (Bérenger) Tranzschel 1935, NOVEL PATHOGEN FOR MYCOBIOTA IN SERBIA

ABSTRACT: During the field studies on a strictly protected plant species *Eranthis hyemalis* (winter aconite) in Serbia, it was noticed that certain individuals were infected by some species of parasitic fungus. According to the structure of spermogonia and aecia, as well as distinctive types of spores recorded in infected leaves of winter aconite, the fungus was determined as *Leucotelium cerasi*, known as a causative agent of rust in *E. hyemalis* and *Prunus* species. Although it was first recorded in Serbia in *Prunus spinosa* as a host (area of Valjevo in 1935), the appearance of this phytopathogen was recorded in foliar tissue of *E. hyemalis* at four localities in Eastern Serbia, near Knjaževac (Podvis, Golemi Kamen, and Ploča) and Zaječar (Zmijanac). It was concluded that plants in natural populations of host species were infected to a very high degree, as the number of infected plants reached half of the total number of individuals in all studied populations. Further research is necessary to determine to what extent this fungus is threatening the survival of host populations and then implement appropriate protection measures.

KEYWORDS: *Eranthis hyemalis*, *Leucotelium cerasi*, pathogen, rust basidiomycete, Serbia, winter aconite.

INTRODUCTION

Genus *Eranthis* Salisb. (fam. *Ranunculaceae,* tribe *Helleboreae*) includes nine species recorded in Europe and Asia (Lee et al., 2012). The primary range of species *E. hyemalis* (L.) Salisb. includes the region of the Southern Alps, the Balkan Peninsula, and certain parts of the Pannonian Plain in Hungary and Serbia (Petrović and Lakušić, 2017). The range of this plant has secondarily extended through the USA, Western Europe, and Romania, where it appears as

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a cultivated, but frequently also as a naturalized or accidental species (Anastasiu and Negrean, 2009; Petrović and Lakušić, 2017). In Serbia, it inhabits shaded forest habitats with fresh humus soils, mostly appearing as an element of oak and hornbeam forests of vegetation alliance *Querco-Carpinion illyrico-moesiacum* (Budak, 1999). Within the territory of Serbia, it also appears at a range of altitudes of 84–600 m above sea level (Panjković et al., 2003; Bogosavljević and Zlatković, 2018).

According to IUCN classification, *E. hyemalis* is a regionally endangered taxon (EN) in Italy and rare (R) in Slovenia, Croatia, and Hungary (Budak, 1999). Until recently, this species has been considered extinct (EX) in Bosnia and Herzegovina, but now it is placed into the category of critically endangered (CR) because four new populations have been discovered (Brujić et al., 2006). *Red* Data *Book* of the *Republic of Bulgaria* includes the taxon *E. bulgarica* (Stef.) Stef., also considered a critically endangered (CR) taxon in Serbia and Bulgaria (Vladimirov, 2015), even though modern floristic literature is treating it either as a variety or a synonym of *E. hyemalis* (Budak, 1999; The Plant List, 2013). Although the recent floristic studies (Petrović and Lakušić, 2017; Bogosavljević and Zlatković, 2018) have shown the presence of several new populations in Serbia, at the regional level this species is still categorized as a critically endangered taxon (CR) (Bogosavljević and Zlatković, 2018).

The threat status of winter aconite is additionally influenced by pathogenic microorganisms, especially microfungi, causing diseases in the host. Therefore, the detection of disease symptoms in natural populations, identification of pathogens, assessment of the degree of pathogen spread, and monitoring of current situation are highly important for the conservation of this species. Some studies have shown that *E. hyemalis* is a host species for pathogenic smut fungus *Urocystis eranthidis* (Pass.) Ainsw. and Sampson, from the class Ustilaginomycetes (Woods et al., 2018) and *Leucotelium cerasi*, rust fungus belonging to the class Pucciniomycetes (Kruse et al., 2015).

L. cerasi, the causative agent of the cherry disease, is a biotrophic plant pathogen. *L. cerasi* is heteroecious. Its life cycle is heteromacrocyclic (0, I, II, III). Corresponding to the ploidy level of the fungus, *E. hyemalis* is the host for the haploid (spermogonium – 0) and dikaryotic stage of the pathogen (aecium – I), while dikaryotic uredium (II) and telium (III) form on hosts of *Prunus* spp. (Riegler-Hager, 2007; Kruse et al., 2015). Host alternation in *L. cerasi* was proven *in vitro* by Tranzschel (1935).

The presence of this pathogenic fungus species in other European countries and other host species was determined according to Helfer (2005), Beenken & Senn-Irlet (2016), and the Fungal Database (Farr and Rossman, 2018). Genus *Leucotelium* Tranzschel consists of three species with almost cosmopolitan ranges: *L. cerasi*, *L. padi* Tranzschel, and *L. pruni-persicae* (Hori) Tranzschel (Kruseet et al., 2015).

According to the *Fungal Database* (Farr and Rossman, 2018), *L. cerasi* was recorded in 7 species of the genus *Prunus* in 9 European countries: *P. avium* (L.)

L. (Bulgaria, Finland), *P. cerasus* L. (Austria, Finland, Hungary, Italy, Slovenia, Spain), *P. domestica* L. (Austria, Finland, Germany), *P. persica* (L.) Batsch (Italy, Portugal), *P. pumila* var. *depressa* (Pursh) Bean (Austria), *P. spinosa* L. (Italy, Finland), and *P. tenella* Batsch (Austria). According to Helfer (2005), this fungus was also recorded in the following species of this genus: *P. armeniaca* L., *P. cerasifera* Ehrh., *P. dulcis* (Mill.) D.A.Webb, *P. fruticosa* Pall., *P. padus* L., *P. virginiana* L., as well as in 7 additional European countries (Bosnia and Herzegovina, Croatia, Czech Republic, Moldova, North Macedonia, Slovakia, and Ukraine). *Eranthis hyemalis* was recorded as a host plant in Austria, Bulgaria (as *E. bulgarica*), and Italy (Farr and Rossman, 2018), while for Switzerland it was cited by Beenken and Senn-Irlet (2016). Also, according to Tranzschel (1935), this fungus appears in *P. cerasus* in the following European countries: France, Hungary, Italy, Switzerland, and Yugoslavia. The records for the territory of Yugoslavia were from the area of Ljubljana in Slovenia and the area of Valjevo in Serbia (Tranzschel, 1935). However, as the cited record for Serbia is more than 80 years old and as this fungus was not recorded in *E. hyemalis* as a host, we believe that our data are new, important records on the appearance of this fungus in Serbia in the host plant *E. hyemalis*. Altogether, *L. cerasi* fungus has been recorded in 19 countries of Europe, including Serbia, appearing in 13 species from the genus *Prunus*, as well as in species *E. hyemalis* (Figure 1).

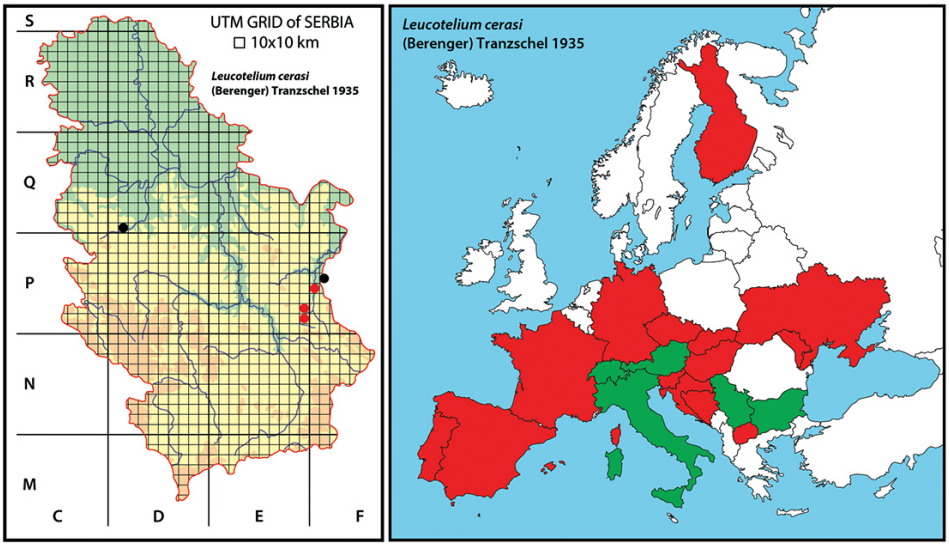

Figure 1. Distribution of *L. cerasi* in Serbia and Bulgaria (left) and in the European countries (right) [compiled according to Tranzschel (1935), Helfer (2005), Beenken and Senn-Irlet (2016), and Farr and Rossman (2018)]. Legend (left): black dots – literature data, red dots – new data; legend (right): red color – present in *Prunus* spp. as a host, green color – present in *Prunus* spp. and *E. hyemalis* as hosts.

MATERIALS AND METHODS

Field studies

Field studies were performed in the period 2017–2020, during the winter and spring seasons of the year. Symptoms of leaf rust were recorded in all natural populations at four localities in Eastern Serbia near Knjaževac (Podvis, Golemi Kamen, and Ploča) and Zaječar (Zmijanac). During the field studies, at least 5 leaf samples of *E. hyemalis* with rust symptoms from different infected individuals were collected, placed in bags, and transported to the Laboratory of the Department of Algology, Mycology and Lichenology at the Faculty of Biology, Belgrade University, for further microscopic analyses and pathogen identification.

New chorological data

Specimen examined and pathogen identified:

Serbia: Surroundings of Knjaževac, Orešac village, Ploča, *Carpino orientalis-Quercetum mixtum*, 600 m, EP91 (Ranđelović, M. and Bogosavljević, S., 10.04.2018. – host plant deposited in BEOU as voucher no.17690); (Ranđelović, M. and Bogosavljević, S., 13.03.2018, field obs.); (Ranđelović, M. and Bogosavljević, S., 21.03.2019, field obs.); (Ranđelović, M. and Bogosavljević, S., 05.03. 2020, field obs.).

Surroundings of Knjaževac, Orešac village, Golemi Kamen, thermophilous oak forests, 365 m, EP92 (Ranđelović, M. and Bogosavljević, S., 10.04.2018. – host plant deposited in BEOU as voucher no.17691); (Ranđelović, M. and Bogosavljević, S., 05.02.2018, field obs.); (Ranđelović, M. and Bogosavljević, S., 27.03.2019, field obs.); (Ranđelović, M. and Bogosavljević, S., 01.03.2020, field obs.).

Surroundings of Zaječar, Vratarnica village, Zmijanac (Provalina), oak forests, 235 m, FP04 (Ranđelović, M. and Bogosavljević, S., 22.04.2018. – host plant deposited in BEOU as voucher no.17692); (Ranđelović, M. and Bogosavljević, S., 24.04.2017, field obs.); (Ranđelović, M. and Bogosavljević, S., 27.03.2019, field obs.); (Zlatković, B. and Bogosavljević, S., 11.03.2020, field obs.).

Surroundings of Knjaževac, Podvis village, 330 m, EP92 (Ranđelović, M. and Bogosavljević, S., 25.04.2018. – host plant deposited in BEOU as voucher no. 17693); (Ranđelović, M. and Bogosavljević, S., 02.03.2017, field obs.); (Ranđelović, M. and Bogosavljević, S., 11.04.2017, field obs.); (Ranđelović, M. and Bogosavljević, S., 05.02.2018, field obs.); (Ranđelović, M. and Bogosavljević, S., 27.03.2019, field obs.); (Ranđelović, M. and Bogosavljević, S., 28.03.2020, field obs.).

Microscopic analyses

Pustules, representing the rust symptoms on leaf surfaces, were sampled from all investigated localities and gently removed using a sterile needle, mounted with lactophenol cotton blue and observed under a light microscope (Nikon Eclipse E200, equipped with camera Bresser MikroCam PRO HDMI). To analyze rust structures in *E. hyemalis* leaves via scanning electron microscope (SEM), sample leaf fragments with visible symptoms were separated using adhesive carbon tape on aluminum cylinders. SEM analyses were performed at the University of Belgrade, Faculty of Mining and Geology, using a JEOLJSM–6610LVSEM (Tokyo, Japan). Samples were gold-coated ($d = 15$ nm, $\rho = 19.2$ g/cm^3) by using a Leica EMSCD005 sputter coater (Wetzlar, Germany). Both light-microscopy and SEM images were analyzed, and identification keys (Helfer, 2005; Riegler-Hager, 2007; Kruseet et al., 2015) were applied to identify pathogens.

Host plant material was preserved and stored in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac" of the Belgrade University (BEOU), with acquisition numbers given in the brackets. The nomenclature and classification of taxa were matched with *The Plant List* database (http://www.theplantlist.org/) and *Species Fungorum* database (http://www.speciesfungorum.org/).

RESULTS AND DISCUSSION

Field studies in Eastern Serbia in 2017 and 2018 revealed the presence of five new populations of a critically endangered plant species *Eranthis hyemalis* (L.) Salisb. (Bogosavljević and Zlatković, 2018). However, symptoms of leaf rust were documented in about a half of all studied specimens of *E. hyemalis* at four out of five new localities (Knjaževac [Podvis, Golemi Kamen, and Ploča] and Zaječar [Zmijanac]). This fungus was not recorded at the locality of Niš, where the plant population is most probably of naturalized origin. Five out of nine (56%) known localities for *E. hyemalis* in Serbia were checked (Niš, Knjaževac [Podvis, Golemi Kamen and Ploča] and Zaječar [Zmijanac]), while symptoms of rust were recorded at four localities (44%).

Infected individuals were light yellow to yellowish-green in color, while their petioles were more elongated than in the healthy individuals. The leaf lobes were also somewhat narrower than in unaffected plants. Similar manifestations in *E. hyemalis* individuals, as a consequence of infection with the same pathogen, were described in papers by Riegler-Hager (2007) and Kruse et al. (2015). The documented symptoms of rust in the host plant were presented in Figure 2.

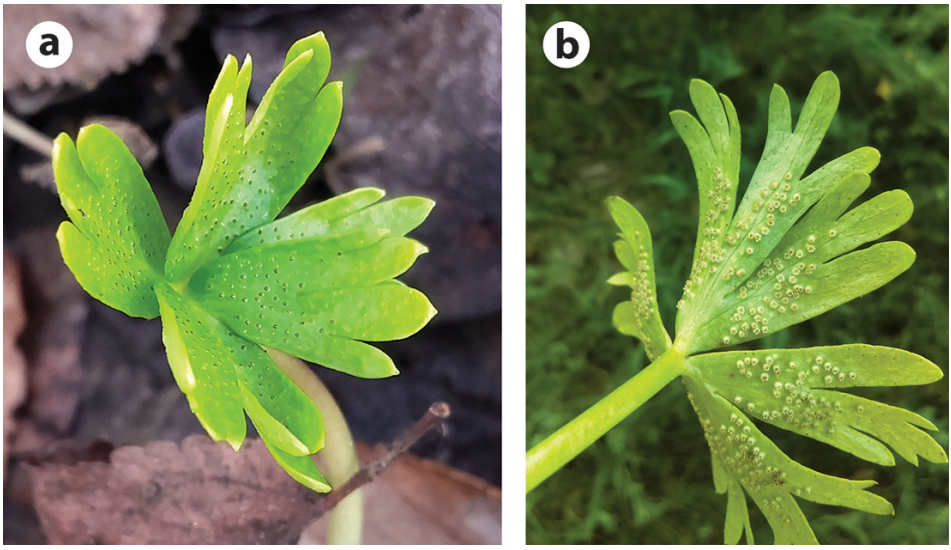

Figure 2. Rust symptoms in *Eranthis hyemalis*; a) Adaxial leaf surface with dark and shiny spermogonia with excudation present in the infected plant from Podvis (photo by S. Bogosavljević, 02.03.2017); b) Abaxial leaf surface with aecia of the infected plant from Podvis (photo by S. Bogosavljević, 25.04.2018).

Microscopic analyses (both light and SEM) performed on *E. hyemalis* leaf samples confirmed the presence of morphological structures of rust fungi (Pucciniomycetes). Volcano-shaped epiphyllous spermogonia (Ø 160 µm, with an ostiole Ø 40 µm) were documented on the adaxial leaf surface (approximate coverage of 10%) (Figure 3a). Due to their subcuticular position and the presence of well-developed peridia and paraphyses, they might be placed into group 6 / type 7 according to spermogonium characterization proposed by Hiratsuka and Cummins (1963). SEM analyses have revealed the presence of numerous rough-walled, ellipsoid, or irregularly shaped spermatia (17.72 × 14.00 – 29.13 × 24.47 µm) in the proximity of spermogonia (Figure 3b, c, d).

Geastrum-shaped aecia (Figure 4 a, b) was documented on the abaxial side of the leaf (approximate coverage of 20%). Pseudoperidia, enclosed in coarse, thick cell-walls, were detached from the aecium body in 4 to 6 irregularly shaped lobes (235.69–354.24 µm). Aecia diameter with lobes ranged from 700 to 1000 µm. Irregularly shaped aecidiospores with thick and verrucose cell walls (Figure 4c) were also documented in tested samples (Ø 12–20 µm). According to Sato and Sato (1985), the documented characteristics of the aecial body and spores indicate that the observed aecia belong to the *Aecidium* type.

According to the spore-bearing structures (spermogonia and aecia) and various types of spores (spermatia and aecidiospores), the pathogen was identified as *Leucotelium cerasi* (Berenger) Tranzschel (Pucciniomycetes, Uropyxidaceae).

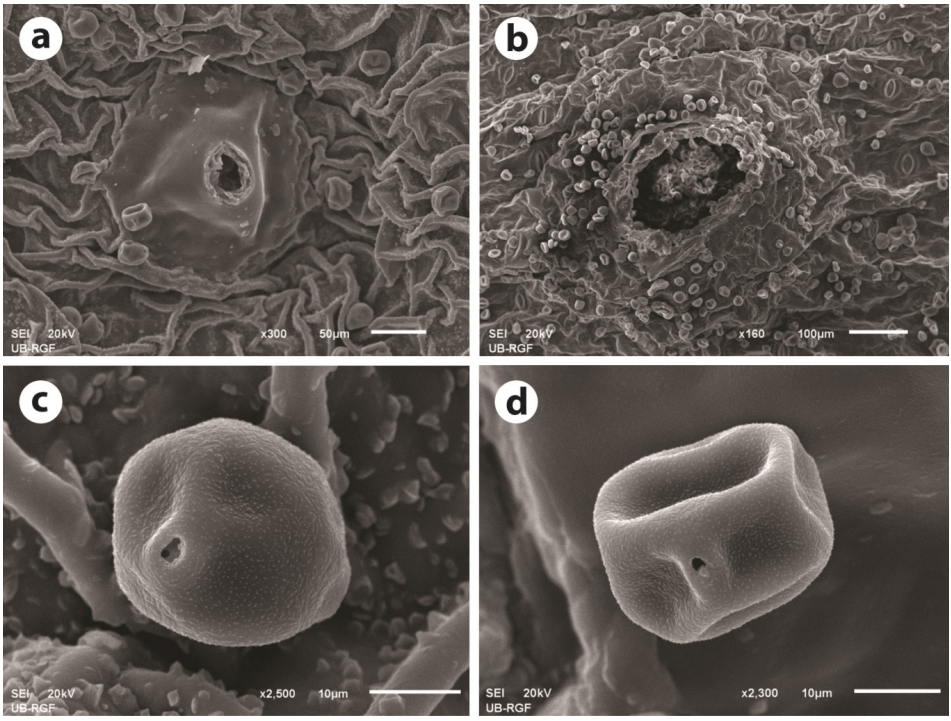

Figure 3. Leucotelium cerasi rust on *Eranthis hyemalis* adaxial leaf surface, SEM: a) Spermogonia; b) Spermatia in mass; c, d) single spermatia.

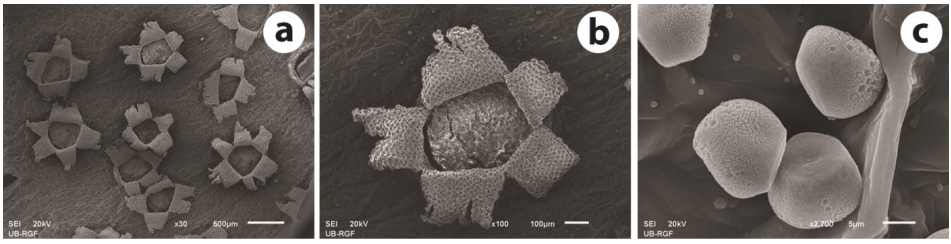

Figure 4. Leucotelium cerasi rust on *Eranthis hyemalis* abaxial leaf surface (SEM) Aecia and aecidiospores: a) Aecia in mass scattered; b) Aecium c) Aecidospores

Leucotelium cerasi (Bérenger) Tranzschel, Riv. Patol. veg. 25: 177 (1935) [syn: *Dicaeoma cerasi* (Bérenger) Kuntze (1898); *Mycogone cerasi* Berenger (1844); *Puccinia cerasi* (Berenger) Cast. (1845); *Sorataea cerasi* (Berenger) Cummins and Y. Hirats. (1983). Gäumann (1959): 799; Majewski (1977): 286; Braun (1982): 230; Poelt (1985): 62; Minkevicius and Ignataviciute (1991): 169].

The presence of *L. cerasi* in *E. hyemalis* (subnom. *E. bulgarica*) in the Balkan Peninsula was first recorded by Denchev (1995) in the western part of Bulgaria at a locality close to the Serbian border. The data in our present study

is the first record of *L. cerasi* in Serbia and the second record on its appearance in the Balkan Peninsula, in populations of *E. hyemalis* at a greater number of localities. Our results show that the appearance of *L. cerasi* in Serbia, due to the number of infected *E. hyemalis* populations and the occupied range, is much more significant than in Bulgaria where its appearance has a local character.

Localities with detected presence of *L. cerasi* fungus are inhabited by several species from the genus *Prunus* (*P. avium*, *P. cerasus*, *P. domestica*, *P. spinosa*, and *P. tenella*), and they are expected hosts for the II and III stages of the life cycle of this fungus. *Leucotelium cerasi* is not lethal for *E. hyemalis* and generally does not appear cyclically each year (Boens, 2015). However, monitoring of *E. hyemalis* populations in Eastern Serbia shown that the pathogen remained present at all localities in continuity for several vegetation seasons (2017–2020). As *L. cerasi* is characterized by perennial mycelium lasting for several seasons, it was concluded that *E. hyemalis* has adapted to this type of fungal infection (Poelt and Zwetko, 1997; Beenken and Senn-Irlet, 2016).

CONCLUSION

Leucotelium cerasi was first recorded in Serbia in its western part during the first half of the 20th century in *Prunus cerasus* as a host. In 2017, it was recorded for the first time in Serbia in *Eranthis hyemalis* as a host, at four localities in Eastern Serbia. This fungus appeared with high frequency in all studied localities for several consecutive vegetation seasons. In Europe, *L. cerasi* was recorded in 20 countries, in 13 species of the *Prunus* genus, as well as in species *E. hyemalis*. The appearance of this fungus in populations of *E. hyemalis* in Serbia recorded in this study is the second record of its appearance on this host species in the Balkan Peninsula. However, the range of this pathogen is probably greater than the available data indicate, and additional studies including molecular examinations are necessary to determine whether it appears on new host species and/or new areas where it was previously absent.

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REFERENCES

Anastasiu P, Negrean G (2009): Neophytes in Romania. In: Rákosy L, Momeu L (ed.), *Neobiota din Romania*, Cluj Napoca: Presa Universitară Clujeană.

Beenken L, Senn-Irlet B (2016): *Neomyceten in der Schweiz. Stand des Wissens und Abschätzung des Schadpotentials der mit Pflanzen assoziierten gebietsfremden Pilze*. Birmensdorf: WSL Berichte, Heft 50.

Boens W (2015): *Eranthis* Update. *Int. Rock Gard.* 62: 19–22.

Bogosavljević S, Zlatković B (2018): Report on the new floristic data from Serbia II. *Biol. Nyss.* 9: 63–75. (DOI: 10.5281/zenodo.2538596).

Brujić J, Stupar V, Milanović Đ, Travar J, Pjanić B (2006): New finding places of winter aconite *Eranthis hyemalis* (L.) Salisb. in Bosnia and Herzegovina. *Bull. Faculty Forest.*, 6: 15–21 (University of Banja Luka).

Budak V (1999): *Eranthis hyemalis* (L.) Salisb. In: V. Stevanović (ed.), *Crvena knjiga flore Srbije. 1, Iščezli i krajnje ugroženi taksoni 1 / The Red Data Book of Flora of Serbia 1*, Ministry of Environment of the Republic of Serbia, Faculty of Biology, University of Belgrade, Instution for Protection of Nature of the Republic of Serbia, Belgrade.

Denchev CM (1995): Bulgarian Uredinales. *Mycotaxon* 55: 405–465.

Farr DF, Rossman AY (2018): Fungal Databases, Systematic Mycology and Microbiology Laboratory, ARS, USDA, 02nd December 2018, Available from: http://nt.ars-grin.gov/fungaldatabases/.

Helfer S (2005): Overview of the rust fungi (Uredinales) occurring on Rosaceae in Europe. *Nova Hedwigia*, 81: 325–370. (DOI: 10.1127/0029-5035/2005/0081-0325).

Hiratsuka Y, Cummins GB (1963): Morphology of the spermogonia of the rust fungi. *Mycologia* 55: 487–507.

Kruse J, Thiel H, Choi YJ, Hanelt D, Jage H, Klenke F, Lutz M, Richter H, Richter U, Kummer V (2015): Bemerkenswerte Funde phytoparasitischer Kleinpilze (5). *Z. Mykol.* 82: 145–191.

Lee CS, Yeau SH, Lee NS (2012): Taxonomic status and genetic variation of Korean endemic plants, *Eranthis byunsanensis* and *Eranthis pungdoensis* (Ranunculaceae) based on nr DNA ITS and cp DNA sequences. *J. Plant Biol.* 55: 165–177. (DOI: 10.1007/s12374-011-9201-8).

Panjković B, Stojšić V, Perić R, Rilak S, Kiš A, Vujasinović A (2015): Mapping the extent of occurrence of winter aconite (*Eranthis hyemalis* (L.) Salisb.) in Special Nature Reserve "Bagremara" (Serbia: Vojvodina). *Balkan Botanical Congress 2015. Book of abstracts Rijeka / Croatia, 14–18. September*, 97.

Petrović I, Lakušić D (2017): Refinding of the critically endangered species *Eranthis hyemalis* (L.) Salisb. in Western and Eastern Serbia. *Bot. Serb.* 41: 79–82. (DOI: 10.5281/zenodo.454889).

Poelt J, Zwetko P (1997): *Die Rostpilze Österreichs 2, revidierte und erweiterte Auflage des Catalogus Florae Austriae, III. Teil, Heft 1*, Uredinales. Biosystematics and Ecology Series, 12. Austrian Academy of Sciences Press.

Riegler-Hager H (2007): Neuere Aufsammlungen von Mikropilzen in Kärnten. 4. Einige bemerkenswerte Rostpilze aus dem Botanischen Garten in Klagenfurt. *Rudolfinum. Jahrbuch des Landesmuseums für Kärnten*, 327–333.

Sato T, Sato S (1985): Morphology of aecia of the rust fungi. Trans. *Br. Mycol. Soc.* 85: 223–238.

The Plant List (2013): Version 1.1.0 2nd February 2020, Available from: http://www.theplantlist.org/.

Tranzschel W (1935): La ruggine del Ciliegio: "*Leucotelium cerasi*" (Béreng.) n. gen. n. comb. ("*Puccinia cerasi*" Cast.) ed il suo stadio ecidiale. *Riv. Pat. Veg.* 25: 177–183.

Vladimirov V (2015): *Eranthis bulgaricus*. In: Peev D. (ed.), *Red Data Book of the Republic of Bulgaria* 1, Plants and Fungi (Electronical Edition). BAS & MoEW, Sofia, 02nd February 2020, Available from: http://e-ecodb.bas.bg/rdb/en/vol1/Erabulga.html.

Woods RG, Chater AO, Smith PA, Stringer RN, Evans DA (2018): Smut and Allied Fungi of Wales. A Guide, Red Data List and Census Catalogue. A.O. Chater, Aberystwyth.

Leucotelium cerasi (Bérenger) Tranzschel 1935, НОВИ ПАТОГЕН ЗА МИКОБИОТУ СРБИЈЕ

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РЕЗИМЕ: Током теренских истраживања строго заштићене биљне врсте *Eranthis hyemalis* (кукурјак) у Србији, примећени су симптоми гљивичне инфекције. Патоген је на основу грађе спермогонија, еција и одговарајућих типова спора детектованих на инфицираним листовима кукурјака, идентификован као *Leucotelium cerasi*, изазивач биљне рђе врсте *E. hyemalis* и врста рода *Prunus*. Иако је први пут забележен у Србији на домаћину *Prunus spinosa* (околина Ваљева, 1935. године), појава овог фитопатогена је регистрована на фолијарном ткиву *E. hyemalis*, на четири локалитета у источној Србији: околина Књажевца (Подвис, Големи камен и Плоча) и Зајечара (Змијанац). Може се рећи да је степен инфицираних биљака у природним популацијама поменуте врсте изузетно велики, при чему се број оболелих у свакој популацији кретао око половине од укупног броја индивидуа. Неопходна су даља истраживања како би се утврдило у којој мери гљивица угрожава опстанак популација и у складу с тим предузети адекватне мере заштите.

КЉУЧНЕ РЕЧИ: биљне рђе, *Eranthis hyemalis,* кукурјак, *Leucotelium cerasi,* патоген, Србија

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EFFECT OF AERATION ON PRODUCTION OF BIOFUNGICIDE USING *Streptomyces hygroscopicus*

ABSTRACT: Apple fruit diseases caused by phytopathogenic species of the genus *Alternaria* have become current in recent years in Serbia as well as in other parts of the world. Due to the fact that pesticides have a number of side effects on the environment and human health, scientists around the world are concerned with finding alternative ways to control this important food. Usage of microorganisms, optimization of the medium and process conditions for their cultivation and production of biofungicides are the actual research direction. The effect of aeration on biofungicide production by *Streptomyces hygroscopicus* was studied in a 3 l stirred tank bioreactor using a previously optimized cultivation medium with glycerol as a carbon source. Aeration rates of 0.5, 1.0, and 1.5 vvm with constant agitation speed of 100 rpm were studied. It was found that the greatest production of antifungal metabolites effective against two *Alternaria alternata* isolates occurred with an aeration of 1.5 vvm and an agitation speed of 100 rpm. Statistical analysis showed that the largest inhibition zone diameters of *A. alternata* KA10 (78 mm) and T1Jg3 (78.67 mm) mycelial growth were achieved under the indicated conditions in 96 h of cultivation.

KEYWORDS: aeration, *Alternaria alternata*, biocontrol, biofungicide, biosinthesis, *Streptomyces hygroscopicus*

INTRODUCTION

Apple fruits are used throughout the year in the diet of babies, children and adults, and their quality and health safety is of great importance. However,

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the presence of various phytopathogenic fungi can impair the quality of this important fruit. *Alternaria* species are known as a group of significant plant pathogens. In addition to cereal crops, *Alternaria* species are also one of the causing agents of apple disease, especially during storage. These phytopathogenic fungi can cause moldy core, fruit spot and dry core rot of the apple fruit, as well as various changes on the apple leaves (Horloch, 2006; Mitrović et al., 2017a). Furthermore, these phytopathogenic fungi are producers of various mycotoxins harmful to human health. Because of all the aforementioned, controlling the presence of this fungus on apple fruit is of great importance. Unfortunately, apple is the food most exposed to synthetic pesticide treatments nowadays.

Application of microorganisms in biological control, in recent years, is a field of interest of many scientists. The reason for this is the overuse of chemicals in plant protection that have a number of adverse effects on the environment and consequently, on human health (Parra et al., 2015; Shi et al., 2018). During the past few decades, several biocontrol agents have been exploited and widely investigated against different post-harvest fungal pathogens (Tadijan et al., 2014). Streptomycetes seem to be a promising tool in biological control of plant diseases and their antifungal activity greatly depends on the medium and condition used for their cultivation (Zheng et al., 2019; Wonglom et al., 2019).

Streptomycetes are among aerobic microorganisms and require oxygen for growth and production of desired metabolites. Different studies have shown that the production of secondary metabolites using streptomycetes in submerged cultivations generally requires a large amount of dissolved oxygen, which is provided by an appropriate agitation and aeration rate (Liang et al. 2008; Martins et al. 2004).

Application of higher agitation speed increases the amount of dissolved oxygen and contributes to better growth of the microorganism producer (Feng et al, 2002). However, cell rupture, slower growth, changes in cellular morphology are just some of the side effects that may result from the application of an agitation speed more or less than optimal (Mitrović et al, 2017b). On the other hand, aeration of 1.5 vvm has been reported in the literature as optimal for the production of secondary metabolites using *S. hygroscopicus* (Yen and Li, 2014).

In the present study, the effect of aeration rate on the production of antifungal metabolite effective against two isolates of *A. alternata* was investigated. Experiments were performed in a 3 l stirred tank bioreactor (Biostat® Aplus, Sartorius AG, Germany) at a constant agitation speed of 100 rpm, by using *S. hygroscopicus*. The aim was to analyze bioprocess parameters of biofungicide production in medium containing glycerol as a carbon source and to examine how an increase in aeration rate affects the production of desired antifungal metabolites.

MATERIALS AND METHODS

Microorganisms

Streptomyces hygroscopicus, isolated from soil sample, was used for the production of antifungal metabolites. This microorganism is stored in the Microbial Culture Collection of the Faculty of Technology Novi Sad, Serbia (GenBank Accession number KT026467) (Mojićević et al., 2017). The bacteria was kept frozen in growth medium containing 20% (w/v) glycerol solution, and propagated twice at 27 °C before use. Growth medium (pH 7.2±0.1) with a composition (g/l): glucose (15.0), soybean meal (10.0), $CaCO_3$ (3.0), NaCl (3.0), $MgSO_4$ (0.5), $(NH_4)_2HPO_4$ (0.5), K_2HPO_4 (1.0), was used for inoculum preparation (Tadijan et al., 2016).

As fungal pathogens, two *Alternaria alternata* isolates marked as KA10 and T1Jg3 were used. Phytopathogenic isolates were obtained from rotten apple fruits, first time during 2012 after four months storage in Ultra Low Oxygen storages in Vojvodina Province, Serbia (localities Kukujevci and Tavankut). The occurrence of these two phytopathogenic species is increasing annually in the apple storage in Serbia. Trials on antagonistic activity were conducted during 2017 and 2018. *Alternaria* isolates were initially grown on Potato Dextrose Agar (PDA: potatoes, infusion from 200 g; dextrose 20 g; agar 20 g; distilled water 1 l) plates for seven days. After seven days, a small amount of mycelium of each isolate was added to flasks containing 50 ml of potato dextrose broth. The flasks were incubated for 48 h on a rotary shaker (150 rpm) at 25 ºC. Before use, culture liquid was filtered through the double layer of sterile cheesecloth.

Bioreactor studies

Biosynthesis of biofungicides effective against *Alternaria* apple pathogens by using *S. hygroscopicus* was carried out in 3 l laboratory stirred tank bioreactor (Biostat® Aplus, Germany) containing 2 l working volume. Three bioprocesses were performed in which the agitation speed was constant, 100 rpm, while the aeration rate was varied: 0.5 vvm, 1 vvm and 1.5 vvm. Bioreactor is equipped with two parallel Rushton turbines and without internal baffles. The ratio of stirrer and vessel diameters (d/Di) is 0.38. In all bioprocesses the medium of the following composition was used (g/l): glycerol, 20.0; $(NH_4)_2SO_4$, 0.25; K_2HPO_4, 1.41; $CaCO_3$, 3.0; NaCl, 3.0 and $MgSO_4$, 0.5. The pH of the medium was adjusted to 7 ± 0.1 (Consort C863, Turnhout, Belgium) prior to sterilization. After sterilization, the bioreactor was cooled to 27 °C and inoculated with 10% v/v of preculture. The temperature was maintained at 27±1 °C during the whole process.

In vitro antagonistic activity assay

In vitro antagonistic activity assay was performed in Petri plates using wells technique (Segy, 1983). In short, two layers of PDA medium were spread in plates. The first layer consisted of 2% PDA medium. After solidification, a new layer composed of 1.2% PDA and filtered fungal culture liquid (35%) was added. Three wells with a diameter of 10 mm represented one treatment. For each treatment, 100 µl of test liquid was added in each well. The treatments included: supernatant (only the liquid phase of the cultivation medium containing antifungal metabolites, without the presence of *S. hygroscopicus*) of *S. hygroscopicus* cultivation medium and sterile distilled water as negative control treatment. After 72 hours of incubation at 27 °C, the diameter (mm) of mycelia growth inhibition zone around wells was measured. The antifungal activity of cell-free culture filtrate was tested in triplicate.

The assessment of antagonistic activity was done after 72 h incubation at 27 °C by measuring diameter of inhibition zones (mm) – zones formed around wells with no visible mycelia growth of *A. alternata* isolates.

Analytical methods

In accordance with the experiment, sampling of the cultivation medium was performed every 12 h of the bioprocess. Sample of cultivation medium was centrifuged at 10,000 rpm for 15 min (Rotina 380 R,Hettich, Germany) and only liquid phase of cultivation medium was used.

Standard method was used to determine phosphorus content (Gales et al., 1966) and total nitrogen content was determined by Kjeldahl method (Herlich, 1990).

Obtained supernatants were filtered through 0.45 µm nylon membrane (Agilent Technologies, Germany) and then analyzed by UHPLC (Thermo Scientific Dionex UltiMate 3,000 series) to determine residual glycerol content. UHPLC instrument was equipped with pump HPG-3200SD/RS, autosampler WPS-3000(T) SL (10-ll injection loop), column ZORBAX NH2 (250 mm 9 4.6 mm, 5 lm) and detector Refracto – Max520. As eluent, acetonitrile (70:30, v/v) was used with flow rate of 1.0 ml/min and elution time of 20 min at column temperature of 30 °C.

Samples (10 ml) were centrifuged at 10,000 g for 10 min at 20 °C. The supernatants were discarded and the cell pellet re-suspended in an equal volume of distilled water and re-centrifuged as above. The biomass was dried at 105 °C overnight and weighed. All determinations were performed in duplicate.

Data analysis

The obtained data were processed by factorial ANOVA using Software *Statistica* 13 (Statistica, 2013). Duncan's multiple range test was used to test significance of differences ($p \leq 0.05$) between mean values of measured diameter of inhibition zones.

RESULT AND DICUSIONS

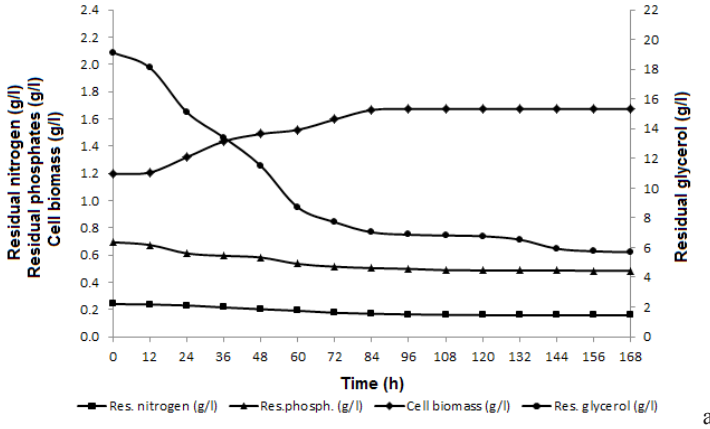
Effect of aeration rate on the substrate consumption and cell growth

In order to monitor the course of the bioprocess, the most important medium nutrients such as carbon, nitrogen and phosphorus and the increase in cell biomass are tested at specified intervals of bioprocess. Figure 1 shows the consumption of the most important nutrients and the increase of biomass during the 7 days of cultivation of *S. hygroscopicus* in the laboratory bioreactor at an agitation speed of 100 rpm and aeration rate of 0.5 vvm (a), 1 vvm (b) and 1.5 vvm (c).

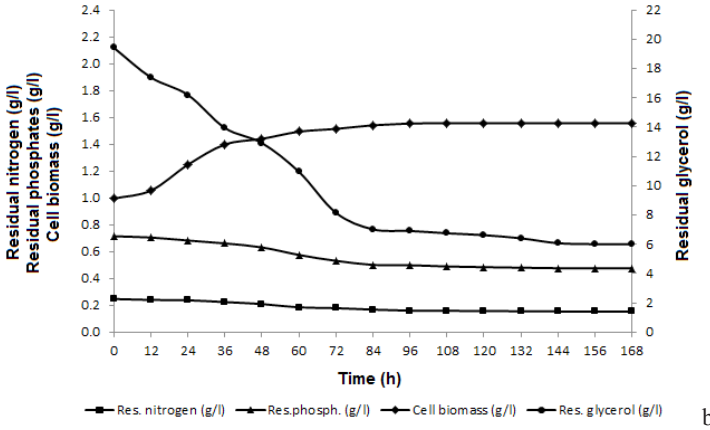
Considering the curve of glycerol, nitrogen and phosphate consumption in Figure 1 (a), it can be concluded that these nutrients are intensively consumed from the first hours of cultivation to 84 h of cultivation, after which the trend of intensive consumption decreases by the end of the bioprocess. The values of residual glycerol, nitrogen and phosphate at 168 h of cultivation were 5.7 g/l, 0.157 g/l, and 0.485 g/l, respectively. This result confirms the fact that carbon, nitrogen and phosphate sources are intensively consumed during the exponential phase and that a decrease in their consumption indicates the beginning of a stationary phase, in which secondary metabolites are produced (Tadijan et al., 2016). The change in the dry weight of the cells content is an indirect indicator of the change in the biomass content of the microorganism producer. From the Figure 1 it can be seen that the amount of dry weight of the cells is practically constant during the first 12 h of cultivation, which is expected since this period includes the lag phase. After 12 h, dry weight of the cells content exponential increase up to 84 h of cultivation. At the end of the bioprocess, the dry weight of the cells content is 1.671 g/l.

Figure 1 (b) shows time-course of substrate consumption and cell growth in a bioreactor with an agitation speed of 100 rpm and aeration rate of 1 vvm. The figure shows that intensive consumption of carbon, nitrogen and phosphorus ends after the third day of cultivation followed by a stationary phase. The residual glycerol, nitrogen, and phosphate values at the end of the bioprocess were 6 g/l, 0.153 g/l, and 0.475 g/l, respectively. In this bioprocess the amount of dry weight of the cells reaches a value of 1.556 g/l by the end of the bioprocess.

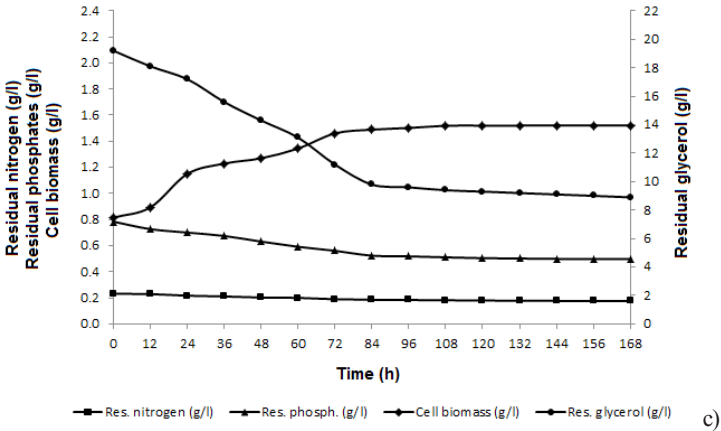
Figure 1 (c) shows the time-course of substrate consumption as well as the increase in biomass over 7 days of cultivation in the bioreactor with an agitation speed of 100 rpm and an aeration rate of 1.5 vvm. The results show that cell biomass intensely increases up to third day after which the growth rate slows down to the end of the bioprocess when it reaches a value of 1,520 g/l. In accordance with the increase in cell biomass, consumption of the most important nutrients is most intense up to 72 h of cultivation. Figure 1 (c) shows intensive consumption of the nutrients during the first three days of cultivation after which the carbon, nitrogen and phosphorus source is consumed by the end of the bioprocess but slower. At the end of the bioprocess the residual values of glycerol, nitrogen and phosphorus were 8.9 g/l, 0.175 g/l and 0.498 g/l, respectively.



a)

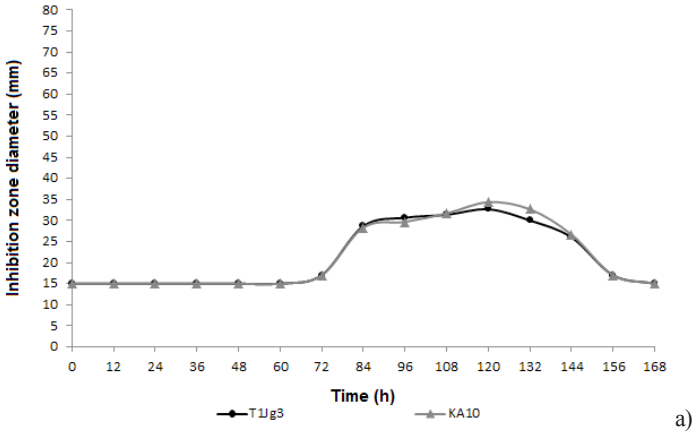


b)

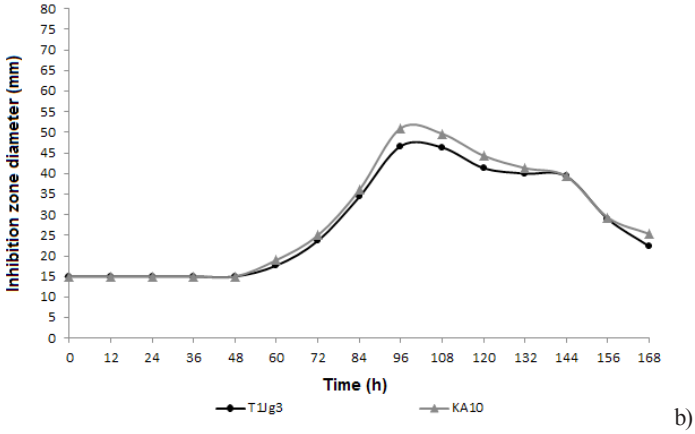


c)

Figure 1. Time-course of substrate consumption and cell growth during 7 days of *S. hygroscopicus* cultivation in bioreactor with agitation speed of 100 rpm and aeration rate of 0.5 vvm (a), 1 vvm (b) and 1.5 vvm (c)



a)



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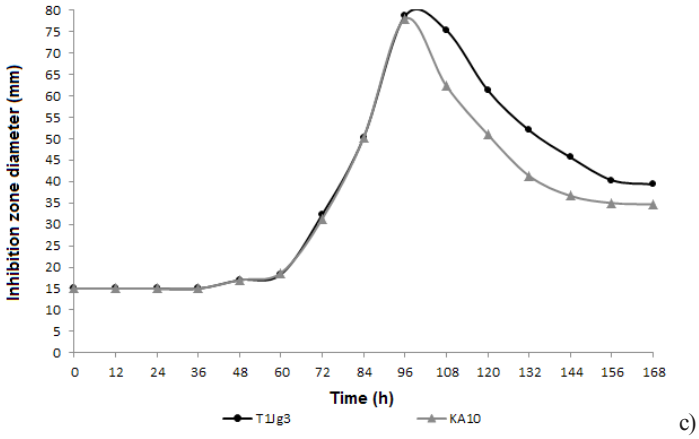

c)

Figure 2. Mean values inhibition zone diameter on *A. alternata* KA10 and T1Jg3 isolate obtained by the action of the *S. hygroscopicus* supernatant in a bioreactor with agitation rate of 100 rpm and aeration rate: a) 0.5 l/l/min b) 1 l/l/min c) 1.5 l/l/min

By analyzing the results it can be concluded that increasing the aeration rate leads to a shortening of the exponential phase and an early stationary phase, where the production of the desired biofungicides occurs (Gottschalk et al., 2003).

Effect of aeration rate on production of biofungicide

The effect of the *S. hygroscopicus* supernatant on two *A. alternata* storage apple pathogens (T1Jg3 and KA10) was tested every 12 h of cultivation by well-diffusion technique. The results of the experiments are shown in Figure 2.

Figure 2 (a) shows the production of biofungicide during the 7 days of cultivation in a bioreactor with an agitation speed of 100 rpm and aeration rate of 0.5 vvm using *S. hygroscopicus*. It can be seen that the maximum production of antifungal metabolites occurs in 120 h of cultivation whereby the largest inhibition zones diameter of *A. alternata* KA10 (34.33 mm) and T1Jg3 (32.67 mm) mycelia growth were formed.

The production of antifungal metabolites effective on *A. alternate* isolates in a laboratory bioreactor with an agitation speed of 100 rpm and an aeration rate of 1 vvm is shown in Figure 2 (b). The stationary growth phase begins during the third day of cultivation (Figure 1 (b)), while the maximum activity of the produced antifungal metabolites is registered in 96 h of cultivation, when the maximum inhibition zones diameters of isolate *A. alternata* KA10 (51 mm) and T1Jg3 (46.67 mm) were formed.

Figure 2 (c) shows the activity of the produced antifungal metabolites on test phytopathogenic isolates during 7 days of bioprocess in a laboratory bioreactor with an agitation speed of 100 rpm and an aeration rate of 1.5 vvm. Isolates of *A. alternata* species are most sensitive to the antifungal metabolites produced in 96 h of cultivation, forming inhibition zone diameters for *A. alternata* T1jg3 and KA10 of 78.67 mm and 78 mm, respectively.

From the presented results, it can be concluded that increasing the aeration rate from 0.5 vvm to 1.5 vvm at the same agitation speed of 100 rpm results in a significant increase in the production of the desired antifungal metabolites (Sousa et al. 2002; Yen and Li, 2014).

Table 1. Mean values of inibition zone diameter and significance of differences at 5% level probability

Conditions	Test phytopathogenic fungi	Mean value of inhibition zone diameter
100 rpm, 0.5 vvm	T1Jg3	32.67 [a]
100 rpm, 0.5 vvm	KA10	34.33 [a]
100 rpm, 1 vvm	T1Jg3	46.67 [b]
100 rpm, 1 vvm	KA10	51.00 [c]
100 rpm, 1.5 vvm	KA10	78.00 [d]
100 rpm, 1.5 vvm	T1Jg3	78.67 [d]

Values followed by the same letter are at the same level of significance

Statistical analysis of obtained results showed that application of different aeration rate had statistically significant ($p<0.01$) influence on mycelia growth inhibition zone diameter (mm) for both test fungi. By analyzing the results presented in the Table 1, it can be seen that the best inhibition of *A. alternata* KA10 and T1Jg3 mycelia growth is formed on a defined medium at agitation speed of 100 rpm and aeration rate of 1.5 vvm by *S. hygroscopicus* (lowercase letters d). Also, by increasing the aeration rate, the stationary phase occurs earlier and the maximum production of the desired metabolites occurs 24 h earlier than in the bioprocess with lower aeration rate (0.5 vvm) (Techapun et al., 2003).

CONCLUSION

The present study showed that aeration is a very important process parameter that is responsible for oxygen transfer, growth, and production of antifungal metabolites in the laboratory bioreactor using *S. hygroscopicus*. The results showed that the application of higher aeration rate contributes to an increase in the production of the desired biofungicides effective against two *A. alternata* isolates (inhibition zone diameters about 78 mm), while reducing their production time. This fact contributes to the cost-effectiveness of the bioprocess because it enables the bioprocess to be shortened from seven to four days.

ACKNOWLEDGEMENT

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REFERENCES

Feng Y, Heb Z, Leong Onga S, Hua J, Zhangb Z, Jern Nga W (2002): Optimization of agitation, aeration, and temperature conditions for maximum-mannanase production. *Enzyme Microb. Tech*. 32: 282–289.

Gales ME Jr, Julian EC, Kroner RC (1966): Method of quantitative determination of total phosphorus in water. *J. Am. Water Works Ass.* 58: 1363–1368.

Gottschalk MFL, Nobrega R, Bon PSE (2003): Effect of aeration on lignin peroxidase production by *Streptomyces viridosporus* T7A. *Appl. Biochem. Biotech*. 105–108: 709–807.

Herlich K (1990): *Official Methods of Analysis of the Association of Official Analytical Chemists*, Arlington: Association of Official Analytical Chemists, pp. 758–759. (fifth ed.).

Horloch C (2006): *Management of Alternaria leaf and fruit spot in apples*. Sydney: Horticulture Australia.

Liang J, Xu Z, Liu T, Lin J, Cen P (2008): Effects of cultivation conditions on the production of natamycin with *Streptomyces gilvosporeus* LK-196. *Enzyme Microb. Tech*. 42: 145–150.

Martins RA, Guimaraes LM, Pamboukian CR, Tonso A, Facciotti MCR, Schmidell W (2004): The effect of dissvolved oxygen concentration control on cell growth and antibiotik retamycin production in *Streptomyces olindensis* So20 Fermentations. *Braz. J. Chem. Eng.* 21: 185–192.

Mitrović I, Grahovac J, Dodić J, Dodić S, Grahovac M (2017a): Effect of nitrogen sources on the production of antifungal metabolites by *Streptomyces hygroscopicus*. *Matica Srpska J. Nat. Sci.* 133: 183–191.
Mitrović I, Grahovac J, Dodić J, Grahovac M, Dodić S, Vučurović D, Vlajkov V (2017b): Effect of agitation rate on the production of antifungal metabolites by *Streptomyces hygroscopicus* in a lab-scale bioreactor. *Acta Period. Technol.* 48: 231–242.
Mojićević M, Grahovac J, Petković M, Vučković I, Dodić J, Dodić S, Vojnović S (2017): Production of nigericin and niphimycin by soil isolate *Streptomyces* sp. MS1: Anti-Candida bioassay guided response surface methodology for the optimized culture medium. *FU Phys. Chem. Tech.* 15: 1–16.
Parra JAC, Ramírez-Lepe M, Romero Cortes T (2015): *Biological Control of Phytopathogenic Fungi. Foster City*, USA, OMICS Group eBooks.
Segy I (1983): *Methods of Soil Microbiology*. Moskva: Mir.
Shi L, Nwet TT, Ge B, Zhao W, Liu B, Cui H, Zhang K (2018): Antifungal and plant growth-promoting activities of *Streptomyces roseoflavus* strain NKZ-259. *Biol. Control.* 125: 57–64.
Sousa MFVQ, Lopes CE, Pereira N (2002): Development of a bioprocess for the production of actinomycin-D. *Braz. J. Chem. Eng.* 19: 277–285.
Tadijan I, Grahovac J, Dodić J, Grahovac M, Dodić S (2016): Effect of cultivation time on production of antifungal Metabolite(s) by *Streptomyces hygroscopicus* in Laboratory-Scale Bioreactor. *J. Phytopathol.* 164: 310–317.
Tadijan I, Grahovac J, Dodić J, Grahovac M, Maširević S, Vučurović D, Dodić S (2014): Effect of carbon sources on the production of the biofungicide by *Streptomyces hygroscopicus*. *Acta Univ. Sapientiae: Alimentaria* 7: 54–62.
Techapun C, Poosaran N, Watanabe M, Sasaki K (2003): Optimization of aeration and agitation rates to improve Cellulase-Free Xylanase production by thermotolerant *Streptomyces* sp. Ab 106 and Repeated Fed-Batch Cultivation Using Agricultural Waste. *J. Biosci. Bioeng.* 95: 298–301.
Wonglom P, Suwannarach N, Lumyong S, Itoc S, Matsui K, Sunpapao A (2019): *Streptomyces angustmyceticus* NR8-2 as a potential microorganism for the biological control of leaf spots of *Brassica rapa* subsp. *pekinensis* caused by *Colletotrichum* sp. and *Curvularia lunata*. *Biol. Control.* 138: 104046.
Yen HW, Li YL (2014): The effects of viscosity and aeration rate on rapamycin production in an airlift bioreactor by using *Streptomyces hygroscopicus*. *J. Taiwan Inst. Chem. Eng.* 45: 1149–1153.
Zheng X, Wang J, Chen Z, Zhang H, Wang Z, Zhu Y, Liu B (2019): A S*treptomyces* sp. strain: Isolation, identification, and potential as a biocontrol agent against soilborne diseases of tomato plants. *Biol. Control.* 136: 104004.

УТИЦАЈ АЕРАЦИЈЕ НА ПРОИЗВОДЊУ БИОФУНГИЦИДА ПРИМЕНОМ *Streptomyces hygroscopicus*

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РЕЗИМЕ: Болести јабука изазване фитопатогеним гљивама рода *Alternaria* постале су актуелне последњих година, како у Србији тако и у другим деловима света. Због чињенице да пестициди имају низ нежељених ефеката на животну

средину и здравље људи, научници широм света фокусирани су на проналажење алтернативних начина за контролу ове важне намирнице. Употреба микроорганизама, оптимизација медијума и услова за њихову култивацију и производњу биофунгицида, представљају актуелан правац истраживања. Утицај аерације на производњу биофунгицида помоћу *Streptomices higroscopicus* проучаван је у 3 l биореактору, користећи претходно оптимизовану подлогу са глицеролом као извором угљеника. Извршено је испитивање производње биофунгицида у три биопроцеса изведена при константној брзини мешања од 100 о/мин а при различитим интензитетима аерације од 0,5 l/l/min, 1 l/l/min и 1,5 l/l/min. Утврђено је да се највећа производња биофунгицида ефикасних против два *Alternaria alternata* изолата остварује применом интензитета аерације од 1,5 l/l/min и брзине мешања од 100 o/min. Статистичка анализа је показала да се највећи пречници зона инхибиције раста мицелије изолата *A. alternata* KA10 (78 mm) и T1Jg3 (78.67 mm) остварује при наведеним условима u 96 h култивације.

КЉУЧНЕ РЕЧИ: аерација, *Alternaria alternata*, биофунгициди, биоконтрола, биосинтеза, *Streptomyces hygroscopicus*

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FLAME-WEEDING: IMPACT ON SOYBEAN PLANTS AND SOIL MICROORGANISMS

ABSTRACT: Flame-weeding is a very useful method for weed control, especially in organic production where the use of herbicides is prohibited. With this method heat suppresses weeds in row within a second. Apart from this, heat also affects growing crop plants and surrounding soil. The aim of this paper was to determine the effect of different propane doses, on photosynthetic and polyphenolic (total flavonoids and anthocyanins) pigments in soybean leaves, as well as the number of microorganisms in the soil. Soybean plants exposed to flame showed a different reaction to high temperature stress, which was reflected in different content of analyzed biochemical parameters, but the most responsive were anthocyanins. Actinomycetes turned out to be the most sensitive group of soil microorganisms affected by weed flaming, while fungi were the most tolerant.

KEYWORDS: pigments, microorganisms, flaming, soybean, temperature stress

INTRODUCTION

In the last decade, organic production has increased considerably. The need for successful, fast and efficient weed control methods without use of herbicides became priority. Thermal suppression of weeds with flame is one of non-chemical methods with great potential for use in practice, especially by organic farmers (Rajković et al., 2015). Compared to chemical measures, flaming has its advantages, such as: quick effect on weeds, no residues in plants and soil, no limits in the crop rotation, decrease in weeds resistance, and as opposed to mechanical measures, there is no inhibition of weed germination and no prob-

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lem with application on wet soil. However, there are some challenges that need to be overcome: slow speed (low efficiency), use of fossil fuels, CO_2 emission, fire danger (in windy conditions), influence on soil organisms and selectivity of crops (heat stress to plants).

Oxidative stress is a part of drought, salt or temperature stress. It is accompanied with diffusion limitations through stomata and mesophyll and the alterations in photosynthetic metabolism (Guidi and Calatayud, 2014). The main cellular components susceptible to damage by reactive oxygen species (ROS) that are produced during oxidative stress are lipids (peroxidation of unsaturated fatty acids in membranes), proteins and enzymes (denaturation), carbohydrates and nucleic acids. However, plants possess a wide range of antioxidant defense systems to counteract oxidative damage by ROS consisting of: (i) antioxidant enzymes, i.e. superoxide dismutase (SOD), catalase and different plant peroxidases, which function as ROS scavengers; (ii) non-enzymatic compounds, i.e. ascorbic acid, α-tocopherol, carotenoids, reduced glutathione (GSH), polyphenols, etc. (Blokhina et al., 2003).

The essential part of flaming application is determination of high temperatures effects on microbiological community of the soil. Microorganisms are one of the key factors responsible for maintaining fertility of the soil, availability of nutrients (Alexander, 1967; Diaz-Ravina et al., 1993) and preservation of the soil aggregates (Brussaard and Kooistra, 1993). Soil microorganisms react strongly to any sudden change in their environment, including the temperature. Extremely high temperature (125 °C) in the surface layer of the soil up to the depth of 2 cm causes sterilization of the soil, transformation of organic matter into ash, evaporation of water and degradation of the soil structure (Attivill and Leeper, 1987). Hence, it is necessary to conduct further research on the effects of high temperatures on soil microorganisms, as well as the possibility of revitalization of the soil afterwards.

Since open flame used in flame weeding gets in contact with the cultivated plants, beside weeds, this may cause damage in plants, i.e. oxidative stress and affect soil microorganisms. Thus, the aim of this study was to examine the influence of flaming on oxidative stress level in young soybean plants by monitoring the change in content of photosynthetic (chlorophylls and carotenoids) and polyphenolic (flavonoids and anthocyanins) pigments in soybean leaves, as well as the number of microorganisms in the soil after flaming.

MATERIALS AND METHODS

Weeds suppression using open flame

Soybean plants (cv. Sava) were grown on experimental fields at the Institute of Field and Vegetable Crops on the location Rimski Šančevi, near Novi Sad (45°16'N and 19°51'E at an altitude of about 80 m). The trial was set in complete randomized block design in four replications. Seeds were sown in 10x3 m plots, with four rows, 75 cm distance between rows and at 4 cm depth.

The dominant soil type was calcareous chernozem with the following basic physical and chemical properties: coarse sand 0.80%; fine sand 39.95%; silt 35.76%; clay 27.49%; density 2.57 g/cm^3; pH in H_2O 6.96; content of humus 2.59%; total nitrogen 0.192% N; light-chain phosphorus 8.4 mg P_2O_5/100 g soil; light-chain potassium 27.3 mg K_2O/100 g soil. Flame-weeding machine (Rajković et al., 2011) is modified four row cultivator with two burners placed on both side of each row to suppress weeds in the row of the crop, while between rows, weeds are suppressed mechanically.

Young soybean plants were treated with flame in three growth stages: the first trifoliate or V1, the three trifoliate or V3 and the six trifoliate stage or V6. The treatment comprised of the control dose (without flaming) and eight different propane doses (20, 30, 40, 50, 60, 70, 80 and 100 kg/ha propane), in four replications. Measured temperatures ranged from 33 °C (18 cm distance from the nozzle; 20 kg/ha propane dose) up to 234 °C (4 cm distance and 100 kg/ha propane dose) (Rajković et al., 2020). Higher propane doses induced higher temperatures on all altitudes. Plants were sampled together with their roots (five hours after treatment) to avoid further stress and then transported to laboratory in a portable refrigerator. Soil samples for microbiological analysis were taken between the rows of soybean 1h and 24h after flaming, at the depth of 2 cm.

Plant pigments determination

Photosynthetic pigments, chlorophylls *a* and *b* (Chl*a* and Chl*b*) and carotenoids were determined by Sairam et al. (2003/2004) and the results were expressed in mg/g of fresh weight. The amount of flavonoids was determined by Marckam (1989) and expressed as mg rutin/g dry weight, while anthocyanins were determined using the pH differential method (Shen et al., 2007) and expressed as mg of cyanidin-3-glucoside/g of dried plant material.

Microbiological analysis of soil

Total number of bacteria on soil agar (10^{-6}), number of actinomycetes in synthetic agar (10^{-4}) and the number of fungi in Czapek's agar (10^{-4}) were determined with the method of forming colonies on selective nutritious substrates with three replications (Jarak and Đurić, 2006). The number of the examined microorganisms was recalculated per gram of absolutely dry soil.

Statistical analysis

Values of the biochemical parameters were expressed as means ± standard error of determinations made in triplicates and tested by ANOVA, followed by comparison of the means by Duncan's multiple range test ($P<0.05$). Data were analyzed using Statistica for Windows version 11. Data from microbiological analysis were logarithmized and processed statistically by using the software Statistica 11 and performing Tukey's test.

RESULTS AND DISSCUSION

In our study, different propane doses used in V1 stage did not show statistically significant differences in Chl*b* and carotenoid content compared to control. Yet, significant accumulation of Chl*a* was observed after the application of 80 kg/ha propane dose (Figure 1), probably due to desiccation of the plant tissue caused by high temperature. In V3 stage, similar was noticed with the Chl*b* and carotenoid contents, i.e. different propane doses did not influence the change in pigments content compared to control. Only doses of 50 (for both Chl*a* and Chl*b*) and 60 kg/ha (for Chl*b*) of propane were stimulative to their content (Figure 2). Similar trend in photosynthetic pigments accumulation was also visible in the next monitored stage of development (V6). Only dose of 60 kg/ha of propane induced accumulation of Chl*b*. Apparent accumulation of pigments in mentioned treatments could be due to desiccation of plant tissue. Higher doses of propane induced plant deterioration and decreased pigment contents (Figure 3).

The most important function of leaves is to produce assimilate through photosynthesis. The leaf chlorophyll content is one of the key factors in determining the rate of photosynthesis and dry matter production (Ghosh et al., 2004). The exposure of weeds to extremely high temperatures obtained by propane burning leads to the rupture of cell membranes structure, protein denaturation, protoplast leakage, dehydration and ultimately, the cell death and plant wilting. Some plant species differ greatly in tolerance to flaming where monocotyledon plants show higher tolerance towards heat of the flame (Ascard, 1998). Furthermore, species with protected apical meristem (at the tip of the stem) are more tolerant compared to species with no protection of meristem, erect habitus and thin leaves (Cisneros et al., 2008). Reaction of plants to open flame depends on burner position, stage of the plant development, leaf moisture, etc. (Knezevic et al., 2012). Different plant organs show different susceptibility to high temperatures which is in close correlation with the presence of protective layers such as hairs, waxes, suberized tissues or water content. All above mentioned indicates the possibility of crop and weed regeneration after flaming. Thus, it is very important to treat weeds with flaming in the most sensitive stages of their development. Weeds are more tolerant in later phenophases, and the most sensitive in the phase of cotyledons. According to Tercé-Laforgue et al. (2004), chlorophyll content in different leaf stages of tobacco was one of the main senescence biomarkers and regardless of the mode of N nutrition, a progressive decrease in chlorophyll was noticed from younger to older leaves. Ascard (1995) found the sigmoidal dose-response and speed-response curves imply that propane dose and the ground speed can be adjusted to the required control effect, the weed flora and the developmental stage of the plants. A 95% reduction in susceptible annual weed species, in phase 0–4 true leaves, was achieved at propane doses of 10–20 kg/ha, and the weeds were completely destroyed at 20–50 kg/ha. Considerably higher doses were needed at later developmental stages and for more tolerant species. According to Stepanović (2013), both corn and soybean were able to tolerate up to two flaming applications with propane dose of 45 kg/ha

without any yield reduction. He suggested that for the best results soybean should be flamed at VC and V4-V5. Combination of both flaming and interrow cultivation applied twice during the season is the most effective weed control treatment in soybean (Stepanović, 2013).

As for total flavonoids content, there were no significant differences between treatments and their controls (Figure 4). Only high doses of propane (60–80 kg/ha) in the V1 and V6 phases caused a slight decrease in flavonoids content, probably due to extended cell destruction. High temperatures may cause inactivation and/or denaturation of the cell proteins (enzymes) which might contribute to decrease in flavonoid biosynthesis.

Many elicitors, both biotic and abiotic, enhance anthocyanins biosynthesis and accumulation: different carbon and nitrogen sources, osmotic pressure, plant hormones, bacteria and yeast, UV light, salicylic acid etc. (Deroles, 2008). In this experiment, all propane doses significantly affected anthocyanins accumulation, except for the lowest propane dose (20 kg/ha) (Figure 5). Intensive accumulation of these phenolic pigments with protective role especially occurred with the propane doses of 30, 40 and 50 kg/ha and they were significantly higher compared to the control. That effect was observed in all three stages of the development. Application of very high propane doses (70 and 80 kg/ha) in V1 and V6 stage resulted in thermal destruction of the cells and the content of anthocyanins was very low. Contrary to this, there was no thermal destruction in V3 stage and all seven treatments with propane showed higher or similar anthocyanins content when compared to the control (Figure 5). Obtained data showed that soybean polyphenolic pigments exhibited different metabolic reaction towards heat induced stress, with anthocyanins as the most responsive pigments to applied abiotic stress.

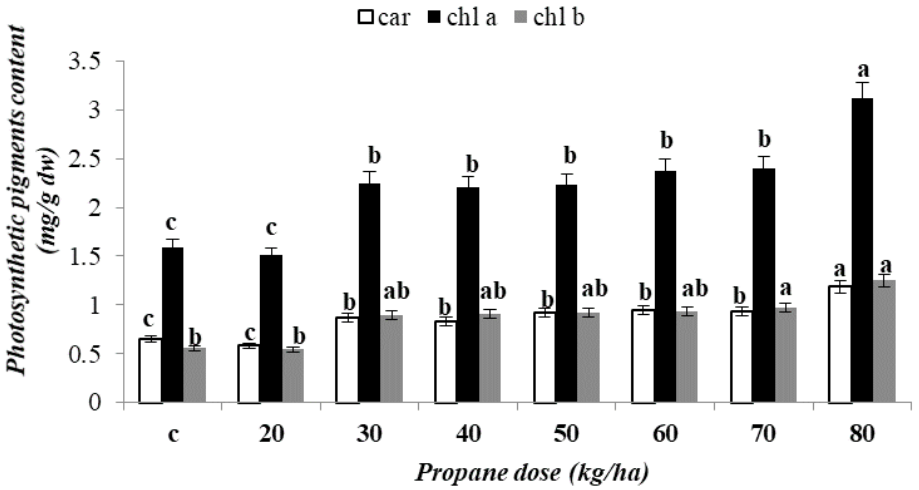

Figure 1. Changes in the photosynthetic pigments content in dependence to propane dose in the stage V1

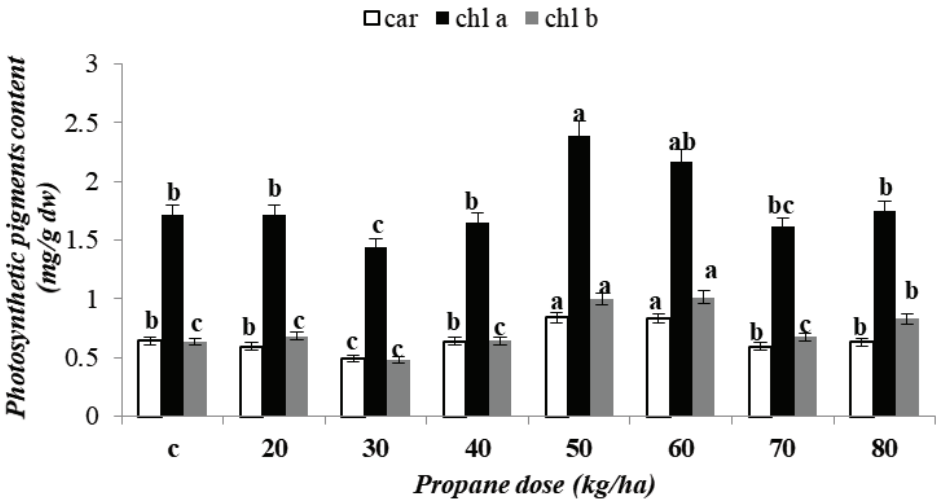

Figure 2. Changes in the photosynthetic pigments content in dependence to propane dose in the stage V3

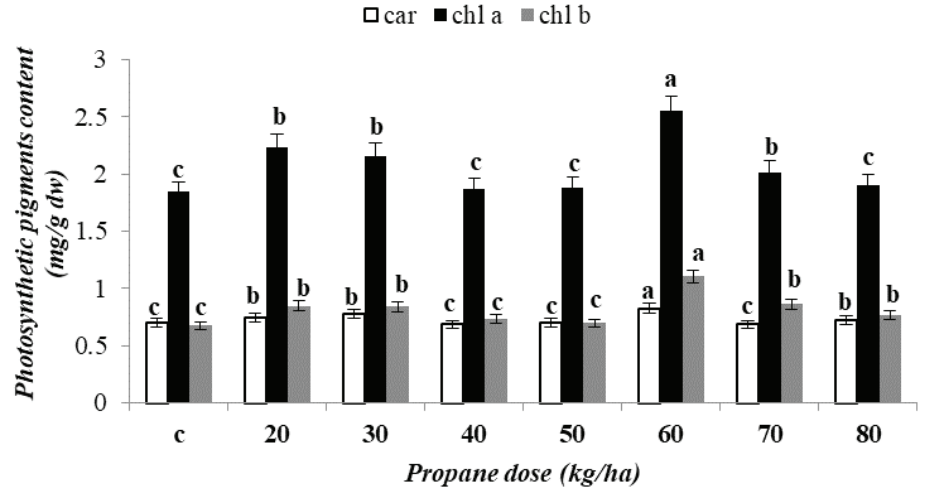

Figure 3. Changes in the photosynthetic pigments content in dependence to propane dose in the stage V6

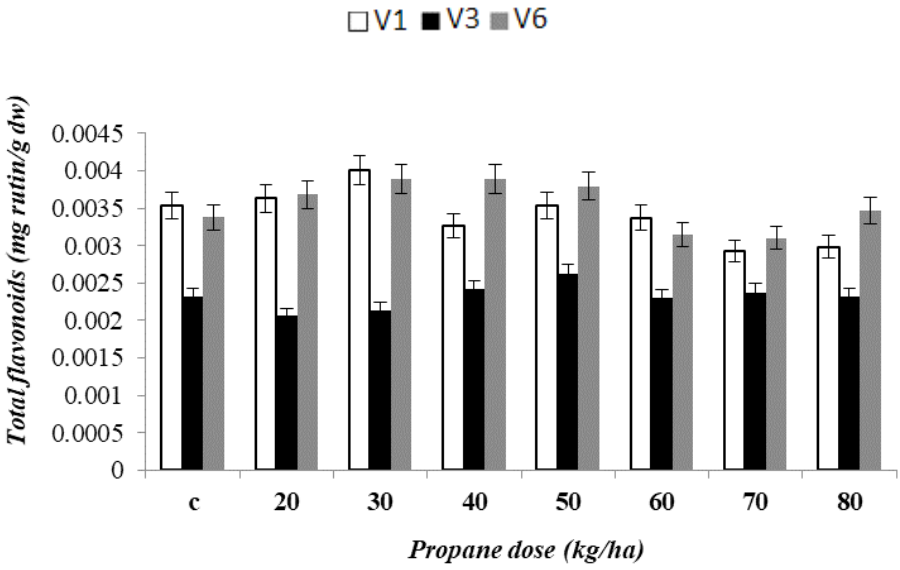

Figure 4. Changes in the total flavonoids content in dependence to propane dose in the stage V1, V3 and V6

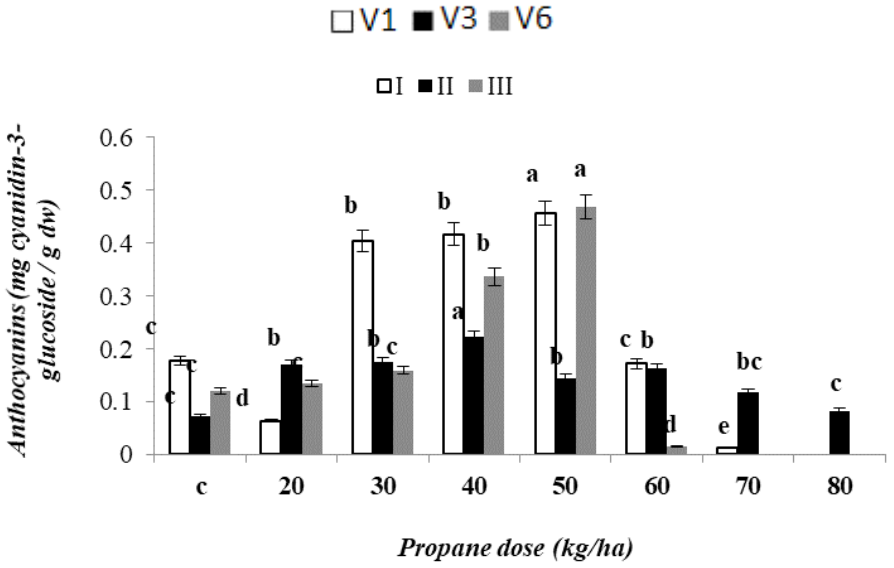

Figure 5. Changes in the anthocyanins content in dependence to propane dose in the stage V1, V3 and V6

The use of different amounts of propane in the soybean crop flaming had affected the total number of bacteria (24h after flaming) and actinomycetes in the soil (24h after flaming), whereas the amount of fungi seemed almost unaffected (Table 1). Bearing in mind the importance of all microbial groups in soil fertility, further research on the effects of high temperatures on soil microorganisms have to include integrative approach with potential soil revitalization methods.

Table 1. The effect of flaming on the total number of bacteria, total number of actinomycetes and total number of fungi (logarithmized number per gram of absolutely dry soil) in the soybean crop 1h and 24h after flame application. a,b,c – different letters indicate significant difference according to Tukey's test of homogeneity of variants.

Variants	Bacteria		Actinomycetes		Fungi	
	1 h	24h	1h	24h	1h	24h
1	6.975 **c**	6.614 **b**	5.607 **a,c**	4.546 **a**	4.915 **a**	4.138 **a**
2	7.256 **b**	7.045 **a,b**	5.333 **b,c**	4.921 **a**	4.818 **a**	4.775 **a**
3	7.203 **b,c**	7.280 **a,b**	5.531 **a,c**	4.746 **a**	5.103 **a**	4.672 **a**
4	7.846 **a**	7.561**a**	5.366 **b,c**	4.694 **a**	4.943 **a**	4.895 **a**
5	7.132 **b,c**	7.228 **a,b**	5.168 **b**	5.062 **a**	5.046 **a**	4.236 **a**
6	7.738 **a**	7.259 **a,b**	5.866 **a**	4.728 **a**	5.184 **a**	4.902 **a**
7	7.666 **a**	6.811 **b**	5.528 **c**	5.110 **a**	5.107 **a**	4.728 **a**
8	7.143 **b,c**	7.149 **a,b**	5.287 **b,c**	4.872 **a**	4.709 **a**	4.872 **a**

CONCLUSIONS

Soybean plants exposed to oxidative stress induced with high temperatures responded by accumulation of anthocyanins almost proportionally with the increase of propane concentrations. Thus, this biochemical parameter could be recommended as stress marker in the field practice due to positive effectiveness/damaging ratio. The use of weed flaming as agrotechnical method in the soybean crop had negative effect on the number of soil actinomycetes, especially 24h after the flaming treatment.

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REFERENCES

Alexander M (1967): *Introduction in Soil microbiology*, New York: Wiley.

Ascard J (1995): Thermal Weed control by Flaming: biological and technical aspects. Swedish University of Agricultural Sciences, Department of Agricultural Engineering, Alnarp. Techical report 200, 1–37.

Ascard J (1998): Flame weeding: Effects of burner angle on weed control and temperature patterns. *Acta Agric. Scand. Section B – Soil Plant Sci.* 48: 248–254.

Attivill PM, Leeper GW (1987): *Forest soil and nutrient cycles*, Melbourne University Press.

Blokhina O, Virolainen E, Fagerstedt KV (2003): Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann. Bot.* 91: 179–194.

Brussaard L, Kooistra MJ (1993): Soil structure and biota interrelationship. *Geoderma* 57: 3–187.

Cisneros J, Zandstra B (2008): Flame weeding effects on several weed species. *Weed Technol.* 22: 290–295.

Deroles S (2008): Anthocyanin biosynthesis in plant cell cultures: A potential source of natural colourants. In: *Anthocyanins*, New York: Springer.

Diaz-Ravina M, Acea MJ, Carballas T (1993): Microbial biomass and its contribution to nutrient concentration in forest soils. *Soil Biol. Biocem*. 25: 25–31.

Ghosh PK, Bandyopadhyay KK, Manna MC, Mandal KG, Misra AK, Hati KM (2004): Comparative effectiveness of cattle manure, poultry manure, phosphocompost and fertilizer-NPK on three cropping systems in vertisols of semi-arid tropics. II. Dry matter yield, nodulation, chlorophyll content and enzyme activity. *Biores. Tech.* 95: 85–93.

Guidi L, Calatayud A (2014): Non-invasive tools to estimate stress-induced changes in photosynthetic performance in plants inhabiting Mediterranean areas. *Environ. Exp. Bot.* 103: 42–52.

Jarak M, Đurić S (2006): *Praktikum iz mikrobiologije*, Faculty of Agriculture, University of Novi Sad.

Knezevic S, Datta A, Bruening C, Gogos G, Stepanovic S, Neilson B, Nedeljkovic D (2012): *Propane-fueled flame weeding in corn, soybean, and sunflower.* University of Nebraska, Lincoln.

Marckam KR (1989): Flavones, flavonols and their glycosides. In: PM Dey, JB Harborne (eds.), *Methods in Plant Biochemistry*. London: Academic Press.

Rajković M, Malidža G, Gvozdenović Đ, Vasić M, Gvozdanović-Varga J (2011): Susceptibility of bean and pepper to flame weeding. *Acta herb.* 19: 67–76.

Rajković M, Malidža G, Vrbničanin S (2015): Flame weeding in soybean, *X Symposium on Plant Protection, 29.11–03.12.2010, Zlatibor*, Book of Abstracts, 81.

Rajković M, Malidža G, Stepanović S, Kostić M, Petrović K, Urošević M, Vrbničanin S (2020): Influence of burners positions on temperature distribution in soybean flaming. *Agronomy* 10: 391.

Sairam RK, Singh DV, Srivastava GC (2003/2004): Changes in activities of antioxidant enzymes in sunflower leaves of different ages. *Biol. Plant* 47: 61–66.

Shen SC, Tseng KC, Chao FT, Wu SB (2007): Color quality of roseliquer. *J. Food Qual.* 10: 202–217.

Stepanović S (2013): *Positioning an innovative flame-weeding technology into crop producton*. Theses, Dissertations, and Student Research in Agronomy and Horticulture 70. Agronomy and Horticulture Department at Digital Commons, University of Nebraska, Lincoln, USA.

Tercé-Laforgue T, Mack G, Hirel B (2004): New insights towards the function of glutamate dehydrogenaserevealed during source-sink transition of tobacco (*Nicotiana tabacum*) plants grown underdifferent nitrogen regimes. *Physiol. Plant* 120: 220–228.

УТИЦАЈ ПРИМЕНЕ ПЛАМЕНА НА УСЕВ СОЈЕ И ЗЕМЉИШНЕ МИКРООРГАНИЗМЕ

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РЕЗИМЕ: Сузбијање корова пламеном је корисна метода за њихову контролу, нарочито у органској производњи где је забрањена употреба синтетичких хербицида. Применом ове методе се, у делу секунде, пламеном сузбијају корови у реду усева. Контакт с пламеном утиче не само на гајену биљку, већ и на земљиште. Циљ овог рада био је утврђивање утицаја примене различитих доза пламена на биљке и земљишне микроорганизме у усеву соје. Мерен је садржај фотосинтетичких и полифенолних (укупних флавоноида и антоцијанина) пигмената у биљкама соје, као и број микроорганизама у земљишту. При сузбијању корова пламеном, биљке соје су показале различиту реакцију на температурни стрес, што се одразило на различит садржај анализираних биохемијских параметара, али највећа промена запажена је у садржају антоцијанина. Најосетљивија група микроорганизама на примену пламена у сузбијању корова су биле актиномицете, док су гљиве биле најтолерантније.

КЉУЧНЕ РЕЧИ: пигменти, микроорганизми, пламен, соја, температурни стрес

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EFFECT OF *Trichoderma harzianum* ON MORPHO-PHYSIOLOGICAL PARAMETERS AND METAL UPTAKE OF TOMATO PLANTS

ABSTRACT: In this study we have investigated the effect of *T. harzianum* on growth, content of chlorophyll and epidermal flavonols and metal distribution in tomato plants. *Trichoderma* strain, isolated from the A horizon (5–30 cm) of agricultural soil used in organic production, was applied near the root in the sixth leaf development phase of tomato. Tomato plants were grown in a growth cabinet up to the stage of 10 leaves. Content of chlorophyll (Chl), epidermal flavonols (Flav) and antocyanins (Ant) were measured *in vivo* non-destructively. The concentration of Cd, Co, Cu, Cr, Fe, Mn, Ni, Se, and Zn was measured in different parts of tomato plants by ICP-OES method. Results have shown that *Trichoderma* application positively affected growth of tomato plants, and significantly decreased nitrogen balance index. In *Trichoderma* treated plants the contents of Cr and Ni were significantly reduced in roots, while content of Cd tended to decrease in all plant parts, significantly in roots. Presented results indicate that investigated isolate is worthwhile testing for plant growth promotion in field conditions, taking in account different supply of macro and micronutrients.

KEYWORDS: ICP-OES, dualex sensor, *Trichoderma*, metals, tomato

INTRODUCTION

Agricultural soils are affected with metal pollution by different means, either with use of sewage sludge compost, mineral fertilizers or recycled water

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(Carbonell et al., 2016). Heavy metals persist in the environment as they are non-degradable, causing crop yield reduction and decreasing quality of crops (Singh et. al., 2011). The increased food demand imposes the need for available productive agricultural land. For tomato cultivars, as one of the most consumed vegetable in the world, it is of great importance to get information on metal content in different plant organs (Ma et al., 2016). As a result of metal toxicity, transfer and bioaccumulation through the food chain, high metal concentrations in soils present a risk for human health. Bio Accumulation Coefficient (BAC) i.e. the ratio of metal concentration in the whole plant to that in soil (Zayed et al., 1998) and the Translocation Factor (TF), the ratio of metal concentration in shoot, to that in roots (Fayiga and Ma, 2006; Zhang et al., 2002), are quantitative parameters of metal distribution in plants. BAC and TF are used to assess metal distribution in plant species. These indexes provide us with information on the translocation and accumulation of examined metals transferred from soil to the edible parts of a plant. Furthermore, they can be used for estimation of possibility to use plants in phytoremediation techniques (Barman et al., 2010; Rodriguez-Iruretagoiena et al., 2015).

Indigenous beneficial microorganisms can decrease soil metal availability to plants and promote plant growth due to positive interaction with roots (Eltlbany et al., 2019).

Fungi that belong to the genus *Trichoderma* are endophytic plant symbionts with beneficial effects influencing plant growth and yield. They colonize plant rhizosphere and through mechanisms of competition, antibiosis and mycoparasitism they are good candidates for the use in agriculture as plant growth promoters and biocontrol agents (Harman et al., 2004).

Flavonoids (e.g., flavones, flavanones, flavanonols, flavanols, isoflavones and lignans) and their derivatives encompass a large group of secondary metabolites specifically induced by symbionts (Hassan and Mathesius, 2012). It is suggested that flavonoids in the root could be involved in the regulation of a temporary defence response in the root triggered by the symbiont invasion.

The non-destructive measurements of epidermal flavonoids based on the fluorescence excitation ratio method (Cerovic et al., 2002), comparing chlorophyll fluorescence induced by ultraviolet (UV) radiation (375 nm) with that induced by red light (650 nm) (Goulas et al., 2004) has been used in many plant species. Positive relationship ($r^2 = 0.97$) between measurements of total flavonoid content from laboratory analysis and amounts detected non-destructively have been confirmed in medicinal plants (Ibrahim and Jaafar, 2012), grapevine (Agati et al., 2008) and white cabbage ($r^2 = 0.93$) (Agati et al., 2016).

It is known that some isolates of *Trichoderma* are known to tolerate higher contents of metals in soil (Tripathi et al., 2013). In this study we have applied *Trichoderma harzianum* SZMC 20660, strain tolerant to increased metal concentrations (Racić, 2017), to investigate its effect on tomato growth, content of epidermal flavonols and chlorophyll in leaves, and metal distribution in roots, stem and leaves of tomato plants.

MATERIAL AND METHODS

Experiment set up

Tomato plants were grown in a growth cabinet at control conditions (LI: 250 µmolm^{-2}s^{-1}, day/night T: 23/17 °C, RH: 60% and PP: 14 h). Experiments started when tomato plants were in the phase of established six leaves and continued until the 10th leaf appeared. The plants were sown in the mixture of soil: peat (1:5 mass ratio) in pots. Ten uniform plants per treatment were used for the experiment.

Fungal suspension

Strain *Trichoderma harzianum* SZMC 20660 (deposited in the Szeged Microbiological Collection (SZMC); Department of Microbiology) was isolated from the A horizon (5–30 cm) of agricultural soil used in organic agriculture. Prior to preparation of fungal suspensions, *T. harzianum* isolate was preincubated at 25 °C in the dark. Suspensions were prepared as follows: pure culture of *T. harzianum* isolate was grabbed from a Petri dish, resuspended in 100 ml of tap water, and shook for 2 h on 50 rpm. Final concentration of *Trichoderma* suspension was 8x10^6 CFU/ml, added in the vicinity of root.

Measurements of plant morpho-physiological parameters

Indices of chlorophyll (Chl), epidermal flavonols (Flav) and antocyanine (Ant) were measured in vivo non-destructively with Dualex sensor (Force-A, France). Plant growth parameters were determined after harvesting the plants at the end of the experiment.

Measurements of metals

The concentrations of heavy metals (Cd, Co, Fe, Cr, Cu, Mn, Ni, Pb Se, and Zn) in the different plant organs were determined by inductively coupled plasma-optima emission spectrometry (ICP/OES system – Thermo iCAP 6500 Duo). Automated system for microwave-assisted digestion (Berghof MSW 3þ) was used for sample preparation, according to the EPA 3052 method. Determination of the metals in soil was performed by EPA 6010 ICP-OES method.

Calculation of metal mobility

To evaluate the mobility of heavy metals from the soil into the plants and the ability to translocate the metals to the harvestable aerial part, the following

factors were used: BioAccumulation Coefficient (BAC, ratio of metal concentration in the whole plant to that in soil) (Zayed et al., 1998) and the Translocation Factor (TF, ratio of metal concentration in shoot, e.g. the aerial part of the plant, to that in roots (Fayiga and Ma, 2006; Zhang et al., 2002).

Statistical analysis

Data are means of at least three replicates from four to ten different plants from each cultivar/treatment combinations. The results were analyzed by Tukey test using GraphPad Prism software.

RESULTS

Plants that were grown in presence of *Trichoderma* had higher fresh weight of leaves (25%) compared to control. However, *Trichoderma* presence did not affect fresh weight of stem and root significantly (Figure 1a). Dry weight of leaves and stem of *Trichoderma* inoculated plants was higher for 40% and 31% respectively, while dry weight of roots was not significantly affected (Figure 1b).

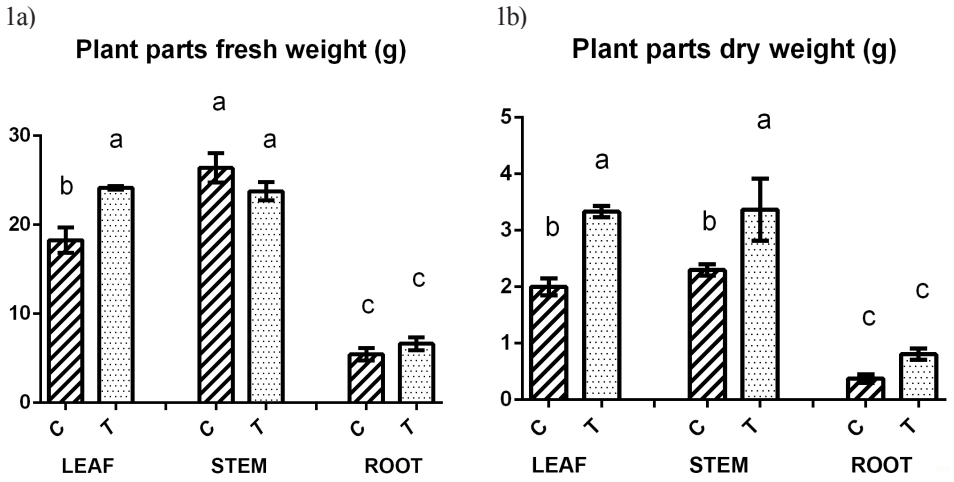

Figure 1. Fresh (a) and dry (b) weight of plant parts in control conditions (C) and after *Trichoderma* application (T). Means with the same letter in the same type of column are not significantly different from each other according to Tukey's test ($p<0.05$). Error lines represent ± standard deviation of the mean.

Both flavonol index and chlorophyll content increased in leaves of *Trichoderma* treated plants, in comparison to the control, but without significant statistical difference. However, the observed decrease of NBI for 16% was statistically significant (Table 1).

Table 1. Mean values of indices of chlorophyll (Chl), epidermal flavonols (Flav), anthocyanin and nitrogen balance index (NBI), as determined on ten plants of examined tomato cultivar per treatment: without (C) or with *T. harzianum* application (T)

	Chl	Flav	Anth	NBI
C	29.17 ±0.80^{c}	0.16 ±0.02^{d}	0.9 ± 0^{e}	188.98 ±26.19^{a}
T	30.52 ±0.95^{c}	0.22 ±0.01^{d}	0.9 ± 0^{e}	158.25 ±13.19^{b}

Means with the same letter in the same column are not significantly different from each other according to Tukey's test ($p<0.05$). Error lines represent ± standard deviation of the mean.

Trichoderma application affected contents of Cr, Ni and Cd in plant parts. Contents of Cr and Ni were lower in roots of tomato plants grown in the presence of *Trichoderma,* however it did not change significantly in other plant parts (Figure 2a and 2b). *Trichoderma* application had significant effect on content of Cd in stem compared to control plants, however it was not changed significantly in leaves and roots (Figure 2c). Contents of Co, Cu, Fe, Se, Zn and Mn were not affected by *Trichoderma* treatment (Table 2).

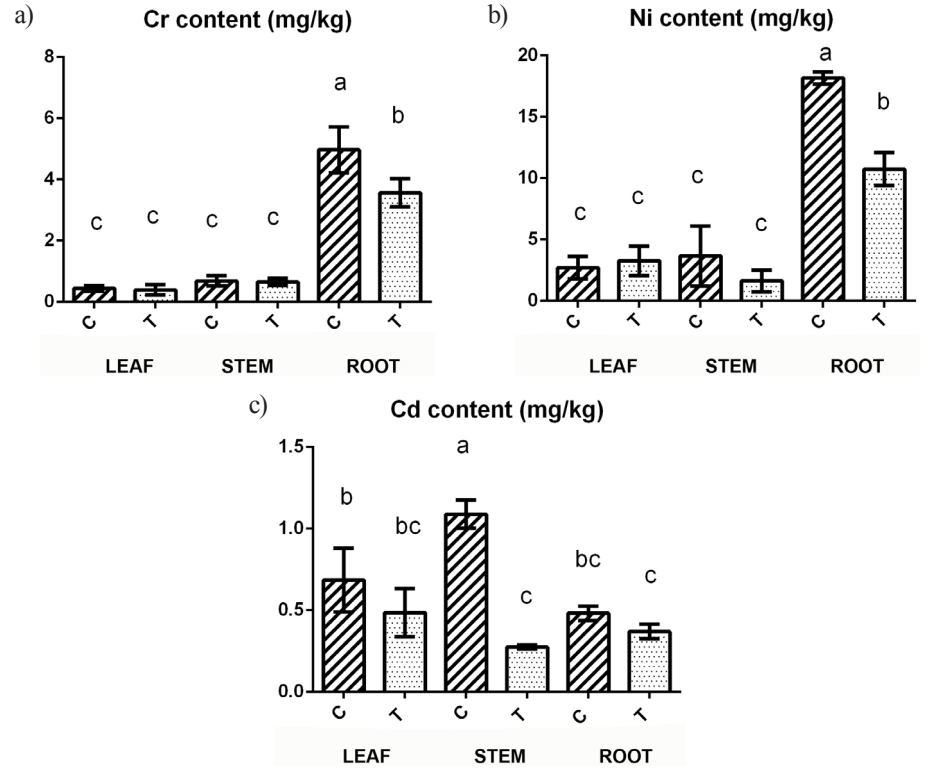

Figure 2a. Chromium, *2b.* nickel and *2c.* cadmium content in different plant organs at different treatments: control plants (C) and plants treated with *Trichoderma* (T). Means with the same letter in the same type of column are not significantly different from each other according to Tukey's test ($p<0.05$). Error lines represent ± standard deviation of the mean.

Table 2. Metal content in different plant organs at different treatments: control plants (C) and plants treated with *Trichoderma* (T)

Metal content/treatment	LEAVES		STEM		ROOTS	
	C	T	C	T	C	T
Co (ppm) ± STDEV	0.14±0.01^{b}	0.16±0.01^{b}	0.18±0.03^{b}	0.18±0.11^{b}	0.94±0.07^{a}	0.89±0.09^{a}
Cu (ppm) ± STDEV	19.15±9.48^{a}	17.77±8.01^{a}	13.54±2.19^{a}	15.41±1.54^{a}	28.44±7.64^{a}	26.33±7.18^{a}
Zn (ppm) ± STDEV	64.80±23.50^{bc}	51.74±10.75^{b}	131.75±1.15^{a}	127.72±37.75^{a}	112.45±20.05^{abc}	81.23±12.8^{abc}
Fe (ppm) ± STDEV	122.30±22.00^{b}	149.45±32.35^{b}	63.20±4.05^{b}	55.65±5.73^{b}	1003.30±61.70^{a}	1023.5±49.5^{a}
Mn (ppm) ± STDEV	51.78±7.09^{a}	55.92±11.24^{a}	17.89±2.97^{b}	16.61±0.06^{b}	41.13±5.11^{a}	44.53±4.28^{a}
Se (ppm) ± STDEV	1.49±1.14^{a}	1.21±0.67^{a}	0.36±0.07^{a}	0.46±0.25^{a}	0.23±0.09^{a}	0.45±0.18^{a}

Means with the same letter in the same column are not significantly different from each other according to Tukey's test ($p<0.05$). Error lines represent ± standard deviation of the mean.

Bioaccumulation and translocation factors were calculated to estimate the uptake and transport of metals from the soil and along different parts of the plant. In control conditions the highest BAC values for Co, Cu, Cr, Fe and Ni were observed in roots, for Mn in leaves and for Cd and Zn in stem (Table 2). *Trichoderma* treatment modulated BAC for Cd and Fe that accumulated mostly in leaves. BAC for Ni in roots has decreased in *Trichoderma* treatment. In control conditions the translocation from root to stem was higher in case of Cd, Co, Cr and Ni, while the translocation to leaves was higher for Cu, Fe and Mn. *Trichoderma* treatment has increased the TL of Co, Cr, Fe, Zn and Ni to leaves (Table 3).

Table 3. Bioaccumulation (BAC) and translocation factors TL for tomato plants grown in control (C) and *Trichoderma* ammended soil (C+T)

metal	tretman	TL/LEAVES	BAC /LEAVES	TL/STEM	BAC/STEM	BAC/ROOTS
Cd	C	1.3969	0.0104	2.2932	0.0167	0.0074
	C+T	1.2831	0.0645	0.7578	0.0408	0.0522
Co	C	0.1500	0.0132	0.2015	0.0176	0.0882
	C+T	0.1834	0.0132	0.2134	0.0154	0.0717
Cu	C	0.6293	0.3698	0.5354	0.2821	0.5642
	C+T	0.6395	0.3821	0.6495	0.3489	0.5759
Cr	C	0.0938	0.0075	0.1466	0.0116	0.0867
	C+T	0.1171	0.0066	0.1810	0.0114	0.0621
Fe	C	0.1210	0.0057	0.0630	0.0029	0.0463
	C+T	0.1448	0.0063	0.0548	0.0023	0.0430
Mn	C	1.2568	0.0643	0.4327	0.0223	0.0511
	C+T	1.2431	0.0577	0.3766	0.0173	0.0461
Ni	C	0.1507	0.0300	0.1974	0.0390	0.1985
	C+T	0.2951	0.0385	0.1432	0.0188	0.1294
Zn	C	0.6336	0.4610	1.2119	0.9254	0.7844
	C+T	0.6746	0.3390	1.5372	0.8134	0.5214

DISCUSSION

Fungi of the genus *Trichoderma* spp. are plant endophytic symbionts possessing beneficial traits such as promotion of plant growth and yield (Harman et al., 2006). It was shown previously that the addition of the *Trichoderma* suspension to the soil has led to an increase growth of the tomato (Azarmi et al., 2011; Khan et al., 2017) and cucumber Yedidia et al. (2001). Tucci et al. (2012) investigated the influence of *Trichoderma* on the root of different tomato genotypes and concluded that the addition of the *Trichoderma* had positive effects on most of the genotypes tested in terms of increasing fresh and dry root mass. The results obtained in this paper are in agreement with the literature data, and show that the addition of *T. harzianum* stimulates growth of tomato plants. However, mechanisms by which *Trichoderma* may influence plant development are still unclear. Yedida et al. (2001) suggested that the improved plant nutritional level may be directly related to a general beneficial growth following *T. harzianum* inoculation.

The content of chlorophylls and epidermal flavonols in the leaves of plants is known to be an important indicator of nitrogen status in a plant (Cartelat et al., 2005). The chlorophyll content is positively correlated with the nitrogen content while content of epidermal flavonols is inversely correlated to nitrogen content (Guller et al., 2016). In our experiment, the higher chlorophyll content in plants grown in the presence of *Trichoderma*, was not statistically significant as compared to control. *Trichoderma* presence significantly affected flavonol content. This is in accordance with the results of Mayo-Prieto et al. (2019). These authors have identified six flavonoids that were significantly increased in bean plants in the presence of *Trichoderma* strain with major stimulation of plant growth. Moreover, the detailed metabolome and transcriptome analysis of Coppola et al. (2019) have shown that *T. harzianum* colonization strongly affects and remodels phenylpropanoid pathway of tomato plants. Our data on decreased NBI in *Trichoderma* treated tomato plants indicates a shift from primary to secondary metabolism and is in accordance with literature.

Metals in soil present in increased concentrations pose a danger to human health and environment. They can be found in soil in two forms: the ionic form or bound to the absorption complex. Plants can absorb them either from an aqueous solution or from a non-specific bound absorption complex, and the degree of absorption depends mainly on its form present in the soil. Vegetable plants have the greatest ability to accumulate heavy metals. For higher plants Cu, Mn, Mo, Ni, Fe and Zn are essential. However, As, Cd, Pb and Sn are only found as trace elements (Alloway, 2013).

Some species belonging to the genus *Trichoderma* are used in the removal of toxic substances from soil (Harman et al., 2004). The greatest variability of *Trichoderma* species is in the soil top horizon, especially in the rhizosphere of different vegetables (Racić et al., 2016). Kredics et al. (2001) have shown that selected strains of these fungi can bind heavy metals and thus make them less toxic to the environment.

In this work we have examined effect of *T. harzianum* on the distribution of different metals in the roots, stem and leaf of tomato plants. Our results on the highest accumulation of Co, Cu, Cr, Fe and Ni in roots, Mn in leaves and Cd and Zn in stem, in control conditions are in accordance with previously published results (Andal and Ching, 2014; Carbonell et al., 2016; Gharabeh et al., 2016; Salem et al., 2006). The results indicated that the presence of this strain significantly reduced the cadmium content in all plant parts, while the chromium and nickel content was significantly lower in the root.

The literature data on the effect of *Trichoderma* inoculation to the plant uptake of nutrients are not consistent. For example, Khan et al. (2017) reported that the concentrations of Cu, Fe, Mn and Zn significantly increased in roots and shoots of tomato plants inoculated with *T. harzianum*. Yedida et al. (2001) also found significant increase in the concentration of mentioned elements in *T. harzianum* – inoculated cucumber roots. In contrast, decreased concentrations of Cu, Mn and Zn were observed in wheat plants grown on a calcareous medium and inoculated with *T. asperellum* (De Santiago et al., 2011). The authors suggest that the decreased concentrations of these elements in plants was due to the competition between plants and *Trichoderma*. Likewise, study on tomato plants grown in hydroponics, with specific nutrient deficiency, indicated that the effect of *T. harzianum* inoculation depended on the deficient element. In case of Fe and Cu-deficiency inoculation was accompanied with the increased uptake of these elements. However, in case of Cu deficiency, the uptake of Cu was suppressed in inoculated plants, due to competition with *Trichoderma*.

CONCLUSION

Based on the results obtained, it is confirmed that the application of *Trichoderma* had a positive effect on the growth and development of tomato plants. Furthermore, nitrogen balance index, when *Trichoderma* applied, influences the transport and redistribution of examined metals in tomato. Most importantly it decreases the uptake of toxic elements (Cd, Ni and Cr) and their content in different tomato parts, suggesting it can reduce heavy metal availability to plants. The results of this paper indicate that *T. harzianum* SZMC 20660 is worthwhile testing for plant growth promotion in field conditions, taking in account different supply of macro and micronutrients.

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REFERENCES

Agati G, Cerovic ZG, Dalla Marta A, Di Stefano V, Pinelli P, Traversi ML, Orlandini S (2008): Optically-assessed preformed flavonoids and susceptibility of grapevine to Plasmopara viticola under different light regimes. *Funct. Plant Biol*. 35: 77–84. (doi:10.1071/FP07178).

Agati G, Tuccio L, Kusznierewicz B, Chmiel T, Bartoszek A, Kowalski A, Grzegorzewska M, et al. (2016): Nondestructive optical sensing of epidermal flavonols and chlorophyll in white head cabbage (*Brassica oleracea* L. var. capitata subvar. alba) grown under different nitrogen regimens. *J. Agric. Food Chem*. 64: 85–94. (doi: 10.1021/acs.jafc.5b04962).

Alloway BJ (2013): Heavy metals and metalloids as micronutrients for plants and animals. In: *Heavy metals in* soils, Dordrecht: Springer, 195–209.

Andal FA, Ching JA (2014): Phytoremediation potential of tomato (Lycopersicon esculentum Mill.) in artificially contaminated soils. In: De La Salle University (DLSU) Research Congress, De La Salle University, Manila, Philippines. Retrieved from: http://www.dlsu.edu.ph/conferences/dlsu_research_congress/2014/_pdf/proceedings/SEE-II-011-FT.pdf.

Azarmi R, Hajieghrari B, Giglou A (2011): Effect of *Trichoderma* isolates on tomato seedling growth response and nutrient uptake. *Afr. J. Biotechnol.* 10: 5850–5855.

Carbonell G, Torrijos M, Rodríguez JA, Ángel Porcel M (2016): Uptake and Metal Transfer from Biosolid-Amended Soil to Tomato (*Solanum Lycopersicum* Mill L.). *Plants. J. Plant Chem. Ecophysiol*. 1: 1002.

Cartelat A, Cerovic ZG, Goulas Y, Meyer S, Lelarge C, Prioul JL, Jeuffroy MH, Gate P, Agati G, Moya I (2005): Optically assessed contents of leaf polyphenolics and chlorophyll as indicators of nitrogen deficiency in wheat (*Triticum aestivum* L.). *Field Crops Res.* 91: 35–49.

Cerovic ZG, Ounis A, Cartelat A, Latouche G, Goulas Y, Meyer S, Moya I (2002): The use of chlorophyll fluorescence excitation spectra for the non-destructive in situ assessment of UV-absorbing compounds in leaves. *Plant Cell Environ*. 25: 1663–1676.

Coppola M, Diretto G, Digilio MC, Woo SL, Giuliano G, Molisso D, Pannacchio Lorito M, Rao R (2019): Transcriptome and metabolome reprogramming in tomato plants by *Trichoderma harzianum* strain T22 primes and enhances defence responses against aphids. *Front. Physiol.* 10: 745.

De Santiago A, Quintero JM, Avilés M, Delgado A (2011): Effect of *Trichoderma asperellum* strain T34 on iron, copper, manganese, and zinc uptake by wheat grown on a calcareous medium. *Plant Soil* 342: 97–104.

Eltlbany N, Baklawa M, Ding GC, Nassal D, Weber N, Kandeler E, Weber N, Kandeler E, Neumann G, Ludewig U, Van Overbeek L, Smalla K (2019): Enhanced tomato plant growth in soil under reduced P supply through microbial inoculants and microbiome shifts. *FEMS Microbiol. Ecol.* 95: 124.

Fayiga AO, Ma LQ (2006): Using phosphate rock to immobilize metals in soils and increase arsenic uptake in Pteris vittata. *Sci. Total Environ.* 359: 17–25.

Gharaibeh MA, Albalasmeh AA, Marschner B, Saleem Y (2016): Cadmium uptake and translocation of tomato in response to simulated irrigation water containing elevated concentrations of cadmium and zinc in clayey soil. *Water Air Soil Pollut*. 227: 133.

Goulas Y, Cerovic ZG, Cartelat A, Moya I (2004): Dualex: A new instrument for field measurements of epidermal ultraviolet absorbance by chlorophyll fluorescence. *Appl. Opt.* 43: 4488–4496. (doi: 10.1364/AO.43.004488)

Guller NS, Pehlivan N, Karaoglu SA, Guzel S, Bozdeveci A (2016): *Trichoderma atroviride* ID20G inoculation ameliorates drought stress-induced damages by improving antioxidant defence in maize seedlings. *Acta Physiol. Plant* 38: 19.

Hassan S, Mathesius U (2012): The role of flavonoids in root-rhizosphere signalling: Opportunities and challenges for improving plant-microbe interactions. *J. Exp. Bot.* 63: 3429–3444.

Harman GE, Lorito M, Lynch JM (2004): Uses of *Trichoderma* spp. to Remediate Soil and Water Pollution. *Adv. Appl. Microbiol*. 56: 313–330.

Harman GE (2006): Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* 96: 190–194.

Ibrahim MH, Jaafar HZE (2012): Dualex 4® reading values as indicator of total flavonoid and chlorophyll content of three varieties of Labisia pumila Benth. (Kacip Fatimah) seedlings under greenhouse condition. *Trans. Malaysian Soc. Plant Physiol*. 20: 102–105.

Khan MY, Haque MM, Molla AH, Rahman MM, Alam MZ (2017): Antioxidant compounds and minerals in tomatoes by Trichoderma-enriched biofertilizer and their relationship with the soil environments. *J. Integr. Agric*. 16: 691–703.
Kredics L, Dóczi I, Antal Z, Manczinger L (2001): Effect of heavy metals on growth and extracellular enzyme activities of mycoparasitic *Trichoderma* strains. *Bull. Environ. Contam. Toxicol*. 66: 249–254.
Ma Y, Oliveira RS, Freitas H, Zhang C (2016): Biochemical and molecular mechanisms of plant-microbe-metal interactions: relevance for phytoremediation. *Front. Plant Sci*. 7: 918.
Mayo-Prieto S, Marra R, Vinale F, Rodríguez González Á, Woo SL, Lorito M, Gutiérrez S, Casquero PA (2019): Effect of *Trichoderma velutinum* and *Rhizoctonia solani* on the Metabolome of Bean Plants (*Phaseolus vulgaris* L.). *Int. J. Mol. Sci*. 20: 549.
Racić G, Körmöczi P, Kredics L, Raičević V, Mutavdžić B, Vrvić MM, Panković D (2016): Effect of the edaphic factors and metal content in soil on the diversity of *Trichoderma* spp. *Environ. Sci. Pollut. Res*. 24: 1–12.
Racić GM (2017): *Variability of indigenous Trichoderma spp. in different soil types and their ecological and biochemical characterization*. University of Belgrade, Faculty of Chemistry (Doctoral dissertation).
Salem NM, Albanna LS, Awwad AM (2006): Toxic heavy metals accumulation in tomato plant (*Solanum lycopersicum*). *ARPN J. Agric. Biol. Sci*. 11: 399–404.
Singh R, Gautam N, Mishra A, Gupta R (2011): Heavy metals and living systems: An overview. *Indian J. Pharmacol*. 43: 246.
Tripathi P, Singh PC, Mishra A, Chauhan PS, Dwivedi S, Bais RT, Tripathi RD (2013): *Trichoderma*: a potential bioremediator for environmental clean up. *Clean Technol. Environ. Policy* 15: 541–550.
Tucci M, Ruocco M, De Masi L, De Palma M, Lorito M (2011): The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. *Mol. Plant Pathol*. 12: 341–354.
Zayed A, Gowthaman S, Terry N (1998): Phytoaccumulation of trace elements by wetland plants: I. Duckweed. *J. Environ. Qual*. 27: 715–721.
Zhang WH, Cai Y, Tu C, Ma QL (2002): Arsenic speciation and distribution in an arsenic hyperaccumulating plant. *Sci. Total Environ*. 300: 167–177.
Yedidia I, Srivastva AK, Kapulnik Y, Chet I (2001): Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant Soil* 235: 235–242.

ЕФЕКАТ *Trichoderma harzianum* НА МОРФО-ФИЗИОЛОШКЕ ПАРАМЕТРЕ И УСВАЈАЊЕ МЕТАЛА КОД БИЉАКА ПАРАДАЈЗА

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РЕЗИМЕ: У овом раду испитан је утицај *T. harzianum* на раст парадајза, садржај хлорофила и епидермалних флавонола, као и расподелу метала у различите делове биљке. Биљке су узгајане у саксијама док нису достигле фазу од 10 листова. Изолат коришћен у експерименту је изолован из А хоризонта (5–30 cm пољопривредног земљишта коришћеном у органској производњи. Садржај хлорофила, епидермалних флавонола и антоцијанина мерен је *in vivo* недеструктивном методом. Садржај метала Cd, Co, Cu, Cr, Fe, Mn, Ni, Se и Zn је у деловима биљака

парадајза измерен ICP-OES методом. Резултати су показали да је примена одабраног изолата довела до смањења индекса баланса азота и да је позитивно утицала на раст биљака парадајза. У биљкама које су третиране такође је примећен и значајно смањен садржај Cr и Ni у корену, а садржај Cd је показао тенденцију смањења у свим биљним деловима, са статистичком значајношћу у корену. Приказани резултати указују на потребу тестирања одабраног изолата у пољским условима, са освртом на различиту доступност микро и макро нутријената.

КЉУЧНЕ РЕЧИ: ICP-OES, dualex сензор, *Trichoderma*, метали, парадајз

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MERCURY POLLUTION OF SEDIMENTS FROM THE RIVER TISA (SERBIA)

ABSTRACT: Mercury (Hg) has been listed as a global high priority pollutant by many international organizations due to its mobility and persistence in the environments and high toxicity to organisms. This research was conducted with the aims to determine: (i) total Hg content (THg) and its spatial distribution in sediments of river Tisa along the river course, (ii) possible sources of THg and (iii) degree of THg pollution in sediments from the river Tisa through different criteria. Total Hg in the sediments ranged from 0.07 to 0.49 mg kg^{-1}, with mean ± S.D. value of 0.26 ± 0.10 mg kg^{-1}. The highest mean value of THg (0.30 mg kg^{-1}) was found in the lower stream, while the lowest (0.13 mg kg^{-1}) was found in the tributary. According to Principal Component Analyses (PCA) strong positive loading of metals in all parts of the river Tisa is mainly controlled from the same sources. These sources are related to anthropogenic activities based on calculated Enrichment Factor (EF) values. Total Hg are higher than background value. According to the Republic of Serbia official standard, THg values of river Tisa sediments were within the range of maximum permissible values. Compared with National Oceanic and Atmospheric Administration (NOAA) guideline, 80.49% of sediment samples indicated that THg in the river Tisa sediments represented minimal and possible risk towards the living organisms. Integrating the results of pollution assessment, it could be concluded that THg in river Tisa sediments in Serbia demonstrates considerable

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contamination according to Geoaccumulation Index (Igeo), and Contaminant Factor (CF), and high pollution risk according to Potential Environmental Risk Index (PERI).
KEYWORDS: mercury, risk assessment, sediments, river Tisa

INTRODUCTION

Mercury (Hg) has been listed as a high priority pollutant by many international organizations due to its persistence in the environments and high toxicity to organisms (Jiang et al., 2006). A global effort to common start in solving this problem has been finalized by the adoption of the Minamata Convention on Mercury (UNEP, 2019). This Convention requires that countries around the world control both new and existing sources of Hg emission and monitor the effectiveness of those controls. The control and management of Hg pollution require both global and regional efforts. Ninkov et al. (2017) studied total Hg content (THg) in agricultural soils from the aspect of soil suitability for the production of healthy and safe food, and its spatial distribution in different parts of Vojvodina province (Serbia). The same authors found the highest THg in alluvial plains of the Danube, Sava and Tisa. In aquatic systems, sediment is usually considered as an ultimate sink of pollutants discharged from land-based sources, such as heavy metals and persistent organic pollutants. Mercury can accumulate in sediments and be released to the surrounding media posing a risk on the living world. In rocks and sedimentary deposits, Hg is characterized by very low natural background concentrations. Thus, an anthropogenic impact can easily be detected by a significant increase of its concentrations. Based on previous research focused on the analysis of background levels of heavy metals in the river Tisa sediments (Štrbac et al., 2018), and in the view of the importance of regional studies of THg, investigation of THg in the river Tisa sediments in this study continued with other aspects of observation. In the present study, a great number of samples were observed, and in addition, a direct analytical method with a lower detection threshold was applied (Nedić et al., 2019). This research was conducted with the aims to determine: (i) THg and its spatial distribution in sediments of river Tisa along the river course, (ii) possible sources of THg and (iii) degree of THg pollution in sediments from the river Tisa through different criteria.

MATERIALS AND METHODS

Study area and sample collection

A total of 41 sediment samples were collected from twenty sites. Thirty-seven samples were taken from river Tisa (13 from upper and 12 each from middle and lower stream). Sampling transect was about 150 km long and stream deposits were collected approximately every 10 km (Figure 1). Four sediment samples were taken from tributaries Begej and Jegrička River and crossed

meander Mrtva Tisa. The sediment samples were taken from depth 0–60 cm depending on the site. The sediment samples were sectioned at 15 cm intervals, taking one to four samples per site. After sampling, the samples were stored in polyethylene bags. The initial quantity of samples was approximately 1.5 kg. The samples were air dried in the laboratory, ground in a mortar and passed through a 0.063 mm mesh in order to achieve consistent physical properties. Sediment samples were collected from September to November 2010. A global positioning system (GPS, Garmin Etrex summit HC, Kansas City, USA) was used to target the different sites.

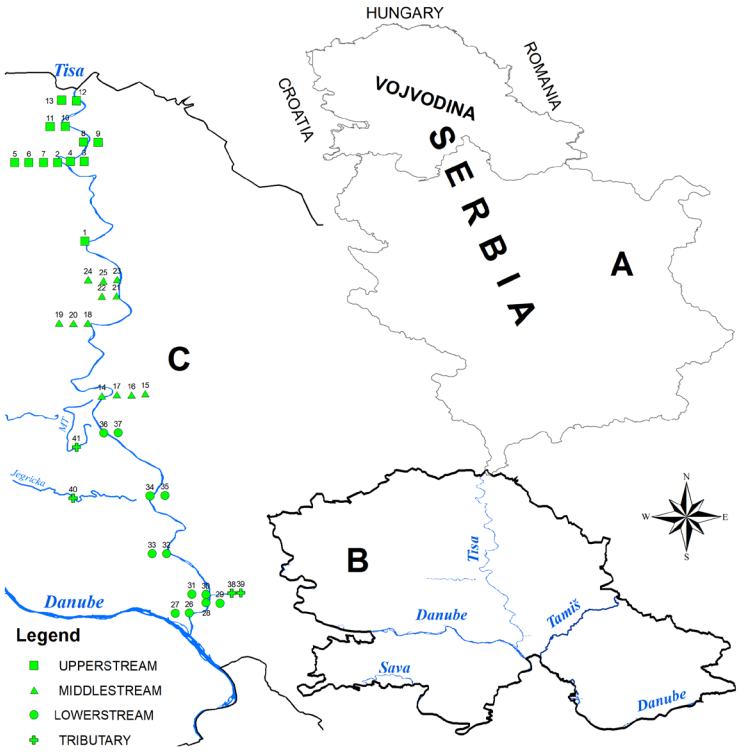


Figure 1. A – Study site; B – Flow of the river Tisa in the Vojvodina province; C – Sites of sediment samples collection.

Laboratory analysis

Particle size distribution was determined in <0.063 mm fraction by the pipette method (Obradović and Vasić 1988). Back-titration with 0.5 N NaOH and phenolphthalein as an indicator was used to determine carbonate content. For THg the samples were analyzed using Direct Mercury Analyzer DMA 80 Milestone as described in Nedić et al. (2019). Three replicates of each sample were used. Quality assurance and quality control (QA/QC) were conducted by

certified reference material BCR 142R (light sandy soil) – EC, JRC, Institute for Reference Materials and Measures. Analysis of BCR samples achieved analytical precision, measured as relative standard deviation, of 0.0025%. The accuracy was within interval 92.84–109.70%, and recovery was 101.11%. The limit of detection (LOD) in our study was 0.0033 mg Hg kg^{-1}. Provided QA/QC procedure verified the validity of applied analytical method.

Assessment of THg contamination in sediment

Background value (Štrbac et al., 2018), national legislation (*Official Gazette of RS* no. 50, 2012), National Oceanic and Atmospheric Administration (NOAA) guideline (Bolaños-Alvarez et al., 2016), and calculation of Enrichment Factor (EF), Geoaccumulation Index (Igeo), Contaminant Factor (CF), and Potential Environmental Risk Index (PERI) were used for the assessment of THg contamination in river Tisa sediments.

According to Ergin et al. (1991) EF is calculated in the following way:

$$EF = (C_{THg}/C_{Al})_{samples} / (C_{THg}/C_{Al})_{background\ area}$$

where $(C_{THg}/C_{Al})_{samples}$ is the ratio of THg (C_{THg}) to the Al concentration (C_{Al})$_{samples}$ in the sediment sample and $(C_{THg}/C_{Al})_{background}$ value is the same ratio in an unpolluted reference sample. The EF classes are: <1 no enrichment; 1–3 minor enrichment; 3–5 moderate enrichment; 5–10 moderately severe enrichment; 10–25 severe enrichment; 25–50 very severe enrichment; >50 extremely severe enrichment (Birch and Olmos, 2008).

The Igeo was used to assess Hg contamination in sediments, and is expressed by Müller (1969) as follows:

$$Igeo = \log_2 C_{THg}/1.5 \times B_n$$

where C_{THg} is the measured THg in the sediment, B_n is the THg geochemical background value of each basin. The constant 1.5 allows us to analyze natural fluctuations in the content of a given substance in the environment and to detect very small anthropogenic influences. Igeo classes are: <0 unpolluted; 0–1 unpolluted to moderately polluted; 1–2 moderately polluted; 2–3 moderately to highly polluted; 3–4 highly polluted; 4–5 highly to very highly polluted; 5–6 very highly polluted (Yaqin et al., 2008).

The PERI was proposed by Hakanson (1980). It has been applied to evaluate the harm of heavy metals in the sediments. The method was described as follows:

1. Contamination Factor (CF) is the ratio obtained by dividing the concentration of metal (C) in the sediment by the background value (Co) (Hakanson, 1980):

$$CF = C / Co$$

According to Hakanson (1980), CF <1 indicates low contamination; 1< CF <3 is moderate contamination, 3< CF <6 is considerable contamination, and CF >6 is very high contamination.

2. The Potential Ecological Risk Index for the single heavy metal pollution (E_i) was described as follows:

$$E_i = T \times CF$$

where T is the toxic-response factor, and is 40 for Hg (Hakanson, 1980), and CF is contamination factor. E_i classes suggested by Håkanson (1980) were: <40 low risk; 40≤ E_i <80 moderate risk; 80≤ E_i <160 considerable risk; 160≤ E_i <320 high risk; ≥320 very high risk.

Statistical analysis

All statistical parameters of descriptive statistics were calculated. The significance of differences in measured parameters between four different part of the river Tisa (upper, middle, lower stream and tributary) was determined using Fisher's LSD test ($p \leq 0.05$). Multivariate analysis performed on the data include Principal Component Analyses (PCA). PCA was used to characterize the concentration of THg in relation to grain size and organic matter (OM) and $CaCO_3$ in the upper, middle and lower streams of the river, as well as the main tributaries and to assess possible contribution sources of THg as well as other heavy metals in sediments of the river Tisa. PCA with varimax normalized rotation was carried out separately for the four different parts of the river Tisa: upper, middle, downstream and tributary to identify the factors influencing each of them. Only factors with an Eigen value greater than 1 were considered significant. Variables with a factor loading greater than 0.7 were interpreted as being meaningfully correlated with the factor. A scatter plot of PCA mean score values was used to graphically display the positions of investigated heavy metals in environmental space. For statistical analysis Statistica version 12 (Dell Inc. 2016) was used.

RESULTS AND DISCUSSION

THg distribution in sediments of the river Tisa along the river course

Total Hg in the river Tisa sediments is shown in Figure 2. Results for the minimum, maximum, mean, and median values, standard deviation, and coefficient of variation of the THg are shown in Table 1.

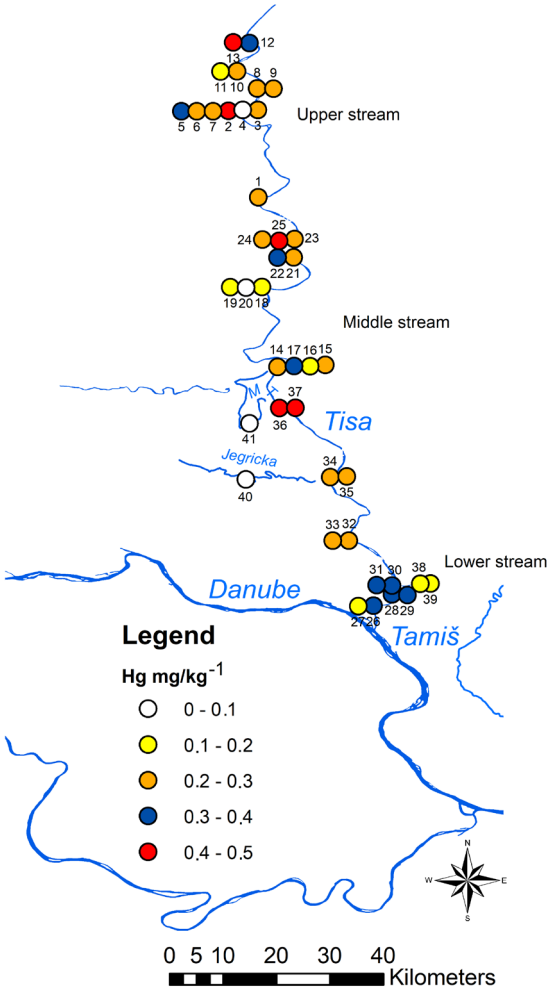


Figure 2. Total Hg (mg kg^{-1}) in the river Tisa sediments along the watercourse

Table 1. Statistical summary of THg concentrations (mg kg^{-1}) in the river Tisa sediment samples

Statistical parameters	Value
Number of samples	41
Minimum value	0.07
Maximum value	0.49
Mean value	0.26
Median value	0.27
Standard deviation (S.D.)	0.10
Coefficient of variation (CV) [%]	39.42

Total Hg in the sediments ranged from 0.07 to 0.49 mg kg^{-1}, with mean ± S.D. values of 0.26 ± 0.10 mg kg^{-1} and median value of 0.27 mg kg^{-1} (Table 1). The lowest concentration (0.07 mg kg^{-1}) was found in the sample 20 in the middle stream, while the highest concentration (0.49 mg kg^{-1}) was found in the sample 37 in the lower stream of the river (Figure 2). The highest mean value of THg (0.30 mg kg^{-1}) was found in the lower stream, while the lowest concentration (0.13 mg kg^{-1}) was found in the tributary. The mean value was obtained based on the measured THg values in the upper, middle and lower stream of the river. Figure 1 shows the distribution of the samples depending on the river flow. Based on THg along the river Tisa streams, tributaries have the lowest content with statistical difference compared to other parts of the river stream. Moreover, the tributaries have the highest percentage of clay and carbonate content (Table 2). Gu et al. (1998) emphasizes that Hg pristine concentrations in igneous and sediment or soil samples vary between 0.08 and 0.4 mg kg^{-1}. According to Ullrich et al. (2001) THg in surface sediments of uncontaminated or less contaminated rivers range from 0.02 to 0.4 mg kg^{-1}, and can be as high as 100 mg kg^{-1} in urban, industrial or mining areas. Total Hg concentrations obtained in this study are lower than Hg contents reported from the river Sava (0.2–0.6 mg kg^{-1}) (Milačič et al., 2010), and the Danube (<0.10–2.37 mg kg^{-1}) (Woitke et al., 2003), and are similar to Hg contents reported from Serbian rivers and artificial lakes (<0.0001–0.72 mg kg^{-1}) (Sakan et al., 2017), and the river Tisa (0.14–0.36 mg kg^{-1}) (Štrbac et al., 2018).

To characterize the THg in relation to grain size, organic matter (OM) and $CaCO_3$ in the upper, middle and lower stream of the river, as well as in the main tributaries PCA was used. PCA defined two groups, which explained 74.22% of the total variation (Figure 3).

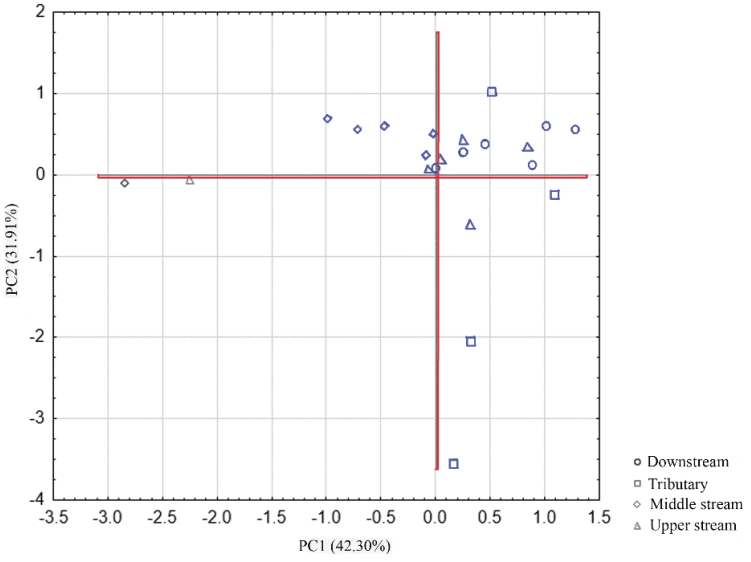


Figure 3. The projection of the cases of the first two components of the PCA

The first principal component explained 42.30% of the variation. It was defined by the concentration of sand, silt and clay. The projection of the cases for the first two components showed that lower stream and tributary could be clearly separated from upper and middle stream (Figure 3). Based on comparison of lower stream and middle stream, in lower stream the significantly higher concentration of silt was found, while in middle stream a significantly higher concentration of sand was found (Table 2).

Table 2. Sediment characteristics of the river Tisa (mean value ± standard error)

	Lower stream	Middle stream	Upper stream	Tributary
THg [mg kg^{-1}]	0.30±0.04 **a**	0.26±0.05 **a**	0.20±0.03 **a**	0.13±0.02 **b**
Sand [%]	14.24±2.96 **b**	44.69±1.83 **a**	27.25±9.29 **ab**	14.31±3.66 **b**
Silt [%]	60.73±1.91 **a**	39.01±0.62 **b**	53.19±1.77 **ab**	52.93±2.44 **ab**
Clay [%]	25.03±2.23 **ab**	16.29±2.02 **b**	19.55±3.15 **b**	32.76±5.62 **a**
$CaCO_3$ [%]	3.59±0.10 **b**	3.78±0.24 **b**	5.13±0.64 **b**	9.12±3.38 **a**
Organic matter (OM) [%]	0.20±0.004 **ns**	0.18±0.006 **ns**	0.19±0.001 **ns**	0.22±0.029 **ns**

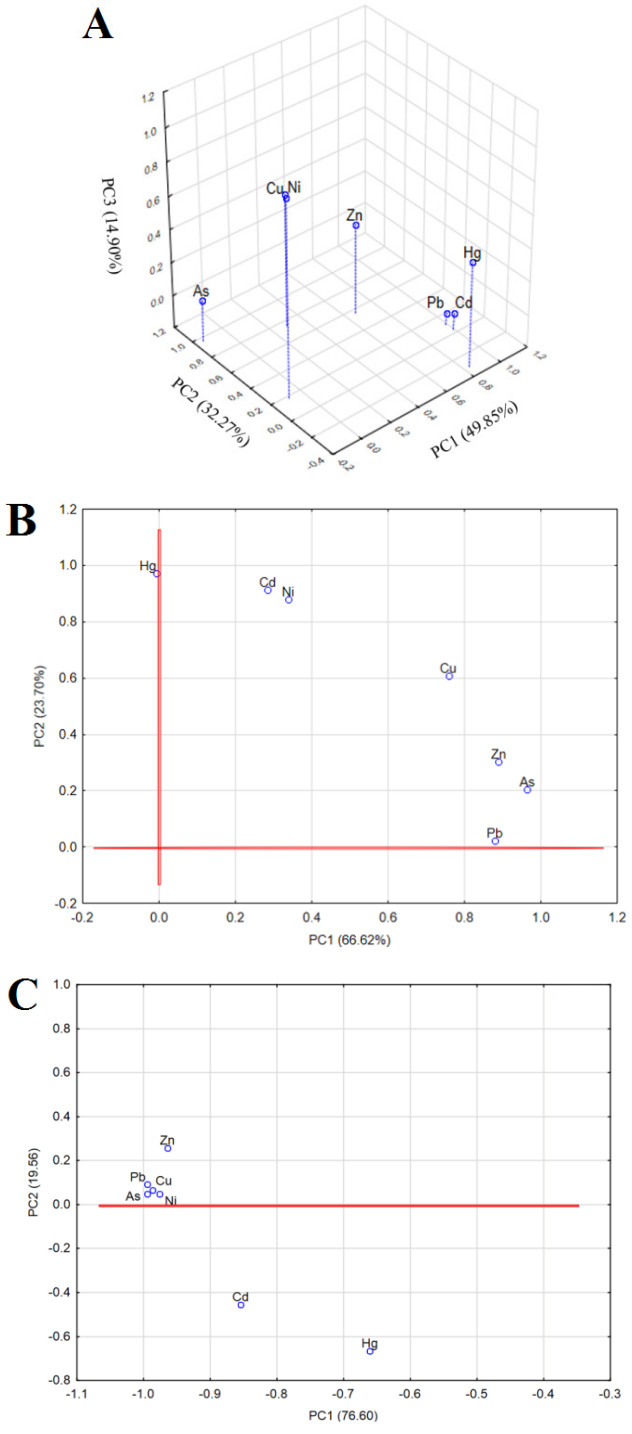
* Different letter indicates that differences between different parts of the river Tisa are significant according to Fisher's LSD test ($p \leq 0.05$), ns – not significant

The second principal component explained 31.91% (Figure 3) of variation due to the variability in $CaCO_3$ and OM and showed clear separation of the middle stream from other investigated parts of the river Tisa. Tributaries have the highest concentrations of clay, $CaCO_3$ and OM. Fisher's test showed no significant differences in OM between different parts of the river Tisa (Table 2). In the river Tisa sediments Hg is likely to be transported primarily by the small particles. Tansel and Rafiuddin (2016) found that heavy metals concentrations were directly correlated with particle size. Fine sediments (<0.106 mm) can accumulate more than 10 times the levels of Hg in comparison to the sediments that are greater than 0.850 mm. The anthropogenic activities in the area of the samples with minimum and maximum values of THg concentration (samples 20 and 37) do not present other likely sources of Hg pollution, variation coefficient CV (39.42 %) points out small heterogeneity of tested sediment samples (Table 1), so the observed increase could be explained by change in the grain size of surface sediments. In the sample 20 percent of clay was 6.67%, and in the sample 37 it was 32.50%. Fine-grained sediments have a higher specific surface area of clay particles than coarser sediments, which can increase the ability of metals to associate (Liu et al., 2017). However, the lowest mean THg was found in tributaries, where the highest percentage of clay was present (Table 2). In addition to the highest percentage of clay, the highest percentage of $CaCO_3$ was also recorded. For remediating pollution of heavy

metals, calcium carbonate-enriched materials (e.g., mussel shells) have recently been introduced (Wang et al., 2019). Peña-Rodríguez (2010) have published the study that high levels of Hg could be removed using calcined mussel shell, although this material contained 7% of aragonite even after calcination (Peña--Rodríguez et al., 2013).

Possible sources of THg content

Total Hg obtained in the upper, middle and lower streams of the river, as well as the main tributaries, were compared with content of As, Cd, Cu, Ni, Pb, and Zn using PCA to establish possible sources of Hg. The contents of As, Cd, Cu, Ni, Pb, Zn in sediments were investigated in samples collected from the same sites during the same year and previously were reported in Štrbac et al. (2018). PCA of the fourth datasets defined three PCs for the lower stream and two PCs for the middle stream, upper stream and tributary which contributed to 97.03%, 90.31%, 96.15% and 98.26% of the total variance. For the dataset referring to lower stream group, the first principal component explained 49.85% of the total variance (Fig. 4A) and has strong positive loadings on Hg, Cd and Pb. The second principal component accounted for 32.27% of variation due to the variability in the concentrations of As, Cu, and Ni. The third principal component explained 14.90% of variation and is formed by Zn. For the second group (middle stream) PC1 (66.62% of total variance) is dominated by As, Cu, Pb and Zn. PC2 has strong positive loadings on Hg, Cd, and Ni explaining 23.70% of total variance (Fig. 4B). PC1 of the upper stream has strong positive loadings on As, Cd, Cu, Ni, Pb and Zn explaining 76.60% of the total variance. The second PC (19.56% of total variance) was related only to the Hg (Figure 4C). In fourth group – tributary, PC1 is dominated by Hg, Cu, Ni, Pb and Zn accounting for 74.68% of the total variance, while PC2 explaining 23.58% due to As (Figure 4D). According to PCA strong positive loading of examined metals in all parts in the river Tisa are mainly controlled from the same sources.


A
PC3 (14.90%)
PC2 (32.27%)
PC1 (49.85%)
Cu
Ni
Zn
Hg
As
Pb
Cd
B
PC2 (23.70%)
PC1 (66.62%)
Hg
Cd
Ni
Cu
Zn
As
Pb
C
PC2 (19.56)
PC1 (76.60)
Zn
Pb
Cu
As
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Cd
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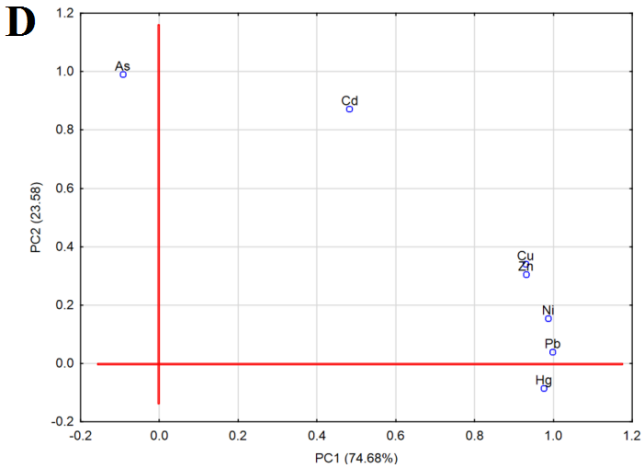

Figure 4. PCA loadings and score plots of metals for groups: A – lower stream; B – middle stream; C – upper stream; D – tributaries.

Degree of THg pollution in sediments from the river Tisa through different criteria

To establish the intensity of anthropogenic influence on the river Tisa sediments, the results of this study were compared with the background value, national legislation, and NOAA guideline. Total Hg were obviously higher than mean background value (0.065 mg kg^{-1}) (Štrbac et al., 2018). According to the official standard on the limit levels of pollutants in the Republic of Serbia, THg in the river Tisa was within the range of maximum permissible values (*Off. Gazette of RS* no. 50, 2012). Maximum permissible value is the concentration of an individual pollutant or group of pollutants above which negative environmental impacts are expected (*Off. Gazette of RS*, no. 50, 2012). Compared with NOAA guideline, 80.49% (Table 3) of sediment samples showed THg between the Effects Range-Low (ERL) and Effects Range-Median (ERM) values, indicating that THg in the river Tisa sediments represented minimal and possible risk towards organisms. The NOAA guidelines classified the sediment: rarely (<ERL), occasionally (≥ERL and <ERM) or frequently associated with adverse biological effects (>ERM) in relation to the concentrations of pollutants. Concentrations lower than ERL represent a minimal effect; those between ERL and ERM represent a possible effect; and those above the ERM represent a probable effect. The ERL and ERM toxicity values for THg as reported by NOAA are 0.170 mg kg^{-1} and 0.486 mg kg^{-1}, respectively (Bolaños-Alvarez et al., 2016). Degree of THg contamination was also assessed by pollution indices EF, Igeo, CF and PERI. EF of THg ranged from 1.06 to 6.75. EF results showed that 31.71% of samples showed minor enrichment, 53.66% moderate enrichment, and 14.63% severe enrichment (Table 3). However, an EF >1.5 indicates that a significant portion of the heavy metals was delivered

Table 3. Degree of THg pollution in sediments from the river Tisa.

NOAA guidelines (Bolaños-Alvarez et al., 2016)							
	<ERL		≥ERL and <ERM			>ERM	
Percentage of examined sediment samples	19.51%		80.49%			–	
EF (Birch and Olmos, 2008)							
	no enrichment	minor enrichment	moderate enrichment	moderately severe enrichment	severe enrichment	very severe enrichment	extremely severe enrichment
Percentage of examined sediment samples	–	31.71%	53.66%	14.63%	–	–	–
Igeo (Yaqin et al., 2008)							
	unpolluted	unpolluted to moderately polluted	moderately polluted	moderately to highly polluted	highly polluted	highly to very highly polluted	very highly polluted
Percentage of examined sediment samples	9.76%	39.02%	51.22%	–	–	–	–
CF (Håkanson, 1980)							
	low contamination		moderate contamination		considerable contamination		very high contamination
Percentage of examined sediment samples	–		34.15%		53.66%		12.19%
Ei (Håkanson, 1980)							
	low risk		moderate risk	considerable risk	high risk		very high risk
Percentage of examined sediment samples	–		12.19%	34.15%	53.66%		–

from non-crustal materials, thus these heavy metals were delivered by other sources, like point and non-point pollution (Barbieri, 2016). In this sense, THg in the river Tisa sediments has enrichment phenomenon with respect to the background value and mainly originates from anthropological sources. The Igeo index for the sampling sites ranged from -0.31 to 1.61. Igeo results showed that 51.22% of all sediment samples were moderately polluted, 39.02% unpolluted to moderately polluted, and 9.76% unpolluted (Table 3). According to the category of CF, 12.19% samples were at a very high contamination level, 53.66% samples were considerably contaminated, while 34.15% were moderately contaminated (Table 3). Specifically, 53.66% of sediment samples were within high pollution risk, 34.15% in considerable pollution risk, and 12.19% in moderate risk according to PERI (Table 3).

CONCLUSION

Total Hg concentrations in the sediments ranged from 0.07 to 0.49 mg kg^{-1}, with mean ± S.D. value of 0.26 ± 0.10 mg kg^{-1}. The highest mean value of THg (0.30 mg kg^{-1}) was found in the lower stream, while the lowest (0.13 mg kg^{-1}) was found in the tributary. In the river Tisa sediments THg is likely to be transported primarily by the small particles. The anthropogenic activities in the area of the samples with minimum and maximum values of THg do not present other likely sources of Hg pollution of the river Tisa, so the observed increase could be explained by change in the grain size of surface sediments in this area. According to PCA, strong positive loading of metals in all parts in the river Tisa is mainly controlled from the same sources. These sources are related to anthropogenic activities based on calculated EF values. EF indicates that THg concentrations in the river Tisa sediment have enrichment phenomenon. Total Hg are higher than mean background value. According to the Republic of Serbia official standard THg in the river Tisa was within the range of maximum permissible values. Compared with NOAA guideline, 80.49% of sediment samples indicated that THg in the river Tisa sediments represented minimal and possible risk towards the living organisms. Integrating the results of pollution assessment, it could be concluded that THg in river Tisa sediments in Serbia show considerable contamination according to Igeo and CF, and high pollution risk according to PERI.

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REFERENCES

Barbieri M (2016): The Importance of Enrichment Factor (EF) and Geoaccumulation Index (I_{geo}) to Evaluate the Soil Contamination. *J. Geol. Geophys.* 5: 1–4.

Birch FG, Olmos AM (2008): Sediment-bound heavy metals as indicators of human influence and biological risk in coastal water bodies. *J. Mar. Sci.* 65: 1407–1413.

Bolaños-Alvarez Y, Alonso-Hernandez CM, Morabito R, Díaz-Asencio M, Pinto V, Gomez-Batista M (2016): Mercury contamination of Riverine sediments in the vicinity of a mercury cell chlor-alkali plant in Sagua River, Cuba. *Chemosphere* 152: 376–382.

Ergin M, Saydam C, Basturk O, Erdem E, Yoruk R (1991): Trace metal concentrations in surface sediments from the two coastal inlets (Golden Horn Estuary and Izmit Bay) of the North–eastern Sea of Marmara. *Chem. Geol.* 91: 269–285.

Gu W, Zhou CY, Wong MK, Gan LM (1998): Orthogonal array design (OAD) for the optimization of mercury extraction from soils by dilute acid with microwave heating. *Talanta* 46: 1019–1029.

Håkanson L (1980): An ecological risk index for aquatic pollution control, a sedimentological approach. *Water Res.* 14: 975–1001.

Jiang GB, Shi JB, Feng XB (2006): Mercury pollution in China. *Environ Sci. Technol*. 40: 3672–3678.

Liu W, Hu L, Lin T, Li Y, Guo Z (2017): Distribution and mass inventory of mercury in sediment from the Yangtze River estuarine-inner shelf of the East China Sea. *Cont. Shelf Res.* 132: 29–37.

Milačič R, Ščančar J, Murko S, Kocman D, Horvat M (2010): A complex investigation of the extent of pollution in sediments of the Sava River. Part 1: Selected elements. *Environ Monit. Assess* 163: 263–275.

Müller G (1969): Index of geoaccumulation in sediments of the Rhine River. *GeoJournal* 2: 108–118.

Nedić A, Pucarević M, Ninkov J, Stojić N, Milić D (2019): Mercury content and distribution in household dust and soil in the town of Šid. *Matica Srpska J. Nat. Sci.* 137: 33–41.

Ninkov J, Marković S, Banjac D, Vasin J, Milić S, Banjac B, Mihailović A (2017): Mercury content in agricultural soils (Vojvodina Province, Serbia). *Environ. Sci. Pollut.* R 24: 10966–10975.

Obradović J, Vasić N (1988): *Sedimentary rock testing methods*. University of Belgrade, Faculty of Mining and Geology.

Peña-Rodríguez S, Fernández-Calviño D, Nóvoa-Muñoz JC, Arias-Estévez M, Núñez-Delgado A, Fernández-Sanjurjo MJ (2010) Kinetics of Hg(II) adsorption and desorption in calcined mussel shells. *J. Hazard. Mater.* 180: 622–627.

Peña-Rodríguez S, Bermúdez-Couso A, Nóvoa-Muñoz JC, Arias-Estévez M, Fernández-Sanjurjo MJ, Álvarez-Rodríguez E, Núñez-Delgado A (2013): Mercury removal using ground and calcined mussel shell. *J. Environ. Sci-China* 25: 2476–2486.

Popov D, Vandenberghe DAG, Marković SB (2012): Luminescence dating of fluvial deposits in Vojvodina, N Serbia: First results. *Quat. Geochronol.* 13: 42–51.

Republic of Serbia (2012): Regulation of limit values pollutant substances into surface water, groundwater and sediments and the deadlines for their attaining. *Official Gazette of the Republic of Serbia*, no. 50.

Sakan S, Sakan N, Anđelković I, Trifunović S, Đorđević D (2017): Study of potential harmful elements (arsenic, mercury and selenium) in surface sediments from Serbian rivers and artificial lakes. *J. Geochem. Explor.* 180: 24–34.

Štrbac S, Kašanin Grubin M, Vasić N (2018): Importance of background values in assessing the impact of heavy metals in river ecosystems: case study of Tisza River, Serbia. *Environ. Geochem. Health* 40: 1247–1263.

Tansel B, Rafiuddin S (2016): Heavy metal content in relation to particle size and organic content of surficial sediments in Miami River and transport potential. *Int. J. Sediment Res.* 31: 324–329.

Ullrich SM, Tanton TW, Abdrashitova SA (2001): Mercury in the aquatic environment: a review of factors affecting methylation. *Crit. Rev. Env. Sci. Tec.* 31: 241–293.

UNEP (2019): United Nations Environment Programme, The Minamata Convention on Mercury. Available from: http://www.mercuryconvention.org/.

Wang J, Xing Y, Xie Y, Meng Y, Xia J, Feng X (2019): The use of calcium carbonate-enriched clay minerals and diammonium phosphate as novel immobilization agents for mercury remediation: Spectral investigations and field applications. *Sci. Total Environ.* 646: 1615–1623.

Woitke P, Wellmitz J, Helm D, Kube P, Lepom P, Litheraty P (2003): Analysis and assessment of heavy metal pollution in suspended solids and sediments of the river Danube. *Chemosphere* 51: 633–642.

Yaqin JI, Yinchang F, Jianhi WU, Tan ZHU, Zhipeng B, Chiqing D (2008). Using geoaccumulation index to study source profiles of soil dust in China. *J. Environ. Sci-China* 20: 571–578.

ЗАГАЂЕЊЕ СЕДИМЕНАТА ЖИВОМ У РЕЦИ ТИСИ (СРБИЈА)

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РЕЗИМЕ: Велики број међународних организација хемијски елемент живу (Hg) означио је као глобално загађујућу супстанцу високог приоритета због своје мобилности и перзистентности у животној средини, као и високе токсичности за живе организме. Истраживање је спроведено с циљем да се утврди: (i) укупни садржај Hg (THg) и њена просторна дистрибуција у седиментима дуж тока реке Тисе, (ii) могући извори THg и (iii) степен загађења седимената реке Тисе примењујући различите критеријуме. Укупан садржај Hg у седиментима реке Тисе кретао се од 0,07 до 0,49 mg kg^{-1}, са средњом вредношћу ± S.D. од 0,26 ± 0,10 mg kg^{-1}. Највећа средња вредност THg (0,30 mg kg^{-1}) утврђена је у доњем току реке, док је најнижа (0,13 mg kg^{-1}) забележена у притокама. На основу анализе главних компонената (PCA) може се закључити да оптерећење седимената металима дуж целог тока реке Тисе има заједничко порекло. Оно се доводи у везу са антропогеним активностима на основу израчунатих вредности фактора обогаћивања (EF). Укупни садржаји Hg у седиментима реке Тисе виши су од природне вредности за тај део слива, али су у границама максимално дозвољених концентрација према званичном стандарду Републике Србије. У поређењу са националним смерницама за океанску и атмосферску управу (NOAA) у 80,49% узорака седимената THg представља минималан и могући ризик за организме. Интегришући резултате процене загађења, може се закључити да THg у седиментима реке Тисе у Србији показује знатну контаминацију на основу геоакумулационог индекса (Igeo) и фактора загађења (CF), као и висок ризик загађења на основу индекса потенцијалног ризика на животну средину (PERI).

КЉУЧНЕ РЕЧИ: жива (Hg), процена ризика, седименти, река Тиса

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SEQUENTIAL EXTRACTION STUDIES ON THE RIVER TISA SEDIMENTS FOR THE ASSESSMENT OF THE METAL POLLUTION

ABSTRACT: The sequential extraction procedure was applied for partitioning of metals in river sediments collected along the course of the river Tisa (Serbia). Eight elements (Sb, Sn, As, Cd, Cu, Hg, Pb and Zn) from twenty-one sampling site were analyzed using the modified BCR sequential extraction procedure in combination with ICP-OES. The results of sequential extraction, statistical analyses and calculation of EF and lithogenic and anthropogenic ratio of metals are similar. In the river Tisa sediments Sn, Cd, Hg, Pb and Zn are of anthropogenic origin, while As, Cu and Sb are of lithogenic and anthropogenic origin. The sediments from the river Tisa show high risk for Cd, medium risk for Hg and Zn, low risk for Sn, As, Cu and Pb, whereas Sb does not show the risk for the aquatic environment.

KEYWORDS: metals, river sediments, sequential extraction, the river Tisa

INTRODUCTION

With rapid urbanization and industrialization, large amounts of metals from anthropogenic sources are discharged into the aquatic environment where they can accumulate in sediments and bio-accumulate by aquatic organisms and even be bio-magnified through the food chain, thus posing potential adverse effects on human health and even the whole eco-system (Wei et al., 2016).

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Investigations concerning the metals present in sediments have increased in recent years. It is accepted that total metals concentrations analyses are the fundamental way to evaluate sediment quality, however they are not sufficient to be able to predict the capacity for mobilization of these elements. The environmental behavior of metals is critically dependent on their chemical form, which influences mobility, bioavailability and toxicity to organisms (Gao and Chen, 2012). Metal ions in sediments are partitioned between different phases, i.e., carbonates, iron and manganese oxides, sulphides, organic matter (OM), and phyllosilicate minerals. In sediments metals are mainly associated with silicates and primary minerals, and therefore have limited mobility. The relative content of metals in the residual phase can be used as a measure of the contribution of natural sources (Singh et al., 2005a). Introduced from human activities, metals show greater mobility and are associated with sediment phases such as carbonates, oxides and sulphides (Medici et al., 2011).

Although the separation of various chemical forms of metals is very difficult, the use of sequential extraction method proves to be an important and effective approach. Sequential extraction technique is one of the most widely used approaches to distinguish between different geochemical associations of many metals and to gain a better insight of geochemical processes occurring in sediments (Venkatramanan et al., 2015). The three-stage sequential extraction procedure proposed by the European Community Bureau of Reference (BCR) has mostly been used (Passos et al., 2010). The sediment fractions obtained using the optimized BCR protocol reflects the mechanisms by which the metals are associated with the sediments. In the first fraction (F1), there are metals associated with carbonates; in the second fraction (F2) there are the ones associated with Fe and Mn oxides; and in the third fraction (F3) there are metals associated with sulphides and OM. The residual fraction (R) contains the primary and secondary minerals derived from natural geological formations, which can contain metals within their crystalline structures (Passos et al., 2010).

To assess the risk and mobility of the non-stable chemical fraction of metals, the Risk assessment code (RAC) was used (Singh et al., 2005a). The RAC considers the percentage fraction of metals that are exchangeable and associated with carbonates. In this fraction, the metals are weakly bound to the sediments, and present a greater environmental risk since they are more available to the aquatic system (Passos et al., 2010).

This study aims to: (1) investigate the distribution of the metal concentrations (antimony (Sb), tin (Sn), arsenic (As), cadmium (Cd), copper (Cu), mercury (Hg), lead (Pb) and zinc (Zn)) in the river Tisa sediments using sequential extraction procedures; (2) identify the sources of metals; (3) assess the ecological risk of metals in the river Tisa sediments.

MATERIALS AND METHODS

Sampling method

A total of 56 sediments samples were collected from 21 sampling sites from the river Tisa in Serbia (Figure 1). Forty samples (0–30 cm) were collected

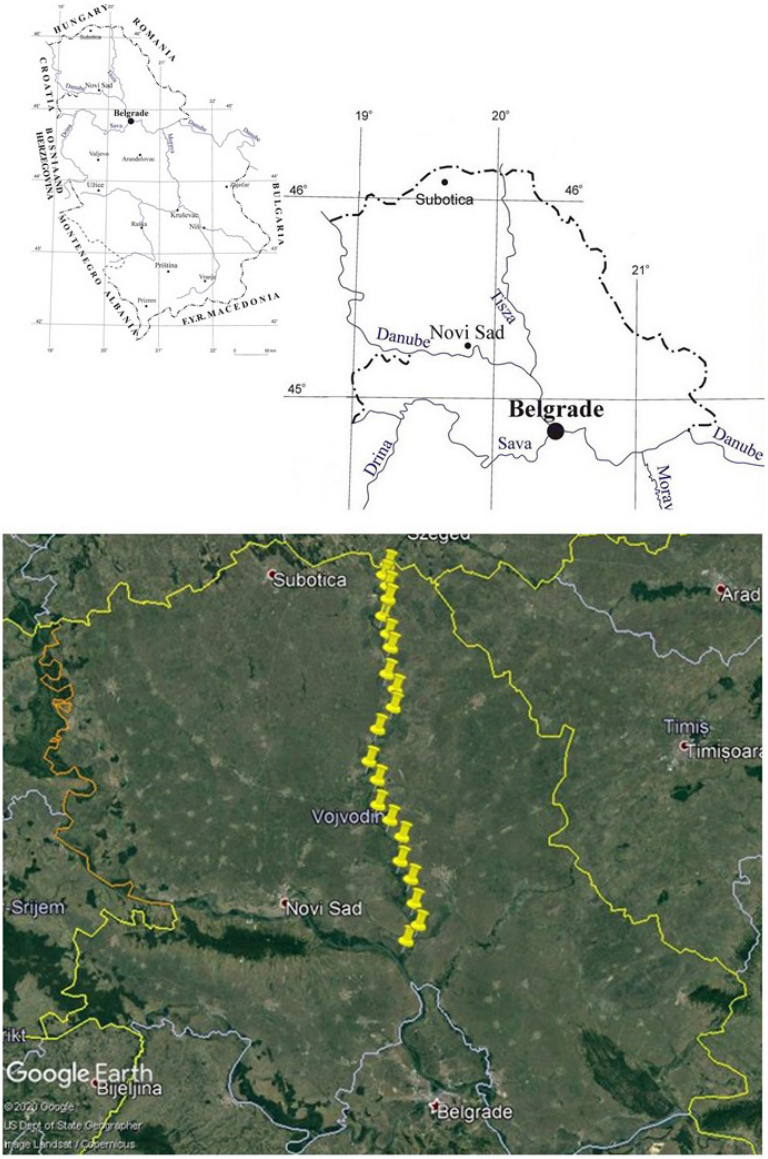

Figure 1. Study area.

from 17 sampling sites and four core (0–80) sediments were collected from 4 sampling sites. Core sediments samples were sectioned at 20 cm intervals. The sediment samples were subjected to a four-step BCR sequential extraction procedure (Mossop and Davidson, 2003). The following extraction solutions were used: phase I (F1): 0.11 M acetic acid (HOAc extractable fraction); phase II (F2): 0.5 mol/dm^3 hydroxylamine hydrochloride adjusted to pH 1.5; phase III (F3): 8.8 mol/dm^3 hydrogen peroxide stabilized at pH 2 and 1 mol/dm^3 ammonium acetate adjusted to pH 2; and phase IV (R): aqua regia 15 ml 37% HCl and 5 ml 65% HNO_3, at 80 °C during 5 h. The concentrations of major and minor elements were determined using an Inductively Coupled Atomic Emission Spectrometer, ICP-OES (Thermo Scientific, UK), model 6500 Duo. The standard reference material: CRM BCR-701 (lake sediment) was employed to check the accuracy and precision of the instruments. The results of the analysis showed good agreement with the certified levels (± 10%). A multielement stock solution containing 1,000 g/L of the elements was used to prepare standard solutions for ICP-OES measurements. All other chemicals used for BCR sequential extraction were of analytical grade and were supplied by Merck (Darmstadt, Germany).

Contamination assessment methods

In order to determine natural or anthropogenic origin of metals enrichment factor (EF), lithogenic and anthropogenic ratio of metals were calculated and statistical analysis (Pearson correlation analysis and Principal component analysis (PCA)) were done. EF represent the ratio of chemical element concentration in the sediments compared to the reference samples. The following formula was used to calculate it (Ergin et al., 2013):

$$EF = (M/Al)_{sample} / (M/Al)_{reference\ sample}$$

where: M_{sample} – metal concentration in the sample; Al_{sample} – concentration of Al in the sample; $M_{reference\ sample}$ – the metal concentration in the reference sample; $Al_{reference\ sample}$ – concentration of Al in the reference sample. The values of EF have the following range (Birch, 2003): < 1 no anthropogenic influence; 1–3 small anthropogenic influence; 3–5 moderate anthropogenic influence; 5–10 moderately severe anthropogenic influence; 10–25 severe anthropogenic influence; 25–50 very severe anthropogenic influence; and > 50 extremely severe anthropogenic influence.

The lithogenic and anthropogenic origin of metals was calculated in the following way (Šparica, 2012):

$$M_{lithogenic} = Al_{sample} \cdot \left(\frac{M}{Al}\right)_{reference\ sample}$$

$$M_{anthropogenic} = M_{total} - M_{lithogenic}$$

where: $M_{lithogenic}$ – concentration of metal of lithogenic origin; Al_{sample} – concentration of Al in the sample; $M_{reference\ sample}$ – concentration of metal in the reference sample; $Al_{reference\ sample}$ – concentration of Al in the reference sample; $M_{anthropogenic}$ – concentrations of metal of anthropogenic origin; M_{total} – total concentration of metal.

Pearson correlation and PCA were performed to aid in identifying the source of the metals. In addition to the concentration of metals, the concentration of major elements (such as K, Al, Fe, Mn, Cr, P, Ti, Si, Na, Ca and Mg) and sediments characteristics (e.g., the grain size distribution, pH and $CaCO_3$) were included in the PCA. The principal components (PCs) are selected on the basis of the criteria of cumulative % variance > 70% and an Eigenvalue > 1.0 (Lin et al., 2016).

Risk assessment code (RAC) has been used to estimate possible harm to benthic organisms and to assess environmental risks caused by contaminated sediments (Passos et al., 2010). RAC was used to estimate the mobility of the non-stable chemical fraction of metals (Singh et al., 2005b), based on the percentage of metal content in the F1 fraction (Passos et al., 2010). In this fraction metals are weakly bound to sediments, therefore they are more readily available to aquatic organisms, and present greater environmental risk. Depending on the percentage value the RAC classification defines: < 1% no risk to the aquatic environment; 1–10% low risk; 11–30% medium risk; 31–50% high risk; > 50% very high risk (Passos et al., 2010).

RESULTS AND DISCUSSION

Table 1 shows the average content of extracted metals by the fraction of sequential extraction. For each extraction fraction, the results are presented in the arithmetic mean ± standard deviation (Table 1). The sums of Sb, As, Cd, Cu, Hg, Pb and Zn concentrations in BCR fractions (ΣF1 + F2 + F3 + R) and the percentages of the sums in ΣF1 + F2 + F3, and ΣF1 are also shown in Table 1. The percentage of the extracted metals in each fraction are shown in Figure 2.

Table 1. The average concentrations of Sb, As, Cd, Cu, Hg, Pb and Zn in each fraction (mg kg^{-1}), the sum ΣF1+F2+F3+R (mg kg^{-1}) and the percentages of the sum ΣF1+F2+F3, ΣF1 (%)

Metal	Fraction 1 (mg kg^{-1})	Fraction 2 (mg kg^{-1})	Fraction 3 (mg kg^{-1})	Residual (mg kg^{-1})	ΣF1+F2+F3+R (mg kg^{-1})	ΣF1+F2+F3 (%)	ΣF1 (%)
Sb	0.03±0.01	1.16±1.91	0.22±0.24	1.72±0.51	3.12±2.67	44.83	0.84
Sn	0.20±0.20	1.23±1.95	0.84±0.39	0.23±0.22	2.50±2.76	90.88	8.12
As	0.14±0.14	2.19±1.05	1.85±2.55	4.18±2.09	8.37±8.83	50.03	1.71
Cd	1.02±0.34	0.50±0.25	0.13±0.09	0.43±0.14	2.07±0.82	79.28	49.04
Cu	10.25±5.01	32.03±13.08	72.60±47.31	75.53±48.65	190.42±114.05	60.33	5.38
Hg	0.04±0.06	0.05±0.06	0.08±0.11	0.03±0.03	0.21±0.25	84.91	19.57
Pb	0.64±0.52	26.15±8.36	3.28±1.51	1.52±1.17	31.58±11.56	95.19	2.01
Zn	49.26±16.71	92.24±30.37	57.21±35.95	62.24±34.63	260.96±117.66	76.15	18.88

The largest Sb content was extracted in the residual (55.17%) and second (37.07%) extraction fraction. Most of Sn was dominantly extracted in the second (49.05%) and third (33.70%) extraction fraction. The largest percentage of As was extracted in the residual (49.97%), while a smaller percentage was extracted in the second (26.18%) and the third (22.14%) extraction fractions. Cd was dominantly extracted in the first exchangeable carbonate fraction (49.04%), while a smaller percentage was extracted in the second (24.15%) and residual (20.72%) extraction fraction. The largest Cu content was extracted in the residual (39.67%) and the third (38.13%) stage of extraction. The most important extraction of Hg occurred in the third (40.24%) and the second (25.10%) stage of extraction. Intended to the river Tisa sediments, the most important was the binding of Pb in the second mobile fraction (82.79%). Zn was mostly extracted in the second extraction fraction (35.35%), then in the residual (23.85%) and third (21.92%) phase of extraction (Figure 2).

Elevated concentrations of metals in the residual fraction indicate that sediments are relatively unpolluted, and that the metals derive mainly from lithogenic origin. Significant proportions of metals have been found insoluble in acid, associated with Fe and Mn oxides, and associated with organic matter and sulphides fractions (the first three extraction fractions) in regions with great anthropogenic inputs (Passos et al., 2010). According to the percentage of metals extracted in the F1+F2+F3 the order was: Pb (95.19%) > Sn (90.88%) > Hg (84.91%) > Cd (79.28%) > Zn (76.15%) > Cu (60.33%) > As (50.03%) > Sb (44.83%) (Table 1). Furthermore, different behaviors of metals in the various phases of sequential extraction reflect different origin of pollution. Regions where metals are weakly associated with sediments, and show an affinity for carbonates, have experienced more recent pollution. Contrary to this, locations where metals are significantly associated with the oxidizable or reducible sediment fractions are influenced by pollution that is not recent (Passos et al., 2010). In this study, Cd was dominantly extracted in the first exchangeable carbonate fractions fraction (Figure 2), indicating recent pollution of investigated sediments by Cd.

The possible anthropogenic and/or lithogenic origin of metals in the river Tisa sediments were explored by obtained Pearson correlation analysis and PCA. Significantly positive correlations ($P < 0.01$) were found between all investigated metals. This result indicated that metals in the river Tisa sediments originate from common sources. PCA was performed by evaluating the principal components and computing the eigenvectors to assist in identifying the source of the metals. When determining the number of components for the analysis of the major components, the latent root criterion has been considered, according to which only those factors with an eigenvalue greater than 1 were taken into account. Based on this criterion, three components that account for 76% of the total variance have been taken into account (Figure 3).

PC 1 explains 44.60% of the total variance and is largely dominated by Sb, Sn, As, Cd, Cu, Hg, Pb and Zn, oxides MnO and Cr_2O_3. PC 2, dominated by Al_2O_3, Fe_2O_3 and clay, explains 19.90% of the total variance. PC 3, dominated primarily by CaO, P_2O_5 and $CaCO_3$, accounts for 11.60% of the total

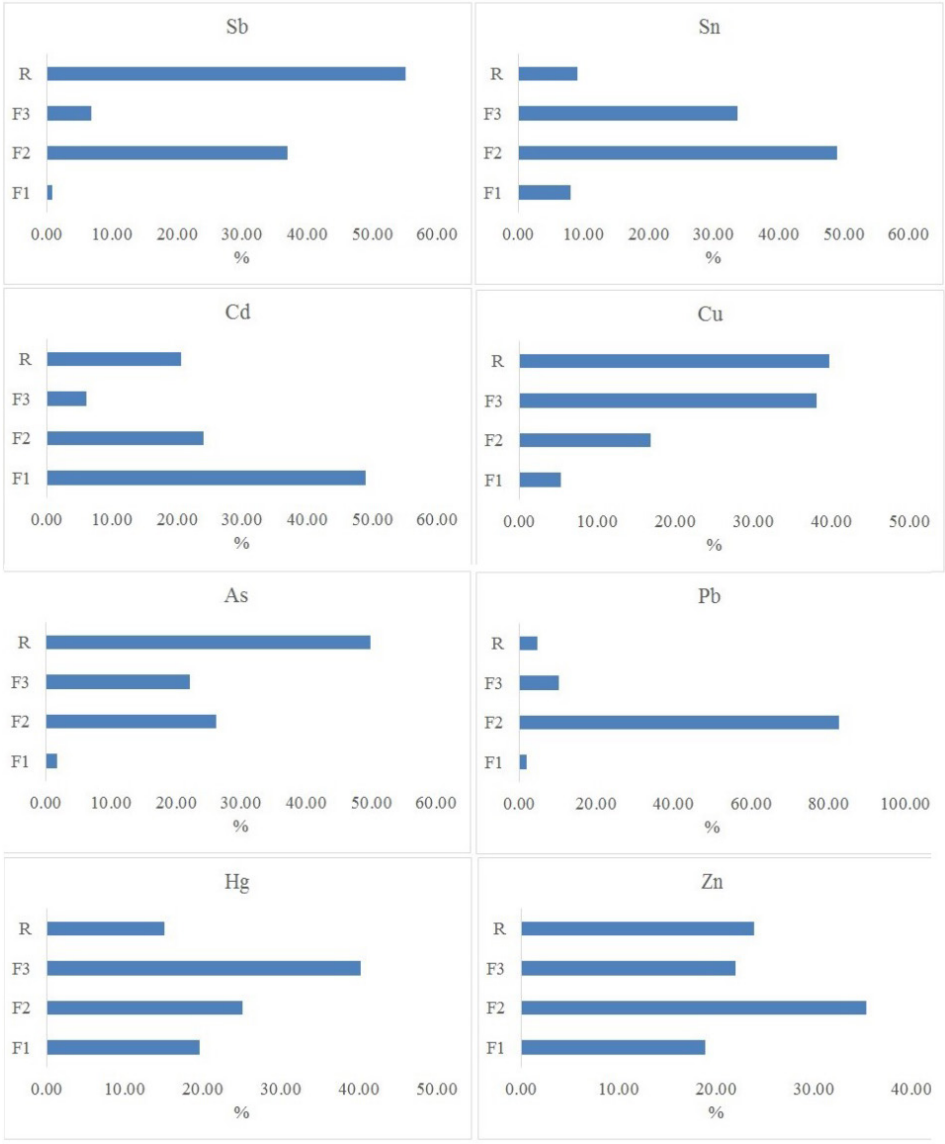

Figure 2. Distribution of Sb, Sn, Cd, Cu, As, Pb, Hg and Zn between different extraction fractions

variance. Positive loading of examined metals in the river Tisa sediments are mainly controlled from common sources. The major sources of metal pollution are attributed to vehicle emissions and commercial and industrial discharges as well as agricultural activities.

The possible anthropogenic and/or lithogenic origin of metals in the river Tisa sediments was also explored by calculated EF and lithogenic and anthropogenic ratio of metals. According to the values of EF, resulting from the ratio

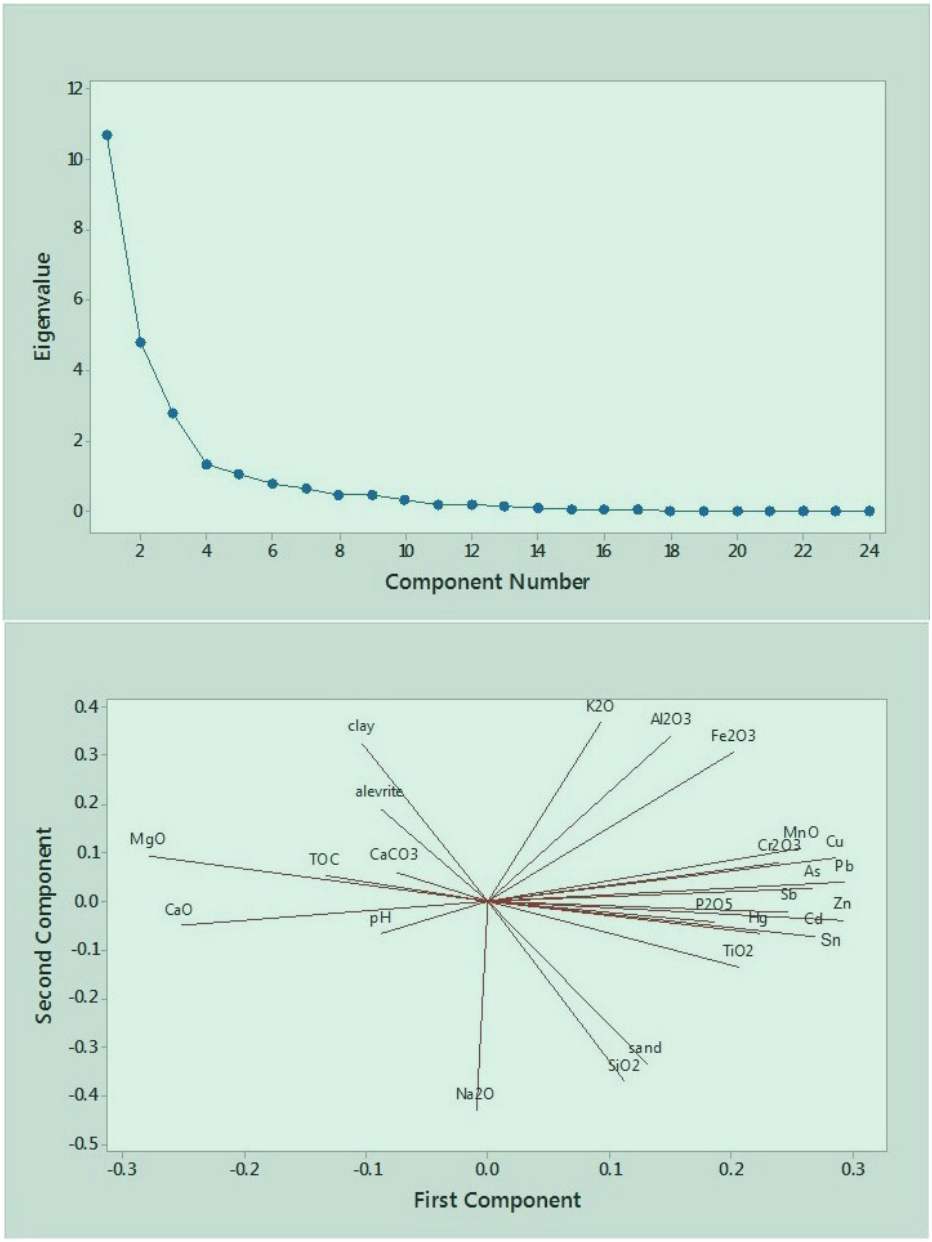

Figure 3. Scree method of analysis of the main components and Biplot analysis of the main components

of metals in the sediments of the river Tisa and the reference samples which were not under anthropogenic influence, the following concentrations were obtained: Sb, As, and Cu were under small anthropogenic influence (EF < 3); Hg was under small and moderate; Pb, Sn and Zn under moderate anthropogenic influence (EF 3–5); while Cd was under moderately severe anthropogenic influence (EF 5–10). By calculating the lithogenic and anthropogenic ratio of metals it could be concluded that the concentrations of As are of lithogenic and anthropogenic origin in the river Tisa sediments, while the concentrations of Sb, Sn, Cd, Cu, Hg, Pb, and Zn are mainly of anthropogenic origin.

Generally, the results of sequential extraction, statistical analyses and calculation of EF and lithogenic and anthropogenic ratio of metals are similar. In the river Tisa sediments Sn, Cd, Hg, Pb, and Zn are anthropogenic origin, while As, Cu and Sb are of mixed, lithogenic and anthropogenic, origin.

In order to determine the intensity of anthropogenic influence on the river Tisa sediments, RAC was calculated. RAC is used to estimate the value of sediment reactivity, based on the differences in strengths with which metals in sediments are bound to fractions (Sundaray et al., 2011). In sediments, metals from anthropogenic sources are bound to the exchangeable carbonate fraction. Metals in this fraction are weakly bonded, and could equilibrate with the aqueous phase and become more bioavailable (Sundaray et al., 2011). When the pH and redox conditions are advantageous, the metals will be particularly soluble and can be taken up by plants or ingested by animals. RAC is defined as the fraction of metals exchangeable and/or associated with carbonates (Nemati et al., 2011), and for this study has been determined based on the percentage of the total metal content that has been found in the first sediment fraction by BCR method (% ΣF1) (Table 1). The sediments from the river Tisa show high risk for Cd with RAC values between 31% and 50%. Medium risk is indicated for Hg and Zn with RAC values between 11% and 30%. Sn, As, Cu and Pb show low risk with RAC values between 1% and 10%, and Sb does not show the risk to the aquatic environment because RAC values are less then < 1%.

CONCLUSION

This study aimed at investigating distribution of the metal concentrations using sequential extraction procedures, to identify metals sources, and to assess the environmental risk of metals in the river Tisa sediments. In order to determine source of metals in the river Tisa sediments EF, lithogenic and anthropogenic ratio of metals were calculated and statistical analyses were done. To estimate possible harm to benthic organisms and assess environmental risks caused by contaminated sediments RAC has been used. Following conclusions are based on the obtained results.

The largest Sb content was extracted in R and F2 extraction fraction. Most of Sn was dominantly extracted in F2 and F3 extraction fraction. The largest percentage of As was extracted in R fraction. Cd was dominantly extracted in F1 extraction fraction. The largest Cu content was extracted in R and F3 stage

of extraction. The most important extraction of Hg occurred in F3 and F2 stage of extraction. Intended to the river Tisa sediments, the most important was the binding of Pb in F2 mobile fraction. Zn was mostly extracted in F2 fraction. Pearson correlation and PCA indicated that metals in the river Tisa sediments originate from common sources. According to the EF results: Sb, As, and Cu were under small anthropogenic influence; Hg was under small and moderate; Pb, Sn and Zn under moderate anthropogenic influence; while Cd was under moderately severe anthropogenic influence. By calculating the lithogenic and anthropogenic ratio of metals in the river Tisa sediments, it could be concluded that the concentrations of As are lithogenic and anthropogenic origin, while the concentrations of Sb, Sn, Cd, Cu, Hg, Pb, and Zn are anthropogenic origin. In the river Tisa sediments Sn, Cd, Hg, Pb, and Zn are anthropogenic origin, while As, Cu and Sb are of mixed, lithogenic and anthropogenic, origin according to the results of sequential extraction, statistical analyses and calculation of EF and lithogenic and anthropogenic ratio of metals. The sediments from the river Tisa show high risk for Cd, medium risk for Hg and Zn, low risk for Sn, As, Cu and Pb, while Sb does not show the risk to the aquatic environment.

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REFERENCES

Birch GA (2003): Scheme for assessing human impacts on coastal aquatic environments using sediments. In: CD Woodcoffe, RA Furness (eds.), *Coastal GIS 2003*, Wollongong University Papers in Center for Maritime Policy, Australia.

Ergin M, Karakaş ZS, Sözeri K, Eser-Doğdu B, KadıoğluYK F-YF (2013): Morphological, drainage and eolian controls on the microtidal Patara Beach sediments (eastern Mediterranean): textural, geochemical and mineralogical investigations. *Quat. Int.* 302: 135–153.

Gao X, Chen CT (2012): Heavy metal pollution status in surface sediments of the coastal Bohai Bay. *Water Res.* 46: 1901–1911.

Lin Q, Liu E, Zhang E, Li K, Shen J (2016): Spatial distribution, contamination and ecological risk assessment of heavy metals in surface sediments of Erhai Lake, a large eutrophic plateau lake in southwest China. *Catena* 145: 193–203.

Medici L, Bellanova J, Belviso C, Cavalcante F, Lettino A, Pasquale Ragone P, Fiore S (2011): Trace metals speciation in sediments of the Basento River (Italy). *Appl. Clay Sci.* 53: 414–442.

Mossop KF, Davidson CM (2003): Comparison of original and modified BCR sequential extraction procedures for the fractionation of copper, iron, lead, manganese and zinc in soils and sediments. *Anal. Chim. Acta* 478: 111–118.

Nemati K, Abu Bakar NK, Abas MR, Sobhanzadeh E (2011): Speciation of heavy metals by modified BCR sequential extraction procedure in different depths of sediments from Sungai Buloh, Selangor, Malaysia. *J. Hazard. Mater.* 192: 402–410.

Passos EA, Alves JC, Santos IS, Alves JP, Garcia CAB, Costa ACS (2010): Assessment of trace metals contamination in estuarine sediments using a sequential extraction technique and principal component analysis. *Microchem. J.* 96: 50–57.

Singh AN, Zeng DH, Chen FS (2005a): Heavy metal concentrations in redeveloping soil of mine spoi under plantations of certain native woody species in dry tropical environment, India. *J. Environ. Sci.* 1: 168–174.

Singh KP, Mohan D, Singh VK, Malik A (2005b): Studies on distribution and fractionation of heavy metals in Gomti river sediments-a tributary of the Ganges, India. *J. Hydrol.* 312: 14–27.

Sundaray SK, Nayak BB, Lin S, Bhatta D (2011): Geochemical speciation and risk assessment of heavy metals in the river estuarine sediments – A case study: Mahanadi basin, India. *J. Hazard. Mater.* 186: 1837–1846.

Šparica M (2012): *Geokemijsko frakcioniranje i mobilnost olova (II) u onečišćenim tlima*. Zagreb: Institut za geološka istraživanja (master's thesis).

Venkatramanan S, Chung SY, Kim TH, Prasanna MV, Hamm SY (2015): Assessment and distribution of metals contamination in groundwater: a case study of Busan City, Korea. *Water Qual Expos. Hea.* 7: 219–225.

Wei M, Chen J, Wang X (2016): Removal of arsenic and cadmium with sequential soil washing techniques using Na2EDTA, oxalic and phosphoric acid: Optimization conditions, removal effectiveness and ecological risks. *Chemosphere* 156: 252–261.

ПРИМЕНА СЕКВЕНЦИЈАЛНЕ ЕКСТРАКЦИЈЕ НА СЕДИМЕНТИМА РЕКЕ ТИСЕ У ЦИЉУ ПРОЦЕНЕ ЗАГАЂЕЊА МЕТАЛИМА

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РЕЗИМЕ: Ова студија имала је за циљ да истражи дистрибуцију концентрација метала применом методе секвенцијалне екстракције, идентификује изворе метала и процени еколошки ризик метала у седиментима реке Тисе. Да би се утврдило порекло метала у седиментима реке Тисе израчунати су фактор обогаћивања, литогени и антропогени однос метала и урађена је статистичка анализа. Да би се извршила процена ризика на животну средину узоркованих седиментима коришћен је RAC (Risk Assessment Code). На основу резултата може се следеће закључити: доминантан садржај антимона (Sb) екстрахован је у R и F2 фракцији. Антимон је доминантно екстрахован у F2 и F3 фракцији. Доминантни садржај арсена (As) издвојен је у R фракцији. Кадмијум (Cd) је доминантно екстрахован у F1 фракцији. Највећи садржај бакра (Cu) екстрахован је у R и F3 фракцији. Најважнија екстракција живе (Hg) била је у F3 и F2 ступњу екстракције. Олово (Pb) се највише екстраховало у F2 фракцији. Цинк (Zn) је углавном екстрахован у F2 фракцији. Резултати секвенцијалне екстракције, статистичке анализе и израчунавања фактора обогаћивања и литогеног и антропогеног односа метала су слични. У седиментима реке Тисе калај, кадмијум, жива, олово и цинк су антропогеног порекла, док су арсен, бакар и антимон литогеног и антропогеног порекла. Кадмијум у седиментима из реке Тисе показује високи ризик, жива и цинк средњи ризик, калај, арсен, бакар и олово низак ризик, а антимон је без ризика за акватичне организме.

КЉУЧНЕ РЕЧИ: метали, речни седименти, секвенцијална екстракција, река Тиса

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BIOLOGICAL SPECTRUM OF THE WEED FLORA IN THE VRŠAC VINEYARDS (SERBIA)

ABSTRACT: Agrotechnical measures are the main factor defining the vineyard weed flora structure and composition, while adequate weed control measures simultaneously ensure that vineyards are being well-managed, thus securing good grapevine health and high quality of wine. Given that the biological spectrum of weeds affects the choice of weed control measures, the aim of this study was to determine the biological properties of the weed flora in Vršac vineyards, by assessing dominant life forms and phenology of the identified weeds. The floristic analysis was conducted during the 2016 vegetation season (March–November) at 60 plots (1 m^2), at three field sites. The presence of 97 plant taxa, belonging to 26 families, was determined. The biological spectrum of the vineyards weed flora has shown a thero-phyto-hemicryptophyte character (therophytes: 57.73% and hemicryptophytes: 34.02%). The scapose herbaceous plants with summer-flowering phenology were dominant within the therophytes and hemicryptophytes. The obtained results have shown a higher weed diversity in vineyards, when compared to previous research of the weed flora in the study area, but similar to more recent studies conducted in the neighbouring countries. Furthermore, the dominant presence of therophytes in the vineyard weed flora was expected, bearing in mind the primarily mechanical weed control measures traditionally applied in vineyards.

KEYWORDS: biological spectrum, life form, phenology, vineyard, Vršac vineyards, weeds

INTRODUCTION

Grapevine is one of the oldest cultivated plants, cultivation of which is considered to have begun in the area between the Black and Caspian seas (Lloret et al., 2011), in the territory of present-day Iran. It is estimated that vineyards occupy more than 5.5 million hectares in Europe, Africa, Australia, New Zealand

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and the United States (Steenwerth and Belina, 2010). According to the last official statistical data, grapevine is grown on about 22.150 hectares in Serbia (Ivanišević and Jakšić, 2014), with a total production of 165.568 tons of fresh grapes and 993 hectoliters of wine in 2017, according to the International Organization of Vine and Wine (OIV, 2019).

Composition of the vineyard weed flora is predominately affected by applied agrotechnical measures (Gago et al., 2007). Weed control is primarily focused on vineyard rows, where weeds compete with the grapevine directly for both water and nutrients (Fredrikson et al., 2011). Nevertheless, weed control in between rows is also of great importance, in order to achieve a seedbank reduction and consequently reduce the need for in-row weed control in the following vegetation period (Fredrikson et al., 2011). Weed control in vineyard rows in done either by mechanical – tillage and harrowing, or chemical measures – herbicide application (Steenwerth and Belina, 2010). However, these physical control measures can actually favor the survival of certain weed groups (i.e. annual weeds) and be inefficient in controlling the rhizomatous weed species (Gago et al., 2007; Fredrikson et al., 2011). Given that vineyard weed communities are rather diverse, due to climate, soil and topographic properties, they should therefore be studied in each area. Knowing their biodiversity and biological spectrum is crucial when choosing appropriate management measures in order to achieve a good vineyard health status and appropriate yield and wine quality (Gago et al., 2007).

Furthermore, certain weed species have a negative impact on grapevine growth, wine quality and health of the vineyard as a whole (Saayman and Huyssteen, 1983; Hulina, 1998; Dujmović Purgar and Hulina, 2004; Jelenić, 2015). Additionally, they can act as natural reservoirs of phytoplasmas and fungi and hosts of potential insect vectors of various plant viruses and phytoplasmas (Cvrković, 2009; Filippin et al., 2009; Agustí-Brisach et al., 2011; Cvrković et al., 2011; Atanasova, 2015). Also, adequate weed control in vineyards reduces the degree of water retention in the field, thus reducing the potential for development of various diseases (Jelenić, 2015).

Bearing all this in mind, the need for keeping vineyards in a good condition and thereby preserving the quality of the grapevine plants and wine, through adequate weed control, is paramount (Lloret et al., 2011). As the biological spectrum of the weed flora is one of the factors affecting the choice of agrotechnical measures which are being applied (Gago et al., 2007; Fredrikson et al., 2011), the aim of the study was to determine the properties of the weed flora in the Vršac vineyards by assessing the prevailing life forms and their morphological and phenological characteristics.

MATERIAL AND METHODS

Study area

The Vršac Mountains (in Serbian Vršačke planine, also known as Vršački breg), are the only mountain area in the Banat region. Their highest point

Guduričkі vrh (641 m a.s.l.) is also the highest point of the Vojvodina Province (Vasiljević, 2015). Their area encompasses 170 km^2, out of which 122 km^2 are located in the territory of Serbia (Papp and Sabovljević, 2010). The study area is characterized by a continental climate, with extremely cold winters and semi-arid summers (PSUZŽS, 2018). Avramov et al. (2000) define the climate of this winegrowing district as sub-humid, with mean annual air temperatures of 11.5 °C, an average annual air humidity of 73% and 659 mm of precipitation, and 86 sunny days per year, on average (Živković, 2014). This area is under strong influence of the southeastern košava winds (avg. speed 4.6 m/s), which are especially prevalent during the winter (PSUZŽS, 2018).

While their northern slopes are steep, southern slopes of the Vršac Mountains are milder and covered in vineyards (Papp and Sabovljević, 2010). Perennial cultures, primarily vineyards, are the main landmark of their eastern and southern slopes, where winegrowing has been one of the most important agricultural activities for centuries (PSUZŽS, 2018). In fact, the area of Vršac Mountains is considered to be the biggest single area under vineyards in Europe (PSUZŽS, 2018). According to the latest national classification, areas under grapevine in Serbia are divided into three winegrowing regions and 22 winegrowing areas (Ivanišević and Jakšić, 2014). Following this, the study area belongs to the winegrowing region of Vojvodina, winegrowing area (sub-region) of Southern Banat and the winegrowing district Vršačko vinogorje.

Field research and data analysis

Field research in Vršački vinogradi (Figure 1) was conducted on three field sites: 1) Magareći breg (lat. +45.096897, long. +21.345669); 2) Izlaz and Šeribl (lat. +45.100456, long. +21.32049); 3) Kozluk and Majdan (lat. +45.150152, long. +21.353299). Field research was carried out during the 2016 vegetation season (from March to November), once per month, in order to include the spring, summer and autumn vegetation aspects.

A combined weed control system was applied in the study area during the vegetation season of 2016, with mechanical weed control measures applied between rows and chemical weed control being applied in the rows. Given the general paucity of available herbicides registered for weed control in vineyards in Serbia (DZBS, 2018), glyphosate-based herbicides were used for chemical weed control in the studied field sites. It is important to highlight that in 2016 the Vršac region experienced meteorological conditions which strongly favored weed germination and growth. High humidity with frequent rainfalls and temperature fluctuations during the spring were followed by a summer period with extremely high temperatures, with intermittent heat waves and an above average precipitation (Radičević et al., 2016).

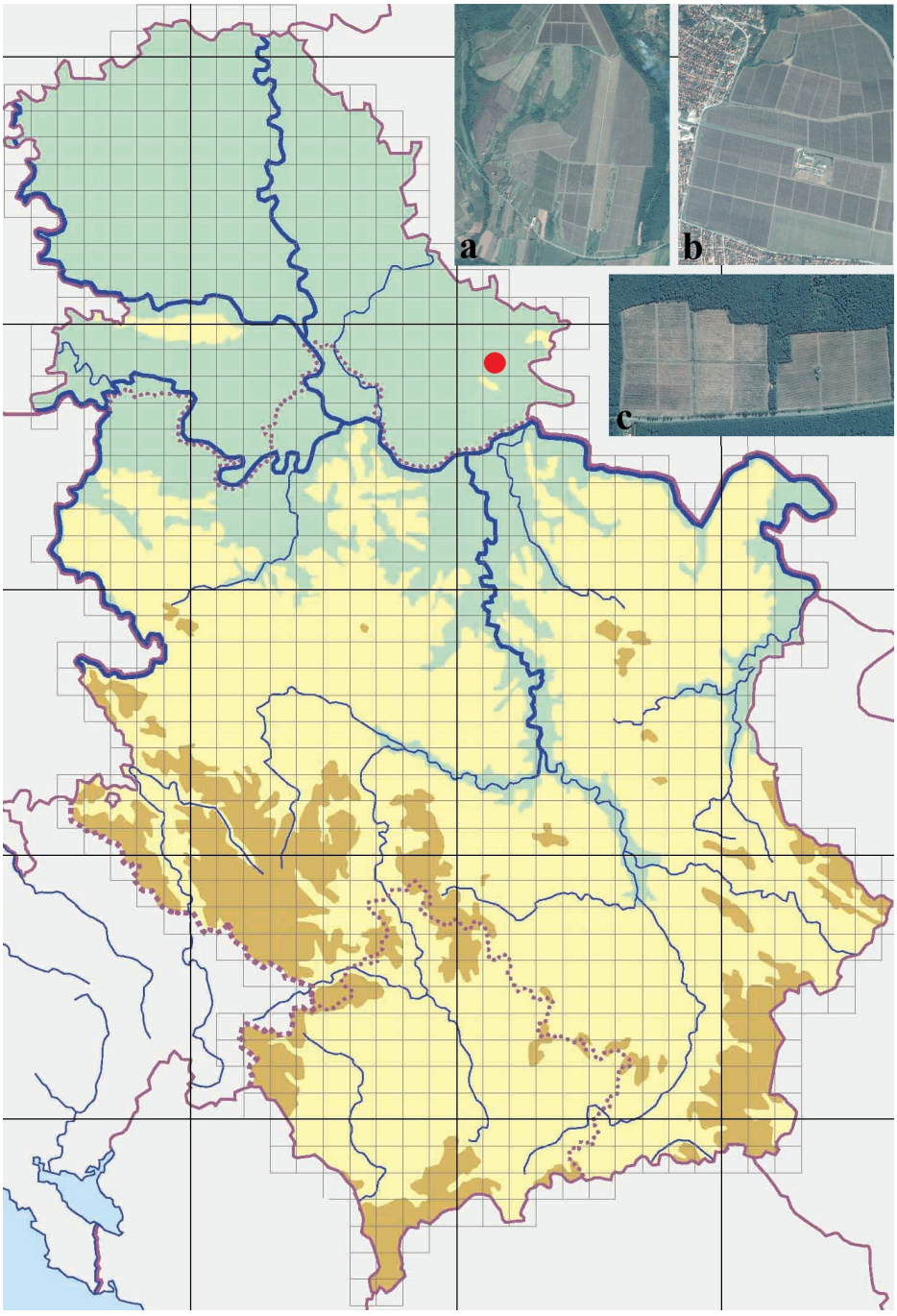

Figure 1. Map of Serbia showing the study area, and satellite images of the three field sites: a) Magareći breg, b) Izlaz and Šeribl, c) Kozluk and Majdan (*source*: Google Earth).

Floristic analysis was carried out at 60 permanent 1 m^2 plots. In each of the three studied field sites, 20 plots were set up (16 plots between the vine rows and four in-row plots). The plant material was identified in the field or collected and then identified in the Laboratory of the Department of Weed Research of the Institute for Plant Protection and Environment, Belgrade. Plants were determined according to Josifović (1970–1977), Tutin et al. (1964, 1968, 1972, 1976, and 1980) and Javorka and Csapody (1975). Taxa nomenclature is in line with the Euro+Med PlantBase (Euro+Med, 2006–2019). Life forms were determined following the Raunkiaer system (Ellenberg and Muller-Dombois, 1976), edited by Stevanović (1992) for the territory of Serbia.

RESULTS

The presence of 97 plant taxa was determined in the studied field sites of the Vršac vineyards during the research period. Recorded species belong to 26 families, with the dominance of Asteraceae (21 species), Poaceae (18) and Fabaceae (12) species.

Analysis of the biological spectrum has shown that weed flora of the studied area has the therophyto-hemicryptophytic character. Therophytes (T) were the dominant life form, followed by a significant presence of hemicryptophytes (H) (Table 1).

Therophytes make up 57.73% of the recorded species (56 species, Table 1). Ratio of the main groups within this life form is given in Table 1, which shows that among the therophytes scapose herbaceous plants (T scap) are the most numerous, making 73.21% of all therophytes. Representatives of annual caespitose life forms (T caesp) make up 8.93% of all the therophytes (four species): *Bromus sterilis* L., *Echinochloa crus-galli* (L.) Beauv, *Hordeum murinum* L. and *Poa annua* L., while representatives of other groups are recorded with a lower number of species (Table 1).

Six species (6.18% of the total number of species, Table 1) belonging to the therophyte-hemicryptophyte transitional life form have also been recorded. Within this T/H group of species, five are biennial scapose plants with no rosette (T/H scap bienn), while one is characterized by the presence of rosette (T/H ros bienn) – *C. bursa-pastoris*.

The therophytes phenology analysis has shown the dominant presence of summer species (a) – 26 species (46.43%), followed by a significant presence of spring-summer (v-a – 16.07%), spring (v – 10.71%) and spring-autumn (v-aut – 10.71%), as well as summer-autumn (a-aut – 8.93%) flowering species (Table 1).

The growth form analysis has shown a significant presence of the medium to large therophytes: Mes-Meg (10 species), Mes-Mac (10 species) and Mes (8 species). Small to medium therophytes (Mi-Mes) were represented by seven species, with the dominance of *Lamium amplexicaule* L. and *L. purpureum* L. The big and tall (Mac-Alt) therophytes were present with six species, while other growth form categories were represented to a lesser degree (Table 1).

Table 1. Biological spectrum of the Vršac vineyards weed flora

life form	morphology	no. spec.	% within the life form	% of the total species number (97)	phenology	no. spec.	% within the life form	% of the total species number (97)	growth form	no. spec.	% within the life form	% of the total species number (97)
therophyte (T)	T scap	41	73.21%	42.27%	a	26	46.43%	26.80%	Mes-Mac	10	17.86%	10.31%
	T/H scap bienn	5	8.93%	5.15%	v-a	9	16.07%	9.28%	Mes-Meg	10	17.86%	10.31%
	T caesp	5	8.93%	5.15%	v-aut	6	10.71%	6.19%	Mes	8	14.29%	8.25%
	T rept	2	3.57%	2.06%	v	6	10.71%	6.19%	Mi-Mes	7	12.50%	7.22%
	ST herb	1	1.79%	1.03%	a-aut	5	8.93%	5.15%	Mac-Alt	6	10.71%	6.19%
	T scap semiros	1	1.79%	1.03%	ver-a	3	5.36%	3.09%	Meg-Alt	4	7.14%	4.12%
	T/H ros bienn	1	1.79%	1.03%	ver	1	1.79%	1.03%	Mac	2	3.57%	2.06%
									Mac-Meg	2	3.57%	2.06%
									Meg	2	3.57%	2.06%
									Mi-Meg	2	3.57%	2.06%
									Mes-Alt	1	1.79%	1.03%
									Mi	1	1.79%	1.03%
									Mi-Mac	1	1.79%	1.03%
total	**therophytes**	**56**	**100%**	**57.73%**		56	100%	57.73%		56	100%	57.73%
hemicryptophyte (H)	H scap	17	51.52%	17.53%	a	19	57.58%	19.59%	Mes-Meg	7	21.21%	7.22%
	H caesp	5	15.15%	5.15%	v-a	9	27.27%	9.28%	Meg-Alt	5	15.15%	5.15%
	H rept	2	6.06%	2.06%	a-aut	2	6.06%	2.06%	Mac-Meg	4	12.12%	4.12%
	H scap bienn	2	6.06%	2.06%	ver-a	2	6.06%	2.06%	Meg	4	12.12%	4.12%
	H ros	2	6.06%	2.06%	v-aut	1	3.03%	1.03%	Mes	4	12.12%	4.12%
	H bienn (T scap)	1	3.03%	1.03%					Mac-Alt	3	9.09%	3.09%
	H scap (T scap)	1	3.03%	1.03%					Mes-Mac	2	6.06%	2.06%
	H scap perenn	1	3.03%	1.03%					Mac	1	3.03%	1.03%
	H scand	1	3.03%	1.03%					Mi-Mac	1	3.03%	1.03%
	SH herb (Hscap)	1	3.03%	1.03%					Mi-Meg	1	3.03%	1.03%
									Mi-Mes	1	3.03%	1.03%
total	**hemicryptophyte**	**33**	**100%**	**34.02%**		33	100%	34.02%		33	100%	34.02%
geophyte (G)	G rhiz caesp	2	40.00%	2.06%	a	4	80.00%	4.12%	Meg-Alt	2	40.00%	2.06%
	G herb rhiz	1	20.00%	1.03%	a-aut	1	20.00%	1.03%	Alt	1	20.00%	1.03%
	G rad	1	20.00%	1.03%					Mac	1	20.00%	1.03%
	G rhiz (H rept)	1	20.00%	1.03%					Mes-Meg	1	20.00%	1.03%
total	**geophyte**	**5**	**100%**	**5.15%**		5	100%	5.15%		**5**	**100%**	**5.15%**
phanerophyta (P)	NP caesp	2	**100%**	**2.06%**	v-a	2	**100%**	**2.06%**	fo dec	2	**100%**	**2.06%**
chamaephyta (Ch)	Ch frut	1	**100%**	**1.03%**	v-a	**1**	**100%**	**1.03%**	Mi-Mes	1	**100%**	**1.03%**

scap – scapose, bienn – biennial, caesp – cespitose, dec – deciduous; fo – forb; rept – creeping, herb – herbaceous, semiros – semirossette, ros – rosette, perenn – perennial, scand – scandetophyta, rhiz – rhizomatous, rad – root, a – summer-flowering, aut – autumn-flowering, v/ver – spring-flowering, Alt – tall, >100 cm, Mac – large, long, Meg – large, robust, Mes – medium, Mi – small.

In the analyzed weed flora, the presence of 33 hemicryptophytes (34.02%) was documented, with the dominance of perennial scapose plants (17 species or 51.52%), Table 1. Within this group species *Achillea millefolium* L., *Cichorium intybus* L., *Galium mollugo* L., *Hypericum perforatum* L., *Rumex crispus* L. and *Sonchus arvensis* L. were the most abundant. Within the hemicryptophyte life forms (Figure 2), the second most represented group (15.15%) were the five perennial caespitose species (H caesp), primarily grasses (fam. Poaceae). Hemicryptophytes with a rosette (H ros), *Plantago lanceolata* L. and *Taraxacum officinale* Weber in Wiggers, creepers (H rept) *Glechoma hederacea* L. and *Ranunculus repens* L. and only one species (*C. sepium*) of the scandetophyte life form (H scan) were also recorded.

When analyzing the phenology of hemicryptophytes, it is evident that plants with a summer flowering period (a) were dominant, with 19 species (57.58%), followed by the group of plants with a spring-summer flowering period (v-a – 27.27%). All other transitional groups (a-aut, v-aut and ver-a) are less represented (Table 1).

Hemicryptophyte growth form analysis has shown a relatively equal presence of individual growth form groups (Table 1). Medium to large (Mes-Meg) growth form group, with 7 species (21.21%) and robust and tall (Meg-Alt) group, with 5 species (15.15%) were characterized by a somewhat higher number of species, when comparing to the others.

The geophytes (G), with five species (5.16%), were the third most represented life form. Four rhizomatous geophytes (G rhiz) and one root-budding geophyte (G rad) were recorded within this life form (Table 1).

Regarding their phenology (Table 1), the recorded geophytes are primarily summer flowering (a – 80%), with the exception of *Sorghum halepense* (L.) Pers. which flowers in the summer-autumn (a-aut) period. Different growth forms are equally represented among the geophytes (Table 1).

Phanerophytes were represented in the study area by two nanophanerophyte species (2.06%, Table 1): *Rosa canina* L. and *Rubus caesius* L., both being deciduous forbs lower than 2 m (life from: fo dec NP caesp) and chamaephytes with only one species – *Thymus vulgaris* L.

DISCUSSION

The recorded diversity of the Vršac vineyards weed flora has doubled, when compared to previous studies in the same locality, conducted 40 years ago by Anđelić (1976) and Šinžar and Živanović (1980), citing the presence of 46 and 35 weed species in the vineyards of the Vršac region, respectively. However, the results of the study are similar to more recent research of vineyards weed flora conducted in Croatia (Dujmović Purgar and Hulina, 2004) and Bosnia and Herzegovina (Kovačević et al., 2015). Therefore, such a discrepancy in weed species numbers in the studied area 40 years ago and today could most likely be a result of different sampling techniques, with previous studies (Anđelić, 1976; Šinžar and Živanović, 1980) possibly sampling a much

smaller study area, or not recording the weed diversity all-year round. Alternatively, it could also result from an increase in weed species numbers, due to consistent disturbances over the past four decades and high propagule pressure from the surrounding agricultural landscape. Given that the recorded diversity has doubled, such a dramatic increase is most likely a result of a combination of both restricted sampling technique in previous studies and an actual increase in weed diversity in the study area over time.

Although hemicryptophytes are best adapted to temperate climate conditions, which many studies have previously confirmed in Serbia (Diklić, 1984; Popović and Obratov-Petković, 2006; Stanković-Kalezić, 2007; Jakovljević et al., 2008; Brković, 2015; Gavrilović, 2016), the obtained results have shown a dominance of therophytes in the vineyard weed flora. This has also been previously observed by Šinžar and Živanović (1980). On the other hand, Dujmović Purgar and Hulina (2004) have recorded a dominance of hemicryptophytes in the vineyards of northwestern Croatia, which is inconsistent with the results obtained. Nevertheless, despite the general prevalent presence of hemicryptophytes in Serbia (Diklić, 1984; Popović and Obratov-Petković, 2006; Stanković-Kalezić, 2007; Jakovljević et al., 2008; Brković, 2015; Gavrilović, 2016) and in Croatia (Dujmović Purgar and Hulina, 2004), our results were expected, as therophyto-hemicryptophytic character of weed flora is also evident in vineyards across the region (Šinžar and Živanović, 1992; Kovačević et al., 2008, 2015; Kovačević, 2013; Rotim, 2016). Furthermore, a similar dominance of annual broadleaf weed species (therophytes), followed by hemicryptophytes, has also been shown to be characteristic for vineyards weed flora in other parts of the world, e.g. in Spain (Buján, 1991; Gago et al., 2007), Czech Republic (Lososová et al., 2003) and North America (Baumgartner et al., 2008; Fredrikson, 2011).

Despite the hemicryptophytic character of the flora of Serbia (Diklić, 1984) and the entire temperate zone (Raunkier, 1934), the documented dominance of therophytes in the weed flora of vineyards is a result of intensive agrotechnical measures (Lososová et al., 2003; Kovačević, 2013; Jelenić, 2015) and microclimatic conditions in vineyards (Asproudi et al., 2016). The prevalence of therophytes is primarily caused by mechanical weed control measures such as soil cultivation – tillage (Lososová et al., 2003; Kovačević, 2013) and therefore frequent ecosystem disturbances (Kovačević, 2013) to which therophytes are well-adapted (Jelenić, 2015). Even though Konstantinović et al. (2012) have concluded that mechanical tillage between the vineyard rows reduces the number of weed species, Lososová et al. (2003) have shown that this management practice actually favors the high proportion of therophytes. Therefore, a high percentage of therophytes which was recorded (57.73%, Table 1) was expected, given that tillage has for years been the main management practice between the grapevine rows in the study area.

In line with the proportion of therophyte groups shown in Table 1, a recent study of the vineyard seedbank (Konstantinović et al., 2012) has also shown a high abundance of seeds of three scapose therophytes (*Portulaca oleracea* L., *A. retroflexus* and *Chenopodium album* L.) in the top (0–10 cm) soil layer. Similarly, the vineyard weed association *Diplotaxis muralis* Kovačević 2013 is

also characterized by absolute dominance (73%) of the T scap life form among the recorded therophytes (Kovačević, 2013), as is the overall vineyard weed flora of the Herzegovina winegrowing region (81.2%; Kovačević et al., 2008). *C. bursa-pastoris* was also the only species of the biennial rosette T/H life form recorded by Kovačević (2013) in the vineyards of the Herzegovina region, which was confirmed in this study.

According to Šinžar and Živanović (1980) a high proportion of hemicryptophytes (and geophytes to some degree) in some vineyards can be correlated with soil types. Namely, their presence is related with more productive soils, which are characteristic for the southern parts of the Vršac region (Vasiljević, 2015). Also, it has been recorded that both hemicryptophytes and geophytes are more abundant in the in-row weed vegetation, while therophytes are more numerous in the spaces between rows (Šinžar and Živanović, 1992), which is expected due to the implementation of different control measures in-row and between the rows during the vegetation season. Also, although *C. sepium* is the only documented species of the scandetophyte hemicryptophytes, its presence is important for vine growers from the phyto pathological point of view as in vineyards *C. sepium* is one of the principal host plants of the cixiid planthopper *Hyalesthes obsoletus* (Langer and Maixner, 2004), which is the main insect vector of the stolbur phytoplasma (Cvrković et al., 2014).

The geophytes were significantly less represented in the study area, compared to therophytes and hemicryptophytes, which was also confirmed by Kovačević (2013) for the vineyards of the Herzegovina region. The results have shown a slight decrease in the number of root geophytes in favor of rhizome geophytes (Table 1), compared to the results of Šinžar and Živanović (1980) in the same study area. A higher incidence of rhizomatous weed species in vineyards can be a consequence of mechanical cultivation practices, which propagate their rhizomes within the field (Fernandez, 2003).

Information pertaining to the phenology of the dominant weed species is relevant for the vine growers, as it enables them to choose appropriate control techniques to achieve good weed control (Gago et al., 2007). The highest proportion of summer flowering plants in the vineyards weed flora was expected, when bearing in mind the climate of the study area. Recent studies have shown that *S. halepense* is one of the most represented summer-autumn flowering weed species in the eastern winegrowing district of Srijem in Croatia (Rac Papak, 2019). Differences in the phenology of the weed life forms between the study area and the vineyards studied in the region of Herzegovina (Kovačević, 2013) reflect the regional uniqueness of the Herzegovina vineyards, primarily related to the climate conditions.

CONCLUSION

Results of the current study have shown that weed flora of the Vršac vineyards is of a therophyto-hemicrypthophytic character, with a strong prevalence of summer flowering medium-large therophyte and tall hemicryptophyte

species. Such results were expected given the common cultivation practices and frequent ecosystem disturbances of these vineyards and should impact the future decision-making of appropriate weed control measures. Seeing how the meteorological conditions during the 2016 vegetation season favored rapid weed development and regrowth, thus making glyphosate-based chemical weed control obsolete in some instances, it would be recommended to include soil-applied herbicides in those vineyards where more persistent weed species are recorded. Soil-applied herbicide which would be appropriate for vineyard application, as it does not affect the grapevine health or the wine quality, while simultaneously efficiently controlling both annual and perennial grass and broadleaf weeds, is flazasulfuron, from the herbicide group of sulphonylureas (Bourdrez and Beraud, 1999). Additionally, its prolonged residual action would enable a fall application of this herbicide to keep the vineyards weed-free for a period of five to eight months. Conversely, if the weather conditions are as favorable for weed growth as they were in 2016, it is possible to also apply it in spring, thus controlling the weed infestation all-year round.

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REFERENCES

Agustí-Brisach C, Gramaje D, León M, García-Jiménez J, Armengol J (2011): Evaluation of vineyard weeds as potential hosts of black-foot and Petri disease pathogens. *Plant Disease* 95: 803–810.

Anđelić R (1976): Neke karakteristike korovske vegetacije Vršačkog vinogorja. Zbornik radova, *Jugoslovenski simpozijum o borbi protiv korova u voćnjacima i vinogradima*, Jugoslovensko društvo za proučavanje i suzbijanje korova, Peć, Jugoslavija, 47–55.

Asproudi A, Petrozziello M, Cavalletto S, Guidoni S (2016): Grape aroma precursors in cv. Nebbiolo as affected by vine microclimate. *Food Chem.* 211: 947–56.

Atanasova B (2015): *Fauna cikada (Hemiptera: Auchenorrhyncha) u vinogradima Makedonije i njihova uloga u epidemiologiji 'Candidatus Phytoplasma solani'*. Doctoral thesis, Faculty of Biology, University of Belgrade.

Avramov L, Nakalamic A, Todorovic N, Petrovic N, Zunic D (2016): Climate of the vineyard zones and the associated vine varieties of Yugoslavia. *J. Agr. Sci.* 45: 29–35.

Baumgartner K, Steenwerth KL, Veilleux L. (2008): Cover-crop systems affect weed communities in a California vineyard. *Weed Sci*. 56: 596–605.

Bourdrez P, Beraud JM (1999): Flazasulfuron: vine herbicide. *Phytoma* 521: 66–68.

Brković D (2015): *Vaskularna flora brdsko-planinskog područja severozapadne Srbije i Šumadije – ekološko fitogeografska studija*. Doctoral thesis, Faculty of Biology, University of Belgrade.

Buján M (1991): *Malas hierbas en viñedos de Galicia.* Doctoral dissertation, University of Santiago de Compostela, Santiago de Compostela.

Cvrković T (2009): *Diverzitet faune cikada u vinogradima Srbije i njihova uloga u prenošenju Bois noir fitoplazme*. Doctoral thesis, Poljoprivredni fakultet, Univerzitet u Beogradu.

Cvrković T, Jović J, Mitrović M, Krstić O, Toševski I (2014): Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of bois noir. *Plant Pathol*. 63: 42–53.
Cvrković T, Jović J, Mitrović M, Krstić O, Krnjajić S, Toševski I (2011): Potential new hemipteran vectors of stolbur phytoplasma in Serbian vineyards. *Bull. Insectol*. 64 (Suppl.): 129–130.
Diklić N (1984): Životne forme biljnih vrsta i biološki spektar flore SR Srbije. U: Sarić M. (ed.), *Vegetacija SR Srbije*, SANU, Posebna izdanja, Beograd, 1: 291–316.
Dujmović Purgar D, Hulina N (2004): Vineyard weed flora in the Jastrebarsko area (NW Croatia). *Acta Bot. Croat*. 63: 113–123.
DZBS – Društvo za zaštitu bilja Srbije (2018): *Pesticidi u poljoprivredi i šumarstvu u Srbiji*. Devetnaesto, izmenjeno i dopunjeno izdanje. Beograd: Društvo za zaštitu bilja Srbije.
Ellenberg H, Muller-Dambois D (1967): A key to Raunkiaer plant life forms with revised subdivisions. *Ber. geobot. Inst*. 37: 56–73.
Euro+Med (2006–2019): Euro+Med PlantBase – The information resource for Euro-Mediterranean plant diversity. Available from: http://ww2.bgbm.org/EuroPlusMed/ [last accessed November 28, 2019].
Fernandez ON (2003): Establishment of *Cynodon dactylon* from stolon and rhizome fragments. *Weed Res*. 43: 130–138.
Filippin L, Jović J, Cvrković T, Forte V, Clair D, Toševski I, Boudon-Padieu E, Borgo M, Angelini E (2009): Molecular characteristics of phytoplasmas associated with Flavescence dorée in clematis and grapevine and preliminary results on the role of *Dictyophara europaea* as a vector. *Plant Pathol*. 58: 826–837.
Fredrikson L (2011): *Effects of cover crop and vineyard floor management on young vine growth, soil moisture, and weeds in an establishing vineyard in the Willamette Valley of Oregon*. MSc Thesis, Oregon State University, Corvallis.
Fredrikson L, Skinkis PA, Peachey E (2011): Cover crop and floor management affect weed coverage and density in an establishing Oregon vineyard. *Horttechnology* 21: 208–216.
Gago P, Cabaleiro C, Garcia J (2007): Preliminary study of the effect of soil management systems on the adventitious flora of a vineyard in northwestern Spain. *Crop Prot*. 26: 584–591.
Gavrilović M (2016): *Ruderalna flora Novog Sada kao potencijalni prirodni resurs lekovitog bilja*. Doctoral thesis, Faculti of Sciences, University of Novi Sad.
Hulina N (1998): *Korovi*. Zagreb: Školska knjiga.
Ivanišević D, Jakšić D (2014): Vinogradarstvo Srbije kroz statistiku i rejonizaciju. *Primena podataka Popisa poljoprivrede*, 113–154.
Jakovljević K, Lakušić D, Vukojčić S, Teofilović A, Jovanović S (2008): Florističke karakteristike Višnjičke kose kraj Beograda. *Arch. Biol. Sci*. 60: 703–712.
Javorka S, Csapody V (1975): *Icanographie der Flora des Sudostlichen Mitteleuropa*. Budapest: Akademiai Kiado.
Jelenić J (2015): Uticaj starosti zasada i mera u suzbijanju korova na zakorovljenost vinograda Belje. *Acta herbol*. 24: 61–76.
Josifović M /ed./ (1970–1986): *Flora SR Srbije*. Beograd: Srpska akademija nauka i umetnosti, Odeljenje prirodno-matematičkih nauka, Vol. I–X.
Konstantinović B, Blagojević M, Samardžić N, Konstantinović B (2012): Banka semena korova u ekstenzivnom gajenju vinove loze. *Acta herbol*. 21: 95–101.
Kovačević Z (2013): Asocijacija *Diplotaxietum muralis* (ass. nova) u korovskoj vegetaciji vinograda rejona Hercegovina. *Agroznanje* 14: 591–606.
Kovačević Z, Kelečević B, Mitrić S (2015): Weed flora of vineyard in Bosnia and Herzegovina. Proceedings, *The 7th Congress on Plant Protection, Plant Protection Society of Serbia*, Belgrade, Serbia, 307–310.
Kovačević Z, Petrović D, Herceg N (2008): The summer aspect of weed flora in the vineyards of Herzegovina. *Herbologia* 9: 9–20.
Langer M, Maixner M (2004): Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP analysis of non-ribosomal DNA. *Vitis* 43: 191–199.
Lloret J, Bosch I, Sendra S, Serrano A (2011): A wireless sensor network for vineyard monitoring that uses image processing. *Sensors* 11: 6165–6196.
Lososová Z, Danihelka J, Chytrý M (2003): Seasonal dynamics and diversity of weed vegetation in tilled and mulched vineyards. *Biologia* 58: 49–57.

OIV – International Organization of Vine and Wine (2019): Statistical report for Serbia. Available from: http://www.oiv.int/en/statistiques/?year=2017&countryCode=SRB [last accessed on November 06, 2019].
Papp B, Sabovljević M (2010): Contribution to the bryophyte flora of the Vršačke Planine Mts., Serbia. *Bot. Serb.* 34: 107–110.
Popović I, Obratov-Petković D (2006): Analiza biološkog spektra flore Divčibara. *Glasn. Šumarsk. Fak.* 93: 143–154.
PSUZŽS – Pokrajinski sekretarijat za urbanizam i zaštitu životne sredine (2018): Prostorni plan područja posebne namene predela „Vršačke planine". Novi Sad: JP Zavod za urbanizam Vojvodine.
Rac Papak M (2019): *Zaštita vinove loze u vinogradima srednje škole Ilok u 2019. godini*. Fakultet agrobiotehničkih znanosti Osijek, Sveučilište Josipa Jurja Strossmayera, Osijek (masters thesis).
Radičević Z, Džingalašević Lj, Bojović J, Milakara S, Radević S (2016): *Agrometeorološki uslovi u proizvodnoj 2015/2016. godini na teritoriji Republike Srbije*. Beograd: Republički hidrometeorološki zavod, Odeljenje za primenjenu klimatologiju i agrometeorologiju.
Raunkiaer C (1934). *The life forms of plants and statistical plant geography; being the collected papers of C. Raunkiaer.* Oxford: Clarendon Press.
Rotim N (2016): Suzbijanje korova u vinogradima. *Glasnik zaštite bilja* 39: 80–85.
Saayman D, Van Huyssteen L (1983): Preliminary studies on the effect of a permanent cover crop and root pruning on an irrigated Colombar vineyard. *SAJEV*, 4: 7–12.
Stanković-Kalezić R (2007): Taksonomska i ekološka analiza ruderalne flore na području Pančevačkog rita. *Acta herb.* 20: 1–13.
Steenwerth KL, Belina KM (2010): Vineyard weed management practices influence nitrate leaching and nitrous oxide emissions. *Agric. Ecosyst. Environ.* 138: 127–131.
Stevanović V (1992): Klasifikacija životnih formi flore Srbije. In: Sarić M. (ed.), *Flora Srbije*, I, (2ed.), Beograd: SANU.
Šinžar B, Živanović M (1980): Životni oblici korovskih zajednica voćnjaka i vinograda u Vojvodini. *Zbornik referata (Proceedings) / Prvi kongres o korovima (First congress on weeds)*, Banja Koviljača, 12–14. VI 1980, 137–145.
Šinžar B, Živanović M (1992): Proučavanje korovske vegetacije vinograda reona Fruška Gora. *Zbornik referata (Proceedings) / Prvi kongres o korovima (First congress on weeds)*, Banja Koviljača, 12–14. VI 1980, 264–272.
Tutin TG, Heywood VH, Burges NA, Charter AO, Edmondson JR, Moore DM, Valentine DH, Walters MS, Webb DA, Akeroyd JR, Newton ME (1964): *Flora Europaea*, I: Lycopodiaceae to Platanaceae. Cambridge: Cambridge University Press.
Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters MS, Webb DA, Ball PW, Chater AO, Ferguson IK (1968): *Flora Europaea*, Vol. 2: Rosaceae to Umbelliferae. Cambridge: Cambridge University Press.
Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters MS, Webb DA, Ball PW, Chater AO, Ferguson IK, DeFilipps RA, Richardson IBK (1972): *Flora Europaea*, Vol. 3: Diapensiaceae to Myoporaceae. Cambridge: Cambridge University Press.
Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters MS, Webb DA, Chater AO, DeFilipps RA, Richardson IBK (1976): *Flora Europaea*, Vol. 4: Plantaginaceae to Compositae (and Rubiaceae). Cambridge: Cambridge University Press.
Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters MS, Webb DA, Ball PW, Chater AO, Richardson IBK (1980): *Flora Europaea*, Vol. 5: Alismataceae to Orchidaceae. Cambridge: Cambridge University Press.
Živković J (2014): *Strategija razvoja turističke destinacije opštine Vršac*, Beograd: Departman za Poslovne sisteme u turizmu i hotelijerstvu, Univerzitet Singidunum (Master thesis).
Vasiljević Đ (2015): *Geodiverzitet i geonasleđe Vojvodine u funkciji zaštite i turizma*. Faculti of Sciences, University of Novi Sad (doctoral thesis).

БИОЛОШКИ СПЕКТАР КОРОВСКЕ ФЛОРЕ ВРШАЧКИХ ВИНОГРАДА (СРБИЈА)

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РЕЗИМЕ: Агротехничке мере представљају основни фактор који одређује структуру и састав коровске флоре у виноградима. Адекватне мере сузбијања корова у исто време обезбеђују добро одржавање винограда, чиме се осигурава добро здравствено стање винове лозе и висок квалитет вина. Имајући у виду да биолошки спектар корова утиче на ефикасност мера контроле, циљ овог истраживања био је да се утврде биолошка својства коровске флоре Вршачких винограда, анализом доминантних животних форми корова и њихове фенологије. Флористичка анализа вршена је током вегетацијске сезоне (у периоду март–новембар) 2016. године, на 60 трајних огледних парцела величине 1 m^2 на три локалитета на подручју Вршачких винограда. Утврђено је присуство 97 врста, у оквиру 26 различитих фамилија. Анализа биолошког спектра показала је да је коровска флора винограда истраживаног подручја терофитско-хемикриптофитског карактера (терофите: 57,73% и хемикриптофите: 34,02%). У оквиру представника животних форми терофита и хемикриптофита, доминантно су заступљене вишегодишње зељасте биљке са стабљиком (T scap), док је у погледу фенолошке динамике највећи број врста које цветају током лета. Резултати до којих се дошло у склопу овог истраживања указују на свеукупно виши диверзитет корова, у поређењу са ранијим истраживањима коровске флоре винограда истог истраживаног подручја. Међутим, приказани резултати су у складу са резултатима новијих истраживања диверзитета коровске флоре винограда спроведених у земљама у региону. Такође, доминантан удео терофита у коровској флори винограда био је очекиван, имајући у виду примарне механичке мере контроле које се у виноградима традиционално примењују.

КЉУЧНЕ РЕЧИ: биолошки спектар, виноград, Вршачки виногради, животна форма, корови, фенолошка динамика

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PROGRAM *R* IN MAPPING OF PROTECTED NATURAL ASSETS IN SERBIA**

ABSTRACT: This paper deals with the tasks of creating maps of protected natural assets (national parks and natural monuments – individual plant specimens) on several case examples in Serbia. The main goal of the paper is to introduce the application of the *R* program in diverse mapping assignments. Even though the R program is commonly used for numerical and statistical analysis, its application in mapping assignments is still relatively new and not widely known. Therefore, this paper aims to promote the use of R in mapping and to present the appearance of maps that can be obtained as a result. The selected examples are shown on different spatial scales, starting from the mapping of national parks on a map of Serbia, continuing with the mapping of a single national park in a narrow spatial context, ending with the mapping of individual protected plants. The same procedure can be repeated for the similar mapping assignments in the future. Creating maps is an integral part of defining adaptive management strategies for protected natural assets, and therefore can greatly impact the process of monitoring and conducting conservation measures for *in situ* plant protection activities.

KEYWORDS: *R* program, mapping, national parks, natural monuments

INTRODUCTION

Protecting plant species can be assisted with three main categories of activities: the measures of legal protection, *in-situ* protection, and *ex-situ* protection (Vujić, 2008). Legal protection is based on implementing different activities defined by legal documents, *in situ* protection implies the protection of species on the spot, while *ex-situ* protection means protecting species away from their natural habitats. Examples of *in situ* protection are national parks, natural monuments, nature reserves, protected landscapes, etc. To define a proper management strategy for a certain protected area, one needs to conduct a comprehensive analysis, including different mapping assignments. Creating

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** BRIEF COMMUNICATION

maps can be done in many computer programs, and among them GIS tools and R are most commonly applied nowadays (Lakicevic et al., 2020b). The main aim of this research is to present and analyze possibilities for mapping the protected natural assets in the R program. For that matter, in this research there were several case examples of protected natural assets in Serbia selected for the procedure of mapping, including both protected areas (natural parks) and protected plant individuals (natural monuments).

Creating maps in R is supported by many packages, starting from the ones that do not require high fluency in R (for example the R package "leaflet") up to more advanced ones that will be shown in this paper (the R packages "ggmap" and "ggplot2"). Mapping of protected areas in R begins with gathering the input spatial data, first of all – geographic coordinates (longitude and latitude) for a specific protected area or protected plant individual. These data are introduced into the R environment and afterwards plotted on different base maps. Once the maps are created, the spatial data can be used for further analysis necessary for the process of defining appropriate management strategies.

In addition to that, the program R provides a valuable support for applying the multi-criteria analysis methods, such as AHP or PROMETHEE, which enables the process of evaluating management strategies and selecting the most appropriate one (Lakićević and Srđević, 2017). Therefore, the program R, aside from mapping, can also be used for numerical analysis and applying multi-criteria analysis methods in the protection of natural assets.

Regarding GIS tools valuable and commonly applied in mapping assignments, this paper points out several advantages of using the R program. One of the advantages is related to the fact that R is a programming language and therefore supports writing and running different codes and the users can write themselves, which gives this program a great flexibility. The other advantage of the R program is the existence of different packages that contain spatial data related to plant species, for example, the package "rgbif". The main disadvantage of the R program is the requirement for, at least, medium proficiency in programming. This being said means that both GIS and R are expected to be intensively applied in the future.

METHODS

For the mapping purposes we have used the R program (version 3.5.1), its interface RStudio (version 1.1.463), and two R packages "ggmap" (Wickham, 2016) and "ggplot2" (Kahle and Wickham, 2013). The mapping assignments were: creating a map presenting national parks in Serbia, creating a map of the national park "Fruška gora", and creating maps of protected plant individuals in Sremski Karlovci. These examples were selected to demonstrate the appearance of maps on different spatial scales. Table 1 contains the input data necessary for plotting the map of national parks in Serbia. The base map for plotting was the plain map of Serbia, and the locations of national parks are displayed as dots.

Table 1. National parks in Serbia

National park	Longitude – λ	Latitude – φ
NP "Fruška gora"	19.65295	45.16670
NP "Đerdap"	21.97583	44.52889
NP "Tara"	19.46583	43.84778
NP "Kopaonik"	20.83333	43.31667
NP "Šar planina"	20.92472	42.20250

For creating a map presenting the boundaries of the national park "Fruška Gora", the coordinates were imported in R from Google Earth Pro (382 pairs of coordinates), and the base map was a satellite map. For creating the maps presenting protected plant individuals in Sremski Karlovci, input data are presented in Table 2.

Table 2. Protected plant individuals in Sremski Karlovci, Serbia

Botanical name	Longitude – λ	Latitude – φ	Protected area [a]
Aesculus hippocastanum L.	19.94025	45.19914	7.5
Morus nigra L.	19.94696	45.19667	2.3
Platanus x acerifolia (Aiton) Willd.	19.93598	45.20214	16.2
Pterocarya fraxinifolia (Poiret) Spach	19.93176	45.20424	3.5
Taxus baccata L. (a, b)	19.93369	45.20222	7.8
Taxus baccata L. (c)	19.93433	45.20361	5.7

Using the data presented in Table 2, two maps have been created. The first one displays the spatial disposition of protected plant individuals on the satellite map, and the second one presents one additional characteristic – the size of the protected area on the so-called "toner" map. The same procedure can be repeated in the future, for diverse assignments related to the mapping of protected areas or protected plant individuals.

It should be noted that the mapping assignments presented in this paper could be done by using the GIS tools, but the goal of this paper was to demonstrate the appearance of maps that have been created by applying the R program. The next section presents the maps created in R but also discusses the possibilities for its application in similar mapping assignments in landscape management and ecology.

RESULTS AND DISCUSSION

Using the package "ggplot2", Figure 1 has been created, previewing the location of national parks in Serbia.

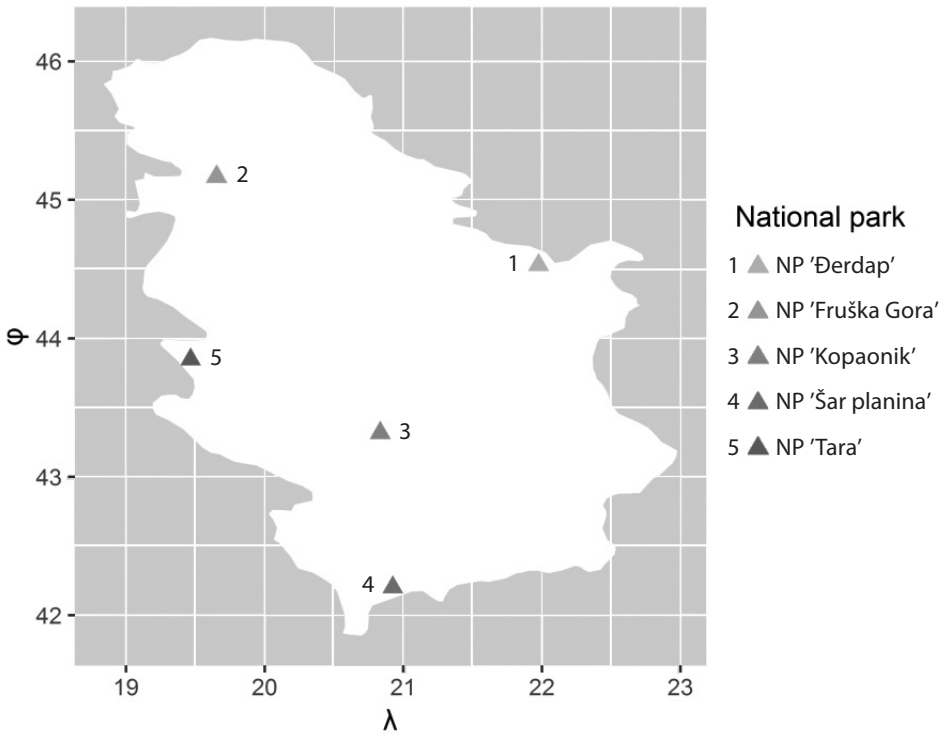

Figure 1. National parks in Serbia

Figure 1 shows the locations of national parks on the plain map of Serbia, in the grid that specifies the value of longitude and latitude, as reference points. For a more detailed preview of the boundaries of national parks, one needs more precise input co-ordinates data, and for example, the map can be plotted on the satellite base map using the package "ggmap". The example for displaying boundaries of a national park is shown in Figure 2, previewing the boundaries for the national park "Fruška Gora".

Figure 2 shows the boundaries of a national park placed in the geographic co-ordinates grid, but if the mapping assignment assigns the mapping of individual plant species, one should work on a different spatial scale, in a more narrow spatial context. For mapping individual plants, Figure 3 has been created, using the "ggmap" package and the satellite base map.

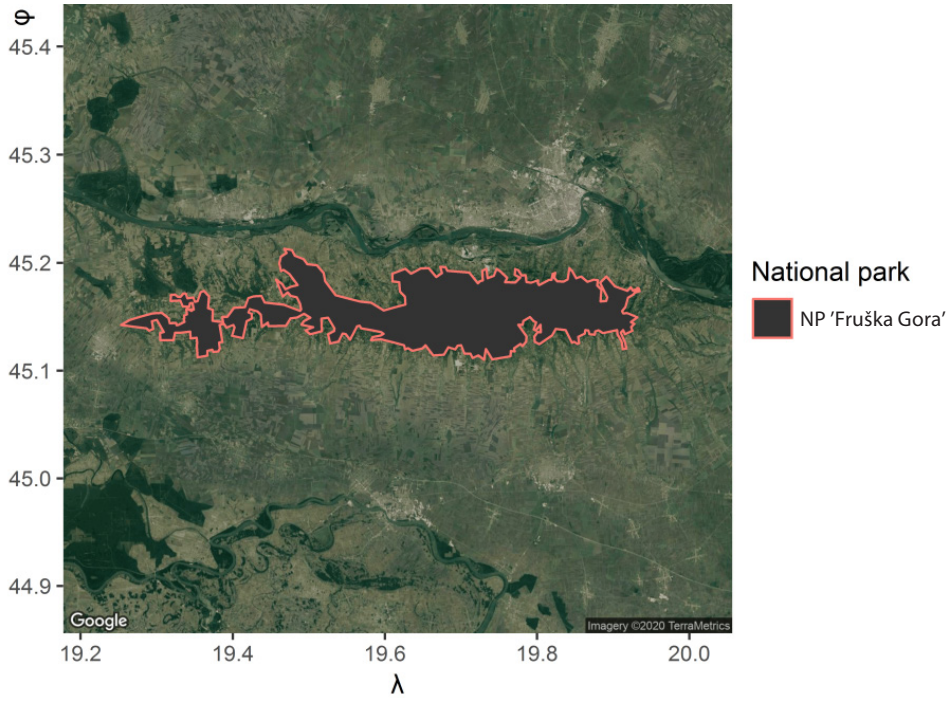

Figure 2. National park "Fruška Gora"

Figure 3. Protected plant individuals in Sremski Karlovci

Figure 3 previews the spatial disposition of protected plant individuals in Sremski Karlovci. As a reference, this map can be compared with the map from the previous research when ArcMap was used as a mapping tool for the same case study example (see Lakićević et al., 2020a). Figure 3 is a basic map and can be upgraded by introducing additional characteristics such as protection status, the size of a protected area, the height of a plant, etc., and that way thematic maps are being created. In this research, we added one characteristic – the size of the protected area to the basic map. A new, thematic map is plotted on the "toner" base map and is presented in Figure 4.

Figure 4 shows spatial disposition and the size of protected plant individuals, divided into three categories. The size of the symbol was defined by the program itself and does not correspond with the actual size of a protected area, because the actual scale of the size of protected plants would be too small for a clear and visible graphic presentation. Instead, the size of symbols operates in the relative ratio and serves for a comparison of the size of the protected area for different plant specimens.

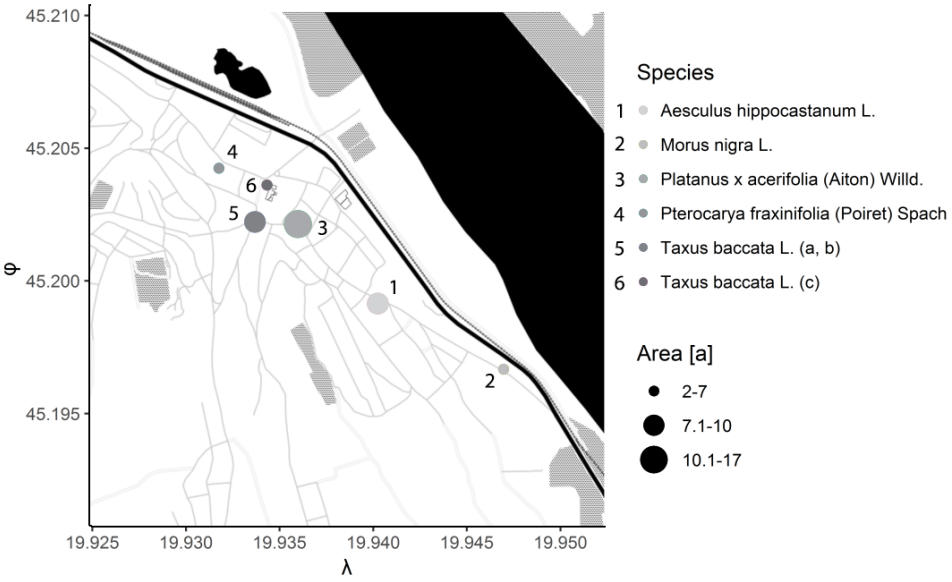

Figure 4. Protected plant individuals in Sremski Karlovci

In this paper, all mapping assignments included importing the spatial data, i.e. coordinates into the R environment and then plotting these on different base maps. As Figure 4 shows, the R program enables mapping procedure taking into account certain conditions or criteria (in here that was the size of the protected area). One of the main strengths of the R program is an easy procedure of filtering and merging large sets of data that can be printed on the map, further on. This feature is essential for creating thematic maps. In addition to that, it

should be noted that there are R packages already containing data related to plant species, for example, "rbgif" or "rredlist". The "rbgif" package contains diverse spatial data related to plant species occurrence (Chamberlain and Boettiger, 2017) which enables an easy procedure of creating horology maps (Lakićević et al., 2018). The "rredlist" package gathers data related to endangered species in accordance with the IUCN Red List (Chamberlain, 2017), and the data extracted from this package can be used for further mapping and spatial analysis. The existence of these "ready-to-use" R packages is the main advantage of R in comparison to GIS tools.

CONCLUSIONS

This paper presents the application of the R program for several mapping assignments related to the *in situ* protection of plant species. When creating maps in R, there are two main possibilities: importing spatial data into the R environment or extracting spatial data from the existing R packages, such as: "rbgif" or "rredlist". Both options provide high-quality maps, and this paper focuses on creating maps by importing spatial data. The selected examples show the procedure of mapping on different spatial scales, and the same concept can be repeated for similar mapping assignments. Even though this paper focuses on the application of R in mapping assignments, the recommendation for future research would be to apply R for multi-criteria assessment of management plans for a protected area, and maps that are previously created in R can serve as a valuable input data.

REFERENCES

Chamberlain S (2017): *rredlist: 'IUCN' Red List Client*. R package version 0.4.0. Available to: https://CRAN.R-project.org/package=rredlist

Chamberlain S, Boettiger C (2017): R Pyton, and Ruby clients for GBIF species occurrence data. *PeerJ. PrePrints*. 5:e3304v1.

Kahle D, Wickham H (2013): ggmap: Spatial Visualization with ggplot2. *The R Journal*. 5: 144–161.

Lakićević M, Mladenović E, Hiel K, Pavlović L (2018): Program R u kartiranju areala biljnih vrsta, *Topola* 201–202: 91–97.

Lakićević M, Orlović S, Mladenović E, Pavlović L, Mlađenović M, Pavlović B (2020a): Mapping of protected areas in Sremski Karlovci. *Ann. Agron*. 44: 26–31.

Lakicevic M, Povak N, Reynolds KM (2020b): *Introduction to R for Terrestrial Ecology*. Springer, Basel.

Lakićević M, Srđević B (2017): Multiplicative version of PROMETHEE method in assessment of parks in Novi Sad. *Matica Srpska J. Nat. Sci.* 132: 79–86.

Vujić A (2008): *Zaštita prirode*. Univerzitet u Novom Sadu, Prirodno-matematički fakultet, Novi Sad.

Wickham H (2016): *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag, New York.

ПРОГРАМ *R* У МАПИРАЊУ ЗАШТИЋЕНИХ ПРИРОДНИХ ДОБАРА У СРБИЈИ

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РЕЗИМЕ: Рад се бави питањем мапирања заштићених природних добара (националних паркова и споменика природе – појединачних биљних индивидуа) на неколико примера у Србији. Главни циљ рада је представљање примене програма R у различитим задацима у вези с мапирањем. Иако се програм R уобичајено користи за нумеричку и статистичку анализу, његова примена у области мапирања је и даље нова и није широко позната. У складу с тим, рад истиче значај примене програма R у мапирању и приказује изглед мапа које се добијају као резултат. Одабрани примери су приказани у различитим размерама: почевши од мапирања националних паркова на карти Србије, затим мапирања националног парка у ужем просторном контексту, и на крају, мапирања појединачних биљних индивидуа. Исти поступак може бити примењен за сличне задатке у будућим истраживањима. Мапирање је неизоставни део дефинисања адаптивних стратегија управљања заштићеним природним добрима и има значајну улогу у поступцима праћења стања и спровођења мера *in situ* заштите биљних врста.

КЉУЧНЕ РЕЧИ: програм *R*, мапирање, национални парк, споменик природе

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Reviewers should alert the Editor to any well-founded suspicions or the knowledge of possible violations of ethical standards by the authors. Reviewers should recognize relevant published works that have not been cited by the authors and alert the Editor to substantial similarities between a reviewed manuscript and any manuscript published or under consideration for publication elsewhere, in the event they are aware of such. Reviewers should also alert the Editor to a parallel submission of the same paper to another journal, in the event they are aware of such.

Reviewers must not have conflict of interest with respect to the research, the authors and/or the funding sources for the research. If such conflicts exist, the reviewers must report them to the Editor without delay.

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Anyone may inform the editors and/or Editorial Staff at any time of suspected unethical behavior or any type of misconduct by giving the necessary information/evidence to start an investigation.

Investigation

• Editor-in-Chief will consult with the Subject Editors on decisions regarding the initiation of an investigation.

• During an investigation, any evidence should be treated as strictly confidential and only made available to those strictly involved in investigating.

• The accused will always be given the chance to respond to any charges made against them.

• If it is judged at the end of the investigation that misconduct has occurred, then it will be classified as either minor or serious.

Minor misconduct

Minor misconduct will be dealt directly with those involved without involving any other parties, e.g.:

• Communicating to authors/reviewers whenever a minor issue involving misunderstanding or misapplication of academic standards has occurred.

• A warning letter to an author or reviewer regarding fairly minor misconduct.

Major misconduct

The Editor-in-Chief, in consultation with the Subject, and, when appropriate, further consultation with a small group of experts should make any decision regarding the course of action to be taken using the evidence available. The possible outcomes are as follows (these can be used separately or jointly):

• Publication of a formal announcement or editorial describing the misconduct.

• Informing the author's (or reviewer's) head of department or employer of any misconduct by means of a formal letter.

• The formal, announced retraction of publications from the journal in accordance with the Retraction Policy (see below).

• A ban on submissions from an individual for a defined period.

• Referring a case to a professional organization or legal authority for further investigation and action.

When dealing with unethical behavior, the Editorial Staff will rely on the guidelines and recommendations provided by the Committee on Publication Ethics (COPE).

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Главни и одговорни уредник / Editor-in-Chief

IVANA MAKSIMOVIĆ

INSTRUCTION TO AUTHORS

1. General remarks

1.1. Matica Srpska Journal for Natural Sciences (short title: Matica Srpska J. Nat. Sci.) publishes manuscripts and review articles as well as brief communications from all scientific fields as referred to in the title of the journal. Review articles are published only when solicited by the editorial board of the journal. Manuscripts that have already been published in extenso or in parts or have been submitted for publication to other journal will not be accepted. The journal is issued twice a year.

1.2. The manuscripts should be written in correct English language regarding the grammar and style. The manuscripts should be submitted electronically as a separate file to vnikolic@maticasrpska.org.rs and enclosed with the author's written consent for the publishing of the manuscript.

1.3. Upon the reception of the manuscript, the author shall be assigned with a manuscript code, which has to be referred to in any further correspondence. The authors will be notified about the manuscript reception within seven days and about the reviewers' opinion within two months from submission. All submitted manuscripts are reviewed and proofread.

2. Planning and preparing of the manuscript

2.1. Type the manuscripts electronically on A4 (21 × 29.5 cm) format with 2.5 cm margins, first line indent, and 1.5 line spacing. When writing the text, the authors should use Times New Roman size 12 font and when writing the abstract, key words, summary, and footnotes use font size 10.

2.2. First name, middle initial and last name should be given for all authors of the manuscript and their institutional affiliations, institution name, and mailing address. In complex organizations, a full hierarchy should be mentioned (e.g. University of Novi Sad, Faculty of Sciences – Department of Biology and Ecology). The institution of employment of each author should be stated below the author's name. The position and academic degrees should not be cited. If there is more than one author, indicate separately institutional affiliation for each of the authors. Put the name and mailing address (postal or e-mail address) of the author responsible for correspondence at the bottom of the first page. If there is more than one author, write the address of only one author, usually the first one.

2.3. Structure the text of the original articles into Abstract, Key Words, Introduction, Material or Methods, or Material and Methods, Results or Results and Discussion, Discussion, Conclusion, References, Summary and Key Words in Serbian language, and Acknowledgement (if there is one). Original articles should not be longer than 10 pages, including the references, tables, legends, and figures.

2.4. Titles should be informative and not longer than 10 words. It is in the best interest of the authors and the journal to use words in titles suitable for indexing and electronic searching of the article.

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(if the article has more than one author, et al. should be used for other authors) and running title of not more than five words.

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3. Structure the Review articles in Abstract, Key Words, Text of the manuscript, Conclusion, and References; submit Summary and Key Words in Serbian language. Review articles should not be longer than 12 pages, including references, tables, legends, and figures.

4. Write brief communication according to the instructions for original articles but not be longer than five pages.

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5.1. List the References alphabetically. Examples:

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(b) Chapters in the book: Last name ED, Last name AS, Last name IP (2011): Title of the pertinent part from the book. In: Last name CA, last name IF (eds.), Title of the book, Vol.4, Publisher, City

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6.1. SI units of measurement (Système international d'unités) should be used but when necessary use other officially accepted units.

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