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ORIGINAL SCIENTIFIC ARTICLE

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ECOLOGICAL ASPECTS OF PEAT, STRAW, AND WOOD ASH APPLICATION FOR ENERGY WILLOW CULTIVATION

ABSTRACT: The utilization of ash obtained as a result of the combustion of fossil fuels (coal, peat) or biomass (straw, wood, solid waste) is an environmental problem that should be optimally solved. The chemical characteristics of ash depend on several factors, mostly on sources of fuel. According to characteristics, ash can be used in agriculture, forestry, or utilized for other purposes. The content of heavy metals (Cd, Ni, Pb, Cr) in peat ash is several times higher than in willow wood ash and straw ash. It means that peat ash application is limited to agricultural crops and its optimal application is one year before planting SRC trees, especially on poor and acid soils. The application of peat ash at a dose of 10 mg dry mass ha^{-1} in willow plantations on post-mining peaty soils changed soil acidity from 5.2 pH to 5.88 pH and stimulated tree growth. Wood and straw ash was applied to willow plantations on arable loam-sandy soils in doses 0.5, 1.0, and 1.5 t dry mass ha^{-1}. The positive effect of ash application in doses 1.0 and 1.5 t dry mass ha^{-1} showed in the second year after the application both for soil and for willow growth.

KEYWORDS**:** bioenergy, willow plantations, peat, straw, wood ash, application

INTRODUCTION

Ash is generated as a result of the combustion of fossil fuels (coal, peat) or biomass (straw, wood, solid waste) for energy production. The utilization of ash, considering its volume and chemical characteristics, is an environmental problem that should be optimally solved. For example, approximately 600,000 tons of wood, peat, and mixed ash are generated in Finland annually as a by-product of energy production (Huotari, 2012). In the nearest future, the ash

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volume might be increased. According to the prognoses of the European Energy Agency (EEA), units of solid biomass for energy production increased from 9.8 Mtoe (megatons of oil equivalent) in 2015 to 13.3 Mtoe in 2020 (Beurskens et al., 2011).

The chemical characteristics of ash depend on several factors, mainly on the sources of fuel.

Fly ash (FA), a result of the combustion of coal at high temperatures, has been regarded as a problematic solid waste all over the world (Jala and Goyal, 2006). However, fly ash is a useful ameliorant that may improve the physical, chemical, and biological properties of problem soils and represents a source of available macro- and micronutrients for plants. In conjunction with organic manure and microbial inoculants, fly ash can enhance plant biomass production from degraded soils. The problem is the high content of chemicals and especially heavy metals in fly coal ash. Fly ash is generally alkaline and contains many toxic metals like Cr, Pb, Hg, As, Cd, and other hazardous metals (Pandey et. al., 2009). Nevertheless, numerous studies revealed that the lower FA incorporation in soil modifies the physical, chemical, biological, and nutritional quality of the soil. However, the higher dosage of FA incorporation results in heavy metal pollution and hinders microbial activity (Pandey and Singh, 2010). As a rule, fly ash has good potential for use in the construction industry. The conversion of fly ash into zeolites has many applications such as ion exchange, molecular sieves, and adsorbents (Ahmaruzzaman, 2010).

Peat, as a fuel for energy production, is used in Finland, Russia, Belarus, and other countries (Moilanen, 2012; Kundas et al., 2015; Rodzkin, 2017). According to statistical data collected by the International Peat Society, energy peat production in Europe in 1999 was 21.5 million tons of air-dried peat. Finland was a leading energy peat producer in terms of volume with around 7.5 million tons. The second was Ireland with 4.7 million tons and the third was the Russian Federation with the production of 3.7 million tons. Belarus, Sweden, and Estonia followed as the next largest producers (Sopo, 2001). As a rule, peat ash is used in forestry, but not in agriculture because of high content of chemicals, and its application has a positive effect on the soil (Nieminen, 2005).

Wood ash is generated in enormous quantities by wood industries and power plants. Several studies have been carried out on the utilization of wood ash in agriculture and forestry (Demeyer et al., 2001; Etiegni et al., 1991; Clarholm, 1994). The properties of wood ash depend on various factors: type of plant, part of plant combusted (bark, wood, leaves), type of waste (wood, pulp, or paper residue), combination with other fuel sources, type of soil and climate, and conditions of combustion, collection, and storage (Zimmermann and Frey, 2002; Voundi Nkana et al., 1998). It appeared that an increase in microbial activity in soil treated with wood ash was accompanied by an increase in the growth rate of soil microorganisms. Wood ash contains all essential nutrients for tree growth, except nitrogen. Therefore, wood ash is an ideal fertilizer for nitrogen-rich peatland forests where a lack of phosphorus or potassium can reduce tree growth (Liimatainen et al., 2017). According to results, fertilization with ash also increases CO_2 emissions of the peat significantly (Traunfeld and Nibali, 2013).

The big potential for bioenergy has cereal straw that occupies the biggest part of arable lands in European countries. In Europe, the leader in using straw for energy is Denmark. The annual utilization of straw for energy in the country is about 1.3–1.4 million tons (Nikolaisen, 1998; Evald, 2011). Ash straw application has a positive effect on soil fertility (Shu et al., 2016; Schiemenz and Eichler-Lobermann, 2010). It was concluded that recycling rice-straw ash had positive effects on soil, which could improve the saturated water-holding capacity of the soil, pH value, the content of available potassium and the content of available phosphorus had little effect on the content of soil organic matter. As the result of ash straw application, special attention was paid to phosphorus (P) availability in soil (Schiemenz and Eichler-Lobermann, 2010). Straw ash can be successfully used both for forestry and agriculture (Sander and Andren, 1997). So, the composition of 79 samples of straw ash from seven heating plants in Sweden was analyzed to evaluate straw ash as a fertilizer and liming agent. Ash from rape straw had a higher Ca content and liming effect compared with ash from cereal straw.

The presented facts give an overview of the big potential of ash application as fertilizer. Nevertheless, the application of ash, especially peat and wood ash, might be problematic for agricultural crops, because of its chemical contents. The prospective direction for ash application is fertilizing of short rotation coppice plantations (trees). The biomass from the plantations can be used for energy purposes, so there are no limitations for heavy metals and other chemicals contents in wood. The climate conditions in the Republic of Belarus, like in other countries of Eastern and Central Europe and North America, are more adapted for willow growing. Bushy *Salix* species with erect stems, rapid growth, and good rooting ability are the most suitable for biomass coppice, with *Salix viminalis* being one of the most widely used species (Ahman and Larsson, 1994). A characteristic of willow, which makes it a very suitable tree for energy, is that it can be frequently harvested by coppicing and yielding as much as 10–15 dry t ha^{-1} $year^{-1}$ (Riddell-Black, 1993). In addition to high biomass productivity, *Salix* trees also have an effective nutrient uptake, high evapotranspiration rate, and a pronounced clone-specific capacity for heavy metal uptake. In Europe, short rotation coppice (SRC) plantations cover an estimated area of 50,000–70,000 ha, with about 12,000 in Sweden and 10,000 in Italy and Hungary. The species most frequently used for energy production are poplars and willows, followed by black locusts (*Robinia pseudoacacia*) and eucalypts (*Eucalyptus spp.*) in Mediterranean areas (Facciottoet al., 2014). Willow biomass is a low-maintenance crop that stimulates rural economies and enhances the environment. A willow plantation can be used for 20–25 years with a period of harvesting every three years (Buchholz and Volk, 2013).

The increase in the number of energy plantations resulted in the generation of huge amounts of tree ash. The optimal utilization of this ash is both an environmental and economic problem. The goal of our investigation was the assessment of the efficiency of ash (wood, straw, and peat) application for short rotation coppice willow production.

MATERIAL AND METHODS

Two experimental fields were established. Experimental field 1 (EF1) was located near Lida Peat Factory (LPF) in Lida, Grodno region, western Belarus. The four experimental plots were laid out on the soil formed after peat extraction. This is badly-drained and low productive soil with the following characteristics: contents of peat ash – 9%; NO_3 – 79.40 mg/kg; P_2O_5 – 20.25 mg/kg; K_2O – 106.40 mg/kg; peat decomposition – 65% and pH – 5.20. The peat ash on EF1 was applied a year before willow planting with a dose of 10 t dry mass ha^{-1}.

Experimental field 2 (EF2) was located at Volma Field Station in Dzerginsk, Minsk region, the central part of Belarus. The soil at the site is sandy-loam with a relatively high base status and with a base saturation of 60–70%. It is well-drained and productive agricultural soil with the following characteristics: contents of P_2O_5 – 60.2 mg/kg; K_2O – 148.5 mg/kg, humus – 2.2%, and pH – 5.6. Willow trees were cutting in the next year after planting in accordance with technology. The ash of straw and wood in EF2 is applied by inter-row application with doses 0.5; 1.0 and 1.5 t dry mass ha^{-1}.

The plots were arranged in a randomized complete block design with 4 blocks. Each elementary plot was 7 m long and 7.2 m wide (50 m^2) and contained 4 double rows of plants. The clone Jorr of *Salix viminalis* was planted.

Peat ash was collected from the storage of LPF, wood ash, and straw ash from the storage of Volma Field Station. Concentrations of K, Ca, Mg, and heavy metals in ash were determined by the X-ray fluorescence method (RFA). The morphological characteristics of willow plants were determined every two weeks during the vegetation season. Woody biomass production (t ha^{-1}) was obtained from harvests of the spacing plots.

A completely randomized design was used to compare elemental concentrations of applied wood ash and straw ash. The experimental data were processed using statistical programs Excel, Statistica 22, and Sigma Plot 11.2.

RESULTS AND DISCUSSION

Ash analyses

By Belarus standards, it is possible to use straw ash for agricultural crops, but peat ash and wood ash can be used in the construction industry or taken to the landfill. There are no regulatory standards for SRC (Short rotation coppice) plantations in Belarus. Nevertheless, the content of chemicals, especially heavy metals in the ash is the key factor for its application. The average results of chemicals content in peat (2009–2010), straw, and willow wood (2011–2013) ash are presented in Table 1. The results show that the contents of Cr, Pb, Cd, and Ni in peat ash are several times higher than in straw and wood ash, but the content of K can be compared with wood ash and Ca with straw ash.

It means that peat ash has a good potential for soil reclamation, but high contents of heavy metals can be a problem for the cultivation of regular agricultural crops.

Table 1. Peat, wood, and straw ash chemical properties

Element	Content in ash, mg/kg					
	Peat	Willow		Straw		
		wood	bark	barley	ray	wheat
Zn	115.3	198.7	186.5	83.2	147.7	61.5
Cu	55.2	154.7	16.9	18.2	34.2	20,2
Cr	25.4	4.6	6.7	11.7	9.2	8.7
Ni	22.5	3.7	2.1	10.9	1.3	7.3
Cd	3.2	0.5	0.1	0.8	0.1	1.1
Pb	12.4	2.4	1.1	16.5	3.8	4.6
K	25230	37002	56693	120912	177171	131963
Ca	59550	195603	293205	97323	81931	99588

For example, in Finland, the utilization of ash as fertilizer is regulated by the Fertilizer Product Act (539/2006) and related decrees (Ministry of Agriculture and Forestry Decree 24/11) (Huotari, 2012). The decree specifies the maximum concentrations allowed for harmful heavy metals: the cadmium concentration of ash, for example, may not exceed 25 mg/kg. In Denmark, the content of cadmium in the ash cannot be more than 0.8 mg/kg, in Switzerland – 3 mg/kg (Nikolaisen, 1998). Nevertheless, peat ash contains plant nutrients, e.g. phosphorus, K, Ca, Mg, and thus it is a potential fertilizer in forestry (Moilanen et al., 2012). It is optimal to use peat ash for fertilizing not arable lands, for example, peat post-mining soils (Huotari, 2011). This type of soil is characterized by a bad structure and fertility, a high level of acidity, and, as a result, poor vegetation cover after finishing peat mining. The application of peat ash improves soil fertility and has a good potential for forestry, including SRC trees. It will not cause environmental problems and pollution of trees, but stimulate the forestation in the area (Huotari, 2011; Moilanen et al., 2012). We came to the same conclusions in our experiment. In our experiment, the wood ash is characterized by greater content of Ca, Cu, Zn, and less content of K compared to straw ash. Similar results were reported by other researchers (Olanders and Steenari, 1995).

Peat ash application on EF1

One of the main problems of peaty soils is their extra high acidity. The optimal condition for the cultivation of most agricultural crops is a neutral level of acidity. The optimal acidity for willow is pH 5–6 (Baum et al., 2002;

Wielgolaski, 2001). As the result of peat ash application, the acidity of peat post-mining soils in our experiment changed from pH 5.20 to pH 5.88. It had a positive effect on the growth of all willow species (Table 2), regardless of their origin: *Salix viminalis, Salix alba*, and *Salix dasyclados.* The same results related to peaty soils were reported for other trees, like birch or pine (Lumme, 1988; Silfverberg and Moilanen, 2008).

Peat ash application on EF2

The experiment with the application of straw and wood ash was conducted on regular farms on arable lands by inter-row application. For this reason, the doses of peat application were several times smaller if compared to peat soils. The application of straw and wood ash in agriculture is regulated. For instance, in Finland there is the "Ministerial order concerning the use of ash from gasification and combustion of biomass and biomass waste for agricultural purposes" – colloquially called the Bio Ash Order with strict rules established to outline how much ash can be spread per hectare (Silfverberg and Moilanen, 2008). Further, we tested the application of the same doses also for potato production (Rodzkin et al., 2014).

Table 2. Morphological parameters and productivity of first-year willow on peat post-mining soils after peat ash application

Clone	Variant	Height of stem, cm	Number of stems per plant	Size of the stem, mm	Willow biomass, g/plant
S. viminalis 330	Ash appl.	184.4	3.8	15.2	248.9
	Control	152.3	3.7	12.1	212.43
S. viminalis. Jorr	Ash appl.	176.1	3.7	13.5	241.6
	Control	135.5	3.5	10.6	205.11
S. alba 1/10	Ash appl.	196.2	3.5	16.4	259.3
	Control	161.4	3.6	12.3	201.45
S. dasyclados 1/47	Ash appl.	211.2	4.2	16.0	267.9
	Control	177.7	3.9	13.1	223.16
LCD_{05}		26.52	0.77	2.42	35.29

The original acidity of the soil was 5.6 pH. After wood ash application it accordingly changed to 5.6, 5.7, and 5.8 with doses of ash 0.5, 1.0, and 1.5 t dry mass ha^{-1}. And after straw ash application pH was 5.6, 5.6, and 5.7 with the same doses. As a result, the ash had a positive effect on willow growing, especially in the second year after application (Table 3).

Table 3. Influence of different doses of wood and straw ash application on morphological parameters of willow clone Volmianka *Salix alba*

Variant	Year	Morphological parameters		
		Height of stem cm	Diameter of stem mm	Number of stems per plant
Control	2012	222.2	12.8	3.2
	2013	405.3	25.2	-
(+0.5 wood ash)	2012	230.1	13.2	3.2
	2013	413.5	26.3	-
(+0.5 straw ash)	2012	227.5	13.4	3.2
	2013	416.6	26.4	-
(+1.0 wood ash)	2012	228.3	13.6	3.3
	2013	428.3	28.4	-
(+1.0 straw ash)	2012	231.4	14.0	3.5
	2013	429.2	29.2	
(+1.5 wood ash)	2012	224.5	13.6	3.3
	2013	434.5	29.3	-
(+1.5 straw ash)	2012	230.7	14.0	3.5
	2013	436.1	32.2	-
LCD_{05}	2012	8.9	0.45	0.35
	2013	19.1	1.74	

In our experiment, the ash had a positive effect with dose application 1.0 and 1.5 mg dry mass ha^{-1} (Parka et al., 2005; Rodzkin et al., 2018; Rodzkin et al., 2019). Another study reported the positive effect of wood ash application on the soil acidity and the size of willow stems, but not on biomass production because the mean number of stems was significantly smaller in the ash-treated plots than in the control plots (Lazdina et al., 2011). The possible reason is nutrient deficiency. On N-rich sites (low C:N ratio), wood ash can increase tree growth and the mineralization and leaching of N in the soil. On N-poor sites (high C:N ratio), N can be immobilized and growth decreased by the application of wood ash. The positive effect of wood ash application on willow and for other trees was also mentioned in other reports (Haveraaen, 2014).

There was no significant difference between straw and wood peat application for willow trees in our experiments. The positive effect of straw ash on soil properties and agricultural perspectives was also reported in several studies (Pitman, 2001).

According to our results and the results of other researchers, the matrix for assessment of potential strength of different types of ash application was created (Table 4).

Table 4. The competitive relevant strength of peat, straw, and wood ash application

Type of ash	Relevant strength when used as		
	Soil melioration	Fertilizer for SRC trees	Fertilizer for agricultural crops
Peat	+++[1]	+	- – -[2]
Wood	++	+++	++
Straw	+	++	++

[1]+++ Maximal strength
[2]- – - Minimal strength

Peat ash has the best competitive capacity for soil melioration. It is more reasonable to use it for this purpose because peat ash contains a high level of several heavy metals like Ni, Cd, Pb, and Cr (Table 3). As a result of it and in accordance with permitted standards, it is not possible to use peat ash for agricultural crops fertilization. It can be probably used as fertilizer for SRC trees, but the dozes of ash application should be controlled. Wood ash has a better capacity for soil melioration when compared to straw ash. It can be explained by higher contents of potassium (Table 4). The results show that the morphological parameters of willows after wood ash application were better than after straw ash application (Table 3). Willow plantations should be close to power plants for wood utilization. It is reasonable to plant willow trees not far from the power plants. Close but not maximal capacities of wood and straw ash application for agricultural crops are defined by contents of heavy metals. It is necessary to control dozes of ash application depending on the content of heavy metals in it and the type of crops.

CONCLUSION

There are two aspects of ash utilization. The first is a large volume of ash generated from fossil fuels and biofuel. Ash volume a has tendency to grow in the future. The second aspect is the potential benefit for soil and plants as the result of ash application. It is necessary to share sources of ash following its chemical contents. Peat ash includes elements useful for soil and plants (Ca, K, Cu, Zn) and heavy metals (Cd, Ni, Pb, Cr) that can pollute the environment. The contents of heavy metals in peat ash are several times higher if compared to willow wood ash and straw ash. It means that peat ash application to arable soils and agricultural crops is limited by legislation. The optimal approach is the application of peat ash to SRC trees, especially on poor and acid soils. In that case, it is possible and necessary to use high doses of peat ash, because it contains several times less Ca compared to wood ash and several times less K compared to straw ash. The peat ash application to soil melioration has a double benefit. Because of its heavy metals content, peat ash cannot be used as fertilizer for agricultural crops. The application of peat as fertilizer for decreasing of acidity of peaty and other soils in a year before crops planting is more

effective. The problem of the high content of heavy metals in the ash can be decreased if it is not applied to agricultural crops, but to trees for energy and other purposes. In our experiments, peat ash application to willow plantations on soils after peat extraction had a positive effect both on soil acidity and the morphological parameters of trees.

Contents of heavy metals in willow wood ash and straw ash were several times lower if compared to peat ash. It can be used as fertilizer for SRC trees and other broad row crops by inter-row application. Of course, the dozes of ash application should be controlled depending on crops. Wood ash has a better capacity for soil melioration compared to straw ash because of the higher contents of potassium. Our experiments show that morphological parameters of willows after wood ash applications were better if compared to straw ash application but without significant differences between variants.

Based on our experiments and the results of other researchers it is possible to ascertain a big potential of ash as fertilizer for agriculture and forestry. Nevertheless, several problems should be solved for the broader application of this method. The additional research of heavy metals and other chemicals accumulation in ash depending on properties of different types of soils should be carried out. Some issues concerning the logistic of ash application should be also investigated.

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ЕКОЛОШКИ АСПЕКТИ ПРИМЕНЕ ПЕПЕЛА ТРЕСЕТА, СЛАМЕ И ДРВЕТА ЗА ЕНЕРГЕТСКИ УЗГОЈ ПЛАНТАЦИЈА ВРБА

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РЕЗИМЕ: Коришћење пепела који настаје као резултат сагоревања фосилних горива (угаљ, тресет) или биомасе (слама, дрво, чврсти отпад) је еколошки проблем који треба решавати оптималним методама. Хемијске карактеристике пепела зависе од више фактора, а највише од извора горива. У складу са карактеристикама пепео се може користити у пољопривреди, шумарству или у друге сврхе. Садржај тешких метала (Cd, Ni, Pb, Cr) у пепелу тресета неколико пута је већи у поређењу с пепелом дрвета врбе и пепелом од сламе. То значи да је примена пепела тресета ограничена за пољопривредне културе, а оптималан приступ је његова примена годину дана пре садње плантација, посебно на сиромашним и киселим земљиштима. Применом тресетног пепела у дози од 10 t суве масе ha^{-1} за плантаже врбе постављене на тресетним земљиштима променила је киселост земљишта са 5,2 pH на 5,88 pH и стимулисала раст дрвећа. Пепео од дрвета и сламе примењен је за засаде врбе на обрадивим иловасто-песковитим земљиштима у дозама 0,5; 1,0 и 1,5 t суве масе ha^{-1}. Позитиван ефекат примене пепела са дозама од 1,0 и 1,5 t суве масе ha^{-1} у другој години након примене показао се како за земљишта тако и за раст врбе.

КЉУЧНЕ РЕЧИ: биоенергија, плантаже врба, тресет, слама, дрво, пепео

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ORIGINAL SCIENTIFIC ARTICLE

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FIRST REPORT OF *Fusarium tricinctum* ON NARROW-LEAVED ASH (*Fraxinus angustifolia* Vahl.) IN SERBIA

SUMMARY: Monitoring the health status of narrow-leaved ash tree seedlings (forest office Morović, locality Vinična) in the early spring of 2015, after the catastrophic floods in May 2014, revealed presence of large dark necrotic areas on 1–2 year old sprouts. The isolation of the fungal pathogen was done by standard phytopathological protocols. Three representative isolates (K41, K42 and K78) were preliminary detected and purified by a single-spore technique for further morphological, molecular analyses and pathogenicity testing. Morphological characteristics classified the isolates as *Fusarium tricinctum.* Tested isolates on narrow leaved ash sprouts caused reddish brown elongated necrotic lesions averaged 20.1 mm. Two marker genes, translation elongation factor 1-alpha (*TEF1-α*) and internal transcribed spacer (*ITS1*), were used in this study. Using the Basic Local Alignment Search Tool (BLAST) searching engine, nucleotide sequences were compared to all related sequences. Alignement score resulted in 98.9% identities with *F. tricinctum* for isolate K78, while isolates K41 and K42 showed 94.1% and 94.3% identities with *F. tricinctum* complex respectively. To the best of our knowledge, this is the first report of *F. tricinctum* pathogen infection on flood stressed narrow-leaved ash trees in Serbia.

KEYWORDS: sprout necrosis, plain forests, floods

INTRODUCTION

Narrow-leaved ash (*Fraxinus angustifolia* Vahl) is one of the most important tree species of lowland floodplain forests in Europe with great ecological and economic importance, due to its rapid development and valuable wood

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(Drvodelić et al., 2016). In Central Europe, the Pannonian Basin and Balkans, narrow-leaved ash occurs mainly in the lowlands, in riparian and floodplain forests along large rivers, where it used to form vast and continuous populations, now with more limited extent. Narrow-leaved ash forests, in the area (southwest part of Srem, Vojvodina, Serbia) of the river Sava lower course, are the most valuable and the best preserved forests of this species in Serbia (Bobinac et al., 2010). They form pure and mixed forests (mainly with common oak), which are conditioned by additional moisture – flood or high levels of underground water (Cvijetićanin et al., 2014).

Over recent years, young plantations, tree seedlings and stands of narrow-leaved ash are endangered by the fungus *Hymenoscyphus fraxineus* (Marković et al., 2016). This fungus is named as a main factor of narrow-leaved ash decline in neighbouring countries (Milotić et al., 2016) and throughout Europe (Kowalski and Holdenrieder, 2009). Furthermore, Kranjec et al. (2017) reported about the possible role of *Pythium* and *Phytopythium* soil borne species in declining of narrow-leaved ash forests in Croatia.

However, forest stands decline is caused by synergy of biotic and abiotic factors. Air pollution, flooding in the vegetation period and the absence of regular winter and spring floods, coupled with consecutive dry periods, are crucial stress factors that exert an adverse impact on narrow-leaved ash forests (Tikvić et al., 2008).

The region of Southwest Srem was exposed to the catastrophic floods by the Sava River during the May 2014. Next year, in the early spring, narrow-leaved ash tree seedlings (aged 4-7 years) were examined in forest office Morović at the locality Vinična (sect. 11 and 16) in a course of routine monitoring of their health status. Beside the necrotic lesions typical for *H. fraxineus,* large dark necrotic areas with bark lesions were observed on some one and two year old sprouts. The aim of this study was to identify the causal agent of these lesions and to test its pathogenicity.

MATERIAL AND METHOD

Isolation of fungi and morphological characterization

Symptomatic branches were collected and ten cuttings of belonging tissue were surface disinfected with 2% sodium hypochlorite solution for 5 min, rinsed three times in sterile distilled water, air dried on sterilized filter paper and plated on potato dextrose agar (PDA) and water agar (WA) amended with streptomycin sulphate. After seven days incubation in the dark at 25 °C, three representative isolates (K41, K42 and K78) were chosen for further analyses and purified by a single-spore technique (Leslie and Summerell, 2006). Colony characteristics of single spore isolates were evaluated on PDA media 14 days after the incubation in the dark at 25 °C. Shape and size of microconidia; size and septation of macroconidia and presence of chlamydospores were observed in two weeks on isolates grown on WA media under the black light. Macroconidia

and microconidia were photographed and measured in a light microscope (DM 1000 LED, equipped with a camera MC190HD and LAS V4.9-imaginig and analysis software, Leica Germany). Per each isolate 100 macroconidia and 50 microconidia were measured and standard deviation was calculated using using software *STATISTICA*, ver. 13.2 (Dell, Inc., USA).

Molecular identification

DNA from the seven-day-old *F. tricinctum* isolates (K41, K42 and K78) used for inoculation as well was extracted using the cetyltrimethylammonium bromide (CTAB) protocol (Permingeat et al., 1998). To confirm morphological characterization of the isolates, several fragments were amplified and sequenced.

IGS rDNA region for the K41 and K42 isolates was amplified using species specific primer pair tri1 (5' CGT GTC CCT CTG TAC AGC TTT GA 3') and tri2 (5' GTG GTT ACC TCC CGA TAC TCT A 3') Kulik (2008). The PCR was carried out in 25 µl volumes containing 40 ng DNA, 1x TaqBuffer (containing KCl, Thermo-Fisher Scientific), 0.5 µM each of forward and reverse primers, 0.2 mM of each nucleotide, 2 mM of $MgCl_2$ and 1.25 units of TaqDNA Polymerase (Thermo-Fisher Scientific). The amplification protocol was as follows: initial denaturation for 5 min at 94 ºC, followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 65 ºC for 30 s and elongation at 72 ºC for 20 s, followed by a final elongation step at 72 ºC for 5 min.

The internal transcribed spacer (ITS) region amplified and sequenced with primers ITS1 (5'CTT GGT CAT TTA GAG GAA GTA A3') and ITS4 (5'TCC TCC GCT TAT TGA TAT GC3') (White et al., 1990). The PCR was carried out in 25 µl volumes containing 30 ng DNA, 1x TaqBuffer (Thermo-Fisher Scientific), 0.2 µM each of forward and reverse primers, 0.2 mM of each nucleotide, 1.5 mM of $MgCl_2$ and 1.20 units of TaqDNA Polymerase (Thermo-Fisher Scientific). Amplification protocol was following: initial denaturation for 5 min at 97 °C, followed by 37 cycles with 94 °C for 30 s, 60 °C for 60 s and 72 °C for 45 s. Final elongation was at 72 °C for 5 min.

Translation elongation factor 1α (TEF1-α) gen for the isolates K41 and K42 were amplified using EF1728F (5'CAT CGA GAA GTT CGA GAA GG3') and EF1986R (5'TAC TTG AAG GAA CCC TTA CC3') primers (Rehner and Buckley, 2005). The PCR was carried out in 25 µl volumes containing 30 ng DNA, 1x TaqBuffer (Thermo-Fisher Scientific), 0.2 µM each of forward and reverse primers, 0.15 mM of each nucleotide, 1.5 mM of $MgCl_2$ and 1.20 units of TaqDNA Polymerase (Thermo-Fisher Scientific). Amplification was done according to the protocol: initial denaturation for 1 min at 97 °C, followed by 36 cycles with 96 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s. Final elongation was at 72 °C for 2 min.

PCR products were separated on a 1.5% agarose gel in 1x TBE buffer, stained with ethidium bromide, and visualized under UV light. The amplification products were purified with QIAEX II Gel Extraction Kit (Qiagen, USA) and sent for sequencing to Macrogen Inc. (Macrogen Europe B.V., the Netherlands).

Pathogenicity test

Pathogenicity tests were conducted on one-year-old narrow-leaved ash sprouts, disinfected with 70% ethanol. Mycelial plugs taken from actively growing PDA colonies of *F. tricinctum* isolates were applied in shallow wounds (0.5 cm in diameter) made by scalpel. Sterile agar plugs were placed in the wounds of a control sprouts. Inoculation was performed on four sprouts per isolate and control respectively, with two cuts per sprout. Inoculated cuts were separately wrapped with moist sterile cotton and covered by aluminium foil to prevent drying. The inoculated branches were planted in plastic containers filled with sterile ground in the open field to incubate for three weeks (April, 2017).

Statistical analysis

The obtained data were analysed using Statistica 13.2 (Dell Inc., USA). The results for pathogenicity were tested by analysis of variance followed by a comparison of means by the Bonferroni test ($P < 0.01$).

RESULTS AND DISCUSSION

Naturally infected sprouts had two types of symptoms. Elongated-diamond shaped lesions brown with discoloration in the bark typical for *C. fraxinea* (Barić et al., 2012) and dark elongated necrotic almost black areas (Figure 1). From these dark necrotic areas *Fusarium* species were isolated.

Figure 1. Symptoms of narrow-leaved ash branches resulting from natural infection

Isolates K 41, K42 and K78 on PDA were growing rapidly and formed abundant dense mycelia that were initially white, but with age became red with yellowish fragments. It also exuded red pigments in the agar (Figure 2).

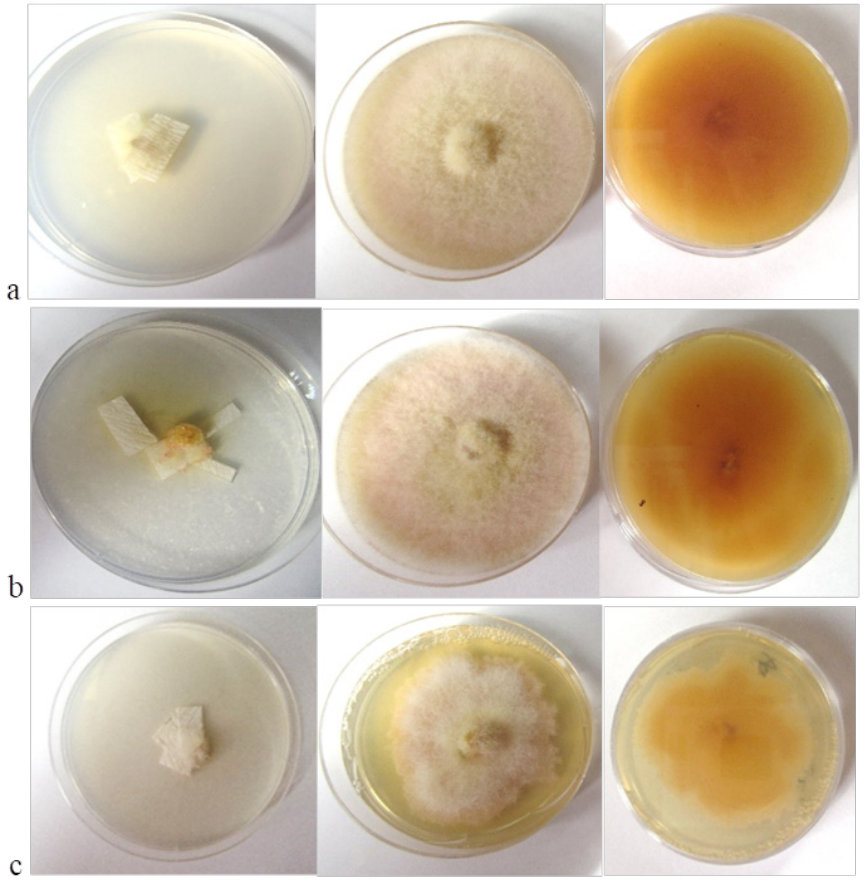


Figure 2. Colony morphology of the isolates a) K41, b) K42, c) K78 on WA and PDA media

On WA medium isolate K-78 formed 3–5 septate macroconidia with curved tapering apical cell and an obviously foot-shaped basal cell. Microconidia were oval, usually non-septate, but occasionally with one septa. Microconidia were clustered on monophialides in small heads. Napiform conidia were rare (Figure 3c). Chlamydospores were absent. Isolates K41 and K42 isolates sporulated on WA medium only after two weeks period under black light. Isolate K41 formed orange sporodochia with the clusters of macroconidia. Macroconidia of isolate K41 were the most slender and did not form microconidia. On the other hand, macroconidia of isolate K42 were sickle shaped, elongated, one-septate microconidia (Figure 3 a, b). The dimensions of different types of conidia are given in the Table 1.

Table 1. Dimensions of macro and microconidia of tested isolates

isolate	conidia type	average dimension±SD* (μm)	min-max dimension (μm)
K 41	macrocinida	45.81 ± 0.79 x 3.17 ± 0.05	(23.4–63.7) x (2.2–4.4)
K 42	macrocinida	52.35 ± 0.89 x 3.36 ± 0.05	(28.8–69.4) x (2.3–4.9)
K 42	microcinida (1 cell)	12.5 ± 0.91 x 2.8 ± 0.4	(11.5–13.7) x (2.3–3,2)
K 42	microcinida (2 cell)	21.95 ± 4.76 x 3.04 ± 0,29	(15.0–26.9) x (2.5–3.3)
K 78	macrocinida	35,24 ± 0,81 x 3,65 ± 0,08	(21.3–52.6) x (1,8–6.2)
K 78	microcinida (1 cell)	12.3 ± 2.8 x 2.7 ± 0.7	(8.5–20.6) x (1.7–5.4)
K 78	microcinida (2 cell)	16.3 ± 2.7 x 3.0 ± 0.7	(9.0–21.3) x (2.2–5.4)

*SD standard deviation

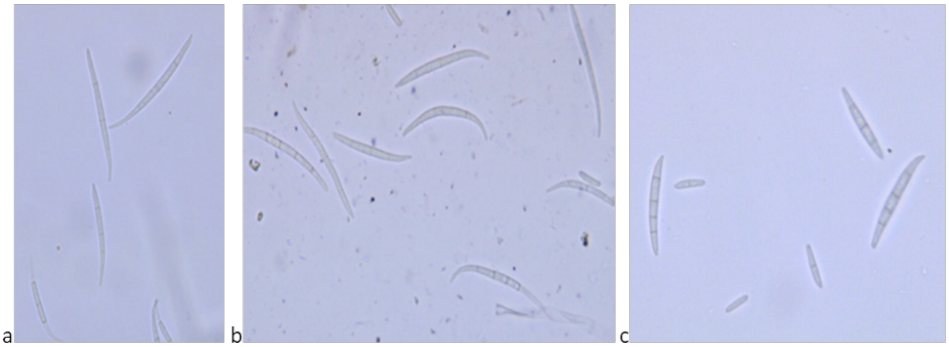


Figure 3. Macro and microconidia of *Fusarium* isolates: a) K-41, b) K-42, c) K-78

Observed morphological characteristics indicated that isolates belonged to *F. tricinctum* (Leslie and Summerell, 2006; Lević, 2008) species which was confirmed by molecular tools.

Based on a BLAST search of the FUSARIUM-ID nucleotide database, the IGS sequences for isolates K41 and K42 (submission no. MZ749901 and MZ749902 respectively) matched with sequence FD_01725_EF-1a (*F. tricinctum* complex) 94.1% and 94.3%, respectively. Based on a BLAST search of the NCBI nucleotide database, the ITS sequence of isolate K78 (GenBank MK928426.1) had 100% identity with *F. tricinctum* strain ZMXR6 (MT446111.1). The TEF1-α sequence of the isolate K78 (MN822227.1) had 98.9% identity with *F. tricinctum* isolate SPF003 (MG704914.1). This was the best match of the obtained isolates with those from the GenBank. Although Kulik (2008) reported that primers specific for *F. tricinctum* amplified aplicons of *F. acuminatum* and *F. nurragi*, the specificity of the primers, in this case, cannot be disputed because molecular identification confirmed the morphological findings.

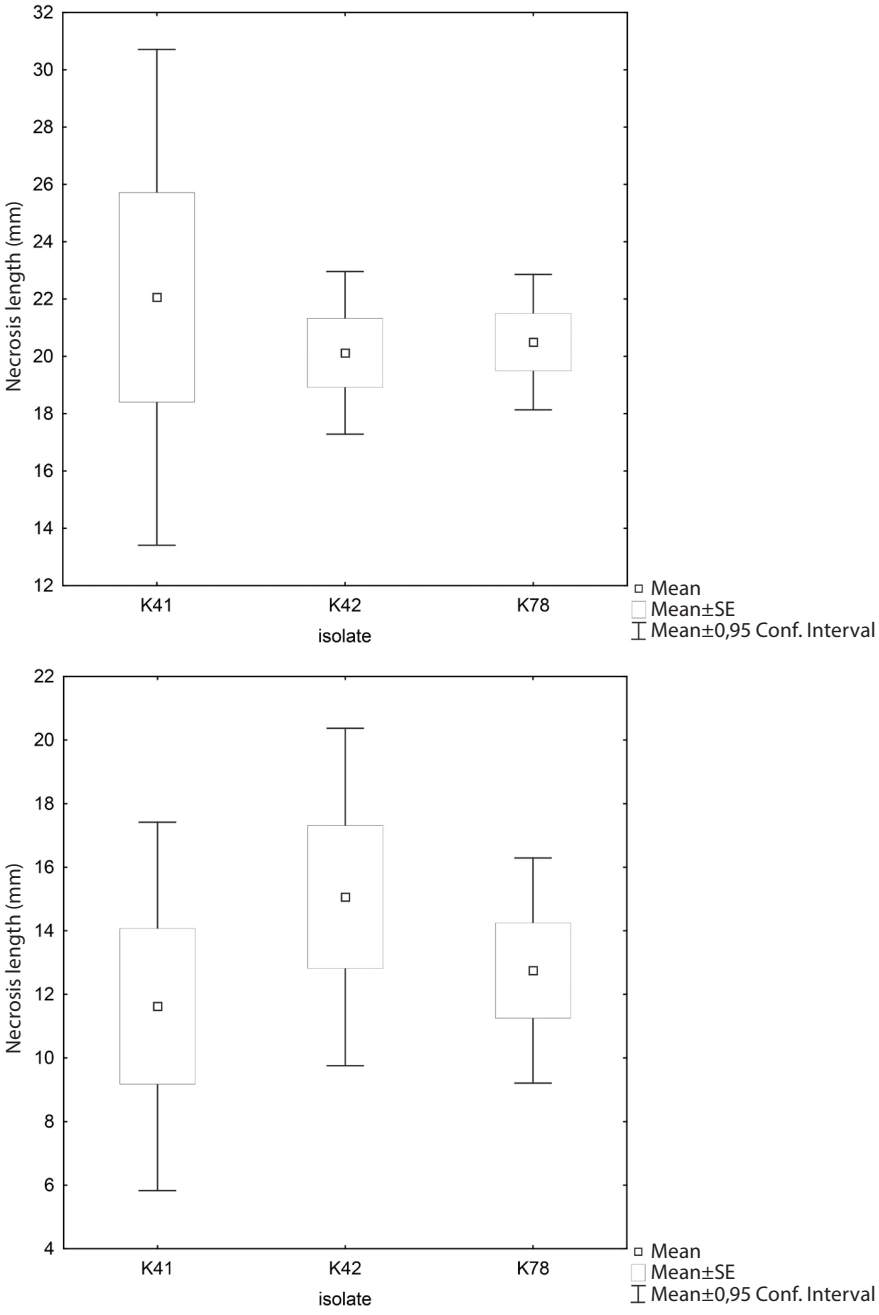
F. tricinctum is worldwide distributed species which usually occurs as a saprophyte or a weak parasite in temperate regions (Leslie and Summerel, 2006). However, recent studies confirmed this species as a causal agent of disease in many agricultural plants such as wheat crown rot (Shikur et al., 2018), pink rot of

onion bulbs (Carrieri et al., 2013), postharvest fruit rot of pumpkin (Aktaruzzaman et al., 2018), and also causal agents of root rots in soybean (Chitrampalam and Nelson, 2014) and alfalfa (Jiang et al., 2020). Taking in the account woody species *F. tricinctum* proved to be weakly virulent or non-virulent to pine seedlings inhibited root growth, but in the greenhouse tests caused small necrotic wounds, which did not differ significantly in size from those observed in the control seedlings (Davydenko et al., 2018). The pathogenicity test was performed in order to check the capability of the isolates to cause the symptoms in the bark. All tested isolates caused symptoms on narrow-leaved ash sprouts in the form of elongated oval shaped lesions with reddish brown border (Figure 4).

Figure 4. Symptoms on sprouts inoculated by *F. tricinctum*: a) K42 isolate, b) K78 isolate

The lesion length and width ranged from 20.1 to 22.1 mm and from 11.6–15.1 mm respectively, but there were no differences in the size of lesions among the isolates. The isolate K-41 showed higher variability in lesion length (Figure 5).

The isolate K-78 caused girdling of an inoculated sprout. Control sprouts inoculated with sterile PDA plug were symptomless. The pathogen was successfully re-isolated from symptomatic sprouts, while no fungi were isolated from control sprouts. Compared to this, the necrosis length on individual one-year-old *F. excelsior* trees of seed origin caused by *Chalara fraxinea* varied from 1.1 to 28.7 cm (on average, 7.2 cm), although about 20% of the trees inoculated with the fungus, necrosis did not exceed 1.5 cm (Bakys et al., 2009a).



F(4, 40)=0.81608, p=0.52252

Figure 5. Necrosis length and width on narrow-leaved ash sprouts caused by *F. tricinctum* isolates

There are not many data in the literature about fungi from the genus *Fusarium* on *F. angustifolia* and *F. excelsior* – European ash. Although it is known that *Fraxinus* species are currently suffering from ash dieback disease caused by the fungus *Hymenoscyphus fraxineus*, there are co-occurrences of large numbers of other fungi with similar endophytic as well as pathogenity among *Fusarium* species (Ivanova et al., 2020). According to Trapiello et al. (2017), one of the most frequently isolated species from European ash leaves with symptoms such as leaf spots and petiole discoloration were *Fusarium* species. *F. lateritium* did not cause any symptoms on stems of *F. excelsior* seedlings, while *F. solani* induced brown discolouration around the inoculation points on two-year-old plants (Przybył, 2002). *Fusarium* sp. was isolated in minor percent in *F. excelsior* petioles and symptomatic shoots (Davydenko et al., 2013). Kranjec-Orlović et al. (2019) among the other species on mycobionta of narrow- leaved ash seed isolated *Fusarium oxysporum*. *Fusarium lateritium* and *F. solani* were isolated from the root and stem base of *F. angustifolia,* whereas the connection to the symptoms of trees crown defoliation up to 60% were confirmed only for *F. solani* (Kranjec-Orlović et al., 2020).

The appearance of the necrosis of *F. tricinctum* on narrow-leaved ash spouts at the locality Vinična (sect. 11 and 16) could be explained by the retention of the water in the stands for almost two months. Symptoms typical for *Fusarium* necrosis appeared sporadically. Forests in Vinična are in the protected area, however, in this case the embankment breaching cause that the entire management unit was flooded. As it was mentioned, the necrosis on narrow ash leaved sprouts were at 50 cm height which was at the level of flood water. According to obtained data it can be assumed that *F. tricinctum* spores were brought by the flood waters of Sava river. Additionally, the *Fusarium* species have not been isolated from the ash sprouts after that period. It can be concluded that young narrow plants exposed to the water stress became susceptible for the fungus colonization.

This conclusion is in the agreement with Pukacki and Przybyl (2005) who isolated fungi from the genus *Fusarium* (*F. avenaceum, F. lateritium, F. solani and F. sambucinum)* from the necrotic buds and shoots of *F.excelsior,* but these authors assumed that stress in this case freezing injury could have been the primary factor, predisposing damaged organs to fungal colonization. This assumption was confirmed by the findings of Bakys et al. (2009b) who reported that the majority of trees inoculated with *Gibberella avenacea*, remained visually healthy, most probably due to favourable growing conditions. The environmental factors and fungal interactions in addition to genetic resistance should be considered as possible modifiers of the pathogenicity of ash mycobiota in nature (Trapiello et al., 2017). Therefore, the possibility cannot be excluded that pathogenicity of certain otherwise naturally occurring endophytic, saprotrophic or opportunistic fungi could be triggered by environmental factors (Bakys et al., 2009b).

CONCLUSION

Fusarium species are known as cosmopolites and pathogenic to vast number of plants, some of them has been reported on ash trees, but yet there has been no formal report of the disease or its causal fungus on narrow leaved ash in Serbia. To the best of our knowledge, this is the first report of *F. tricinctum* on narrow-leaved ash trees in Serbia. Considering the fact that *Fusarium* species, in this particular case *F. tricinctum,* under stress conditions could cause necrosis in the narrow ash sprouts, their occurrence and pathogenicity should be monitored.

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ОРИГИНАЛНИ НАУЧНИ РАД

ПРВИ НАЛАЗ *Fusarium tricinctum* НА ПОЉСКОМ ЈАСЕНУ (*Fraxinus angustifolia* Vahl.) У СРБИЈИ

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РЕЗИМЕ: Пољски јасен (*Fraxinus angustifolia* Vahl.) је дрвенаста врста распрострањена у низијским шумама у Европи. Регион југозападног Срема где се налазе највредније шуме ове врсте у Србији, био је изложен катастрофалним поплавама у мају 2014. Праћењем здравственог стања садница јасена (шума Моровић, локалитет „Винична") у рано пролеће 2015. године откривено је присуство великих тамних некротичних подручја на избојцима старим 1–2 године. Са оболелих избојака, стандардним фитопатолошким поступком, урађена је изолација патогена. Према морфолошким карактеристикама добијени изолати су детерминисани као *Fusarium tricinctum*. За даље анализе су узета три репрезентативна моноспорна изолата (К41, К42 и К78). BLAST претрагом NCBI нуклеотидне базе утврђено је 98,9% сличности TEF1-α секвенце изолата К78 (MN822227.1) са *Fusarium tricinctum* и 100% поклапања ITS секвенце овог изолата (GenBank MK928426.1) са изолатом *F. tricinctum* ZMXR6 (MT446111.1). IGS секвенце изолата К41 и К42 (MZ749901 и MZ749902) имале су 94,1% и 94,3% сличности са *F. tricinctum* комплексом, на основу BLAST претраге FUSARIUM-ID нуклеотидне базе. Тестови патогености на избојцима јасена старим годину дана показали су црвенкасто смеђе издужене некротичне лезије просечне дужине 20,1 mm. Патоген је реизолован из избојака са симптомима. Према нашим сазнањима, ово је први налаз *F. tricinctum* на стаблима пољског јасена у Србији.

КЉУЧНЕ РЕЧИ: пољски јасен, *Fusarium tricinctum*, некроза избојака

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ORIGINAL SCIENTIFIC ARTICLE

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QUECHERS APPROACH TO THE *Alternaria* MYCOTOXINS DETECTION IN WHEAT: THE RECOVERY STUDY

ABSTRACT: The performance of the QuEChERS extraction followed by the LC-MS/MS analytical method was evaluated in terms of *Alternaria* mycotoxins recovery from the wheat. The alternariol (AOH), tentoxin (TEN) and alternariol monomethyl ether (AME) were analyzed using the ESI+ (electrospray positive ionization) by multiple reactions monitoring mode (MRM). In order to determine the recovery, the blank wheat samples were spiked at two spiking levels (0.01 and 0.1 mg/kg) in six replicates. The obtained average recoveries and precisions (expressed as the RSDr, %) were as follows: 107.6% (RSDr of 6.84%) for alternariol, 108.0% (RSDr of 6.78%) for tentoxin and 110.1% (RSDr of 6.50%) for alternariol monomethyl ether. The results of this study were in accordance with the Commission Decision 2002/657/EC and Commission Regulation (EC) No 401/2006.

KEYWORDS: *Alternaria* mycotoxins, wheat, QuEChERS, LC-MS/MS

INTRODUCTION

Alternaria is a fungal genus that includes saprophytic and pathogenic species and is widespread in nature. *Alternaria* spp. can infect a wide variety

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of crops in the field and in the postharvest stage causing considerable losses due to fruit and vegetable decay. The most common *Alternaria* species include: *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. radicina*, *A. brassicae*, *A. brassicicola*, and *A. infectoria* (Escriva et al., 2017).

In 2011, in the light of the concerns for human and animal health, the European Food Safety Authority (EFSA) performed a risk assessment of some of the main *Alternaria* toxins, i.e. alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), and tenuazonic acid (TeA). The conclusions were that AOH and AME were genotoxic in bacteria and mammalian cells *in vitro* (EFSA, 2011). As regards *in vivo* systems, only the limited information was available and the indications of precancerous changes were reported in oesophageal mucosa of mice. In 2016, EFSA collected occurrence data for AOH, AME, TEN, and TeA to estimate the chronic dietary exposure to these mycotoxins (EFSA, 2016; De Berardis et al., 2018). The *Alternaria alternata* species group alone is recorded as causing disease on over 100 host plants. This includes economically essential crops including cereals, ornamentals, vegetables and fruits, with the losses incurred through direct crop damage, postharvest spoilage or through the contamination with mycotoxins.

Today, some of the mycotoxins extraction techniques used for various matrices are as follows: a liquid-liquid extraction (LLE), pressure liquid extraction (PLE), supercritical fluid extraction (SFE), solid phase extraction (SPE), matrix solid-phase dispersion (MSPD), ultrasound and homogenizing extraction with various organic solvents mixtures (Vuković et al., 2017). The "Green Analytical Chemistry" represents the trend resulting from the aspiration to make the chemical analyses more environmentally friendly (Breidbach, 2017). Due to that trend, the QuEChERS (Quick Easy Cheap Effective Rugged Safe) method has been considered to be the most current extraction and extract purification procedure. In the field of the mycotoxins research, this method has drawn enormous attention because of how simple and effective it is for the mycotoxins isolation from the complex matrices, being environmentally friendly at the same time (Vuković et al., 2019, Wenbo et al., 2019).

In the present study, the performance of a QuEChERS extraction followed by LC-MS/MS analytical method was evaluated, aiming to the developed and validated method for the determination of the TEN, AOH and AME *Alternaria* mycotoxins in wheat.

MATERIALS AND METHODS

Chemicals and reagents

The analytical standards of the AOH, TEN and AME were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). The standards were reconstituted with 1.0 mL of the methanol (MeOH) to obtain 0.1 mg/mL stock solutions. All stock solutions were kept at 4 °C. The mixtures of all the *Alternaria* toxins were prepared in acetonitrile (MeCN) in the final concentrations

of 10 and 1 μg/mL. These solutions were used for spiking the blank samples for the recovery analyses. The MeOH and MeCN were LC-MS grade obtained from Sigma-Aldrich. The ammonium formate was analytical grade purchased from Merck (Darmstadt, Germany). The products Dispersive SPE 15 mL, Fatty samples (EN) (part no. 5982–51565) and QuEChERS extraction kit Original method (part no. 5982–7550) were purchased from Agilent Technologies (USA).

Instrumentation

The HPLC Agilent 1290 Infinity II chromatograph equipped with a quaternary pump, multisampler and column compartment thermostat was used for the *Alternaria* toxins detection. The HPLC system was coupled to an Agilent 6470B LC/TQ triple quadrupole mass spectrometer with AJS ESI (Jet Stream Technology Ion Source). A Zorbax Eclipse Plus C18 column (2.1x50 mm; 1.8 μm) was used for the chromatographic separation. The column temperature was held at 35 °C and the injection volume for the LC system was 2 μL. The chromatographic separation of the AOH, TEN and AME was carried out with the mobile phase consisting of water (A) and acetonitrile (B), both containing 10 mM ammonium formate, in a gradient mode and flow rate of 0.3 mL/min. A gradient elution started at 5% of B and held for 1 min. This composition was increased to 40% B at 7 min, 90% B at 8 min and held for 2 min. The composition of the mobile phase returned to the initial conditions in 1 min and the system was equilibrated during 2 min. The total running time was 11 min. The ESI source was used with the following settings: drying gas (nitrogen) temperature 200 °C, drying gas flow rate 16 L/min, nebulizer pressure 30 psi, sheath gas temperature of 300 °C, sheath gas flow 12 L/min and capillary voltage 3,000 V. The detection was performed using the multiple reactions monitoring mode (MRM). The Agilent MassHunter software (version B.10.0 SR1 Agilent Tehnologies, 2006–2019) was used for the optimization and quantification.

Spiking samples and extraction

The homogenized blank wheat samples were spiked at two levels 0.01 and 0.1 mg/kg in six replicates.

About 5.0 ±0.1 g of the sample was weighed in a falcon tube of 50 mL. A total of 10 mL of MeCN (with 1% of formic acid) was added. The sample was vortexed for 10 min on 2,000 rpm. Afterwards, the QuEChERS extraction kit Original method (containing 4 g of $MgSO_4$ and 1 g of NaCl) was added and the samples were put on vortex (10 min/2,000 rpm) and centrifugated for 5 min/6,000 rpm. A mixture – QuEChERS Dispersive Kit, 15 mL which contains 400 mg of PSA, 400 mg of C18EC and 1,200 mg of $MgSO_4$ was added to 6 mL of extract. The extracts were vortexed for 10 min on 2,000 rpm and centrifugated for 5 min/10,000 rpm on 4 ºC. The gained supernatant was diluted with water (1:1, v/v), filtrated through syringe filter (pore size of 0.45 μm) and injected in LC-MS/MS.

RESULTS AND DISCUSSION

Analytical method

The AOH, TEN and AME were analyzed using ESI+ (electrospray positive ionization) by multiple reactions monitoring mode (MRM). The fragmentation of the protonated AOH, TEN and AME ions yielded 2 product ions, respectively (Tab. 1). The intense MRM transition was monitored for the quantification and the second one was used for the confirmation (Vuković et al., 2018).

Table 1. LC-ESI-MS/MS parameters for the analysis of AOH, TEN and AME in MRM mode

TA*	Molecular formula	Molecular weight (g/mol)	Retention time (min)	Precursor ion [M+H*] (m/z)	Product ion (m/z)	Fragmentation voltege (V)	Collision energy (V)
AOH	$C_{14}H_{10}O_5$	256.0	4.35	257.0	213.0 215.0	170 170	23 23
TEN	$C_{22}H_{30}N_4O_4$	412.3	4.54	413.3	141.0 271.0	170 170	23 17
AME	$C_{15}H_{12}O_5$	270.0	5.54	271.0	256.0 227.0	135 135	23 30

*Target analytes

The MRM (multiple reaction monitoring mode) was used for the quantification and the confirmation of the AOH, TEN and AME. TIC chromatogram of spiking sample (level 0.1mg/kg) and MRM chromatograms are shown in Figure 1. All the obtained results for the recovery and precision when the blank samples were spiked at 0.01 and 0.1 mg/kg in six replicates are shown in Table 2.

Table 2. Recovery data and RSD (%)

Mycotoxin	Recovery (%RSD) for n=6					
	0.01 mg/kg	%RSD_1	0,1 mg/kg	%RSD_2	Av. recovery (%)	% RSDr
AOH	109.0	6.64	106.2	1.67	107.6	6.84
TEN	106.3	4.81	109.7	4.77	108.0	6.78
AME	112.1	5.72	108.1	3.09	110.1	6.50

*The results represent average of both spiking levels per replicates

The obtained average recoveries and precisions (express as RSDr, %) for all investigated mycotoxins were in accordance with the Commission Decision 2002/657/EC and Commission Regulation (EC) No 401/2006.

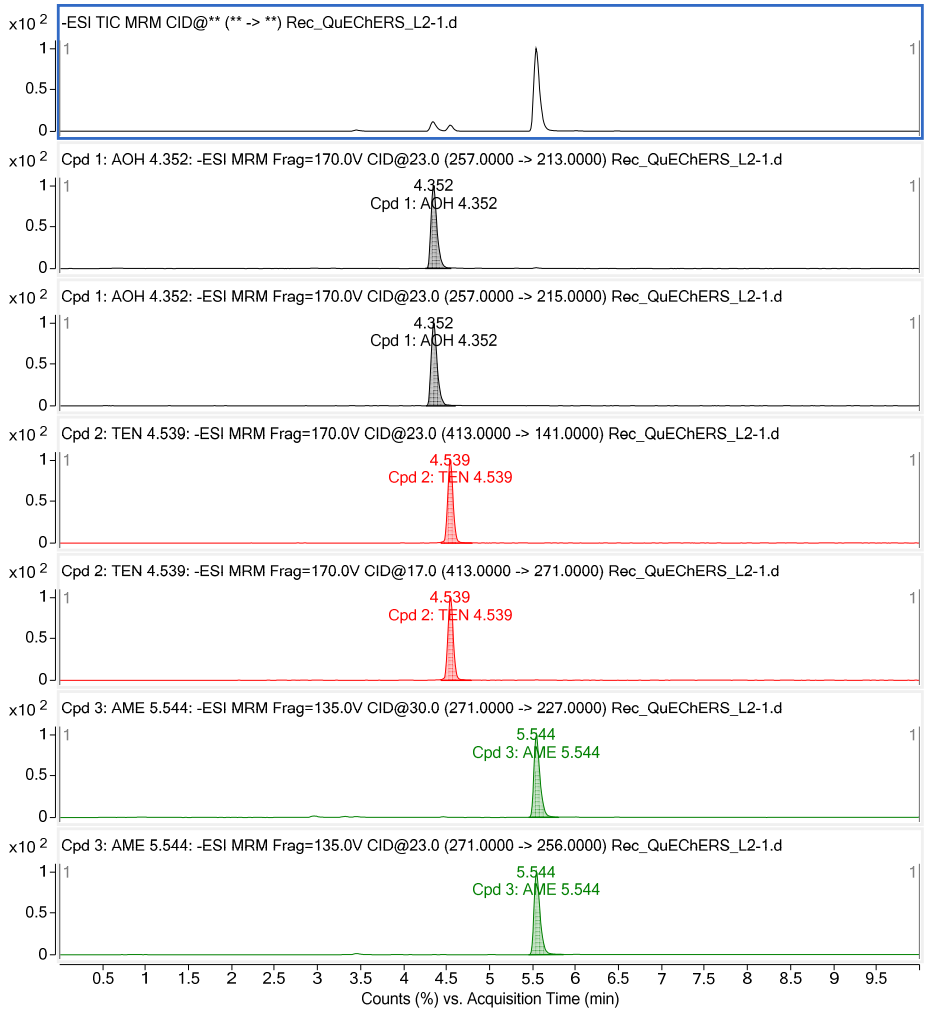

Figure 1. LC-MS/MS chromatogram of spiking sample at 0.1 mg/kg

Comparing the obtained recoveries using QuEChERS method with the literature data, it can be concluded that the obtained recoveries (in different matrices) are in accordance with the available research throughout the world. Namely, Fontana et al. (2016) developed a reliable, simple, fast, inexpensive and robust sample preparation approach for tenuazonic acid (TA) determination in grapes by liquid chromatography with ultraviolet detection (HPLC-UV). The method was based on a modified QuEChERS (using ethyl acetate as extraction solvent). They obtained average recovery of 91.7% for grape samples spiked at 0.05, 0.5 and 5 mg/g.

De Berardies et al. (2018) used the QuEChERS method for the extraction of AOH, AME, TEN and TeA from tomato and fruit based samples. Namely, in

case of tomato based samples the AOH, AME, TEN and TeA recoveries were as follows: 85, 78.5, 83.3 and 106.5% respectively (for low spiking concentrations). Somewhat lower recoveries were obtained for the high spiking concentrations, i.e. 64.4% for AOH, 69.3% for AME, 64.2% for TEN and 98.8% for TeA. The recoveries for the fruit based samples were as follows: 63.1% for AOH, 66.3% for AME, 76.2% for TEN and 100.3% for TeA.

Guo et al. (2019) evaluated a simple and reliable analytical method for the simultaneous determination of AOH, ALT, TEN, ALS, TA and AME in grapes using ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) and a modified QuEChERS by sodium chloride and anhydrous magnesium sulfate. The obtained recoveries were in the range from 77.8 to 101.6%.

CONCLUSION

The possible applicability of the developed QuEChERS – LC-MS/MS analytical method for the *Alternaria* mycotoxins detection in wheat was demonstrated through the obtained average recoveries and precisions: 107.6% (RSDr of 6.84%) for alternariol, 108.0% (RSDr of 6.78%) for tentoxin and 110.1% (RSDr of 6.50%) for alternariol monomethyl ether, which were in accordance with the Commission Decision 2002/657/EC and Commission Regulation (EC) No 401/2006. These results are speaking in favour of the presented method for mycotoxins detection since it is simple and highly sensitive, while allowing easy sample preparation because it can exclude the sample cleanup and pre-concentration.

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ОРИГИНАЛНИ НАУЧНИ РАД

QUECHERS ПРИСТУП У ДЕТЕКЦИЈИ *Alternaria* МИКОТОКСИНА У ПШЕНИЦИ: ПРИНОС ЕКСТРАКЦИЈЕ

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РЕЗИМЕ: Примена QuEChERS екстракције праћене LC-MS/MS аналитичком методом процењена је у погледу приноса екстракције *Alternaria* микотоксина из пшенице. Алтернариол (AOH), тентоксин (TEN) и алтернариол монометил етар (AME) су анализирани коришћењем ESI+ (јонизација електро-спрејом) помоћу

режима праћења вишеструких реакција (MRM). Као би се одредио принос екстракције, „бланк" узорци пшенице су обогаћени на два нивоа обогаћења (0,01 и 0,1 mg/kg) у шест понављања. Утврђени просечни приноси екстракције и стандардне девијације (изражене као RSDr, %) су: 107,6% (RSDr 6,84%) за алтернариол, 108,0% (RSDr 6,78%) за тентоксин и 110,1% (RSDr 6,50%) за алтернариол монометил етар. Резултати овог истраживања су у складу са Одлуком Комисије 2002/657/ЕЗ и Уредбом Комисије (ЕЗ) број 401/2006.

КЉУЧНЕ РЕЧИ: *Alternaria* микотоксини, пшеница, QuEChERS, LC-MS/MS

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ORIGINAL SCIENTIFIC ARTICLE

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TOXIGENIC POTENTIAL OF *Alternaria* SPECIES FROM CEREALS

ABSTRACT: Toxigenic potential of four and one isolate of *A. alternata* and *A. tenuissima*, respectively, on durum wheat cultivar Dušan (*Triticum durum* L.) and common wheat cultivar Barbee (*T. vulgare* L.) were tested. Three different wheat / isolate genotype combinations were used for artificial inoculation of grains under laboratory conditions and toxins production. Alternaria toxins alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), tenuazonic acid (TeA) and altenuen (ALT) concentrations were determined by LC-MS/MS. Cultivar Barbee proved to be a more suitable substrate for toxin production, whereby AOH, AME and TeA were present in highest concentrations. These results underline the possibility of fungal infection and mycotoxin production by *Alternaria* species in field and under storage conditions. Further research is needed for official regulation of acceptable levels of *Alternaria* mycotoxins in food and feed.

KEYWORDS: *Alternaria,* toxin production, wheat

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INTRODUCTION

Wheat is one of the main crops in the Republic of Serbia, with a total production area of about 560.000 ha (Statistical Yearbook, RS, 2021). However, as extreme weather conditions are becoming increasingly prevalent due to climate change and global warming, the risk of mycotoxin contamination has also heightened. As indicated by Vučković et al. (2012), the resulting greater occurrence of phytopathogens on small grains may have negative consequences on the quality and safety of food and feed.

Species of the genus *Alternaria* are significant contaminants of cereals that, in addition to mycotoxins production, cause product degradation during transport and storage. *Alternaria* species produce more than 70 secondary metabolites, some of which e.g., alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), tenuazonic acid (TeA) and altenuen (ALT) are classified as mycotoxins due to their harmful effects on humans and animals (EFSA, 2011, 2016; Escrivá et al., 2017; Gashgari et al., 2019). Moreover, more than one mycotoxin can be found on an infected substrate (Zain, 2011). During the recent years, *Alternaria* species, especially *A. alternata*, have been found as contaminants of wheat and wheat-based products in the Vojvodina Province (Janić Hajnal et al., 2015). For the 2010−2015 period, the EFSA (2016) reported presence of the highest levels of *Alternaria* toxin TeA in tomatoes, nuts, oilseeds, cereals, and fruit. More recent findings reported by Mujahid et al. (2020) further indicate that as emerging mycotoxins, *Alternaria* toxins are candidates for regulation by European authorities. Monitoring and regulation of the permitted levels of *Alternaria* toxins should thus be initiated throughout the EU, according to the SANTE/11356/2019 draft issued by the European Commission.

Mycotoxins produced by *Alternaria* spp. on humans and animals exhibit prolonged and chronic toxic effects, as their intake can be harmful even in low doses. The most common symptoms include nervous system damage, brain bleeds, liver damage and cancer, hyperestrogenism, reproductive system cell degeneration, epithelial cell damage, as well as adverse changes to the mucous membranes, intestinal tract, immune system, and respiratory tract (Liu et al., 1992; European Commission, 2006).

Considering the importance of *Alternaria* as a pathogen and small grain cereals contaminant, the aim of this study was to evaluate the toxigenic potential of *A. alternata* and *A. tenuissima* isolates obtained from wheat and rye by artificially inoculating wheat kernels under laboratory conditions.

MATERIAL AND METHODS

Alternaria isolates and inoculum preparation

Alternaria alternata (isolates A2, A3, A4 originating from wheat) and *A. tenuissima* (isolate A6 originating from rye) isolates were obtained from CRC Genbank of Small Grain Cereals and Microorganisms, Cereal Research Non-

profit Company, Szeged, Hungary. One *A. alternata* isolate (SOR1IIIZA1) obtained from wheat grain grown in the Vojvodina Province was also included in the analyses. To obtain conidia, all isolates were grown on CPA (Carrot = Potato Agar) in Petri dishes of 90 mm diameter for 10 days, under dark conditions at 25 °C. Presence of conidia was monitored on a daily basis. After 10 days, conidia were harvested by washing the culture surface and macerating the medium in sterile distilled water (SDW), after which the concentration was adjusted to 2×10^5 conidia/ml.

Artificial inoculation of wheat kernels

Two wheat genotypes were inoculated under laboratory conditions with different combinations of *Alternaria* isolates: (1) Durum wheat (*Triticum durum* L.) inoculated with isolates A2+SOR1IIIZA1; (2) common wheat cultivar Barbee (*T. vulgare* L.) inoculated with isolates A3+A4+A6; and (3) Durum wheat inoculated with isolates A2+A3+A4+A6+SOR1IIIZA1. Prior to the treatment, 500 g of wheat kernels was weighed and was placed into glass bottles and sterilized at 121 °C for 60 min. After autoclaving and cooling the kernels, 200 ml of the fungal conidia suspension was added and homogenized. Inoculated kernels were incubated at 25 °C in the dark for 20 days. The bottles containing inoculated kernels were shaken on a daily basis for 30 minutes. After the incubation time had lapsed, the glass bottles were exposed to 45 °C for 30 minutes, closed with screw caps and placed into cold storage (at -20 °C) until required for analysis.

Determination of toxin concentrations in artificially inoculated wheat kernels

The analyses were performed using LC-MS/MS methodology adapted from Scott (2001). Briefly, 10 g of the artificially inoculated seed sample was weighed and, after extraction with acetonitrile/water, the resulting aliquot was subjected to solid phase clean-up. The *Alternaria* toxins were then eluted with methanol and quantitatively determined by HPLC-MS/MS after changing the solvent. The *Alternaria* toxin content in the samples was corrected to obtain the required recovery rates. The following toxins were analyzed: (AOH), (AME), (TEN), (TeA) and (ALT) (Table 1).

Table 1. Analyzed toxins produced by *Alternaria* isolates

Toxins	LOQ (µg/kg)	LOD (µg/kg)
Tenuazonic acid (TEA)	10	3
Altenuen (ALT)	10	3
Alternariol (AOH)	2	1
Alternariol monomethyl ether (AME)	2	1
Tentoxin (TEN)	10	3

RESULTS AND DISCUSSION

Alternaria species are common saprophytes or pathogens of a wide range of plants, whereby infection can occur in field as well as during storage. The most frequently analyzed toxins are AOH, AME and TeA, followed by ALT, produced by *A. alternata* or *A. tenuissima*, and TEN toxin produced by *A. alternata*. Due to the occurrence of *Alternaria* species, these mycotoxins can be present as contaminants throughout the entire food and feed chain, especially in cereals, vegetables, fruit and oil seeds (Zwickel et al., 2016).

As a part of the present study, the toxigenic potential of *Alternaria* isolates (*A. alternata* and *A. tenuissima*) obtained from wheat and rye was evaluated, whereby different wheat kernels were subjected to artificial inoculation. As shown in Table 2, all inoculated wheat samples were successfully contaminated with *Alternaria* toxins (AOH, AME, TeA, ALT, and TEN) in high concentrations.

On the durum wheat samples inoculated with the *Alternaria alternata* isolates originating from wheat grown in Hungary (A2) and Serbia (SOR1IIIZA1), the toxin concentration was the lowest. Additionally, analyses related to this combination showed the presence of AOH (>2,000 μg/kg), AME (>1,000 μg/kg), TeA (>9,000 μg/kg), ALT (760±300 μg/kg) and TEN (57±23 μg/kg).

When inoculation was performed on kernel of wheat cultivar Barbee with isolates originating from wheat (A3, A4; *A. alternata*) and rye (A6; *A. tenuissima*), the highest concentrations of the *Alternaria* toxins were recorded, i.e. >30,000 μg/kg, >10,000 μg/kg, >250,000 μg/kg, >5,000 μg/kg and >2,000 μg/kg were maxima attained for AOH, AME, TeA, ALT and TEN, respectively.

Using a mixture of all tested *Alternaria* isolates (A2+A3+A4+A6 and SOR1IIIZA1) in the inoculation of durum wheat kernel resulted in a lower (higher) toxin production (Sample 1) compared to Sample 2. The highest recorded values were as follows: >10,000 μg/kg, >3,000 μg/kg, >50,000 μg/kg, >2,000 μg/kg and 170±68 μg/kg for AOH, AME, TeA, ALT and TEN, respectively. When all results were compared, TeA emerged as the most abundant toxin in all wheat samples, while TEN was present in the lowest concentrations. Finally, the most suitable substrate for *Alternaria* toxin production was wheat Barbee.

Given the high toxicogenic potential of *Alternaria* isolates from small grain cereals and the widespread occurrence of *Alternaria* species, there is a need for the legislation of maximum allowable concentrations of *Alternaria* toxins in food and feed.

Table 2. Determination of toxin concentrations in wheat samples artificially inoculated with *Alternaria* isolates

Wheat samples, mixture of Alternaria isolates	*Toxin*	*Concentration* (μg/kg)
1) Durum, A2 (*Aa*) + SOR1IIIZA1 (*Aa*)	Alternariol (AOH)	>2,000
	Alternariol monomethyl ether (AME)	>1,000
	Tenuazonic acid (TeA)	>9,000
	Altenuen (ALT)	760±300
	Tentoxin (TEN)	57±23
2) Barbee, A3 (*Aa*) + A4 (*Aa*) + A6 (*At*)	Alternariol (AOH)	>30,000
	Alternariol monomethyl ether (AME)	>10,000
	Tenuazonic acid (TeA)	>250,000
	Altenuen (ALT)	>5,000
	Tentoxin (TEN)	>2,000
3) Durum, A2 (*Aa*) + A3 (*Aa*) + A4 (*Aa*) + A6 (*At*) + SOR1IIIZA1 (*Aa*)	Alternariol (AOH)	>10,000
	Alternariol monomethyl ether (AME)	>3,000
	Tenuazonic acid (TeA)	>50,000
	Altenuen (ALT)	>2,000
	Tentoxin (TEN)	170±68

Aa – Alternaria alternata; *At – Alternaria tenuissima*.

CONCLUSION

Fungal species of the genus *Alternaria* represent a potential hazard for food safety owing to their toxin-producing potential. Under laboratory conditions, *A. alternata* and *A. tenuissima* isolates produced a wide range of mycotoxins in high concentrations. As shown in the present study, significant differences exist among wheat types and cultivars in terms of their suitability as a toxin production substrate. Field infections of wheat by these *Alternaria* species can provide conditions conducive to further fungal development during storage and may thus result in significant toxin contamination.

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ТОКСИГЕНИ ПОТЕНЦИЈАЛ ВРСТА ИЗ РОДА *Alternaria* ИЗОЛОВАНИХ СА СТРНИХ ЖИТА

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РЕЗИМЕ: У раду је испитан токсигени потенцијал изолата врста из рода *Alternaria – A. alternata* и *A. tenuissima* у лабораторијским условима на дурум пшеници сорте „Душан" (*Triticum durum* L.), као и на сорти пшенице „Barbee" (*T. vulgare* L.). Током вештачке инокулације у лабораторији коришћене су три различите комбинације генотип пшенице/изолати. Путем LC-MS/MS методе испитан је садржај алтернариа токсина алтернариола (AOH), алтернариол мометилетра (AME), тентоксина (TEN), тенуазоничне киселине (TeA) и алтенуена (ALT). Сорта „Барби" показала се као најпогоднији супстрат за продукцију алтернариа токсина. У испитиваним узорцима утврђена је највиша концентрација токсина AOH, AME и TeA. Резултати ових истраживања указују на могућност остварења инфекције стрних жита од стране *Alternaria* врста током складиштења и на ризик од потенцијалне контаминације од стране микотоксина и уласка отровних једињења у ланац исхране. Даља истраживања и потреба званичне регулације максимално дозвољених количина *Alternaria* токсина у циљу су смањења ризика од тровања секундарним метаболитима које стварају гљиве из рода *Alternaria*.

КЉУЧНЕ РЕЧИ: *Alternaria*, продукција токсина, пшеница

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REVIEW SCIENTIFIC ARTICLE

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REVIEW OF TRENDS IN ESSENTIAL OILS AS ALTERNATIVES TO ANTIBIOTICS IN BOVINE MASTITIS TREATMENT

ABSTRACT: Bovine mastitis is an important disease in the dairy industry responsible for the welfare and significant economic losses in dairy cows. The treatment of choice for mastitis is the administration of antibiotics. However, this therapeutic choice has some disadvantages including presence of antibiotics residues in the milk, low cure rate as well as rapid increase in antibiotic-resistant pathogens. Therefore, new alternative approaches to antibiotics were investigated by different groups of researchers in order to find an effective approach for bovine mastitis therapy. This review was conducted in order to analyze different publications on usage of essential oils in relation to bovine mastitis. There are many *in vitro* studies for evaluating the antimicrobial efficacy of essential oils against many mastitis associated pathogens. In addition, numerous of tested essential oils have shown good efficacy with a wide range of minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs). On the other hand, only several *in vivo* studies have focused on therapeutic effects of essential oils. Moreover, recent studies indicate the possibility of using essential oils in the fight against biofilm which could be promising fight against bovine mastitis since unsuccessful antibiotic treatment can be associated with the presence of biofilms.
KEYWORDS: antibiotics, bovine mastitis, essential oils, *in vivo*, *in vitro*, biofilm

PHARMACOLOGICAL ACTIVITY OF ESSENTIAL OILS

Essential oils (EOs) are aromatic oily liquids obtained from plant materials, proved to be good sources of bioactive compounds. EOs possess various

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applications mainly in health, cosmetic, agriculture and food industry (Raut and Karuppayil, 2014). Compared to conventional drugs, these compounds are of natural origin, lower side effects and low resistance after prolonged exposure (Montironi et al., 2016). The pharmacological activities of these oils have been well known since ancient times and they include antimicrobial, antiviral, antimutagenic, anticancer, antioxidant, anti-inflammatory, immunomodulatory and antiparasitic activities (Bakkali et al., 2008; Cho et al., 2020; Nehme et al., 2021). Due to bactericidal, fungicidal and virucidal effects, EOs are used in veterinary medicine in the treatment of diseases caused by microorganisms, but also in the treatment of non-infectious diseases because they have hypolipidemic, hypotensive, anticancer, anticoagulant, sedative, analgesic, anticoagulant, antirheumatic and other effects (Vučinić et al., 2011).

The compounds present in EOs are probably related to their pharmacological activities (Burt et al., 2004; Nehme et al., 2021). The greatest advantage of compounds of plant origin in relation to synthetic agents lies in the fact that plants have a larger pharmacological complex and can affect several different diseases. While synthetic agents are designed to inhibit or stimulate one of the pathways of pharmacological effects, natural compounds can act on a number of different pathways without adverse effects (Nikolić, 2015).

Antibacterial activity of EOs

Antibiotic therapy is one of the most important tools used in the fight against infectious diseases (Yap et al., 2014). Irrational use of antimicrobial drugs is a long-standing problem, both in human and veterinary medicine. In recent years, the development of antimicrobial resistance to a number of antibiotics has increased, thus endangering the control of infectious diseases (Grundmann et al., 2011; Puvača et al., 2021). Besides, bacterial resistance to antibiotics and lack of new antibiotics on the drug market have encouraged research of antibacterial activity of non-antibiotic alternatives, including EOs.

A large number of EOs are known to have antimicrobial properties and in many cases this activity is due to the presence of different classes of monoterpenes (Stanković et al., 2011). The antimicrobial activity of EOs and their components can vary from partial to complete inhibition of bacterial growth, so that EOs exhibit bacteriostatic or bactericidal activity (Stanković et al., 2011). A large number of *in vitro* studies have assessed the antimicrobial activity of the EOs against pathogenic bacteria (Mimica-Dukić and Božin, 2007; Mullen et al., 2014; Motlagh et al., 2014; Piotr et al., 2018; Kovačević et al., 2021). Some studies have demonstrated that whole EOs usually have higher antibacterial activity than the mixtures of their major components, suggesting that the minor components are critical to the synergistic activity, though antagonistic and additive effects have also been observed (Bassolé and Juliani, 2012).

The mechanism of action of the EOs depends on their chemical composition, and their antimicrobial action cannot be attributed to a single mechanism, but represents a cascade reaction involving the whole bacterial cell (Nazzaro

et al., 2013). In many cases, antimicrobial activity is the result of a complex interaction among different classes of compounds such as phenols, aldehydes, ketones, alcohols, esters, ethers or hydrocarbons found in the EOs. The greatest antimicrobial effect is exerted by phenols, which are mainly present with the highest percentage in EOs, followed by alcohols, aldehydes, ketones, ethers, while the antibacterial effect of hydrocarbons is very low (Dorman and Deans, 2000; Bassole and Juliani, 2012). The antimicrobial activity of phenol depends on the applied concentration. It means that at lower concentrations, they cause structural changes in the cell membrane by inhibiting cellular respiration, while at higher concentrations they lead to more severe membrane damage, complete disruption of homeostasis and cell death (Carson et al., 2002). The high antimicrobial activity of *Thymus* and *Origanum* species is attributed to their phenolic components such as thymol and carvacrol (Lambert et al., 2001; Oussalah et al., 2007). *In vivo* study conducted by Radinović et al. (2018) showed that thyme EO can be used in the prevention and treatment of mastitis in dry cows.

The interaction between EO compounds can produce four possible types of effects: indifferent, additive, antagonistic or synergistic (El-Tarabily et al., 2021). The combination of phenol (thymol with carvacrol and both components with eugenol) produced synergistic effects on several microorganisms, and they are especially active against *E. coli* strains (Bassole and Juliani, 2012). Although in other studies they have shown an additive effect (Lambert et al., 2001), as well as an antagonistic one (Gallucci et al., 2009).

The antibacterial activity of the EOs depend not only on chemical characteristics, but also on the type of bacteria (Ebani and Mancianti, 2020). Namely, gram-negative bacteria are more resistant to EOs than gram-positive bacteria (Trombetta et al., 2005). The lower susceptibility of gram-negative bacteria is explained by the difference in cell wall structure that limits the diffusion of hydrophobic compounds through the lipopolysaccharide envelope (Burt, 2004). The cell structure of gram-positive bacteria allows hydrophobic molecules to easily penetrate cells and act on both the cell wall and the internal cytoplasm. Due to their hydrophobic nature, EOs interact with the lipid membrane of the bacterial cell increasing its permeability but also with mitochondria (Chouhan et al., 2017). The permeability of the cell membrane occurs as a result of a change in the membrane potential, the collapse of the proton pump and the release of ions from the cell, which results in lysis and death of the cell. This mechanism is thought to be most responsible for bacterial cell damage (Burt, 2004; Bakkali, 2008). EOs can also cause coagulation of bacterial cell cytoplasm (Gustafson et al., 1998) and affect the biosynthesis of lipids, including unsaturated fatty acids. EOs in the bacterial cell, even in a concentration below the minimum inhibitory concentration (MIC), decrease the level of unsaturated fatty acids that are generally responsible for the membrane fluidity (Nehme et al., 2021).

EO components react not only on membrane lipids, but also on proteins such as the enzyme adenosine triphosphatase (ATPase) (Burt, 2004). Eugenol has been shown to inhibit the activity of the following enzymes: ATPase, histidine decarboxylase, amylase and protease (Hyldgaard et al., 2012). Research has

shown that the EOs can also affect other enzymes involved in energy regulation or responsible for the synthesis of structural components of the cell (Burt, 2004). The EO can exert its antibacterial action by preventing the process of transcription and translation of microorganisms. The main active component of tea tree extract, terpinen-4-ol, has been shown to directly affect the regulation of 10 *Staphyloccocus auerus* genes and prevent their transcription (Cuaron et al., 2013).

The antimicrobial activity of EOs is determined by the method of diffusion, dilution or bioautographic method. The diffusion method is most often used to determine the existence of antimicrobial activity of EOs, while the agar dilution method is used to determine the strength of antimicrobial activity (Burt et al., 2004).

The influence of EOs and their constituents on the biofilm of bacteria

Biofilm-forming microorganisms represent a serious medical problem, as they protect themselves from antibiotics and the hosts' immune response. In this structure, microorganisms show a 10 to 1,000 time higher tolerance to antimicrobial agents than the same cells in planktonic form (Pedersen et al., 2021; Iseppi et al., 2021). Additionally, these formations favour the exchange of genes located at the plasmid level responsible for antibiotic resistance (Iseppi et al., 2021).

Chronic biofilm infections are notoriously known to be difficult to eradicate with antibiotics and biofilm formation could be a possible explanation for mastitis cases that are not resolved by standard treatment. Pedersen et al. (2021) reported that plenty of *in vitro* studies investigated the biofilm forming abilities of mastitis pathogens isolated from milk samples, but only few have focused on biofilm formation inside udders from dairy cows with mastitis. Furthermore, the treatment of mastitis is difficult, especially caused by *S. aureus*. Therefore, the connection between this form of mastitis and *S. aureus* biofilm presence and many virulence factors that are upregulated during mastitis infections should be further investigated (Zecconi et al., 2006; Oogai et al., 2011).

The ability of EOs to inhibit biofilm formation has been poorly explored; however, some reports suggested their utilization as potent inhibitor of biofilm formation (Jia et al., 2011; Caputo et al., 2020; Martínez et al., 2021). When it comes to mastitis pathogens, Aiemsaard et al. (2011) reported that lemongrass oil and its major components demonstrated an inhibitory effect on clinically isolated bovine mastitis pathogen *S. aureus* biofilm formation. Furthermore, study conducted by Budri et al. (2015) observed strong antibiofilm activity for *Syzygium aromaticum* and *Cinnamomum zeylanicum* and their major component cinnamaldehyde against *S. aureus* isolated from cases of subclinical bovine mastitis.

Essential oils *in vitro* efficiency against bovine mastitis pathogens

Various *in vitro* studies have shown promising results regarding antibacterial efficiency against the most common mastitis pathogens, which is

summarized in Table 1. The most tested EOs in different studies were obtained from *Thymus vulgaris* L., Lamiaceae and *Origanum vulgare* L., Lamiaceae (Table 1).

Table 1. Antibacterial activity of EOs against bovine mastitis pathogens – *in vitro*

EOs	Mastitis pathogens	Study
Menthae piperitae and *Melissa officinalis*	*Streptococcus* spp., *Streptococcus* spp. β heamoliticus, *Escherichia coli*, *Enterobacter sakazakii*, *Klebsiella oxytoca*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Staphylococcus* spp. coagulase negative and *Staphylococcus aureus*	Tomanić et al., 2022
Origanum vulgare, *Satureja montana*	*Streptococcus spp.*, *Escherichia coli*, *Streptococcus* spp. β haemoliticus, *Staphylococcus aureus*, *Staphylococcus* spp. coagulase negative, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Klebsiella oxytoca*, *Cronobacter sakazakii*	Kovačević et al., 2022
Eugenia caryophyllata, *Origanum vulgare,* and *Cinnamomum cassia*	*Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*	Barreiros et al., 2022
Thymus vulgaris, *Thymus serpullum*	*Escherichia coli*, *Streptococcus* spp., *Staphylococcus* spp. *coagulase negative*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Klebsiella oxytoca*, *Enterobacter sakazakii*	Kovačević et al., 2021
Ocimum sanctum, *Origanum onites*	*Staphylococcus aureus* and *Escherichia coli*	Aydin et al., 2021
Juniperus virginiana, *Cinnamomum cassia*, *Pelargonium graveolens*, *Leptospermum scoparium*, *Pogostemon cablin* and *Thymus vulgaris*	*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus xylosus*	Piotr et al., 2018
Minthostachys verticillata	*Escherichia coli*, *Bacillus pumilus*, *Enterococcus faecium*	Cerioli et al., 2018
Mentha pulegium, *Nepeta cataria*, *Melissa officinalis*, *Agastache foeniculum*, *Lavandula angustifolia*, *Origanum vulgare*, *Althaea officinalis*, *Plantago lanceolate*, *Artemisia absinthium*, *Populus nigra*, *Evernia prunastri*	*Staphylococcus xylosus*, *S. intermedius*, *S. chromogenes*, *S. hyicus, S. aureus*, *Vibrio fluvialis*, *Serratia liquefaciens*, *Escherichia coli*, *Lactococcus lactis* ssp. *lactis, Enterobacter intermedius*, *Bacillus cereus*, *Yersinia ruckeri*, *Aeromonas hydrophila/caviae*, and *Kytococcus sedentarius*	Pașca et al., 2017
Minthostachys verticillata	*Streptococcus uberis*	Montironi et al., 2016
Thymus vulgaris and *Lavandula angustifolia*	*Staphylococcus and Streptococcus*	Abboud et al., 2015
Syzygium aromaticum, *Cinnamomum zeylanicum*	*Staphylococcus aureus*	Budri et al., 2015

Cinnamomum zeylanicum, *Citrus bergamia*, *Eucalyptus globulus, Foeniculum vulgare*, *Origanum majorana*, *Origanum vulgare*, *Rosmarinus officinalis*, *Satureja montana, Thymus vulgaris*	*Staphylococcus aureus*, *Staphylococcus chromogenes*, *Staphylococcus sciuri*, *Staphylococcus warneri*, *Staphylococcus xylosus* and *Escherichia coli*	Fratini et al., 2014
Thymus vulgaris	*Staphylococcus aureus*, *Staphylococcus epidermis*, *Escherichia coli* and *Staphylococcus agalactiae*	Motlagh et al., 2014
Cinnamomum zeylanicum and *transcinnamaldehyde (TC)*	*Staphylococcus* spp.	Dal Pozzo et al., 2012
Lemongrass and *Oregano*	*Staphylococcus aureus*	Choi et al., 2012
Origanum vulgare, *Thymus vulgaris*, *Lippia graveolens, Rosmarinus officinalis*, *Salvia officinalis*, *Ocimum basilicum*, *Zingiber officinale*	*Staphylococcus* spp.	Dal Pozzo et al., 2011
trans-cinnamaldehyde *(Cinnamomum verum)*, eugenol *(Eugenia caryophillis)*, carvacrol, and thymol *(Origanum glandulosum)*	*Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Staphylococcus aureus*, *Escherichia coli*	Bascaran et al., 2009

The remarkable antimicrobial activity of *O. vulgare* and *T. vulgaris* against *Staphylococcus spp.* from different studies has been confirmed (Dal Pozzo et al., 2011*;* Kovacevic et al., 2021; Kovacevic et al., 2022). Furthermore, MIC values (0.16–10 µl/ml) for EOs obtained from *O. vulgare* (Norby and Halbert, 2016) and MBC values (6.25 mg/ml) (Kovacevic et al., 2022) show significant antimicrobial activity against *Staphylococcus* spp. Similar activity against *Staphylococcus* spp. is confirmed for EOs obtained from *T. vulgaris*; MIC values from 0.8 to 3.2 mg/ml and MBC values from 1.6 to 3.2 mg/ml (Dal Pozzo et al., 2011).

Most *in vitro* studies against bovine mastitis have focused on *S. aureus* isolates. This bacteria is considered a main pathogen of bovine mastitis and well-known biofilm producer highly resistant to antimicrobial agents which makes it difficult to control (Lopez et al., 2020; Pedersen et al., 2021). *T. vulgaris* and *O. vulgare* EOs have shown promising results against these bacteria; MIC values from 0.05 to 6.25 µl/ml for *T. vulgaris* EO and MBC values from 0.1 to 6.25 µl/ml (Piotr et al., 2018). Additionally, Choi et al. (2012) reported MIC values from 1 to 4 mg/ml for EO obtained from *O. vulgare*.

It is established that EOs and their major components could exhibit inhibition of initial biofilm formation. However, some studies have shown that low concentrations of EO, such as tea tree oil and cinnamaldehyde, can increase bacterial metabolic activity in relation to biofilm production due to environmental stress in *S. aureus* and *Pseudomonas aeruginosa*, respectively (Kwiecinski et al., 2009). Interestingly, the strong antibiofilm activity for *Syzygium aromaticum* and *Cinnamomum zeylanicum* EO and cinnamaldehyde against *S. aureus* recovered from cases of subclinical bovine mastitis was reported by Budri et al. (2015). Furthermore, carvacrol, one of the main antibacterial components of oregano oil and other EOs, was reported to inhibit biofilms of *S.*

aureus and *S. typhimurium* in the initial growth phase and prevented the formation of mature biofilms (Knowles et al., 2005).

Essential oils *in vivo* efficiency against bovine mastitis pathogens

It is always challenging to transfer *in vitro* results in *in vivo* study models. Hence, there are only a few studies that tested and confirmed *in vivo* activity of EOs based phytopharmaceuticals targeting udder tissue of dairy cows with diagnosed subclinical and clinical form of mastitis. Actually, beside different kind of EOs, different pharmaceutical formulation and dosage are used. In addition, the most common way of application of the EOs based pharmaceuticals was intramammary, followed by application of the semisolid pharmaceutical formulations (gel or ointment) (Table 2). Besides, Hase et al. (2013) evaluated therapeutic efficacy of herbal spray and gel. The results indicated that herbal therapy by potentiating the udder immunity, not only eliminates udder infection in subclinical form of mastitis, but also there were no side effects observed. On the other hand, use of ointment derived from oregano EO suggest that it may be a useful alternative to antibiotics in the control of subclinical form of bovine mastitis caused by *S. aureus* and/or *E. coli* (Cho et al., 2015).

Table 2. Antibacterial activity of EOs against bovine mastitis pathogens – *in vivo*

EOs	Pharmacological form	Study
Origanum vulgare, *Lavandula angustifolia* and *Rosemary*	Gel	Pașca et al., 2020
Thymus vulgaris, *Gaultheria procumbens*, *Glycyrrhiza uralensis*, *Angelica sinensis*	IMM infusion	Mullen, 2020
Angelica dahurica and *Rheum officinale extracts*	IMM infusion	Yang et al., 2019
Minthostachys verticillata	IMM infusion	Montironi et al., 2019
Salvia officinalis	IMM infusion	Alekish et al., 2017
Origanum vulgare	Ointment	Cho et al., 2015
Thymus vulgaris and *Lavandula angustifolia*	intramammary injection, external application or massage of a solution	Abboud et al., 2015
Thymus vulgaris, *Gaultheria procumbens*, *Glycyrrhiza uralensis*, *Angelica sinensis*	IMM infusion	Pinedo et al., 2013
Cedrus deodara, *Curcuma longa, Eucalyptus globules*, *Glycyrrhiza glabra*	Herbal spray	Hase et al., 2013
Cedrus deodara, *Curcuma longa*, *Eucalyptus globules*, *Glycyrrhiza glabra*	Gel	Hase et al., 2013
Lippia salvifolia and *Lippia sidoides*	Gel, cream, ointment and solution.	Soares et al., 2013
Thymus vulgaris, *Gaultheria procumbens*, *Glycyrrhiza uralensis*, *Angelica sinensis*	IMM infusion	McPhee et al., 2011
Thymus vulgaris, *Rosmarinus verbenone*, *Laurus nobilis*	IMM infusion	Lefevre et al., 2008

Mullen et al. (2020) and McPhee et al. (2011) reported no visible signs of udder irritation and good tolerance of EOs formulations of two commercial products known as Cinnatube® and PhytoMast® in cows with mastitis. Contrary to this, Alekish et al. (2017) reported significant decrease in somatic cell count (SCC) post treatment with intramammary infusion of sage EO. Similar results on SCC were observed by Hase et al. (2013) and Cho et al. (2015). On the other hand, Yang et al. (2012) tested the therapeutic effectiveness of *Angelica dahurica* and *Rheum officinale* extracts in bovine mastitis therapy and they pointed out lower probability for the causative agents to develop drug resistance post treatment.

Interestingly, the duration of treatment lasted differently depending on the type of preparation used in the study. Usually, treatment duration varied from three days (Pinedo et al., 2013; Cho et al., 2015; Alekish et al., 2017) to five days (Hase et al., 2013).

Natural formulations used in *in vivo* studies listed in Table 2 suggested good efficacy in the treatment of bovine mastitis measured trough clinical and/or bacteriological cure of this disease. Additionally, medicinal plant extracts appear to be a safe, efficient, and low-cost option for treating this type of disease, minimizing economic losses, but also affecting the safety of animal products consumption.

CONCLUSION

Possibility of using phytochemicals is gaining more and more attention in light of replacement of different synthetic drugs. EOs can replace antibiotics, being immune enhancers and fight bacterial infections without any potential side effects and leaving harmful residues in the animal body. Furthermore, various *in vitro* and *in vivo* studies have shown promising results regarding the use of EOs in prevention and treatment of bovine mastitis. As alternative to antibiotic treatment, EOs based phytochemicals still need to clarify composition and mechanism of action in order to assess the appropriate dose and dosage regimen that should be safely used in cows with diagnosed subclinical and clinical form of mastitis. Since there is a gap in field, especially in non-antibiotic treatments for mastitis in organic dairy herds, tested and standardized EOs phytochemicals could find their place in different pharmaceutical forms regarding the site of the pharmacological activity.

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ПРЕГЛЕДНИ НАУЧНИ РАД

ПРЕГЛЕД ИСПИТИВАЊА ЕТАРСКИХ УЉА КАО АЛТЕРНАТИВЕ АНТИБИОТИЦИМА У ЛЕЧЕЊУ МАСТИТИСА КРАВА

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РЕЗИМЕ: Маститис је, као озбиљно обољење присутно у млекарској индустрији, одговоран за добробит и значајне економске губитке млечних крава. Примена антибиотика представља једну од главних стратегија за контролу маститиса. Међутим, овај приступ има неке недостатке укључујући присуство резидуа антибиотика у млеку, ниску стопу излечења, као и пораст броја патогена резистентних на антибиотике. Из наведених разлога, велики број научника бави се налажењем алтернатива антибиотицима како би пронашли ефикасан приступ у терапији маститиса крава. Циљ овог ревијалног рада јесте анализа различитих публикација о употреби етарских уља у терапији маститиса крава. Постоје многа *in vitro*

испитивања антимикробне ефикасности етарских уља против различитих патогених узрочника маститиса. Многобројна етарска уља која су испитана показала су добру антимикробну активност са широким распоном минималних инхибиторних и минималних бактерицидних концентрација. С друге стране, само неколико *in vivo* истраживања бавило се испитивањем терапијских ефеката етарских уља. Поред тога, недавна истраживања указују на могућност употребе етарских уља с циљем смањења формирања биофилма, што може дати одличне резултате у борби против маститиса крава, обзиром да се неуспешна антибиотска терапија може повезати са присуством биофилма.

КЉУЧНЕ РЕЧИ: антибиотици, маститис крава, етарска уља, *in vivo*, *in vitro*, биофилм

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REVIEW SCIENTIFIC ARTICLE

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ANTIMICROBIAL AGENTS IN LAYING HENS

ABSTRACT: The European Union permitted 6 antimicrobial agents that can be used in laying hens. These are colistin, tyrosine, neomycin, oxytetracycline, chlortetracycline, and erythromycin. Antimicrobial drugs are used today primarily for the prevention and treatment of diseases in poultry and often (not in the EU) to stimulate growth. Because these drugs are often used irrationally, there are good chances that their residues will be found not only in poultry meat but also in the eggs within a certain period after the termination of treatment. In addition to the administration of authorised VMPs, the residues in eggs can be the result of erroneously applied medicated food, the contamination of the food with some antimicrobial drug in the mixing unit, as well as "extra-label" use of drugs in poultry. The antimicrobial agents are distributed in the body and deposited in the eggs, mainly in the yolk where they persist longer than in the albumen. Drugs that are poorly absorbed from the gastrointestinal tract (aminoglycosides, aminocyclitols, polymyxins) cannot be detected in the eggs, while the residues of some antimicrobial drugs can be detected for up to two months (chloramphenicol) after the last treatment. The rational use of drugs in veterinary medicine has manifold significance. When using drugs only when they are really necessary (indicated), in the right dose and route of administration, the potential damage can be reduced and efficiency increased, while the risk of microorganism resistance development would be significantly decreased. All of this becomes more important when these drugs are used in food animals.

KEYWORDS: antimicrobials, eggs, laying hens, residues, yolk, albumen

INTRODUCTION

In the clinical practice of human and veterinary medicine throughout the world, a large number of antimicrobial drugs are used. Likewise, many scientists

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intensively work on the discovery and synthesis of new drugs with a broader antimicrobial spectrum, stronger action, and a more satisfactory safety profile. Unfortunately, these drugs are often not rationally used. Despite permanent indications for all failures and harmful consequences of such use, it is present in everyday clinical practice (Ćupić and Dobrić, 2003).

Non-rational use of these drugs in veterinary medicine, as well as the need for control of their use, become greater problems regarding their use in food-producing animals. In that case, there is a possibility that minimal quantities of the drugs and their metabolites (residues), which remain in the edible tissues and animal products (meat, milk, eggs, honey) can induce some harmful effects in people as potential consumers of that food (Adams, 2001; Giguere et al., 2013; Ćupić and Živanov, 1990; Ćupić, 1997, Ćupić and Teodorović, 1997).

Because of their toxicity, both for animals (to whom are applied) and people as potential consumers of the products derived from these animals, the Food and Drug Administration (FDA) has banned the use of some antimicrobials, as well as some other drugs in food for animals. These are chloramphenicol, nitroimidazoles, nitrofurans, quinoxalines, fluoroquinolones, sulfonamides, glycopeptides, ionophores, cephalosporins, diethylstilbestrol, dipyrone, phenylbutazone, clenbuterol, and some antiviral drugs in poultry (Payne et al., 1999; Davis et al., 2009).

The use of antibiotics in laying hens has always been the subject of discussion because there are people who support their use and those who do not support it. In the Republic of Serbia and some neighboring countries, no antimicrobial drugs are used for the treatment of diseases in laying hens. However, antimicrobials are used in some countries. This relates mainly to those antibiotics that absorb from the digestive tract little or not at all (Goetting et al., 2011).

The European Union has approved 6 antimicrobial agents that can be used in laying hens. These are colistin, tylosin, neomycin, oxytetracycline, chlortetracycline, and erythromycin. In some countries of the European Union, USA, Australia, and Canada, the following antimicrobial drugs are approved: bacitracin, chlortetracycline, lincomycin and spectinomycin, neomycin, and tylosin (Australia); chlortetracycline, neomycin, oxytetracycline, and penicillin G (Canada); chlortetracycline, colistin, erythromycin, phenoxymethylpenicillin, tiamulin, and tylosin (Ireland); colistin, erythromycin, phenoxymethylpenicillin, tiamulin, and tylosin (England) and bacitracin, erythromycin, hygromycin B, nystatin, and tylosin (USA) (Goetting et al., 2011).

Although laying hens lay eggs every day (every 24 h), each egg takes several days to develop *in vivo* (and some egg components several months). It is considered that the period from the 10th to 14th day before the egg is laid (this is the period of intensive development of the yolk) is the most suitable for the deposit of residues of drugs in eggs. Just during this period the largest amount of lipoproteins arrives from the liver with circulation and takes part

in the final stage of the formation of the yolk. If individuals are treated in this period, then the greatest chances are that lipoproteins are contaminated with drug residues (Goetting et al., 2011).

Depending on the physicochemical properties, the drugs are distributed in different concentrations in the body and deposited in the yolk and albumen. The largest number of drugs (probably because of the longer development of the yolk) achieve higher concentrations in the yolk than in the albumen. The retention length of certain antimicrobial drug residues in the eggs after the treatment is different (Goetting et al., 2011).

Drugs that are poorly absorbed from the gastrointestinal tract (aminoglycosides, aminocyclitols, polymyxins) cannot be determined in the eggs, while the residues of some antimicrobial drugs that are absorbed can be detected for up to two months (chloramphenicol) after the last treatment (Goetting et al., 2011).

This paper gives an overview of the pharmacokinetics of some groups of antimicrobial drugs: *aminoglycosides, amphenicols, tetracyclines, and macrolides*, with special emphasis on the possibility of depositing these drugs in eggs.

ANTIMICROBIAL DRUGS

Aminoglycosides

Aminoglycosides (like aminocyclitols) act on gram-negative and some gram-positive but not anaerobic bacteria. They are very poorly absorbed from the digestive tract. After oral administration, these drugs are mostly excreted in mammals by feces. In birds, aminoglycosides after oral administration, are eliminated by feces also (Botsoglou and Fletouris, 2001; Adams, 2001; Brown and Riviere, 1991).

Because of the poor absorption from the digestive tract, it is rare to find a residue of these drugs in the eggs after oral administration. When aminoglycosides are given parenterally for the treatment of systemic infections, the main route of elimination of these drugs in mammals is the kidney.

However, in mammals and birds, the systemic application is limited due to the toxic effects (nephrotoxicity, ototoxicity) of these drugs. Although in birds there are no data on the pharmacokinetics of systemically administered aminoglycosides, a nephrotoxic effect is also expected and it is believed that the main pathway of elimination is the kidney (Bennett et al., 2001).

When aminoglycosides are applied to laying hens intramuscular or subcutaneous, gentamicin and dihydrostreptomycin are deposited in yolks and albumen and the residues persist for a long time in the yolk (Roudaut, 1989b; Filazi et al., 2005) (Table 1).

The retention length of these drug residues in eggs is shown in Table 1.

Table 1. Residues of aminoglycosides in chicken eggs after parenteral and oral administration to laying hens (Goetting et al., 2011)

Drug	Status	Maximum residue limit	Dose and mode of application	Hen age (months)	Treatment duration (days)	The time from last treatment until the residue no longer detected (days)
Gentamicin	EU: Not approved	None	25 mg/kg b.w. (s.c.)	7,5	1	Y: 10 A: 4 WE: 10
			50 mg/kg b.w. (s.c.)	7,5	1	Y:12 A: 5 WE: 12
			10 mg/kg b.w. (i.m.)	7,5	1	Y: 7 A: 3 WE: 7
Neomycin	EU: approved USA: Not approved	500 µg/kg	0.25 g/l p.o. (in drinking water)	13-18	5	WE: 0
			25 mg/kg b.w. p.o. (in drinking water)	13-18	5	WE: 0
Kanamycin	EU: Not approved	None	20 mg/kg b.w., p.o. (in food)	10	7	Y: 0 A: 0 WE: 7
			1,000 mg/kg food, p.o. (in food)	10	7	Y: 0 A: 0
			4,000 mg/kg food, p.o. (in food)	10	7	Y: 0 A: 0
			8,000 mg/kg food, p.o. (in food)	10	7	Y: 0 A: 0

WE – whole egg; Y – yolk; A – albumen

This table shows that gentamicin persists longer in egg yolk than in albumen and the retention length is dependent on the dose.

As already said, neomycin belongs to a group of antimicrobials that are approved in the EU. Because of that, a maximum residue limit is determined. However, this drug is poorly or not absorbed and consequently, it cannot be determined in eggs.

This table shows that kanamycin persists longer in egg yolk than in albumen regardless of the dose and retention length is dependent on the dose.

Amphenicols

Amphenicols effectively act against rickettsia, chlamydia, anaerobic and gram-positive aerobic bacteria, as well as intestinal bacteria. Since it can cause irreversible bone marrow suppression in humans, the use of chloramphenicol

is banned or restricted in animals which are used for human consumption in many countries (Ćupić et al., 2003, 2019). Amphenicols are given orally in food or drinking water to poultry (Bishop, 2001; Papich and Riviere, 2001; Botsoglou and Fletouris, 2001; Dorrestein et al., 1984).

After oral administration to chickens, absorption is rapid but incomplete. They are rapidly distributed throughout the body, and the pathways of excretion vary depending on the drug. Studies performed in most mammalian species have shown that chloramphenicol is metabolized in the liver and excreted via the urine and the bile. The pathways of chloramphenicol excretion in birds are not described. In chickens, thiamphenicol is eliminated through both systems (bile and kidney). Florfenicol and its metabolite florfenicol amine are deposited in significant amounts in the liver and kidneys (Anadon et al., 1994a; Bennett et al., 2001).

A small number of studies carried out in laying hens, examining the elimination of amphenicol residues, showed that the residue can be found in the yolk and the albumen a few days (and more) after oral administration.

The persistence of residues of these drugs in eggs is shown in Table 2.

Table 2. Residues of amphenicols in chicken eggs after oral administration to laying hens (Goetting et al., 2011)

Drug	Status	Maximum residue limit	Dose and mode of application	Hen age (months)	Treatment duration (days)	The time from last treatment until the residue no longer detected (days)
Chloramphenicol	Not approved	None	40 mg/l p.o. (in drinking water)	Not specified	5	Y: > 5 A: 4
			500 mg/l p.o. (in drinking water)	3	8	WE: > 17
			1,000 mg/l p.o., (in drinking water)	3	6	WE: > 19
			60 mg/kg b.w., p.o., (in drinking water)	10	10	WE: > 72
Thiamphenicol	Not approved	None	40 mg/kg t.m., p.o. (in capsules)	6	1	Y: 10 A: 2
			40 mg/kg t.m., p.o. (in capsules)	6	5	Y: 8 A: 1

WE – whole egg; Y – yolk; A – albumen

Table 2 shows that chloramphenicol persists in eggs for a very long time, even more than 72 days.

Unlike chloramphenicol, the residues of thiamphenicol remain much shorter in eggs.

Tetracyclines

Tetracyclines are a typical example of antimicrobials with a broad spectrum of action. They are used to prevent and treat diseases, as well as to improve growth in animals whose products are used to feed people in countries where such use is legal (Giguere et al., 2013). They are effective against a large number of gram-positive and gram-negative bacteria, mycoplasmas, chlamydia, and rickettsia. The most common mode of using tetracycline in poultry is oral (in food or drinking water) (Botsoglou and Fletouris, 2001; Chopra and Roberts, 2001).

Generally, tetracyclines are moderately absorbed from the digestive tract in mammals, but absorption is incomplete in birds. Tetracyclines have a high affinity for ionic metals, such as calcium, iron, magnesium, and zinc, which hinder absorption if present in the food or digestive system (Anadon et al., 1994b; Botsoglou and Fletouris, 2001).

When tetracyclines are absorbed, they are distributed throughout the body and concentrated in the liver and kidneys. Tetracyclines are also deposited in egg-laying hens. After administration, the residues of these drugs appear more rapidly in the albumen than in the yolk but the concentrations in the yolk are higher and persist longer. The achieved levels of residues and the degree of their decrease in eggs depend on the mode of administration, the dose, and the drug that is applied (Frazier et al., 1995; Yoshida et al., 1973c).

When administered in the same dose and in the same way, doxycycline is deposited in eggs at higher concentrations than tetracycline, and tetracycline achieves higher concentrations than oxytetracycline. Variations in the persistence of residues in eggs are a direct consequence of the difference in drug absorption. Doxycycline can be detected in eggs for almost a month after discontinuation of the drug, while after a similar dosage regimen, oxytetracycline residues can be detected within 4–10 days after administration (Nogawa et al., 1981; Roudaut et al., 1989).

The persistence of residues of these drugs in eggs is shown in Table 3.

Table 3. Residues of tetracyclines in chicken eggs after oral and parenteral administration to laying hens (Goetting et al., 2011)

Drug	Status	Maximum residue limit	Dose and mode of application	Hen age (months)	Treatment duration (days)	The time from last treatment until the residue no longer detected (days)
Oxy-tetracy-cline	EU: Approved USA: Not approved	200 µg/kg	0.1 g/l (10 mg/kg b.w.) p.o. (in drinking water)	Not specified	5	Y: 0 A: 0
			0.25 g/l (25 mg/kg b.w.) p.o. (in drinking water)	–	5	Y: 4 A: 3
			0.4 g/l p.o. (in drinking water)	12	7	Y: 3 A: 0
			30 mg/kg b.w., i.m.	Not specified	3	Y: 11 A: 9
			200 mg/kg b.w., i.m.	Not specified	5	Y: 12 A: 5
Chlor-tetracy-cline	Approved in EU and USA	200 µg/kg (EU) 0.4 mg/kg (USA)	0.5 g/l p.o. (in drinking water)	Not specified	7	WE: 6
Doxycy-cline	EU: Not Approved USA: Not Approved	None	0.5 g/l p.o. (in drinking water)	Not specified	7	Y: 27 A: 25

WE – whole egg; Y – yolk; A – albumen

Table 3 shows that the residues of doxycycline (when compared to chlortetracycline and oxytetracycline) remain the longest in eggs.

Macrolides

These antibiotics are effective against mycoplasmas and gram-positive microorganisms (streptococci and staphylococci), while they are less active against gram-negative bacteria. The oral route is the most common route of administration of these drugs to chickens. After the absorption, in birds and mammals macrolides are widely distributed in the body and penetrate everywhere into the tissues and cells, deposited mostly in the yolk (Adams, 2001; Papich and Riviere, 2001; Anadon and Reeve-Johnson, 1999; Botsoglou and Fletouris, 2001; Ćupić et al., 2019).

The persistence of residues of these drugs in eggs is shown in Table 4.

Table 4. Residues of macrolides in chicken eggs after oral administration to laying hens (Goetting et al., 2011)

Drug	Status	Maximum residue limit	Dose and mode of application	Hen age (months)	Treatment duration (days)	The time from last treatment until the residue no longer detected (days)
Tylosin	Approved in EU and USA	200 µg/kg (EU and USA)	0.5 g/l p.o. (in drinking water)	5	5	WE: 8
			0.5 g/l p.o. (in drinking water)	7–16	5	Y: 0 A: 0
			0.5 g/l p.o. (in drinking water)	Not specified	7	Y: 6 A: 3
			0.529 g/l p.o. (in drinking water)	Not specified	3	WE: 6
Spira-micin	EU: Not Approved	None	100 mg/kg b.w., p.o. (in food)	10	7	WE: 2
			200 mg/kg food, p.o. (in food)	10	7	WE: 1
			400 mg/kg food, p.o. (in food)	7–16	7	Y: 7 A: 15

WE – whole egg; Y – yolk; A – albumen

Table 4 shows that the length of retention of residues of the tylosin and spiramycin in chicken eggs depends on the length of treatment and the age of the animals being treated with the drug.

CONCLUSIONS

The wide and irrational use of drugs in animals whose products are used for human consumption (in addition to all other adverse effects) inevitably leads to an increased risk that a certain amount of these drugs remains in foods of animal origin.

Since residues of antimicrobial drugs can also be deposited in eggs, special attention should be devoted to the use of these drugs in poultry, especially laying hens.

In the European Union, 6 antimicrobial drugs were approved for laying hens. These are neomycin, erythromycin, tylosin, oxytetracycline, chlortetracycline, and colistin.

In the Republic of Serbia and some neighboring countries, no antimicrobial drugs are used for the treatment of diseases in laying hens. However, there are cases when antimicrobials are used. This relates mainly to the antibiotics that are very poorly absorbed from the digestive tract.

The question arises whether we are fully aware of the real situation in the field and whether the prohibition on the use of antimicrobial drugs is being respected.

We suppose it is not being fully respected. That was the reason for the presentation of this paper.

Therefore, we think that this issue should be dealt with properly and that some of the drugs approved in the EU should be also approved in Serbia, in the first place those that are not absorbed from the digestive tract.

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ПРЕГЛЕДНИ НАУЧНИ РАД

АНТИМИКРОБНИ ЛЕКОВИ КОД КОКА НОСИЉА

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РЕЗИМЕ: Европска унија је одобрила шест антимикробних лекова који се могу користити код кока носиља конзумних јаја. То су: колистин, тилозин, неомицин, окситетрациклин, хлортетрациклин и еритромицин. Антимикробни лекови се данас користе првенствено за превенцију и лечење болести код живине, а

често (не у ЕУ) за стимулацију раста. Узимајући у обзир чињеницу да се ови лекови често користе нерационално, постоје велике шансе да ће се њихови остаци наћи не само у месу перади већ и у јајима у одређеном периоду након престанка лечења. Поред примене одобрених лекова, остаци у јајима могу бити резултат погрешно примењене лековите хране, контаминације хране неким антимикробним леком у мешаонама хране, као и „ехтра-лабел" коришћења лекова код живине. Антимикробни лекови се дистрибуирају у организму и депонују у јајима, углавном у жуманцету где се задржавају дуже у односу на беланце. Лекови који се слабо апсорбују из гастроинтестиналног тракта (аминогликозиди, аминоциклитоли, полимиксини) не могу се утврдити у јајима, док се остаци неких антимикробних лекова који се апсорбују могу детектовати и до два месеца (нпр. хлорамфеникол) након последњег третмана. Рационална употреба лекова у ветеринарској медицини има вишеструки значај. Употреба сваког лека, само када су заиста неопходни (индиковани) у правој дози и начину примене, потенцијална штета од њихове употребе би се смањила, а ефикасност повећала, те би се ризик од развоја резистенције код микроорганизама значајно смањио. Све ово постаје још важније када се ови лекови користе код животиња чији се производи користе за исхрану људи.

КЉУЧНЕ РЕЧИ: антимикробни лекови, јаја, коке носиље конзумних јаја, резидуе, жуманце, беланце

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ORIGINAL SCIENTIFIC ARTICLE

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CITIZEN SCIENCE – SCIENCE – INDUSTRY: A CASE STUDY IN SELF-DRIVING CAR

ABSTRACT: The paper discusses the connection between citizen science, science and industry in the field of STEM technologies. A methodology is established for connecting non-expert and expert groups on certain research topics in STEM with the aim to achieve better dissemination of knowledge from universities and research laboratories and industry. On the other hand, the CS projects give the response, recommendations, opinions, etc. to new technological achievements, which should correct and direct the further scientific research and innovation activities. The paper gives a case study of the CS-science-industry project realized among students of Faculty of Technical Sciences of Novi Sad and experts, about self – driving car. The aim of the project was to assess the degree of acceptability of this technical solution and, in correlation with experts, to make a recommendation for finishing or improving this vehicle according to the requirements of future users. CS actors were informed by experts about the importance and characteristics of these vehicles, as their introduction into public transport is expected in the near future. In coordination with the experts, the CS actors formed a questionnaire in which they asked questions that should indicate the degree of acceptability of the vehicle, but also gave the opportunity to express opinions and views on the vehicle. The answers on the questionnaire were put on a MS Teams platform. The analysis of the results of the CS project pointed to the low readiness of young people to drive a self-driving car of the 5th level (without human-driver), and gave recommendations and ideas to experts on finishing and modifying the vehicle. The work of CS and experts enabled CS actors, as future engineers and bearers of technical progress, to realize the importance of innovation and STEM technologies, and experts to gain better insight into the reaction and opinion of non-experts about their product.

KEYWORDS: citizen science, innovation, knowledge dissemination, non-expert group, self-driving car

INTRODUCTION

This paper deals with the possibility of incorporating the so-called "citizen sciences" (CS) into a science that is being developed and researched within

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scientific and professional institutions, primarily universities and institutes. The coupling of CS is shown, i.e, the results of research by amateur scientists and research conducted by scientists and highly qualified experts. Citizen science means the participation of the public in scientific research, the outcome of which is the improvement of scientific knowledge as well as the improvement of public understanding of science. It can be considered as a subset of science where scientific research is conducted in whole or in part by amateur scientists. The importance of working on CS projects is that these projects contribute to improving the lives of citizens, and the entire community. It has two basic tasks: the first, to provide insight into the opinion and attitude of people about modern technologies and to involve CS activities as intensively as possible in modern science, and the second task is to provide CS activities with a large amount of data that will be the input base for further research by experts. Involving a large number of citizens – volunteers in the project allows getting a lot of data in a cheap way. People in CS projects volunteer to contribute to scientific improvement by gathering and managing information. The arrival of the 21st century has led to the rapid development of the CS initiative in uniting scientists and peoples with the aim of raising awareness and finding solutions to social problems, environmental protection tasks including health, defining social trends, predicting human mobility, technology impact such as exchanging information on natural resources, living conditions, as well as on species protection. Nowadays, volunteers can quickly locate CS projects by subject of interest and easily access them, because human-computer interaction is made possible by simply connecting to projects that were not possible before. Thus, every citizen can have a vital impact on research, from providing information to experts to participating in decision-making. In order to realize the connection of CS with science, it is necessary to organize and coordinate between citizens, state institutions, agents in industry and science and local groups. Depending on the content of the research, basic scientific principles must be met, as well as well-designed methods of data collection and methods for their evaluation, accompanied by explicit instructions and research questions, and providing feedback as a reward for participating in the project.

Sensors and meters are used for data collection, which, owing to the most modern technology, can provide data with a rather high accuracy compared to professional devices. The cost of the device is relatively low (significantly lower than the equipment used for research in professional laboratories) and the process of measuring and collecting data is rather short. This is made possible by the development of new technologies, especially smartphones with various sensors and applications that allow the detection of various physical quantities and their monitoring and storage in a brief period of time.

The data obtained within the CS must be distributed in a brief period of time. This is made possible by special applications that provide easy and fast data transfer to central servers or other information collectors. Transferring data from the phone of CS participants to the server or any center must be as simple as possible, because it has been shown that if there is a small difficulty or if the transmission requires a long time, modern man gives up, because time is money

and it is a rather important life factor. In recent years, a lot of methods have been developed that allow data to be transmitted just by touching the screen, and a lot of new software programs have been developed that enable the realization and availability of data to those who will use it.. CS projects enable the population to be involved in scientific research and to provide data. Despite the increased interest in CS, the number of participants in some projects has decreased, because people are not ready to waste time filling out forms or sending some reports according to complicated procedures. In order to eliminate this shortcoming, new applications for mobile phones have been developed, which transfer data to the collection center in the simplest possible way, just by touching the screen, which further processes them. The paper presents as many as three such new techniques, their advantages and disadvantages (Younis et al., 2019).

The most common areas of CS's interest are in the field of environmental science, biodiversity, health, climate change, etc. In all fields of activity, CSs raise the awareness of the population about the need for science in order to improve the quality of life.

Data collection is most often performed by measuring data of a physical nature such as noise, radiation, magnetism, or chemical character (toxicity). The measured data can be used for the production of distribution maps, but also as input data for further scientific research. By installing convenient sensors in smartphones and installing appropriate application software, it is possible to generate various physical data of high quality and at a low price. This provided an opportunity for application in the new CS and mass application for monitoring these physical parameters for innovative research. Noise was measured in the CS project in Denmark (Sonne and Alstrp, 2019). About 100,000 children and adults took part in the project, measuring the noise level on their smartphones using a special application. Based on the data obtained by the volunteers, a map of noise pollution was made (Sonne and Alstrp, 2019). In addition to noise, radioactivity and magnetism can be measured from physical quantities (Odenwald, 2021). In order to obtain information on the toxic effect of plants on humans, microscopic HAB detectors have been developed and connected to mobile phones. Such detectors can, based on the analysis of the number of flower cells in a small sample of water, determine its harmfulness to human respiratory organs (Hardison et al., 2019) and enable the creation of a map by classifying the levels. Measurement of quantities according to the GM project can also be used to test scientific results. Thus, the onomatopoeia program for the identification of bird call was verified by non-experts within the CS project. It has been shown that the data collected within CS projects indicate the need to expand the method and are of special importance for scientists for further development and better understanding of bio-acoustic phenomena (Vella et al., 2021).

Another aspect of CS relates to the transfer of scientific knowledge to a large number of people. Thus, in Denmark, the collection of plastic waste as an environmental pollutant was organized within the CS (Sonne and Alstrp, 2019). 100,000 children and 50,000 adults participated in the project. This has

proven to be one of the best ways to disseminate knowledge and raise awareness about environmental pollution. Within the Horizon2020 project, CS activity was realized on school children aged 7–8 in primary school in Ljubljana, Slovenia (Kocman et al., 2020). The children were introduced to noise by experts, actively measured noise, participated in the formation of the project and gave an assessment of the results together with experts in the field. This interaction between science and CS has been shown to give extremely good results. Using the suggestions given within this project, GM was made in the field of STEM (science, technology, engineering and mathematics) technology. The research was conducted on the topic of self-driving car (SDC).

The paper has four parts. After the introduction, based on the known method for CS-science interaction, a modified and extended procedure for connecting CS-science – industry in the topic of STEM is developed. The method is tested on the project dealing with the self-driving car. Chapter 3 presents the results of the research. The paper ends with conclusions.

METHOD OF INVESTIGATION

As it is shown in the Introduction, the main aim of CS – science projects which are already realized is to obtain a large number of data which would be of interest for experts on the topic. However, for STEM technologies it is of interest to enlarge the project by introducing also the experts of industry who are working in research institutions or dealing with the problem of sale and distribution of new products. Based on results of CS project, the scientific investigation and production activities in industry need to be modified. At the other side, there is the need for the feedback of experts to participants of CS to increase their technical and scientific literacy. The public survey already done is only in one direction, from respondents to scholars (Payre et al., 2014; Schoettle and Csirak, 2014; Penmetsa et al., 2019) and not to experts in industry, and there is no flow of information in the opposite direction. In this procedure it is suggested the project activities to be divided into 4 phases: a) identification and definition of problems, b) coding of research activities and formulation of hypotheses and a questionnaire, c) data collection and d) presentation and transparent discussion of results. As part of the identification, a recording of the current situation regarding the knowledge between non-experts in CS on the topic have to be made. After that, training on the topic in STEM has to be performed by experts. Usually, it is suggested to be in the form of the presentation put on the project platform. In the second phase, based on the suggestions of the project participants, a list of hypotheses has to be compiled and a questionnaire has to be formed for obtaining of the opinion of CS participants on the topic. In the third phase, data have to be collected by filling in a list of questions and the views of non-experts on the benefits, advantages and disadvantages of the suggested topic are obtained. Within the last phase, the analysis of the answers has to be performed and conclusions have to be formed by experts. The results of the analysis have to be distributed to CS actors and

experts in the field who should use this material to deepen certain aspects that are important for the adoption of the object of topic in the near future as the latest technical achievement important for human life.

The method of work is designed so that CS participating would be active subjects in research. Participants are given the opportunity to get acquainted with one of the results of modern research, and to be acquainted with scientific work and work on innovation within the modern economy and get an idea of what innovation is and how it should be treated.

PROJECT OF SELF-DRIVING CAR

The developed methodology of CS-science-industry is tested on the topic of the self-driving car (SDC) (Techopedia, 2017) which represents the recent type of cyber-physical system (Putnik et al., 2019). The aim of the research is to obtain public opinion on the acceptability of SDC as a future mean of transport. The result of the research should enable experts to improve and refine the SDC in order to increase the degree of acceptability. On the other hand, the public will be much better informed about the new type of vehicle as a new technical achievement.

The CS project included 123 first-year students at the Faculty of Technical Sciences in Novi Sad and a group of experts in the field of SDC. The short text about self-driving car (SDC) of the 5th level of automation (SAE, 2018) is prepared by experts and put on the platform of CS. The text contains the information that every SDC-user needs to know (how to take the car, which personal data are necessary, to whom to turn with questions) and also some technical specifications (the car is without a human driver and usually without steering wheel, driving is in Auto-pilot mode, driving decision is done by computer with AI, control of SDC is done by the operating center).

The concept of self-driving car (SDC) which is considered within this project is such that it does not require a man-driver (SAE, 2018) but has the highest (5th) level of automation. This type of vehicle should provide significantly greater safety in traffic, but also improve the functioning of transport and thus people's lives. In an effort to equate the technological advancement of the digital age with the physical world, SCD uses information and communication technologies such as embedded sensors, GPS, and devices to collect and analyze data and integrate them. Then the physical environment is transformed into a dynamic source of information, which is used by the 'intelligent' vehicle. Based on the adaptation of data from networking, the system provides passengers with tools, resources and services to benefit from this data flow. By installing intelligent systems in the SDC, environment can be divided into 3 parts: a) Internet of Things (IoT) which facilitates the connection of physical and virtual devices using a communication protocol, b) Internet of Services (IoS) consisting of merging different applications into explanatory services and c) Internet of People (IoP) which involves the interaction between citizens who are the end users of the system. However, in order to realize the application of

SDC in ordinary life, it is necessary to harmonize between end users and producers of SDC, i.e. industry and economy. CS activities are extremely important for the realization of that request.

Based on the text, the participants pose different questions. The most important ones are about safety and security in SDC. The questions are dealing with physical safety and security during driving according to the fact that the decision is made by computer and not by human driver. There is the dilemma about cyber security and data protection, as well. Namely, the SDC user needs to give some personal data like ID, name, address, travel destination, etc. and the question is about these data protection, then how the SDC can be prevented from hacking and the level of the cyber security. The participant in CS were wondering about the benefits and lacks of SDC connected to traffic accidents and traffic jams, environmental protection, re-organization of infrastructure in towns, possible activities during riding, length of driving, etc. However, the main question asked by experts is: 'Is the population ready for self-driving vehicles?' (Page and Krayem, 2017).

Using the participants' comments in CS and also the experts' requirements, the questionnaire is formed and put on the CS platform.

RESULTS AND DISCUSSION

In the first group of questions, CS students answered the question whether they were familiar with SDC and filled out lists with personal data related to gender, place of residence and whether they were involved in traffic as drivers. The results of the survey showed that 33% of project participants are familiar with the automated vehicle, however, less than 5% are informed about SDC of the 5th degree of automation without man-driver and the movement of the vehicle guided by Autopilot. Male students are more informed about SDC than female students. That is why an online lecture on MS Teams platform was held where students were introduced to SDC. Explanations are given about the physical specifics of the vehicle: built-in sensors, perception systems, navigation systems, but also about decision-making systems with AI artificial intelligence. Aspects of the SDC study are: safety (Taeihagh and Lim, 2019) and cybersecurity (Petit and Shladover, 2015), energy consumption, environmental protection (Taiebat, 2018), sociological and economic aspects (Ryan, 2020), suitable infrastructure, etc. After that course on SDC by the professor, the students participated in the formation of hypotheses and the design of protocols for data collection and the formation of a list of questions by submitting their suggestions. This cooperation produced a questionnaire with the aim of examining whether SDC is acceptable as a means of transport by CS participants. Thus, the students expressed their attitude and opinion on traffic safety, comfort when traveling, activities while driving, seeing the new urban plan of cities and infrastructure, and gave their individual comments. The results of the CS project are: more than 80% of participants believe that the introduction of SDC and the elimination of conventional vehicles will reduce the number

of accidents, especially those with a fatal outcome. About half of the respondents believe that environmental pollution will be reduced, because transport will be more rational, so more people will be in the car at the same time and fuel consumption will be lower. However, there is also the opposite opinion, i.e, that there will be no improvement in the area of environmental pollution. Namely, it is expected that the number of rides with SDC would increase (driving would be available to the disabled, children, the elderly and infirm, and those who do not have a driver's license). The problem of lithium batteries and pollution was especially mentioned.

The opinion of 66% of CS participants is that the time spent in travel would decrease due to the fact that the traffic on the roads would reduce, there is no need to find the parking place, etc. Even 82% of respondent are ready to use SDC for long-distance drive rather than airplane due to much higher comfort and less time loss (going to the airport, spending time on checking, traveling according to a time-table). The participant of CS are sure that they would make the good use of time during driving: by performing their working activity, working on laptop, reading and sending messages, playing games and watching TV or Internet, making telephone calls, sleeping, eating, etc. However, some of them are asking whether this working activity would be regulated as working time.

Furthermore, 28% of CS participants are wondering about social aspects of SDC. Namely, introduction of SDC would eliminate some professions (like drivers) and reduce the number of auto mechanics (number of individual cars would reduce). However, some new professions would be increased, for example IT programmers and operators.

Economic aspects

More than 75% of project participants do not believe in AI decisions and would feel insecure in such a vehicle. Female participants are in the lead. As many as 97% of them are not ready to sit in a car that does not have a steering wheel. The analysis of the results demonstrated that the number of respondents who would be ready to ride with SDC is below 4%. This is a serious warning to the industry that it must take adequate and intensive actions to increase vehicle acceptance.

At this moment, insisting on a vehicle without a steering wheel and without presence of a man-driver is unacceptable even in the youth population (that is aged 20 years). The attitude of CS actors is that they do not believe the decisions of AI and in Auto-piloting without possibility of reconnection to human driver. This information forces experts in science that it is necessary to make additional efforts to improve and enrich software in order to improve.

Summarizing the results, all participants in the project (the CS participants but also experts from science and industry) reviewed the level of success of the project and gave conclusion that progress has been made with the following outcome:

1. CS participants are introduced to one of the latest developments in technology,
2. Dissemination of knowledge in the field of SDC and training on that topic were performed,
3. Experts from science and industry received information about the attitude of the general population about their technical innovation.

The CS project participants concluded that they are still interested in participating in projects of this type and that the method of co-creation and design of the project is a special pleasure, and that the project results stimulate them to think and engage in future creative work.

CONCLUSION

Based on the above, it can be concluded:

1. CS represents an adequate approach to involving broad layers of non-professional scientists in the process of research and consideration of the latest scientific or technical achievements. The strength of CS, which is recognized in the field of earth science, nature sustainability as well as the science of agriculture and food, should have been extended to STEM technologies. This has been done with the project presented in this paper. If CS projects are conceived in the right way, a large number of people can be involved without making a visible contribution to research and development through their CS activities.
2. It is convenient to acquaint the population with the new results of technical development with the action of forming a CS project related to science and industry. CS participants receive timely and accurate information on STEM technology innovations. On the other hand, science and industry receive a response to their innovative endeavors and opinions from people whose activity is outside that domain, and which can be a signpost for improvement, modification, and for directing towards new research. The implemented concept of activities proved to be suitable for connecting CS participants with experts in order to achieve communication and exchange of views and opinions on SCD. CS student participants gained knowledge of SDS but also had the opportunity to express their agreement or disagreement with this vehicle. Experts who benefited from working on this project gained insight and opinion of non-experts as useful advice for further work on SDC.
3. The integration of CS projects aims not only to increase scientific accessibility but can also serve as a framework in which students will be able to participate in the development of science. Students gain insights into what science is, how science works, why we need science, and what are the crucial aspects of raising scientific literacy. CS participants understand what research is, what is defined as science, who are scientists, who are innovators, what will be their future mission as an engineer. These issues seem too complicated for all those who have not been involved in CS so far.

4. The general conclusion is that the benefit of integrating people into CS projects is improving their power of critical thinking, gaining practical experience around working in a group, as well as ways to approach problem solving. Activities at CS contribute to the following two aspects: 1. getting to know and approaching the results of new technologies that are available and applicable today, 2. not only expanding knowledge about the topic they dealt with, but also allows to increase work experience and CS. Therefore, it is recommended that younger generations, even high school students, be included in the CS, especially in STEM field projects.

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ОРИГИНАЛНИ НАУЧНИ РАД

ГРАЂАНСКА НАУКА – НАУКА – ПРИВРЕДА: СТУДИЈА СЛУЧАЈА САМОВОЗЕЋЕГ АУТОМОБИЛА

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РЕЗИМЕ: У савременом свету све већа пажња поклања се тзв. грађанској науци (ГН) и пројектима ГН који се баве темама најчешће из области заштите животне средине, здравља популације, климатских промена, али и биодиверзитета. Посебна специфичност ових пројеката је да окупља врло велики број људи који су спремни активно да учествују у решавању проблема дајући одређене информације или мерећи одређене физичке величине, на пример буку, радијацију, магнетизам. Мерачи су уграђени и додати савременим паметним телефонима и применом специјалних апликација директно се пребацују у централни сервер где се и обрађују и ускладиште. Учесници у пројектима ГН обично су волонтери који нису експерти за област па су процедуре за добијање података и њихов једноставан и брз трансфер били од приоритетног значаја за научнике. Циљ ГН пројеката је да се на основу измерених података, који су прослеђени научним

институцијама и експертским организацијама, допринесе побољшању и унапређењу или решавању одређених проблема. Чак врло често, ови подаци могу бити и путоказ за даља истраживања. С друге стране, актери ГН пројекта имају бенефит да се правовремено и на валидан начин информишу о проблемима, науче да користе нове апликације и да врше мерења, али и да сазнају шта је то наука и како се врши истраживање. На тај начин се подиже и техничка свест широких слојева становништва. У овом раду разматрана је могућност проширења ангажовања ГН и на област СТЕМ технологија. Разрађена је организација повезивања грађанске науке, научних истраживања и привреде. Предложена је методологија успостављања конекције између неекспертских и експертских група по техничкој теми истраживања с циљем остварења боље дисеминације знања са универзитета и истраживачких лабораторија и из привреде. С друге стране формирањем пројекта ГН добија се одзив од популације о новом технолошком достигнућу, мишљење и сугестија који треба да коригују и усмере даља истраживања и иновативну делатност. У раду је дат осврт на ГН пројекат реализован међу студентима ФТН са темом самовозећих аутомобила 5. нивоа аутоматизације. Циљ пројекта био је да дâ оцену о степену прихватљивости овог техничког решења и да у корелацији са експертима дâ препоруку за дораду или побољшање овог возила према захтевима будућих корисника. Актери ГН су од стране експерата информисани о значају и карактеристикама ових возила чије се увођење у јавни саобраћај очекује у скорој будућности. У координацији са експертима актери ГН су формулисали упитник у којем су поставили питања на основу чијих одговора треба да се покаже став и мишљење неексперата о возилу. Анализа резултата пројекта ГН указала је на неспремност младих да се возе самовозећим аутомобилом без возача па и возила без волана, и дала је препоруке и идеје експертима око дораде и модификације возила. Рад на пројекту ГН са експертима омогућио је да актери ГН, као будући инжењери и носиоци техничког прогреса, спознају значај иновације и СТЕМ технологија, а научницима и стручњацима у области да добију бољи увид у реакцију и мишљење неексперата о њиховом производу.

КЉУЧНЕ РЕЧИ: грађанска наука, дисеминација знања, неекспертска група, самовозећи аутомобил, техничка иновација

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• 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.

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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.

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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.

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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.

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