



University of Novi Sad - Univerzitet u Novom Sadu
Faculty of Agriculture - Poljoprivredni fakultet



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POSTWEANING ESTRUS REACTION OF SOWS AFTER THE TREATMENT WITH eCG IN WARM AND COOL SEASONS*

BLAGOJE STANČIĆ, IVAN STANČIĆ,
IVAN RADOVIĆ, ALEKSANDAR BOŽIĆ¹

SUMMARY: Numerous studies show significantly reduced sow fertility in the warm period of the year. The aim of this study was to investigate the effect of sow treatment with eCG preparation, 24h after weaning, on the estrous reaction level. A significantly ($p < 0.01$) higher number of sows, treated with single eCG injection of 1,000IU (primiparous) or 1,500IU (older sows), manifested estrus within 7 days after weaning (83.7%) in comparison with the sows which were not treated (63.9%). In addition, the treatment with eCG results in a high level of estrus synchronization, both in the warmer and in the cooler season of the year. Namely, on day 4 and day 5 after weaning, estrus was detected in 81% of eCG treated sows within the cooler season, and in 78% of sows treated in the warmer season. These values were significantly ($p < 0.01$) lower in the control (untreated) sows (41.1% in the cooler, vs. 33.3% in the warmer season). The obtained results show that the treatment with placental gonadotropin can be an effective method of increasing sow fertility in the warmer season of the year.

Key words: fertility, season, eCG-treatment, weaning, sow.

INTRODUCTION

The herd reproductive efficiency in the industrial pig production is measured by the number of weaned piglets per sow per year. This value, however, can vary greatly due to the influence of many genetic and paragenetic factors. However, as the heritability for the average number of weaned piglets per litter was about 10% (See, 2002), the phenotypic value of this parameter is significantly affected by many paragenetic factors. The main

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parameters of fertility such as the weaning-to-estrus interval, farrowing rate, embryonic or fetal survival rate, live born piglets per litter and the survival rate of piglets during the lactation period are the most important factors which influence the number of weaned piglets per sow per year (Nielsen, 1981a; Tomes et al., 1982; Stančić, 1994).

The value of these parameters of fertility can be modified by the influence of numerous factors, of infective and non-infective etiology (Vanroose et al., 2000; Stančić et al., 2011). Non-infective causes of reduced fertility are numerous and, in production, mostly seem to interact so it is very difficult to define precisely the individual effect of each. However, these factors are: nutrition, housing, insemination technology, body condition, treatment with placental gonadotropin (eCG, and hCG) and general health status of sows (Stančić, 2005, Stančić et al., 2010; Stančić et al., 2011). Under modern production conditions, a phenomenon known as “seasonal infertility of pigs” is the most important factor in reducing sow fertility parameters. Moreover, during the last 40 years, significantly lower values of the sow fertility parameters have been recorded in the warmer part of the year (Almond, 1992). The studies in Eastern Europe have shown a measurable extension of weaning-to-estrus interval, reducing farrowing rate and litter size, as well as an increase in the number of regular and irregular return (rebreeding) rate, during the warmer periods of the year (Almond and Bilkei, 2005). It has been shown that treatment with placental gonadotropins (eCG, and hCG), immediately after weaning, can substantially increase sow fertility in the warm period of the year (Almond and Bilkei, 2005; Taker i sar., 2008; Stančić i sar., 2010).

Therefore, the aim of this study was to investigate whether it is possible to reduce the weaning-to-estrus interval and increase the farrowing rate in sows after the treatment with placental gonadotropin in the warmer period of the year.

MATERIALS AND METHODS

The experiment was conducted on one large pig farm unit in the AP Vojvodina (Serbia), in a one year period. The farm capacity was about 5,000 breeding sows (purebreds Large White, German Landrace, Dutch Landrace, Duroc and Hampshire, as well as their crosses F1 and F2 generations). The sows were kept in enclosed buildings, except the weaned sows up to the time of successful insemination. The pregnant sows were kept for about 30 days in individual boxes, and later, up to about 7 days before farrowing, in the group boxes with outlets. During lactation, lasting about 30 days, the sows were housed in individual pens. Sow feeding was adequate. The estrus detection was performed in the presence of a teaser boar once a day. The artificial insemination was carried out by liquid diluted semen, with 100 ml volume doses, containing about 5×10^9 progressively motile sperm. The artificial insemination was performed few hours after estrus detection, and about 24 hours later. The control of rebreeding was performed in the facilities for inseminated (pregnant) sows, starting about 14 days after insemination.

The hormonal treatment was carried out about 24 hours after weaning litters. Placental gonadotropin preparation eCG (equine chorionic gonadotropin), formerly known as PMSG (“Sugonal” Veterinary Institute Subotica), was used. Each sow was treated with a single intramuscular injection of 1,000 IU eCG (first farrowing) or 1,500 IU eCG (older sows), during the warmer (May - September) and cooler (October - April) seasons of the year. The

data for this study were obtained from the farm reproductive records, for a total of 2,535 sows (455 first parity and 2,080 higher parity sows). The values of these parameters of reproductive performance were analyzed in relation to parity and the season.

The data were analyzed by the software package “Statistics 12”.

RESULTS AND DISCUSSION

In the warmer months of the year, a significantly lower ($p < 0.01$) number of sows had a short duration of weaning-to-estrus interval (WEI: ≤ 4 , and 5 to 6 days), while the number of sows with longer WEI duration (7 days) significantly increased. Thus, in the cooler season, within the first 4 days after weaning, estrus was manifested in 27.9%, and in the warmer season in 18.7% of sows, while these values for WEI 5 to 6 days were 46.7% (cool) and 37.2% (warm). However, the number of sows which manifested estrus 7 days after weaning was significantly higher ($p < 0.01$) in warmer (44%) compared to the cooler season of the year (25.4%) (Table 1).

Table 1. Distribution of estrus reaction within first 7 days after weaning

Parity		Weaning-to-estrus interval (days)								
		≤ 4		5 - 6		7		Total		Total (C+W)
		C	W	C	W	C	W	C	W	
1.	n	33	12	50	14	92	69	175	95	258
	%	18,8 ^a	12,6 ^b	28,5 ^a	14,7 ^b	52,6 ^a	72,6 ^b			
$\geq 2.$	n	310	115	525	238	221	229	1056	582	1596
	%	29,3 ^a	19,7 ^b	49,7 ^a	40,9 ^b	20,9 ^a	39,3 ^b			
Total	n	343	127	575	252	313	298	1231	677	1854
	%	27,9 ^a	18,7 ^b	46,7 ^a	37,2 ^b	25,4 ^a	44,0 ^b			

C – Cool season; W – Warm season.

^{a, b} Values with different superscripts, within same row and interval, significant differ ($p < 0,01$).

It was also found that a significantly ($p < 0.01$) lower number of the first parity sows react with estrus within the first 4 days after weaning (18.8% in the cool and 12.6% in the warm season) compared to the higher parity sows (29.3% in the cool and 19.7% in the warm season). Conversely, 7 days after weaning, estrus manifested over 72% of the first parity sows in the warmer and 52.6% in the cooler season, while in these WEI range, were 39.3% higher parity sows in the warmer, and 20.9% sows in the cooler season. The differences were statistically significant ($p < 0.01$) (Table 1).

Approximately 24 hours after weaning, the sows were treated with a single intramuscular injection of eCG (older sows 1,500 IU, and first parity sows 1,000 IU) in the cooler and warmer season of the year. The objective was to determine whether the treatment with gonadotropin preparations may increase the degree of sow estrus reaction, within the first 7 days after weaning, during the warmer season of the year.

It has been shown that a significantly ($p < 0.01$) larger number of the total sows treated with eCG (83.7%) manifested estrus within the first 7 days after weaning in comparison with the control (non-treated) sows (63.9%). A significant increase in estrus reaction, within the first 7 days after weaning, after treatment with the eCG compared to the control sows during the warm period of the year, were also found in the first parity sows (76.5% vs. 49.7%) and in the older sows (85.2% vs. 67%) (Table 2).

Table 2. Sows estrual reaction within first 7 days after weaning

Parity		Treatment				
		eCG		Control		
		C	W	C	W	
1.	Treated		132	98	264	191
	In estrus	n	107	75	175	95
		%	81,1 ^a	76,5 ^a	66,3 ^b	49,7 ^c
≥ 2.	Treated		684	492	1211	869
	In estrus	n	618	419	1056	582
		%	90,3 ^a	85,2 ^a	87,2 ^a	67,0 ^b
Total	Treated		816	590	1475	1060
	In estrus	n	725	494	1231	677
		%	88,8 ^a	83,7 ^a	83,4 ^a	63,9 ^b

C – Cool season; W – Warm season.

^{a, b} Values with different superscripts, within same row and interval, significant differ ($P < 0,01$).

It is important to point out that the number of estrus sows, treated with eCG in the warmer, compared to the cooler season, was reduced by only 5% ($p > 0.05$) both in the primiparous sows (76.5% vs. 81.1%) and in the older sows (85.2 vs. 90.3%). However, this value in the control sows, significantly ($p < 0.01$) decreased in the warmer seasons by 16.6% in the primiparous sows (49.7% vs. 66.3%) and by 20.2% in the older sows (67% vs. 87.2%) (Table 2). Furthermore, the treatment with eCG results in high level of postweaning estrus synchronization, both in the warmer and cooler season of the year. Namely, on day 4 and day 5 after weaning, estrus was induced in 78% sows treated in the warmer and in 81% sows treated in the cooler season of the year. In this same weaning-to-estrus interval, estrus was detected in 41.1% of untreated (control) sows in the cooler and 33.3% sows in the warmer season of the year (Figure 1).

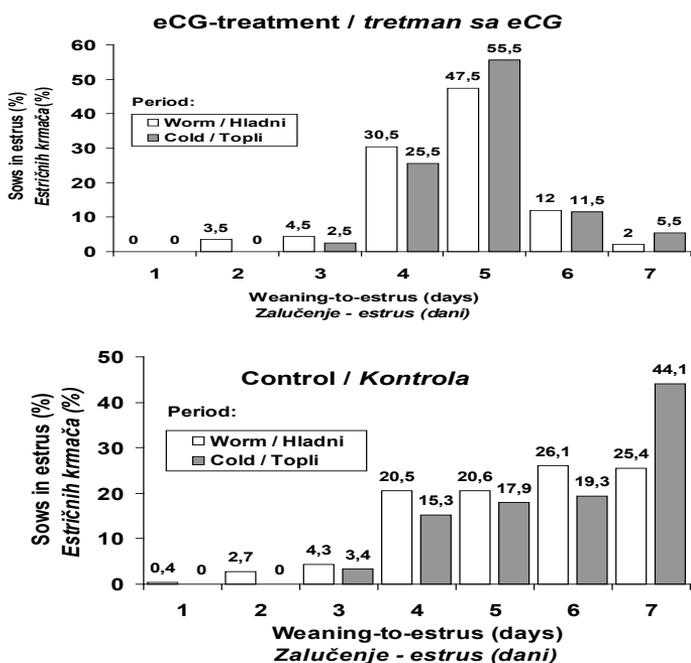


Figure 1. Distribution of sows estrus reaction after eCG treatment in worm and cold season

Seasonal infertility is well-known phenomenon in the pig reproduction. It is manifested with lower values of the basic parameters of sow fertility during the summer months (Stančić et al., 2011). Some of these parameters are: increased occurrence of postweaning anestrus, extended weaning-to-estrus interval, lower values of conception rate, increased number of rebreeding, abortions and pseudopregnancy, as well as the lowered number of vital live-born pigs (Love, 1978; Rozeboom et al., 2000). This is associated with the negative effects of high ambient temperature, on the hormonal mechanisms (release the pituitary FSH and LH) that re-establish postweaning estrus response, as well as on the establishment and/or maintenance of pregnancy (lower conception rate and increase embryonal and foetal mortality) (Britt et al., 1983; Xue et al., 1994; Stančić, 1994; Prunier and Quesnel, 2000; Bassett et al., 2001; Peters and Pitt, 2003; Stančić et al., 2011).

The results of our study show that, after a single eCG injection, 24h after weaning, during the warm period of the year, within 7 days after weaning, a significantly ($p < 0.01$) higher number of sows manifested estrus (83.7%), compared to sows which were not treated (63.9%). Similar results were obtained by other authors (Almond and Bilkei 2005; Bracken et al., 2006; Tucker et al., 2008; Stančić et al., 2010). Based on these results, it can be concluded that the treatment with placental gonadotropin preparations on the day of weaning can be an effective method of increasing and synchronizing the sow postweaning estrus reactions. Therefore, it can contribute to the reduction of the level of sow infertility during the warm summer months.

CONCLUSION

Based on the obtained results, it can be concluded that the treatment of sows with placental gonadotropin, 24 hours after weaning, can significantly increase the number of sows in estrus within the first 7 days after weaning, during the warm season of the year.

These results demonstrate that postweaning gonadotropins treatment can contribute to the reduction of the negative influence of warm season on sow fertility in the intensive pig production herds.

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ESTRUSNO REAGOVANJE KRMAČA TRETIRANIH SA eCG POSLE ZALUČENJA U TOPLOJ I HLADNOJ SEZONI

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Izvod

Brojna istraživanja pokazuju značajno smanjen fertilitet krmača u toplom periodu godine. Cilj rada je bio da se ispita uticaj tretmana krmača preparatom eCG, 24h posle zalučenja, na stepen i sinhronizaciju estrusnog reagovanja. Pokazalo se da značajno ($p < 0.01$) veći broj krmača tretiranih sa 1,000ij do 1,500ij eCG, manifestuje estrus unutar 7 dana po zalučanju (83.7%), u odnosu na krmače koje nisu bile tretirane (63.9%). Osim toga, tretman sa eCG dobro sinhronizuje pojavu estrusa, kako u toploj tako i u hladnoj sezoni godine. Tako je, 4. i 5. dana po zalučanju, estrus manifestovalo 81% krmača tretiranih sa eCG u hladnoj i 78% krmača u toploj sezoni. Ove vrednosti su bili značajno ($p < 0.01$) niže kod kontrolnih krmača (41.1% u hladnoj i 33.3% u toploj sezoni). Dobijeni rezultati pokazuju da tretman sa placentalnim gonadotropinima može biti efikasan metod povećanja fertiliteta krmača u toplom periodu godine.

Ključne reči: fertilitet, sezona eCG-tretman, zalučenje, krmača.

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REPRODUCTIVE EXPLOITATION OF AI BOARS ON THE INTENSIVE FARM UNITS IN AP VOJVODINA (SERBIA)*

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SUMMARY: Lower fertility of artificial inseminated (AI) sows, compared with natural breeding is often problem in pig farm practice. Overextension of ejaculate for AI doses formation was frequently demonstrated as the factor for these lower AI sows fertility. The aim of the present study was to investigate the number of AI doses per ejaculate and the ejaculate dilution proportion on the intensive pig production farms unit in the AP Vojvodina (Serbia). Data from the farm reproduction records were used, for investigation 28 AI boars on the two farm units. Average 50.3 ejaculates per boar per year were taken (ie. one ejaculate per week, or average 611 ejaculates per boar per year). Average 12 AI doses were formed per ejaculate, with average 1:3 dilution proportion of native ejaculate. The obtained data demonstrate low number of ejaculates per boar per year; low AI dose number per ejaculate and not high ejaculate dilution proportion. The values of the examined parameters indicate that they are probably not the primary reason for the low fertility of AI sows on the intensive pig farms in AP Vojvodina (Serbia).

Key words: AI, boar, exploitation, intensive production, pig.

Original scientific paper / *Originalni naučni rad*

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INTRODUCTION

Artificial insemination is widely used in the modern intensive pig production, with the doses of fresh diluted semen, which contain 3 to 6×10^9 of progressively motile spermatozoa (Stančić et al., 2011). On average, one boar ejaculate provides approximately 20 insemination doses (Singelton, 2001; Stančić et al., 2002). However, intensive pig production requires increasing the efficiency of reproductive exploitation of genetically superior boars in the artificial insemination (AI) technology. In practice, this can be achieved by the increased number of the insemination doses per ejaculate. Formation of a larger number of AI doses, involves a significant increase the natural ejaculate dilution extension. Unfortunately, overextension of native ejaculate seminal plasma, result in reduce the potential fertility of each AI dose (Roseboom et al., 2000). This suggests that seminal plasma has a significant impact on the boar sperm fertility (Stančić et al., 2013; Flowers, 2014). Because, overextension of native ejaculation, for forming the larger number of AI doses per ejaculate, can be the significant reason for often lower reproductive performance of artificially inseminated sows, in farm practice, than that achievable with natural breeding (Spronk et al., 1997; Stančić, 2000). Fertility of sows on the intensive pig farms in Serbia is lower by 10% to 20% of that in developed countries of EU and USA (Maletić, 2012; Stančić et al., 2013).

Therefore, the aim of this study was to investigate the frequency of boar ejaculate taking, number of insemination doses per ejaculate and the degree of ejaculate dilution, as parameters that can significantly affect fertility of artificially inseminated sows on the pig farms in AP Vojvodina (Serbia).

MATERIAL AND METHODS

The study was conducted on a three commercial pig farms in AP Vojvodina. The study included 28 Swedish Landrace boars, aged 1.5 to 2.5 years, which were used in the artificial insemination (AI) for a minimum of one year. The following data for boars used in artificial insemination were collected from farms reproductive records: (a) ejaculate taken per boar per year, (b) ejaculate taken frequency (days), (c) ejaculate volume (ml), (d) sperm progressive motility (%), (e) number of AI doses per ejaculate and per boar per year and (f) dilution proportion of ejaculate for AI doses formation.

RESULTS AND DISCUSSION

Average parameters of boars reproductive exploitation, for three investigated farms, are shown in Table 1. Ejaculate was taken from boars average once per week (aver. 50.3 ejaculates per boar per year). Average ejaculate volume was 287 ml (277 to 306), with 77% (74% to 80%) sperm progressive motility. Average 12 (9 to 15) AI doses was formed per one ejaculate, ie. average 611 (483 to 791) AI doses per boar per year. To formation these 12 doses, each ejaculate was diluted in 1:3.5 (1:2 to 1:4) proportion. 35.4% of total ejaculate number were diluted in proportion $\geq 1:4$.

Table 1. Some parameters of boars reproductive exploitation

Parameter A B		F a r m			Average (A+B+C)
		C			
Boar nubers		10	9	9	28
Ejakulates per boar per year (n)		47	52	52	50.3
Ejaculate frequency taken (days)		6.7 (1-28)	7 (1-15)	7.8 (1-20)	7.2 (1-28)
Ejaculate volume (ml)		277 (120-420)	280 (200-450)	306 (116-629)	287 (116-629)
Progressive motility (%)		77 (65-90)	80 (65-95)	74 (60-90)	77 (60-95)
AI doses number	per ejaculate	11.9 (4-20)	9.4 (6-18)	15 (4-32)	12.1 (4-32)
	per boar per year	559	483	791	611 (416-1019)
Ejaculate dilution proportion		1:3.5 (1:2-1:7)	1:2 (1:1.5-1:4)	1:4 (1:1-1:9)	1:3.5 (1:1-1:9)
Ejakulates diluted in proportion $\geq 1:4$ %	n	216/475	29/468	255/468	500/1411
		45.5	6.2	56.5	35.4

Some recent data show that today's average insemination dose volume is 80 ml, with an average of 3.25×10^9 spermatozoa. Average five ejaculates is taken from each boar per month (60 ejaculates per year) and form average 20 AI doses per ejaculate, or 1200 AI doses per boar per year (Singelton, 2001). Our research shows almost double lower number of AI doses per ejaculate (12) and per boar per year (611). This is not significant high level of boar reproductive exploitation, and it is unlikely to be the main cause for the relatively low value of sows farrowing rate (75% do 80%) at the intensive pig farms in AP Vojvodina (Maletić, 2012; Stančić et al., 2013). However, in our farms practice 100 ml volume AI dose are using, which requires a higher degree of ejaculate dilution. Furthermore, our results demonstrated that 35% of ejaculates were extended $\geq 1:4$, which needs increasing extension proportion of native ejaculate. This result with a significant reduction (3 to 5 times) the contents of bioactive substances in the seminal plasma. For example, seminal plasma proteins maintain a high degree of sperm progressive motility and maintain their fertilization ability *in vitro*. (Caballero et al., 2008; Mogielnicka-Brzozowska and Kordan, 2011). On the other hand, it has been shown that estrogens, oxytocin and prostaglandin $F_{2\alpha}$, which contain the natural seminal plasma, play an important role in the stimulation of the myometrial contraction (Roseboom et al. 2000). The inadequate sperm transport within the uterus results in decreasing the sow fertility (Langendijk et al., 2005). In order to stimulate myometrial contractions and thus enhance the sperm transport through the uterine horns, oxytocin, estrogens, prostaglandins, as well as the synthetic seminal plasma Predil MR-A® can be added to the AI dose (Levis, 2002;

Castaneda Morreno, 2002; Dimitrov, 2012; Stančić et al., 2013). It has been shown that a two-phase insemination in combination with the synthetic seminal plasma Predil MR-A® increases sow fertility parameters (the farrowing rate and litter size) (Martin Rillo et al., 1996; Lyczynski, et al., 2000; Garcia Ruvalcaba et al., 2008; Garcia Ruvalcaba et al., 2009). Finally, it has been frequently shown that ejaculates of only 20% to 25% boars tolerate dilution proportion 1:4 and maintain the fertilization ability *in vitro* up to 72h (Johnson et al., 2000; Komisurd et al., 2002; Stančić et al., 2002; Stančić et al., 2003b; Katanić, 2004; Stančić, et al., 2012). Therefore, it is very important to determine the optimum degree of ejaculate dilution for each single boar. In this way, it is possible to significantly increase the fertility of artificially inseminated sows.

CONCLUSION

On the intensive pig farms in AP Vojvodina, the ejaculate is collected on average every week, ie. average of 50.3 ejaculates per boar per year. Average 12 AI doses is formed from one ejaculate, ie. 611 AI doses per boar per year. This is not a high degree of boar reproductive exploitation, and probably not the main reason for the low sows fertility. However, a higher degree of dilution of the ejaculate can reduce sperm fertilization ability. This should be kept in mind, because ejaculates from only 20% to 25% boars tolerate high dilution proportion and storage for up to 72 hours *in vitro*. Therefore, it is important to determine the optimum degree of ejaculate dilution for each individual boar, as a factor to increase fertility rate in the artificial inseminated sows.

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REPRODUKTIVNO ISKORIŠTAVANJE NERASTOVA ZA VO NA INTENZIVNIM FARMAMA U AP VOJVODINI (SRBIJA)

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Izvod

Niža reproduktivna performansa veštački osemenjenih krmača, u porednju sa prirodnim osemenjavanjem, dosta je čest problem u praktičnoj intenzivnoj proizvodnji svinja. Preveliko razređenje ejakulata, radi formiranja većeg broja VO doza, navodi se kao čest faktor smanjene reproduktivne performanse krmača. Cilj ovog rada je bio da se ispita frekvencija uzimanja sperme, broj inseminacionih doza po ejakulatu i stepen razređenja ejakulata na vojvođanskim farmama intenzivne proizvodnje svinja. Analizirani su podaci reproduktivne eksploatacije 28 nerastova, na osnovu evidencije dve farme u AP Vojvodini (Srbija). Prosečno se dobija 50.3 ejakulata po nerastu godišnje. Od svakog ejakulata se pravi prosečno 12 inseminacionih doza, odnosno 611 doza po nerastu godišnje. Prosečan stepen razređenja ejakulata je 1:3. Ovi parametri ne ukazuju na preteranu reproduktivnu eksploataciju nerastova, što, samo po sebi, nebi trebalo da bude značajniji razlog niskog fertiliteta krmača na našim farmama.

Ključne reči: VO, nerast, eksploatacija, intenzivna proizvodnja, svinje.

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**DETERMINATION OF SOME ESSENTIAL ELEMENTS
IN HERBAL TEAS FROM SERBIA
USING ATOMIC SPECTROMETRY (AAS)***

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SUMMARY: Levels of Fe, Mn, Cu and Zn were determined in 14 medicinal plants, which are widely used in phytopharmacy as herbal teas. The following plants were investigated: mountain yarrow (Achillea millefolium L.), basil (Ocimum basilicum L.), St. Jon's wort (Hypericum perforatum L.), peppermint (Mentha x piperita L.), field horsetail (Equisetum arvense L.), stinging nettle (Urtica dioica L.), thyme (Thymus serpyllum L.), maize silk (Zea mays L.– Maydis stigma), hibiscus (Hibiscus sabdariffa L.), marshmallow (Althaea officinalis L.), chamomile (Matricaria chamomilla L.), rosehip/dogrose (Rosa canina L.), winter savory (Satureja montana L.) and spearmint (Mentha spicata L.). A total of 16 samples of different parts of medicinal plants (root, leaf, flower, whole plant) were examined, whereby 13 samples were delivered in original package and three samples were loose leaf herbs. Samples were prepared using the microwave digestion technique, and measurements were performed applying the atomic absorption spectrometry. Average contents of microelements in the examined samples were Mn (108.06±109.66 mg/kg); Fe (274.83±204.46 mg/kg); Cu (13.11±4.92 mg/kg) and Zn (38.53±23.26 mg/kg). According to determined amounts of Fe, Mn, Cu i Zn, the investigated samples of herbal teas are considered safe for human consumption.

Key words: herbal teas, essential heavy metals, AAS.

Original scientific paper / Originalni naučni rad

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INTRODUCTION

Mineral elements are of a unique and manifold importance both for the plant world and human beings. Content of minerals in plant dry matter averagely ranges between 1 to 6 %. Minerals are found in plants as ions, inorganic and organic salts or are incorporated into diverse organic compounds (Kastori and Maksimović, 2008). Mineral elements are involved in a range of chemical reactions in plants. According to their proportional representation in the plant composition, they are grouped into macro, micro and ultra-microelements. Concentration of microelements (Cu, Zn, B, Mn etc.) in plant dry matter is less than 1 mg/g and usually above 1 µg/g (Kastori, 1998). It should be emphasized that some essential microelements (Fe, Zn, Cu, Mn, Co etc.), may have toxic effects at higher concentrations. Such a negative effect on plants is manifested as the impairment of one or several metabolic and/or chemical reactions (Kastori et al., 1997). Variations in chemical composition and mineral matter content in plants are due to a variety of factors, including plant species, plant age, pedological features of soil and implementation of agrotechnical measures (Živkov-Baloš et al., 1999; Maksimović et al., 1998; Radanović et al., 2006; Savić et al., 2007)).

Plants are either direct or indirect source of minerals in human diet. Of particular importance are species used for obtaining a range of phytopreparations in pharmaceutical industry, supplied as monocomponent teas or tea blends that are widely applied in traditional medicine. Sources for obtaining medicinal raw material are wildcrafted plants (over 200 species) or cultivated plants (around 30 species) (Kovačević, 2002). Medicinal herbs are mainly used in the form of herbal teas. Chemical composition of teas is very complex, encompassing flavonoids, alkaloids, enzymes, minerals, trace elements, etc (Ražić and Kuntić, 2011). In Serbia, herbal teas are mainly prepared from aromatic herbal species that contain etheric oils, which are not only remedial but also have a very pleasant scent and aroma.

Human body needs appropriate concentrations of different minerals to maintain the normal function and sustain life. In that respect, deficiency or excess of essential heavy metals (Fe, Cu, Zn, Co, Mn, Mo) in the diet can induce some adverse effects (Vučetić and Krstić, 2000; Trajković-Pavlović et al., 1996). Thus, the content of heavy metals is an important criterion when using plant material in the production of traditional remedies and herbal infusions (Kostić et al., 2011). Intensive agrotechnical measures in modern agriculture, vicinity of industrial plants, mines, traffic roads inevitably lead to contamination of the soil and plants with pesticides and heavy metals. In that respect, continuous and planned monitoring of hygienic safety of plants used as raw material in pharmaceutical industry is critical.

Analysis of samples of different herbal teas obtained from retail establishments in the territory of Novi Sad as well as samples obtained from the natural habitat (region of eastern Serbia, localities I and II) was aimed at determining contents of microelements, including essential heavy metals (Fe, Cu, Zn, Mn), in order to establish the health-safety of these phyto products, having in mind their wide application in folk medicine.

MATERIAL AND METHODS

Material: Herbal tea samples in original package (samples No. 1 – 13) were collected from the retail shops in the territory of Novi Sad, whereas three samples of medicinal plants (samples No. 14, 15 and 16) were collected directly from the natural habitat (East Serbia region, localities I and II). According to Table 1, it is obvious that all samples are herbal teas, which are widely applied and popular among Serbian population and in folk medicine practices.

Table 1. The names of herbal teas and plant parts used in research
(Kovačević, 2002; Kovačević and Jančić, 2003)

No.	Plant	The Latin name of the plant (family)	Parts with medicinal properties
1	Mountain yarrow	<i>Achillea millefolium</i> L. (<i>Asteraceae</i>)	whole plant
2	Basil	<i>Ocimum basilicum</i> L (<i>Lamiaceae</i>)	whole plant
3	St. Jon's wort	<i>Hypericum perforatum</i> L. (<i>Hypericaceae</i>)	whole plant
4	Peppermint	<i>Mentha x piperita</i> L. (<i>Lamiaceae</i>)	leaf
5	Field horsetail	<i>Equisetum arvense</i> L. (<i>Equisetaceae</i>)	whole plant
6	Stinging nettle	<i>Urtica dioica</i> L. (<i>Urticaceae</i>)	root
7	Stinging nettle	<i>Urtica dioica</i> L. (<i>Urticaceae</i>)	leaf
8	Thyme	<i>Thymus serpyllum</i> L. (<i>Lamiaceae</i>)	whole plant
9	Maize	<i>Zea mays</i> , <i>Maydis stigma</i> (<i>Poaceae</i>)	silk
10	Hibiscus	<i>Hibiscus sabdariffa</i> L. (<i>Malvaceae</i>)	flower
11	Marshmallow	<i>Althaea officinalis</i> L. (<i>Malvaceae</i>)	root
12	Chamomile	<i>Matricaria chamomilla</i> L. (<i>Asteraceae</i>)	flower
13	Rosehip, dog rose	<i>Rosa canina</i> L. (<i>Rosaceae</i>)	fruit
14	Winter savory	<i>Satureja montana</i> L. (<i>Lamiaceae</i>)	whole plant
15	St. Jon's wort	<i>Hypericum perforatum</i> L. (<i>Hypericaceae</i>)	whole plant
16	Spearmint, English mint	<i>Mentha spicata</i> L. (<i>Lamiaceae</i>)	whole plant

Methods: The samples were prepared using the microwave digestion method (Milestone, 2000) and Ethos, Microwave Labstation. The amounts of Manganese ($\lambda=279.5$ nm), Iron ($\lambda=248.3$ nm), Copper ($\lambda=324.7$ nm) and Zinc ($\lambda=213.9$ nm) were determined using atomic absorption spectrophotometry applying Varian SpectrAA-10 and D₂-lamp for background correction. The measurements were performed in triplicates

for each particular sample. The obtained values for the content of elements in herbal tea samples were expressed in mg of material per kg of dry matter. The measurement was performed subsequent to determining the moisture content in each herbal tea by drying certain amount of the sample at 105 °C

Statistical analysis: Encompasses determination of basic parameters of statistical analysis: arithmetic mean, standard deviation (SD) and coefficient of variance (Cv %). The analysis was performed using a software package STATISTIKA-10, Stat Soft, Inc.

RESULTS

In Table 2, the results on contents of Mn, Fe, Cu and Zn in examined tea samples are displayed.

Table 2. Content of microelements in the herbal tea samples (mean \pm SD), mg/kg dry matter

Sample No.	Mn [mg/kg]	Fe [mg/kg]	Cu [mg/kg]	Zn [mg/kg]
1.	77.90 \pm 3.59	67.24 \pm 0.54	15.56 \pm 0.19	28.48 \pm 1.02
2.	71.98 \pm 0.36	539.87 \pm 18.90	24.46 \pm 0.19	38.09 \pm 0.15
3.	80.86 \pm 1.05	63.71 \pm 3.44	10.93 \pm 0.25	22.18 \pm 1.10
4.	111.97 \pm 4.02	443.90 \pm 12.42	17.15 \pm 0.50	26.86 \pm 0.54
5.	254.70 \pm 5.09	617.46 \pm 20.37	7.76 \pm 0.35	27.9 \pm 0.67
6.	28.18 \pm 0.65	673.00 \pm 18.18	12.71 \pm 0.48	22.75 \pm 0.54
7.	57.84 \pm 1.05	303.00 \pm 11	13.23 \pm 0.28	29.14 \pm 1.60
8.	127.06 \pm 3.18	445.78 \pm 12.93	8.94 \pm 0.01	44.26 \pm 0.44
9.	31.60 \pm 0.60	193.21 \pm 2.32	8.10 \pm 0.61	59.80 \pm 2.87
10.	453.71 \pm 14.07	219.82 \pm 4.18	7.80 \pm 0.31	46.24 \pm 1.61
11.	23.86 \pm 0.43	114.16 \pm 1.48	15.69 \pm 0.53	23.59 \pm 0.87
12.	76.14 \pm 1.67	130.26 \pm 4.55	14.25 \pm 0.21	34.67 \pm 0.45
13.	70.99 \pm 2.49	61.87 \pm 1.11	6.68 \pm 0.06	16.11 \pm 0.82
14.	54.22 \pm 1.03	155.83 \pm 10.59	10.76 \pm 0.27	51.11 \pm 0.41
15.	170.78 \pm 0.51	217.32 \pm 8.47	19.86 \pm 0.24	113.81 \pm 4.89
16.	37.24 \pm 1.56	150.85 \pm 2.42	15.87 \pm 0.22	31.43 \pm 1.82

The content of Mn in herbal teas determined in our research ranged from 23.86 \pm 0.43 mg/kg (marshmallow-root) to 453.71 \pm 14.07 mg/kg (hibiscus-flower), with an average

value of 108.06 mg/kg and coefficient of variance 101.48 %.

The levels of Fe established in samples of herbal teas ranged from 61.87 ± 1.11 mg/kg (rosehip) to 673 ± 18.18 mg/kg (stinging nettle - root), with an average value of 274.83 ± 204.46 mg/kg and coefficient of variance 74.39 %.

Average content of Cu in herbal tea samples was 13.11 ± 4.92 mg/kg, with the coefficient of variance of 37.53%. Maximum Cu level was established in basil (whole plant) being 24.46 ± 0.19 mg/kg, while minimum Cu content (6.68 ± 0.06 mg/kg) was determined in rosehip fruit.

Average Zn level determined in herbal tea samples was 38.53 ± 23.26 mg/kg. Maximum Zn content obtained in our research was 113.81 ± 4.89 mg/kg (St. Jon's wort - locality II), whereas minimum concentration was obtained in rosehip fruit, being 16.11 ± 0.82 mg/kg.

DISCUSSION

A group of microelements includes the essential heavy metals, which are required by all living organisms (Fe, Zn, Cu, Mn, Co) and which, if present at high concentrations, can manifest toxic effects on plants and humans (Kastori et al., 1997). Microelements are essential for the activity of enzyme and protein transportation systems (Trajković-Pavlović et al., 1996). The content of microelements in plants depends on plant species, soil type, content of particular element in the soil and its physicochemical properties, as well as the fertilizers and plant protection agents used.

Mn contents in herbal teas: peppermint, chamomile, stinging nettle (leaf), stinging nettle (root), St. Jon's wort, mountain yarrow and basil correspond with the results of other authors (Ražić et al., 2005, 2006, 2008). In the marshmallow, Ražić et al. (2005) reported a low Mn content, of 9 ± 1 mg/kg, which is in accordance with our finding (23.86 mg/kg). Ražić and Kuntić (2011) measured the essential microelements and toxic metals in 33 herbal tea samples. The values obtained for Mn in three samples of peppermint tea (packed in tea bags) were above 200 mg/kg, whereas Mn content in the fourth (loose-leaf) sample was 120.4 mg/kg that is in accordance to our finding (111.97 mg/kg). Manganese content in chamomile tea (tea bags) was 69.3 mg/kg, which corresponds with our result of 76.14 mg/kg. It is to emphasize that Mn content obtained by aforementioned authors in rosehip tea (*Rosa canina L.*) was 1,585.9 mg/kg, which is multifold higher than our result, being 70.99 mg/kg. The authors themselves pointed out the high manganese levels in herbal teas obtained in their research. When it comes to our research, we need to emphasize elevated content of manganese in hibiscus tea (453.71 ± 14.07 mg/kg) as compared to other samples. Szentmihályi et al. (2006) have measured microelements and toxic metals in a range of medicinal plants collected at different localities. Manganese content in stinging nettle sample collected from natural habitats (Transylvania) was 52.73 ± 0.21 mg/kg, which corresponds with our result 57.84 ± 1.05 mg/kg (stinging nettle - leaf). Stef et al. (2010) measured the levels of microelements and toxic metals in 33 medicinal plants from the territory of Romania. The content of minerals was determined using flame atomic absorption spectrometry, and organic matter destruction was performed in the oven at 650 °C. The obtained levels of Mn in chamomile (65 ppm), St. Jon's wort (75 ppm) and stinging nettle (28 ppm) fairly correspond with our results, whereas Mn contents in peppermint (77 ppm) and rosehip (42 ppm) were lower than values obtained

in our research. Gentscheva et al. (2010) determined levels of essential microelements in plants originating from the region of Bulgaria and Macedonia using the method of flame atomic absorption spectrometry (FAAS). Mn contents in chamomile and hibiscus flower were higher than our results, being 137 ± 5 and 717 ± 15 mg/kg, respectively. The authors pointed out the high level of Mn in hibiscus flower, which is in accordance with our finding.

The iron content in the following herbal teas: basil, peppermint, field horsetail, stinging nettle – root and thyme, was very high. Moreover, higher Fe concentration was observed in the peppermint leaf obtained from a retail establishment as compared to the samples of spearmint (whole plant) obtained from the locality II (eastern Serbia). This could be explained by its function in chlorophyll synthesis in the leaf. Sample of St. Jon's wort (whole plant) obtained from locality II revealed higher content of Fe as compared to the St. Jon's wort from the retail. Fe levels established by Ražić et al. (2005) in the following herbal teas: peppermint (leaf), stinging nettle (leaf), St. Jon's wort (whole plant), marshmallow (root), mountain yarrow (whole plant) and basil (whole leaf) compare well with our results with some minor differences.

Copper concentrations in chamomile, stinging nettle (leaf), stinging nettle (root), St. Jon's wort (whole plant) and marshmallow (root) are in accordance with the results of other authors (Ražić et al., 2005), whereas Cu levels in peppermint (leaf), mountain yarrow (whole plant) and basil (whole plant) obtained in our research were somewhat higher than those reported by aforementioned authors. Szentmihályi et al. (2006) established 12.09 ± 0.21 mg Cu/kg in stinging nettle, which corresponds with our findings. Kékedy-Nagy and Yonescu (2009) have measured Cu content in samples of peppermint, chamomile and stinging nettle. Concentration of Cu in chamomile, being 10.20 mg/kg, is in accordance with our results, whereas contents in peppermint (4.45 mg/kg) and in stinging nettle (23.5 mg/kg) were notably lower and higher as compared to our results, respectively.

Average Zn level determined in herbal tea samples was 38.53 ± 23.26 mg/kg, which corresponds with data from the literature reporting that Zn concentrations in plant dry matter can range from 30 to 150 mg/kg, and predominantly 20-50 mg/kg (Kastori and Petrović, 1993). Maximum Zn content obtained in our research was 113.81 ± 4.89 mg/kg (St. Jon's wort - locality II), whereas minimum concentration was obtained in rosehip fruit, being 16.11 ± 0.82 mg/kg. The obtained maximum Zn level was still below the toxic concentration of 200 µg/g for cultivated plants (Kastori et al., 1997). According to Table 2, the concentrations of zinc were fairly consistent among the tea samples, with the exception of St. Jon's wort from the locality II (eastern Serbia) that revealed maximum content of Zn. This value is five times higher than that measured in St. Jon's wort sample originating from the territory of Novi Sad (22.18 ± 1.10 mg/kg). Potential reasons for such difference are most probably the soil type, i.e. the content of Zn and its antagonists in the soil. Zn concentration in agricultural soils varies between 10-300 mg/kg (Alloway, 1990); however, in many countries, maximum permissible Zn content in the soil is 300 mg/kg (Bogdanović et al., 1997). Our results on Zn content in herbal teas: peppermint (leaf), chamomile (flower), stinging nettle (leaf), stinging nettle (root), St. John's wort (whole plant), marshmallow (root), mountain yarrow (whole plant) and basil (whole plant) correspond with the results of other authors (Ražić et al., 2005). Among other parameters, Ražić and Kuntić (2011) measured zinc concentrations in mint, chamomile and rosehip. The values they obtained for mint samples (23.6; 20.1 and 21.8 mg/kg) well

correspond with our result, being 26.86 ± 0.54 mg/kg. Furthermore, zinc level of 13.7 mg/kg determined in rosehip (*Rosa canina L.*) is comparable with our result - 16.11 ± 0.82 mg/kg. The levels obtained for chamomile (24.4 and 21.9 mg/kg) are somewhat lower than our results (34.67 ± 0.45 mg/kg). Gentscheva et al. (2010) investigated Zn contents in samples of chamomile, mint, rosehip and hibiscus. The obtained zinc concentrations were 45 ± 2 , 34 ± 2 , 4 ± 0.5 and 33 ± 2 mg/kg, respectively. The results correspond with our findings, except for rosehip sample.

CONCLUSION

Sixteen samples of 14 different herbal teas widely consumed among Serbian population were analyzed for concentration of microelements, with an aim of establishing the mineral status and hence the health safety of medicinal plants used for very popular herbal teas. The contents of the examined elements were within the ranges reported in the literature, with some variations associated with plant species, applied agricultural measures and pedological features of soil. Large-scale variability in the concentration of microelements was observed according to the species of medicinal plants and the availability of microelements in the soil. The highest contents of essential heavy metals were established for Fe. Maximum Fe level was observed in stinging nettle root (673 ± 18.18 mg/kg). Our analysis revealed that basil, peppermint, stinging nettle (root), field horsetail and thyme are rich in iron. Thus, these plants are beneficial Fe source for humans. The highest variations are established for Mn content ($C_v = 101.48\%$). The obtained results on concentrations of Mn, Fe, Cu and Zn indicated that the investigated samples are safe for human consumption.

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**ODREĐIVANJE NIVOVA ESENCIJALNIH TEŠKIH METALA U UZORCIMA
BILJNIH ČAJEVA
IZ SRBIJE METODOM ATOMSKE APSORPCIONE SPEKTROMETRIJE
(AAS)**

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Izvod

Fe, Mn, Cu i Zn, su određivani u 14 vrsta lekovitih biljaka, koje imaju veliki značaj u fitofarmaciji u formi biljnih čajeva, kao napitka. To su: hajdučka trava (*Achillea millefolium L.*), bosiljak (*Ocimum basilicum L.*), kantarion (*Hypericum perforatum L.*), pitoma nana (*Mentha x piperita L.*), rastavić (*Equisetum arvense L.*), kopriva (*Urtica dioica L.*), majčina dušica (*Thymus serpyllum L.*), kukuruzna svila (*Zea mays L. – Maydis stigma*), hibiskus (*Hibiscus sabdariffa L.*), beli slez (*Althaea officinalis L.*), kamilica (*Matricaria chamomilla L.*), šipak, divlja ruža (*Rosa canina L.*), vrijesak (*Satureja montana L.*) i divlja nana (*Mentha spicata L.*). Ispitano je ukupno 16 uzoraka, od različitih delova lekovitih biljaka (koren, list, cvet, cela biljka), pri čemu se 13 uzoraka nalazilo u originalnom pakovanju, a tri uzorka u rastresitom obliku. Uzorci za merenje pripremljeni su metodom mikrotalasne digestije, a pri merenju korišćena je metoda atomske apsorpcione spektrometrije. Izmereni prosečni sadržaji mikroelemenata u ispitivanim čajevima su: Mn (108,06±109,66 mg/kg); Fe (274,83±204,46 mg/kg); Cu (13,11±4,92 mg/kg) i Zn (38,53±23,26 mg/kg). Ispitivani uzorci biljnih čajeva su bezbedni za ljudsku upotrebu s obzirom na izmerene količine gvožđa, mangana, bakra i cinka u njima.

Ključne reči: biljni čajevi, esencijalni teški metali, AAS.

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GENETIC VARIABILITY OF PORCINE CIRCOVIRUS TYPE 2 (PCV2) STRAINS IN CROATIA

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SUMMARY: Porcine circovirus type 2 (PCV2) has been present for the last 2 decades in Croatia, with its highest incidence level in 2004. The clinical features of the disease have altered within this period. Infections until 2008 were mostly accompanied by severe signs such as enteritic and respiratory disorders, erythematous skin lesions that showed a tendency to progress to dermal necrosis accompanied by a high mortality rate of primarily 4 to 16 week-old pigs. Later PCV2 infections were milder and mostly manifested as waste loss and poor growth performance as well as reproductive failure in pregnant sows. In both cases the disease had a direct negative impact on the pig production. Since the infection is continuously present in some regions of Croatia, the heterogeneity of detected PCV2 strains prior to 2008 with strains isolated in 2012 were compared for a better insight in the epidemiological situation. The results of phylogenetic analysis revealed that the viral strains found in 2012 genetically differ from those detected in earlier years. This indicates that new entries into the pig population appeared probably due to pig trade.

Key words: PCV2, genetic diversity, clinical manifestation, Croatia.

INTRODUCTION

PCV2 infections have been recognized through several syndromes such as PMWS (post-weaning multisystemic wasting syndrome), PDNS (dermatitis and nephropathy syndrome), respiratory disease complex, granulomatous enteritis, exudative epidermis, necrotizing lymphadenitis, congenital tremor (Chae, 2005) and reproductive failure (Hansen et al., 2010). The causative agent, porcine circovirus (PCV) is a small, non-enveloped virus with a single-stranded circular DNA of 1.76 kb (Tischer et al., 1982.,

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Crowther et al., 2003) belonging to the genus *Circovirus* within the family *Circoviridae*, along with other animal viruses such as chicken anaemia virus and beak and feather disease virus (Todd et al., 2005). PCV genome contains three open reading frames (ORF), ORF1 encoding a replicase, ORF2 encoding the capsid protein and ORF3 encoding a protein related to cell apoptosis (Mankertz et al., 2004). Two types of PCV have been described, namely PCV1 and PCV2. Their DNA sequence homology is 68 to 76% (Meehan et al., 1998). PCV1 is not pathogenic, and has been detected in cell culture of pig origin used even for vaccine production (Jemeršić et al., 2005), while PCV2 has been defined as the causative agent of pathological syndromes in pigs. Until today, PCV2 isolates are genetically divided into three groups, PCV2a, PCV2b and PCV2c (Opriessnig et al., 2007., Olvera et al., 2007). Boissesson et al. (2004) found that the variations among the PCV2 genomic sequences are mainly due to the variability within ORF2, while ORF1 is highly conserved.

PCV2 infections have been described in Croatia in 1997 and 2004 on large, small and medium size pig farms in several west and eastern Croatian counties (Jemeršić et al., 2004). The morbidity in all studied farms was from 15-30%, while the mortality ranged from 9.3% to 23.8%. Due to high losses in the pig industry, vaccination was introduced to prevent the spread of infection and viral shedding of subclinically infected pigs. From 2008 the clinical signs of disease became less severe in most epidemics recorded, but still remained causes of great losses in the pig industry. Alterations in the predominant PCV2 strains in Croatia or their genetic changes as well as introducing vaccination may have influenced the clinical features of PCV2 infection.

Therefore, we present the sequencing results of the amplified ORF1 of PCV2 strains from 2012 to determine genetic heterogeneity of the virus isolates and to carry out the comparison with PCV2 strains collected until 2008.

MATERIALS AND METHODS

Serum samples of fattening pigs (3-6 months of age) were collected in 2012 from two pig farms in Osijek-Baranja County with a history of PCV2 infection. The samples were pooled (5-10 samples per pool) and kept frozen at -70°C. A total of 10 pools, 5 containing samples collected from one PCV2 affected farm (CRO-PCV2-1 to CRO-PCV2-5) and 5 pools from the second PCV2 affected farm (CRO-PCV2-6 to CRO-PCV2-10) were examined for the presence of PCV2 DNA.

An amount of 200 µl of sera was used for viral DNA purification carried out by QIAamp® DNA Mini kit (Qiagen, USA) according to the manufacturer's instructions. For detection of PCV2 DNA a specific primer pair for the amplification of a 360 bp fragment within the ORF1 region was used, according to a previously described protocol by Yang et al., (2003). Reaction mixtures lacking a DNA template were used as negative controls, while a Croatian PCV2-positive sample was used as a positive control. The thermal profile of the amplifications contained an initial denaturation step at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and primer extension at 72°C for 30 sec. The final extension was for 3 min at 72°C.

The amplification products were separated by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized by UV transillumination.

Prior to sequencing PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA) and sent for direct sequencing in both directions to Macrogen Inc., Amsterdam, the Netherlands.

The sequence comparison with the reference strains from the GenBank (Table 1) was performed by algorithm BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Sequencer 4.6. (<http://www.genecodes.com>, Genes Codes Corporation). Alignment was carried out by ClustalX. For the reconstruction of phylogenetic trees the Neighbor-Joining (NJ) method with Kimura-2 Parameter Model followed by MEGA 5 were used. The clustering stability of the NJ tree was evaluated by bootstrap analysis with 1000 replicates. The PCV2 sequences from Croatia were registered in the GenBank under accession numbers KF498717-KF498720 (CRO_PCV2_1, 3, 4, 6).

Table 1. PCV2 reference sequences used for phylogenetic analysis retrieved from the GenBank.

GenBank Accession No.	Original name	Country
HQ591366	PCV2, isolate 90-08-21	Croatia, 2008
HQ591367	PCV2, isolate 110-08-2	Croatia, 2008
AY256460	PCV2, strain 375	Hungary, 2003
AY424405	PCV2, isolate AUT5	Austria, 2003
HQ591368	PCV2, isolate 126-07-5	Croatia, 2007
HQ591369	PCV2, isolate 147-07-7	Croatia, 2007
HQ591370	PCV2, isolate 161-08-2	Croatia, 2008
HQ591365	PCV2, isolate 70-08-2	Croatia, 2008
AY180397	PCV2, strain Pingtung-5	Taiwan, 2002
AY484410	PCV2, isolate NL-control-4	Netherlands, 2003
AF311296	BFDV	Australia, 2000
AF071879	PCV1	SAD, 1998

RESULTS

The amplification of PCV2 genome regions from samples originating from the two farms with clinical signs of PCV2 (CRO-PCV2-1 to CRO-PCV2-10) resulted in clear PCR products of 360 bp. All strains showed to be members of phylogenetic group 1 or PCV2b group (Figure 1). The seven PCV2 sequences (CRO-PCV2-1, CRO-PCV-2, CRO-PCV2-5, CRO-PCV2-7 to CRO-PCV2-10) were found to be 100% identical among themselves in the ORF1 region. Sequences CRO-PCV2-3 and CRO-PCV2-4 differed in one (0.33%), whereas sequence CRO-PCV2-6 differed in two (0.66%) nucleotides. The obtained phylogenetic clustering shows that the PCV2 isolates differ from the previously published Croatian strains from the year 2007 HQ591368 and HQ591369 in 4.4% of nucleotides whereas when compared to strains from 2008 (HQ591366, HQ591367, HQ591365, HQ591370) in 2.3%, 3.0% and 4.4% (last two) of nucleotides, respectively. The closest genetic similarity of Croatian strains has shown to be with strains detected in Hungary and Austria.

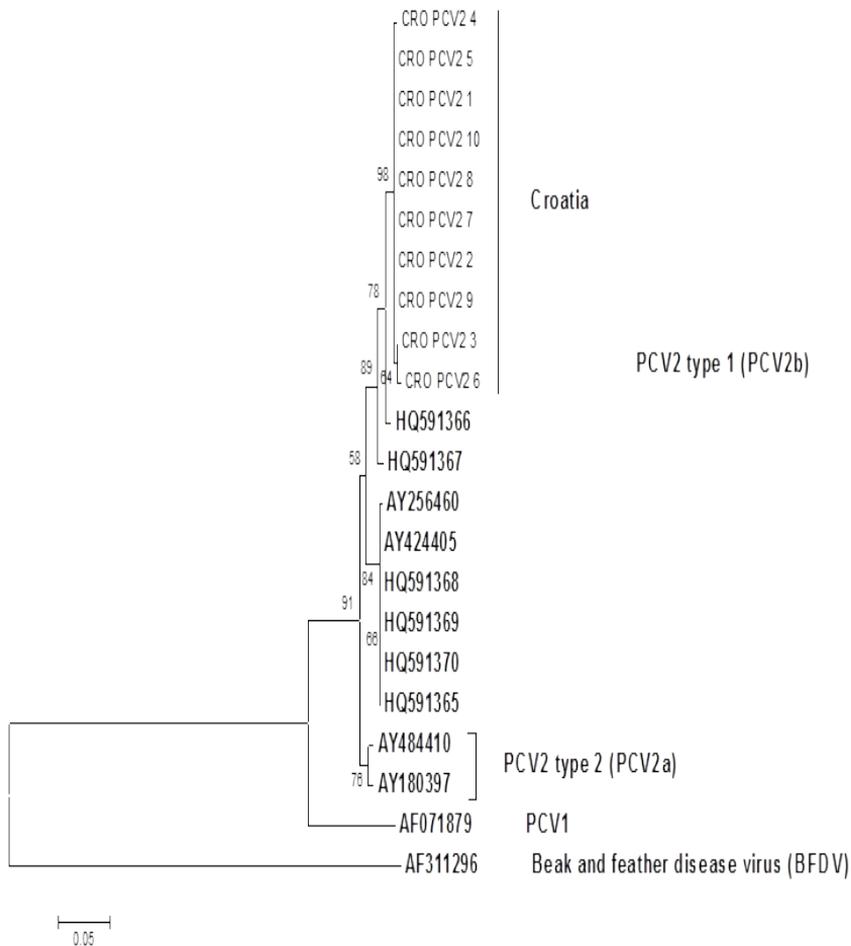


Figure 1. Neighbor-joining phylogenetic tree obtained by the analysis of the partial ORF1 region (297 bp) of Croatian PCV2 samples. Reference sequences included in the analysis are marked in bold.

Bootstrap values are presented next to tree nodes. The bar represents 0.05 nucleotide substitution per site.

DISCUSSION AND CONCLUSIONS

Due to the potentially high economic impact PCV2 infections may be considered as one of the most important diseases affecting pig production worldwide (Baekbo et al., 2012). It is thought that PCV2 has been present in pig herds for decades if not centuries before the disease emerged, and the virus slowly mutated into disease-causing forms.

Porcine circovirus type 2 (PCV2) has been detected in pigs with various clinical conditions in different regions of Croatia. The signs that predominated in the affected pigs were skin lesions, loss of weight, respiratory and digestive disorders followed by an increase in the mortality rate. Genetic analyses showed that PCV2 strains from 1997

and 2002 clustered into PCV2a and PCV2b groups, respectively (Jemeršić et al., 2004). Within this study, samples were collected during 2012 from two farms in Croatia with a history of high PCV2 seroprevalence and clinical features of the infection. The 2012 year strains showed clustering into PCV2b group and were more related to those from the year 2002. However, nucleotide differences found between them suggest the appearance of some novel strains. Similar findings were reported and recognised in countries neighbouring Croatia, such as Slovenia (Toplak et al., 2002), Hungary (Kiss et al., 2006), Austria (Schmoll et al., 2002), Serbia (Becskei et al., 2010, Savić et al., 2012), and Italy (Martelli et al., 2009). Interestingly, previously found strains belonging to group PCV2a were not detected in 2012 indicating that this viral group has been eliminated from the tested farms probably as a result of implementing effective biosecurity measures and/or vaccination. Vaccination against PCV2 infection in Croatia was introduced rather late, in 2009/2010. Only commercially available inactivated and subunit vaccines are registered, therefore no chimeric PCV2 was expected to be found during this study, as has been recorded in Canada (Gagnon et al., 2010). However, the milder clinical manifestation of disease recorded from 2008 may be a result of systemic vaccination carried out on farms in combination with the genetic variations found within novel PCV2 strains in Croatia.

Generally, the seroprevalence regarding PCV2 infection is highest in Osijek-Baranja County when compared to other regions in Croatia (Roić et al., 2013) and also contains the highest pig breeding density. Therefore, these were the main reasons to collect the samples from this region.

The detection of novel PCV2 strains and the alteration of existing ones suggest that PCV2 is still being introduced into Croatian pig breeding farms, probably due to pig trade. Therefore, apart from vaccination and biosecurity measures, testing of pigs that will be introduced to pig farms would highly contribute in controlling PCV2 infection.

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GENETSKA RAZNOLIKOST SOJEVA CIRKOVIRUSA TIPA 2 (PCV2) U HRVATSKOJ

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Izvod

Cirkovirus tipa 2 (PCV2) je prisutan u svinjogojstvu Republike Hrvatske (RH) tijekom protekla dva desetljeća s najvišim stupnjem pojavnosti u 2004. godini. Klinička slika bolesti je tijekom datog razdoblja promijenjena. Infekcije PCV2 su do 2008. bile većinom praćene teškim znakovima bolesti poput crijevnih i respiratornih poremećaja, crvenila kože s razvojem nekroze okrajnih dijelova tijela svinje, te značajnim povećanjem pomora u prasadi (naročito one starosti od 4. do 16. tjedna). Kasnije infekcije bile su blaže i očitovale su se gubitkom težine, slabim rastom prasadi i reproduktivnim poremećajem u krmača. U oba opisana razdoblja, infekcija je dovela do značajnih gubitaka i šteta u svinjogojstvu proizvodnji. Obzirom na kontinuiranu pojavu PCV2 infekcije u RH i sa svrhom dobivanja boljeg uvida u stvarnu epizootiološku situaciju u državi, sojevi virusa izdvojeni iz svinja do 2008. uspoređeni su sa sojevima izdvojenima u 2012. Rezultati filogenetske analize ukazuju na različitost sojeva iz 2012. godine u odnosu na ranije izdvojene sojeve. Naši rezultati upućuju na unos 'novih' PCV2 sojeva u svinjogojstvu proizvodnju RH moguće kao posljedica trgovine svinjama.

Ključne riječi: PCV2, genetska raznolikost, klinička slika bolesti, Republika Hrvatska.

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MICROBIOLOGICAL ANALYSIS OF WATER IN THE CANAL DANUBE-TISA-DANUBE

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SUMMARY: Contaminated water has a very negative impact on the entire agroecosystem. Surface water is usually polluted through the discharge of pollutants of various origins such as wastewater from industries and households, wastewater from livestock, washing harmful chemicals from the soil into the groundwater, oil spills and more. In this water, there are a variety of harmful and toxic substances, as well as pathogenic microorganisms. The consequences can be disastrous and large-scale. One way of removing unwanted and harmful substances from the wastewater is biological. Biological control means separation of harmful substances through the metabolism of microorganisms. The aim of this work was to estimate the microbiological quality of water in the canal Danube-Tisa-Danube (DTD) at two different locations and the possibility of using this water in agricultural production. Water samples for microbiological analysis were taken from one locality near the village Čelarevo, and two localities near Vrbas, outside of direct influence of wastewater flows and its tributaries. Water samples were taken according to the guidelines for taking samples of surface water from rivers and streams ISO 5667-6. Time up period was from January to June 2013. Each sample containing 100ml of water was transferred in the laboratory. Microbiological analysis included the determination of total number of bacteria, the number of Salmonella sp., Echerichia coli and the number of sulphite reducing clostridia (genus Clostridia). Large number of saprophytic bacteria as well as the fluorescent Pseudomonas sp. were found at both localities in the investigated time period.

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The number of Salmonella spp. and E. coli was high indicating the presence of water pollution. Sulphite reducing clostridia were recorded in small numbers, only at the end of the test period, which points to a lack of anaerobic conditions in the canal water. The presented results suggest that the water from the tested localities need to be treated before it is used in agricultural purpose.

Key words: *Salmonella* sp., *Echerichia coli*, *Clostridia* sp., water, canal Danube-Tisa-Danube.

INTRODUCTION

Contaminated water have a very negative impact on the entire agroecosystem. Surface water is usually polluted through the discharge of pollutants of various origins such as wastewater from industries and households, wastewater from livestock, washing harmful chemicals from the soil into the groundwater and more. Also, the anthropogenic factor has a significant impact on water pollution, throwing various types of waste into the water (Fan et al., 2009; Sapers et al., 2009). Consequences can be disastrous and large-scale. In contaminated water there are a variety of hazardous and toxic materials, substances and pathogenic microorganisms. The list of pathogens includes bacteria *Campylobacter* sp., enterohemorrhagic *Escherichia coli*, enterotoxigenic *Staphylococcus aureus*, enterotoxigenic *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella* sp., *Shigella* sp., *Yersinia enterocolitica*, protozoa *Cryptosporidium* sp., *Cyclospora cayetanensis*, *Giardia* sp., *Entamoeba histolytica*, and viruses, in particular, adenoviruses, enteroviruses, noroviruses, and rotaviruses (Warriner et al., 2009).

Because of the wastewater negative effects, it is necessary to invest a lot of effort to protect the environment. One way of removing harmful substances from the wastewater is bioremediation. Bioremediation is an alternative method that has emerged in recent years to treat the wastewater. The applications of microorganisms, such as bacteria, fungi, algae, dead microbial biomass and other biomaterials, are the hot topics in this research realm (Cabatingan et al., 2009; Bingol, et al. 2004; Aravindhana et al., 2004).

In our country, the sanitary quality of irrigation water sources is unknown currently. Therefore, the objective of this work was to estimate the microbiological quality of water in the canal Danube-Tisa-Danube (DTD) at two different locations and the possibility of using this water in agricultural production.

MATERIAL AND METHODS

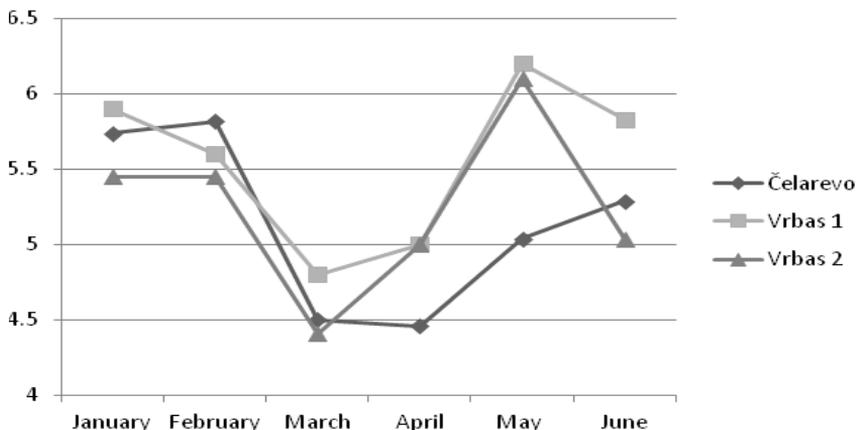
Water samples for microbiological analysis were taken from one locality near the village Čelarevo, and two localities near Vrbas, outside of direct influence of wastewater flows and its tributaries. Water samples were taken according to the guidelines for taking samples of surface water from rivers and streams ISO 5667-6. Sampling period was from January to June 2013.

Each sample containing 100 ml of water was transferred in the laboratory. Microbiological analysis included the determination of total number of bacteria, the number of *Salmonella sp.*, *Echerichia coli* and the number of sulphite reducing clostridia (genus *Clostridia*). The number of microorganisms was determined, using the dilution method (Trolldenier, 1996). Appropriate nutrient media were used: nutrient agar for the total number of bacteria, HiCrome Salmonella agar for the number of *Salmonella sp.* and *Echerichia coli*, and medium with pepton (pepton 15 g l⁻¹, extract yeast 9 g l⁻¹, NaSO₃ 0.5 g l⁻¹, agar 15 g l⁻¹; after medium sterilization, 20 ml of 7% solution of FeSO₄ was added) for the sulphite reducing clostridia.

Graphically displayed results are presented as logarithm of cell number.

RESULTS AND DISCUSSION

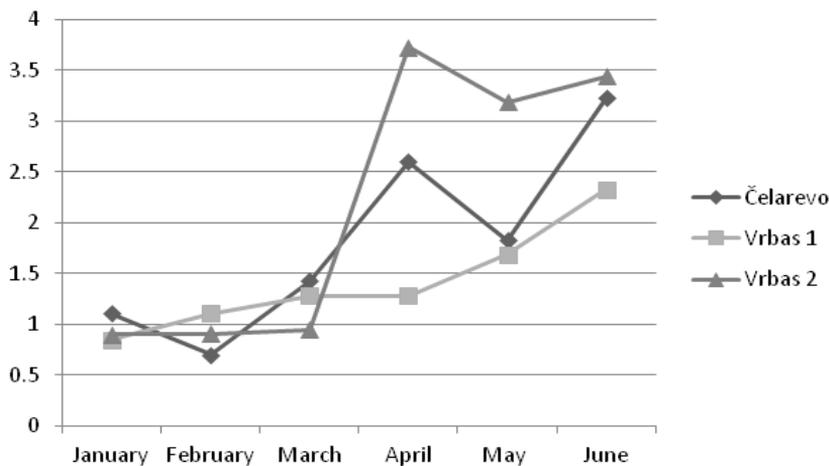
Saprophytic bacteria play an important role in the process of water purification. In this study, the number of saprophytic bacteria during the six months of testing at all three sites was very high. The highest number was recorded in May at the site Vrbas 1 and it was 15.97 x 10⁵ CFU/ml, while the minimum was in March at the site Vrbas 2, 0.26 x 10⁵ CFU/ml (Graf 1). Also, in the water samples at locality Vrbas 1, colonies of fluorescent *Pseudomonas sp.* were identified. These bacteria have capability to use a variety of pollutants as a source of nutrients.



Graph.1. Number of saprophytic bacteria in the water of canal DTD from January to June 2013th (cells number logarithm)

The results obtained for the site Čelarevo show that the largest number of bacteria in the water was in January and February, and then decreased. In the spring months, it was noted a slight increase in the number of saprophytic bacteria. The number of saprophytic bacteria was high during winter months although the water temperature was at border of the minimum for survival of these bacteria. It could be explained by the increased influx of organic matter in the water of canal DTD. At sites Vrbas 1 and 2 number of bacteria decreased from winter to spring, and reached its peak in May.

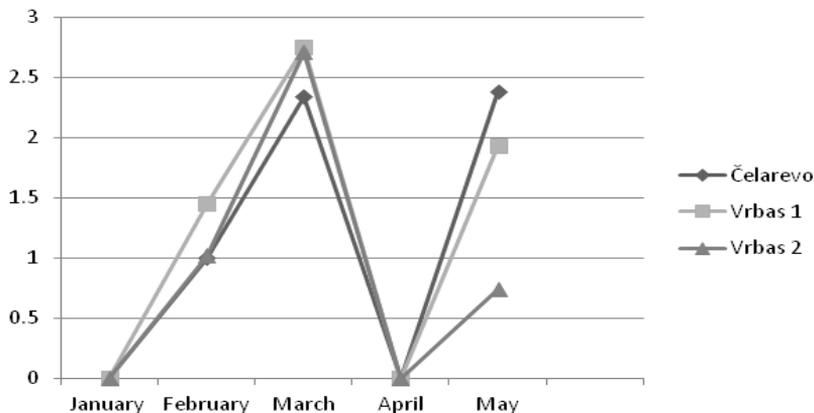
Escherichia coli inhabits the intestines of animals, as well as humans, where is intensively involved in the process of digestion. This bacterium causes many human diseases such as diarrhea, urogenital tract infection, sepsis and other. It also serves as an indicator of water quality (McLain and Williams, 2008; Higgins et al., 2009). The presence of enterobacteria *E. coli* in the water of canal DTD at all locations during the six month, indicates the flow of raw sewage into the water canal, while a relatively high abundance of this bacteria indicates a large contamination of the canal (Graph. 2). The largest number of *E. coli* was recorded at the site Vrbas 2 in June, and it was $2,80 \times 10^3$ CFU/ml. Contrary to that, no presence of the bacteria was determined in the canal water at the same site in February.



Graph. 2. Number of *Escherichia coli* in the water channel DTD from January to June 2013th (cells number logarithm)

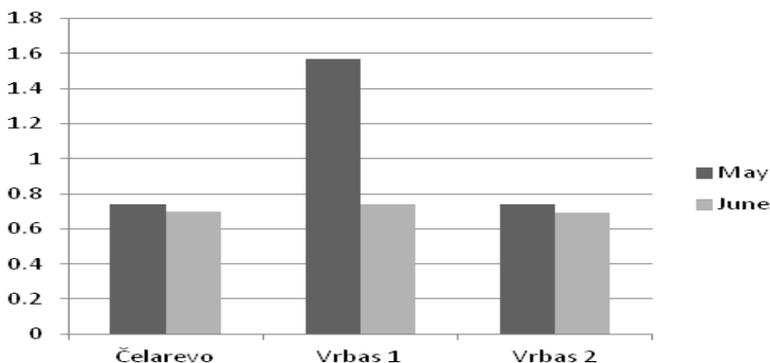
The above graph shows that the numbers of *E. coli* during the winter months, January and February, were lower than in the spring and summer months. The highest number was recorded in June. This trend in the number of *E. coli* in the water during the examined period can be explained by the growth of optimal temperature for this bacteria which is 37°C.

Salmonella sp. is the causative agent of various diseases in the human. It causes infection of the gastrointestinal tract or salmonellosis, thyroid and parathyroid fever and also food poisoning. The number of *Salmonella* sp. in the canal on the three examined sites was relatively low (Graph. 3). The highest number was recorded in March at the site near the Vrbas 1 and it was 560 cells per ml. Also, there were periods when this bacterium was not identified.



Graph. 3. Number of *Salmonella* sp. in the water of canal DTD from January to June 2013th (cells number logarithm)

Bacteria of the *Clostridium* genus are anaerobic, Gram-positive, rod-shaped, endospore-forming bacteria. Causing a variety of diseases in humans such as gas gangrene, botulism, sepsis and etc., bacteria from the *Clostridium* genera are commonly present in natural environment, e.g. they live in dust, soil, water, bottom sediments and in human and animal alimentary canals (Moriishi et al., 1996). Species of the genus *Clostridium* can synthesize strong exotoxins which can be lethal to humans and animals (Beckers et al., 2010). The total number of sulphite reducing clostridia in the water of canal DTD, at the tested sites was very low (Graf 4). The highest number was found in May at the site Vrbas 1.



Graph. 4. Number of *Clostridium* spp. in the water of canal DTD (cells number logarithm)

According to Matavuly et al. (2003), the water of DTD canal at almost all tested localities belonged to slightly polluted waters, II class according to Kohl (1975). Few exceptions were recorded in the spring season when water quality were categorized as polluted - III class.

On the basis of the results of our research, it can be concluded that the water from the tested localities need to be treated before it is used in agricultural purpose.

CONCLUSION

A large number of saprophytic bacteria and the presence of the fluorescent *Pseudomonas* sp. indicates the presence of large amounts of organic matter in the water of canal DTD which is probably due to contamination. The number of *Salmonella* sp. and *Escherichia coli* is also high, especially in the spring months (April, May, June), which is also evidence of water pollution. A small number of sulphite reducing clostridia in water and only at the end of the test period, suggests a lack of anaerobic conditions in the water of canal DTD. Results presented in this paper suggest that the water in the canal DTD near Vrbas and Čelarevo should be remediate and purify before using in agricultural purposes.

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MIKROBIOLOŠKI SASTAV VODE KANALA DUNAV-TISA-DUNAV

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Izvod

Zagađene vode imaju vrlo negativan uticaj na celokupan agroekosistem. Zagađivanje površinskih voda najčešće se vrši putem ispuštanja zagađujućih materija različitog porekla kao što su otpadne vode iz industrija i domaćinstava, otpadne vode sa stočnih farmi, ispiranjem štetnih hemijskih materija iz zemljišta u podzemne vode, izlivanjem nafte i drugo. Posledice mogu biti katastrofalne i velikih razmera. Jedan od načina da se uklone štetne materije iz otpadnih voda jeste biološkim putem. Cilj ovog rada je da se na osnovu mikrobiološkog kvaliteta vode kanala DTD, na dva različita lokaliteta, proceni mogućnost upotrebe te vode, u okviru poljoprivredne proizvodnje. Uzorci vode za mikrobiološke analize uzeti su iz kanala DTD sa jednog lokaliteta kod naselja Čelarevo, i sa dva lokaliteta kod Vrbasa, van zone direktnog uticaja uliva otpadnih voda i pritoka. Uzorci vode uzimani su prema smernicama za uzimanje uzoraka površinskih voda iz reka i potoka SRPSISO 5667-6. Vremenski period praćenja bio je od januara do juna 2013. godine. Po 100 ml svakog uzorka vode donešeni su u laboratoriju gde su vršene mikrobiološke analize na ukupan broj bakterija, broj roda *Salmonella sp.*, vrste *E. coli* i zastupljenosti sulfid redukujućih klostridija (rod *Clostridium sp.*). Na oba lokaliteta zabeležen je velik broj saprofitnih bakterija i prisustvo fluorescirajućih *Pseudomonas sp.* Ovi podaci ukazuju na prisustvo velike količine organske materije u vodi kanala, što je verovatno posledica zagađenja. Brojnost roda *Salmonella sp.* i vrste *E. coli* je takođe visoka, naročito u prolećnim mesecima, što je takođe dokaz prisutnog zagađenja vode. Sulfid redukujuće klostridije su u vodi registrovane u malom broju i samo na kraju ispitivanog perioda, što ukazuje na odsustvo isključivih anaerobnih uslova u vodi kanala DTD. U pogledu korišćenja vode kanala DTD u poljoprivredne svrhe, voda sa ispitivanih lokaliteta morala bi se prethodno prečistiti na šta upućuju izneti rezultati.

Ključne reči: *Salmonella sp.*, *E. coli*, *Clostridia sp.*, voda, kanal Dunav-Tisa-Dunav.

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DISTRIBUTION OF WEED SEED BANK FLOODED SOILS WITH REFERENCE TO INVASIVE WOODY WEED SPECIES *Amorpha fruticosa*, L.

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SUMMARY: Determination of soil weed seed bank is especially important for study of weed population dynamics as well as for planned weed control. During 2013 and 2014 soil sampling and determination of the number of weed species seed was performed on the flooded areas of the Danube. Analysis of the soil weed seed bank from locality Futog, showed that in the top soil layer of 0-10 cm, seeds of weed species Amorpha fruticosa L. had the highest abundance, comparing to other two soil layers, numbering 1276 seeds per m².

Key words: seed bank, *Amorpha fruticosa* L., bush, invasive species.

INTRODUCTION

Weed seeds from plant reach the soil surface where they represent future soil weed seed bank (Konstantinović, 2008). Seed quantity and density in great extent depend on soil type, preceding crops, soil tillage, as well as on herbicide use (Konstantinović et al., 2008). Soil weed seed bank impacts on distribution of annual (Steinmann and Klingebiel, 2004), as well as perennial weed species (Blumenthal and Jordan, 2001), which has effect to the spread of weed community over the years. In perennial agrophytocenoses, such as vineyards it is less likely that dramatic changes would occur in composition of weed seed bank. Reduced soil tillage and use of mineral fertilizers, as well as permanent weed composition are restrictions that beside compact soil and inconvenient soil water regime can effect to the occurrence of weeds and weed seed bank composition (Le'ge `re et al., 2005, Smith and Gross, 2006). During 2012 in canals of the Public Water Management Company "Vode Vojvodine" phytocenological screenings confirmed presence of woody

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weed species on canal banks and slopes such as *Amorpha fruticosa* L., *Prunus spinosa* L., *Morus spp.*, *Crataegus laevigata* (Poir) DC, *Populus nigra* L., *Populus alba* L., *Populus tremula* L. and *Rubus caesius* L., while on certain localities occur also *Sambucus nigra* L. and *Salix alba* L. Weed species *Amorpha fruticosa* L. is a bush with a lot of grey branches. It flowers by the end of May and in June. *Amorpha fruticosa* L. (Fabaceae) is a bush of 1-3 (6) m in height, it can be tree like, branches are thin and erect. Purple flowers are in spiked inflorescences on the top of the branches. It grows well on embankments, roads and railways slots, it is used for reforestation of salt marsh, it is important for beekeeping. Flowering period lasts 20 to 25 days, immediately after Acacia, during the period without other significant bee pasture. It belongs to the honey weed species, which in the last five to ten years are also suitable for bees, which indicates great distribution of this woody weed species on the territory of Vojvodina. Acacia is suitable for reforestation of escarpments, slopes and landslides, because the root binds soil well. It is also used for windbreaks belts, hedges and as ornamental shrubs in parks. Root system of this weed species is surface, anastomosis like. During germination of "seeds" (monograin beans), from radicle first develops tiny root out of which grows axial root with lateral roots that anchor to the soil out of which they absorb water and minerals (Tucović and Isajev, 2001). On the flooded meadows *A. fruticosa* L. prevents development of existing vegetation (Botta-Dukat, 2005). This weed species is also described as invasive weed species of Central Europe (Weber and Gut, 2004; Dumitrascu et al., 2010).

Weed species *Amorpha fruticosa* L. is usually found in swamps, on the canals and ponds, as well as in flooded forests (Fargione, 2005) or purely vegetated banks, reeds and high halophyte habitats (Anastasiu et al., 2007). On canal banks and slopes grass weeds are necessary for prevention of erosion on certain canal sections, but broadleaved weeds are necessary to be controlled. According to the study of Pantelić et al. (2003) dominant weeds near canal network are *Amaranthus retroflexus*, *Erigeron canadensis*, *Ambrosia artemisiifolia*, *Daucus carota*, *Convolvulus arvensis*, *Sorghum halepense*. On canal banks and slopes there are numerous woody weed species such as *Amorpha fruticosa*, *Rubus caesius* and *Prunus spinosa* (Pantelić et al., 2003).

At ruderal sites, weed species *Amorpha fruticosa* L. is efficiently controlled by the following herbicides: glyphosate (products Glifosat-Zorka, Glifol, Roundup, Cidoherb, Glifosat SL-480, Glifosav-480, Pirokor, Glitotal-480, Agroglifosat, Agroglifosat eco, Agrototal, Glifosat BN-480, Sirkosan, Glifosol-48, Cosmic-36, Boom-efekt, Clinic 480-SL, Glyfos, Glifomark, Bingo-480, Fozat-480, Dominator, Uragan/Sistem-4, Glyphogan 480-SL, Titan, Touč-down 4-LC) and triclopyr (the product Garlon 4) (Konstantinović, 2011). Knowledge on capability of fast vegetative propagation of emergent and woody species and their huge biomass, suggests that mechanical control is costly and inadequate control measure. Besides uneconomical manual mowing, mechanical mowing cuts plants to smaller parts, and plant regeneration causes repeated formation of huge phytomass. Hitherto studies on control of aquatic, especially emergent and woody weeds suggest that there exists need for several years lasting herbicide use, as the most efficient and the most economical way of their elimination (Konstantinović, 2011). Biological weed invasion represents great danger for keeping of biological diversity, causing extinction of autohton species and change of ecosystem functions. Among numerous factors, success of invasion depends upon biological properties of the invasive weed, as well as on availability of the habitat resources (Rejmanek et al., 2005). Wetlands are especially prone to invasion due to

highly changeable environmental humidity regime (Pino *et al.*, 2006) and light dispersal of propagules by water (Pysek *et al.*, 2004). Invasive wetland plants have significant impact to soil structure and biodiversity (Zedler & Kercher, 2004), but it should have in mind that wetlands are less prone to changes, at least in terms of dramatic ecosystem transformations (Pysek *et al.*, 2004).

MATERIAL AND METHODS

The soil sampling was performed at localities Futog and Begeč, on the coastal area of Danube (flood zone before the dam). The both of the studied localities consist of alluvial soil type, and they are flood zones due to high Danube water level. At the chosen locality that was about 10 ha, soil sampling was done up to depth of 0-30cm with ten replications per diagonal. Soil sampling was performed by a probe that was 4.5 cm in diameter. Soil samples were taken from depths of 0-10, 10-20 and 20-30 cm, and each sample from three soil depths contained about 3 kg of the soil (Smutný and Křen, 2002). The soil was sieved through a series of copper sieves of 0.25 mm in diameter. After that followed weed seed separation from soil samples, and their determination. Determination and number of weed species seeds was accomplished by microscope, as well as by weed classifiers (Janjić and Kojić, 2003). The classified weed seeds were disinfected in 0.1% solution of fungicide benomyl, and then placed into climatic chamber for germination in controlled conditions. Climatic chamber parameters were set to 12 h illumination period at 25 °C ($52.4 \mu\text{mol}\cdot\text{m}^{-2} \cdot \text{s}^{-1}$) and to 12 h at 18°C without illumination, air humidity was 65%. Petri dishes 100x90mm in size were covered by polyethylene leaves in order to avoid evaporation. Check on number of germinated determined seeds was performed each two days. After 28 days aboveground and underground parts of each germinated seed were measured.

RESULTS AND DISCUSSION

At the studied localities, in weed seed bank the total of 19 weed species were identified: *Amorfa fruticosa*, *Amaranthus retroflexus*, *Ambrosia artemisiifolia*, *Brassica rapa*, *Chenopodium album*, *Chenopodium hybridum*, *Urtica dioica*, *Euphorbia helioscopia*, *Galium aparine*, *Mentha arvensis*, *Portulaca oleracea*, *Polygonum aviculare*, *Polygonum lapatifolium*, *Rumex crispus*, *Setaria glauca*, *Setaria viridis*, *Stellaria media*, *Solanum nigrum*, and *Trifolium repens*. The greatest number of weed species was identified in the first two soil layers of 0-10 and 10-20cm. *Amorfa fruticosa* seed were also determined only in the first two layers at the both of the studied localities. In the first layer of 0-10 cm at locality Futog, 1276 seeds of weed species *Amorpha fruticosa* were found per m², in the second layer 877 seeds were determined, and in the third studied layer there were 319 seeds per m². At locality Futog the seed of weed species *Stellaria media* in the soil layer of 10-20 cm had the highest distribution, numbering 2951 plants per m² (Figure 1). In climatic chamber germinated seeds of the following weed species: *Amaranthus retroflexus*, *Amorpha fruticosa*, *Stellaria media*, *Urtica dioica* and *Setaria glauca*.

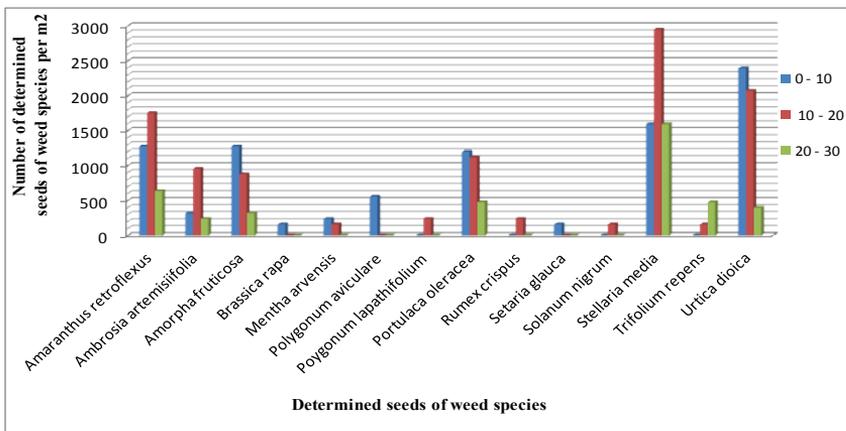


Figure 1. Number of weed species seeds per m² at locality Futog

Low variation percentage (V%) suggests that seeds of certain weed species were evenly distributed in all three studied soil layers. Extremely high variation percentage shows that seeds of one weed species occur unevenly in soil layers, i.e. that in some soil layer there is greater number of them than in other, or it cannot be found. In Table 1 the lowest variation coefficient of 31.22% was recorded for weed species *Stellaria media*, while the highest value of 141.42% was recorded for weed species *Brassica rapa*, *Polygonum aviculare*, *Polygonum lapathifolium*, *Setaria glauca* and *Rumex crispus*.

Table 1. Statistical data processing by standard deviation and variation coefficient at locality Futog

Weed species	Number of weed seeds in samples from different soil depths X			Σ 0-30	Standard deviation	V%
	0 - 10	10 - 20	20 - 30			
<i>Amaranthus retroflexus</i>	16	22	8	46	5.73	37.40
<i>Ambrosia artemisiifolia</i>	4	12	3	19	4.03	63.59
<i>Amorpha fruticosa</i>	16	11	4	31	4.92	47.63
<i>Brassica rapa</i>	2	0	0	2	0.94	141.42
<i>Mentha arvensis</i>	3	2	0	5	1.25	74.83
<i>Polygonum aviculare</i>	0	7	0	7	3.30	141.42
<i>Polygonum lapathifolium</i>	0	0	3	3	1.41	141.42
<i>Portulaca oleracea</i>	0	14	6	20	5.73	86.02
<i>Rumex crispus</i>	0	3	0	3	1.41	141.42
<i>Setaria glauca</i>	0	2	0	2	0.94	141.42
<i>Solanum nigrum</i>	0	2	6	8	2.49	93.54
<i>Stellaria media</i>	20	37	20	77	8.01	31.22
<i>Trifolium repens</i>	0	3	5	8	2.49	93.54
<i>Urtica dioica</i>	30	26	5	61	10.96	53.92
TOTAL	91	140	61	292		

Standard deviation indicates the average deviation of the number of seeds of each weed species from arithmetic mean of the number of seeds of the given weed in all three depths of the sampled soil. The highest value of standard deviation of determined weed seeds at locality Futog was recorded for weed species *Urtica dioica*, while the lowest value was found for weed species *Brassica rapa* and *Setaria glauca*. Table 1 also shows presence of the highest number of weed seeds in the soil layer of 10-20cm, which was to be expected because the studied soils were overflowed soils in which the top soil layer in great measure changes in terms of weed seed bank composition.

At locality Begeč, 17 seeds of weed species were determined, and according to the number of seeds weed species *Stellaria media* with 2472 seeds per m² proved to be the most numerous in the soil layer 10-20cm (Figure 2). At locality Begeč, in the top soil layer of 0-10 cm, 1037 seeds of weed species *Amorpha fruticosa* were found per m², in the second layer were found 558 seeds and in the third 160 seeds per m². Figure 2 shows greater presence of highest number of seeds of weed species in the soil layer of 10-20cm, and the lowest in the soil layer of 20-30 cm.

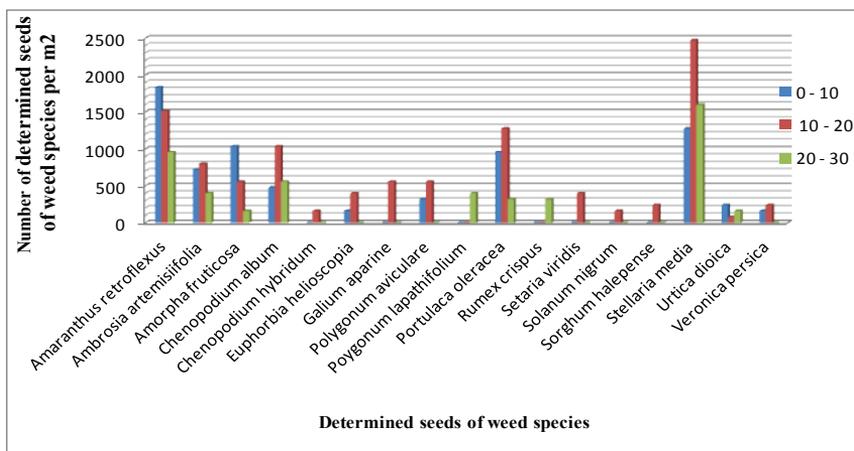


Figure 2. Number of seeds of weed species per m² at locality Begeč

In Table 2, the lowest value of variation coefficient was from 25.26% and 27 % , recorded for weed species *Amaranthus retroflexus* and *Ambrosia artemisiifolia*, while the highest value of 141.42% was recorded for weed species *Chenopodium hybridum*, *Polygonum lapathifolium*, *Galium aparine*, *Setaria viridis* *Solanum nigrum*, *Sorghum halepense* and *Rumex crispus*. The lowest value of variation coefficient had seeds of three weed species, distributed only in one of three studied soil layers. For weed species *Amorpha fruticosa* at locality Begeč did not exist uniform value of determined seeds of this species at all three studied soil depths. Value of variation coefficient for weed species *Amorpha fruticosa* at locality Begeč of 61.32% (Table 2) was higher than at locality Futog where it was 47.63%, i.e. at locality Begeč there exists higher concentration of weed seeds in some of the layers, and at locality Futog that difference was significantly lower. Table 2 also shows that the medium soil layer of 10-20cm had the greatest number of weed seeds (138) which was for more than 50% higher in comparison to the deepest soil layer.

Table 2. Statistical data processing by standard deviation and variation coefficient at locality Begeč

Weed Species	Number of weed seeds in samples from different soil depths (X)			Σ 0-30	Standard deviation	V%
	0 - 10	10 - 20	20 - 30			
<i>Amaranthus retroflexus</i>	23	19	12	54	4.55	25.26
<i>Ambrosia artemisiifolia</i>	9	10	5	24	2.16	27.00
<i>Amorpha fruticosa</i>	13	7	2	22	4.50	61.32
<i>Chenopodium album</i>	6	13	7	26	3.09	35.67
<i>Chenopodium hybridum</i>	0	2	0	2	0.94	141.42
<i>Euphorbia helioscopia</i>	2	5	0	7	2.05	88.06
<i>Galium aparine</i>	0	7	0	7	3.30	141.42
<i>Polygonum aviculare</i>	4	7	0	11	2.87	78.20
<i>Polygonum lapathifolium</i>	0	0	5	5	2.36	141.42
<i>Portulaca oleracea</i>	12	16	4	32	4.99	46.77
<i>Rumex crispus</i>	0	0	4	4	1.89	141.42
<i>Setaria viridis</i>	0	5	0	5	2.36	141.42
<i>Solanum nigrum</i>	0	2	0	2	0.94	141.42
<i>Sorghum halepense</i>	0	3	0	3	1.41	141.42
<i>Stellaria media</i>	16	31	20	67	6.34	28.40
<i>Urtica dioica</i>	3	1	2	6	0.82	40.82
<i>Veronica polita</i>	2	3	0	5	1.25	74.83
TOTAL	103	138	63	304		

CONCLUSION

The highest number of germinated weed seeds, determined at locality Futog had weed species *Amaranthus retroflexus* and *Stellaria media*. At locality Begeč the highest number of germinated seeds had three determined weed species *Amaranthus retroflexus*, *Chenopodium album* and *Stellaria media*. At both of localities those were weed species that were dominant in weed seed bank. At locality Futog, number of seeds of weed species *Amorpha fruticosa* was 2472 per m² in all three soil layers. In the second soil layer of 10-20 cm seeds of this weed species prevail, numbering 140 seeds, taking into consideration that it is flooded soil in which seeds of weed species are hardly present in the top layer. At locality Begeč, where the soil is also flooded, number of seeds of this weed species was 1755 per m². At locality Begeč seeds of *Amorpha fruticosa* were present mostly in the top soil layer numbering 1037 seeds per m². At both localities, soil is often flooded. Flooded soils can have more or less concentration of seeds in the top layer, due to great impact of water regime.

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**ZASTUPLJENOST BANKE SEMENA KOROVA PLAVNIH ZEMLJIŠTA S
OSVRTOM NA INVAZIVNU DRVENASTU KOROVSKU VRSTU
Amorpha fruticosa, L.**

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KONSTANTINOVIĆ BOJAN

Izvod

Determinacija zemljišne banke semena korovskih biljaka je od izuzetnog značaja kako za izučavanje dinamike populacije korova tako i za plansko suzbijanje korova. Tokom 2013. i 2014. godine urađeno je uzorkovanje zemljišta, kao i određivanje brojnosti semena korovskih vrsta na plavnom području Dunava. Analizom banke semena korovskih vrsta istraživanih u blizini lokaliteta Futog, konstatovano je da u površinskom sloju zemljišta 0-10cm postoji najveća brojnost semena korovskih vrsta *Amorpha fruticosa* L., koja iznosi 1276 po m², u odnosu na druga dva dublja sloja zemljišta.

Key words: banka semena, *Amorpha fruticosa* L., žbun, invazivna vrsta.

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DETERMINATION OF THE IDEAL AND THE REAL GROWTH FOR ROE DEER (*Capreolus Capreolus Linnaeus, 1758*) IN THE HUNTING GROUNDS OF VOJVODINA*

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SUMMARY: By investigations of so-called ideal growth in conditions of Vojvodina hunting grounds, a large number of non-fertilized roe does was found. The average number of fetuses-embryos per doe was 0.65, while for pregnant females it was 1.48. The average value obtained by our investigations evidently significantly differs, compared to the other authors, so these investigations should be continued for several more years. The growth of population, real growth or pre-hunting growth should be determined every year, especially after spring counting, based on previous data on general catch in specific hunting grounds. The growth of local roe deer population may considerably vary from year to year. For instance, in Novi Bečej hunting grounds it ranged from 0.42 to 0.91, with average of 0.70 fawns per doe older than 2 years. In 2013, in this hunting grounds it was 0.49, and for all four hunting grounds where the trial was set the average was 0.48. If during a five or more years large differences in planned and achieved growth are repeated, then coefficients of planned growth must be changed during revision of hunting grounds base document.

Key words: roe deer, ideal growth, real growth, embryo.

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INTRODUCTION

Roe deer (*Capreolus capreolus* Linnaeus, 1758) is a big game species belonging to ungulates (*Artiodactyla*) that inhabits more than 90% of productive hunting areas in Serbia for this type of game (Popović et al., 2007). This means that the roe deer is the most numerous and most widely spread large game species managed by hunters associations, with over 90% in total game fund of Serbia. It is estimated that presently there is between 110,000 and 130,000 specimens of roe deer in hunting grounds of Serbia. Its productive hunting area is about 4,000,000 hectares, so its population density is ranging between 2.75 and 3.35 specimens per 100 hectares. In Vojvodina, in total productive hunting area of 1,500,000 hectares, estimated roe deer number is about 45,000 individuals, which is in average 3.00 specimens per 100 hectares. However, there are certain hunting grounds where population density is over 10 specimens per 100 hectares (1 km²) and in those hunting grounds our trial has been carried out (Nova Crnja, Novo Miloševo and Novi Bečej).

Ecologically, it is a species adaptable to various habitat conditions. A century ago, it populated habitats with forests and meadows, but since then it had gradually adapted to mostly agricultural habitats of plain landscapes, where it occupies large stretch of agricultural fields without trees. In such fields, especially if sown with barley, wheat, clover, soy etc., roe deer has a good view and a control so it can easily hide. In such conditions it shows extraordinary properties and gives individuals with excellent conditions and extraordinary trophies. Therefore planning management for this type of game must be approached with extreme seriousness and competence (Ristić, 2014). The basic aim in breeding big game for hunting, in this case roe deer, is to attain maximal trophy in male individuals, respecting breeding age and capacity of the hunting grounds, i.e. to obtain optimal number with most favorable sex structure. In order to reach such goal, it is necessary to plan rational management at all levels. For breeding roe deer game, preparations of hunting grounds base document and management plans must objectively appreciate all facts from the field. Management of roe deer game is discussed by Kučančanin et al. (1994), pointing that planning must be based on several elements: a) real population growth, b) real sex structure and c) real age structure. The real population growth is the number of young individuals added to the basic number of game. It is expressed as a percent from the number of all mature roe deer in basic herd. Normal losses are not taken into account (Ristić, 2014).

Therefore the aim is not to hunt but to preserve and attain the optimal number of game in the hunting ground. In order to do that, it is necessary to hunt only the real growth, and this is to be realized if the spring count, as a way to establish the basic number, is realistically shown (Urošević et al., 2012). Otherwise unrealistic hunting may occur, which brings catastrophic consequences. Regarding the age structure, population should consist of 50% fawns, 20% immature individuals, 20% middle-aged or breeding animals and about 10% mature ones. This is certainly hard to obtain, but these are percents to aspire to. In central Serbia, real population growth is 70-85% of real number of fertile females (Urošević et al., 2012). Possibility for constant increase of production of roe deer game is limited. Having in mind interests of forestry and agriculture, it is necessary to regulate growth and limit hunting (Fišer, 1977).

MATERIAL AND METHODS

Regular breeding season of roe deer begins in second half of July, and ends mid-August. Because of delayed embryo implantation or embryonic diapause that lasts about four months, roe deer embryos are not starting development until the end of December (Linnell et al., 1998). Therefore determination of fertilization in does after breeding season (July-August) and until December-January has been done by embryo observation in does caught.

Real growth of population was established based on data from spring number of roe deer at hunting areas in Nova Crnja, Novo Miloševo, Novi Bečej and Senta. By counting roe deer, the total number was established as well as their sex and age structure. Based on total number of fawns and the parameters for fawn shot in previous hunting season, total number of fawns was calculated by adding these two values together. Also, by adding total number of roe deer counted in 2014 and shooting of roe deer in previous hunting season (2013/14), the total number of roe deer was computed for every hunting grounds. By dividing number of fawns by number of does, the real growth of roe deer population was calculated for every hunting grounds.

Investigations of embryo number were carried out in following hunting grounds: Novi Bečej, Nova Crnja, Novo Miloševo, Kanjiža, Futog and Rusko Selo. Samples were taken during a roe deer hunting season in these hunting grounds and embryos were noted. During investigations of population growth at hunting areas in Vojvodina (Nova Crnja, Novo Miloševo, Novi Bečej, Senta) number of fawns per doe before hunting season was considerably higher than planned growth rate in April for all trial sites. It is important to emphasize that population growth was planned as 60% from total number of females. This parameter occurred to be considerably lower in all three sites. Planned (expected) population growth was between 60% and 80% and it was low in comparison to realized growth before hunting season. From our results, and from results of other authors, it is notable that realized growth may considerably vary from year to year at the same area.

RESULTS AND DISCUSSION

Real population growth in trial areas was established per doe older than two years for following hunting grounds: “Košťanac” Nova Crnja – 0.50 fawns per doe, “Senčanski salaši” Senta – 0.46; “Miloševski rit” Novo Miloševo – 0.47 and “Arača” Novi Bečej – 0.49 fawns. The average real population growth for 2013 per doe older than 2 years was 0.48.

In several hunting grounds in Vojvodina (Novi Bečej, Novo Miloševo, Nova Crnja, Futog, Rusko Selo and Kanjiža) 376 shot does were inspected during 2013/14 hunting season, and results were: 86 (22.87%) does with 1 embryo, 79 (21.01%) does with 2 embryos and 211 (56.12%) does without embryos.

If a total number of does inspected is considered (376) and total number of embryos (158 from 79 two-embryo does and 86 from one-embryo does, which adds to 244 embryos), a large number of non-fertilized does is apparent. If number of embryos (244) is divided to number of does inspected (376), average number of embryos per doe was only 0.649 (rounded to 0.65). If the same number of embryos is divided to number of pregnant does

(165), average number of embryos per pregnant doe was 1.478 (rounded to 1.48). It is worrisome that more than half of shot does was without embryos (56.12%), so in further investigations we will try to find the cause of such high percent of non-fertilized does. When inspecting bagged roe deer, we will pay special attention to age and to sex ratio for each particular hunting grounds, as well as genetic traits of shot roe deer. All data are given in Table 1.

Table 1. Number of embryos in several hunting grounds in Vojvodina, hunting season 2013/14

Hunting ground/hunting organisation	Total roe deer	Gravid does	No. embryos	Fecundity per doe	Fecundity per gravid doe	Percent of Non-gravid does
Novi Bečej	22	22	44	2.00	2.00	0.00
Novo Miloševo	83	63	66	0.80	1.05	24.10
Nova Crnja	184	40	60	0.33	1.50	78.26
“Panonija” - Futog	16	6	7	0.44	1.17	62.50
“Kapetanski rit” Kanjiža	51	16	32	0.63	2.00	68.63
“Zec” Rusko Selo	20	18	35	1.75	1.94	10.00
Ukupno Total	376	165	244	0.65	1.48	56.12

In investigation by Popović et al. (2007), the average number of embryos in 2-year-old does was established as 1.14 embryos per bagged 2-year-old doe and 1.33 embryos per pregnant 2-year-old doe. In older does, average number of embryos had increased up to 2.0 embryos in 5-year-old does. Average potential fertility of does in this sample was relatively low (1.40 embryos per doe and 1.54 embryos per pregnant doe). Results obtained by Stubbe et al. (1982) are lower than in investigations by Kurt (1968), who had found 1.85 embryos per pregnant doe. Results obtained by Pielowski (1984) shows that Stubbe and Passarge (1979) had found 1.90 embryos; Strandgaard (1972) between 1.57 and 2.08, and Bobek et al. (1974) found 1.7 embryos etc.

Based on presented results of Stubbe et al. (1982) investigations of embryo number per pregnant doe were done in England by Harnilton et al. (1960) who found 1.8 embryos; Short et al. (1966) – 1.8; Chapil et al. (1966), Prior (1968), Chapman et al. (1971) – 1.9; Holmes (1973) – 1.7; in Danish hunting grounds Andersen (1953) found 1.9 embryos; in Sweden Essen (1966) – 2.3 and Borg (1970) – 2.2; in Hungarian hunting grounds Fodor et al. (1979) – 2.0 and Szederjei et al. (1971) – 1.4, etc. The population growth rate is an important element of population dynamics, and it is determined by the time of reaching sexual maturity, the ratio of females participating in breeding and the average number of fawns per doe. Rate of fertilization (number of embryos per doe), brood (number of fawns immediately after birth), fawn losses in first 5 or 6 months and realized/fall number before hunting season are important parameters that should be known and respected in management of roe deer population. In several European countries, a high fertilization rate was confirmed in roe deer, using method of inspection of embryos (Danilkin, 1996). For instance, in Lithuania only 3.4% females were not fertilized, and in Poland between 1.6%

and 14% (Danilkin, 1996). In Denmark, percent of unfertilized females was between 0 and 5% (Strandgaard, 1972), in Romania 14% (Almasan, 1967). Regarding the number of fawns immediately after birth, in roe deer it is mostly two fawns, occasionally one fawn and very rarely three. In Romania, investigations confirmed that in 78% cases doe has two fawns, in 19.4% cases one fawn and in 2.6% cases three fawns (Almasan, 1967). All these data regarding reproductive potential of roe deer are impressive, but the most important parameter in management of roe deer population is the number of fawns in comparison to total number of does in fall (September-October) before the start of the hunting season, i.e. so-called real growth.

Number of fawns per doe in fall is considerably lower than number of fawns born (May-June). Results presented by Nikolandić and Degmečić (2007) indicate that rates of survival of fawns between spring and fall ranges from 78% in Denmark (Strandgaard, 1972), 68% in England, 53% in Switzerland, 57% to 93% in Germany to between 38% and 84% in France (Galliard et al., 1998). Mortality in roe deer is distinctly high during the first spring/summer period, even up to 50% (Strandgaard, 1972). In Germany, during a first week of life up to 10% fawns is lost (Stubbe, 1997) and in Poland total mortality between May and November was 37.6% (Danilkin, 1996).

In Vojvodina, based on investigations in four year period in five hunting grounds (Ristić, 1999), population growth had varied, depending on hunting grounds and year, between 0.45 and 0.90 fawns per breeding doe, and in average for the whole period and all hunting grounds it was 0.63% (per breeding doe older than two years) which is only 31% of total number of roe deer game. Average growth in Czech Republic, according to Nečas (1972) was from 80% to 120%, although in more severe conditions brood of fawns was not higher than 40-50%.

After investigations by Popović, et al. (2007), the real population growth varies, depending on year and the hunting grounds, between 0.65 and 1.1 fawns per doe (2 years old or older). Average real growth for period investigated was 0.86 fawns per breeding doe. In paper by Ristić et al. (2013) for 1994-2012 period in Novi Bečej hunting grounds, average coefficient of real growth was 0.7 fawns per doe (2 years old or older); maximum was noted in 1997 – 0.91 fawns per doe, and minimum in 2001 and 2012 – only 0.42 fawns per doe. In 2012, year that will be noted as the most arid year in these parts, it was confirmed that drought had significant effect on survival of fawns, but increased number of predators also had effect in such low growth of population. Regarding sex structure, it ranged between 1:1.19 and 1:1.71 in favor of females, and for the whole period it was 1:1.43 which is quite satisfactory. When analyzing real growth in roe deer after counting (by method of total count) in spring at the whole trial area (March-April), adding shot does (who had birth) and shot fawns for every hunting season between 1994 and 2012, somewhat lower growth is noted – 0.69 fawns per doe older than 2 years.

CONCLUSION

Based on the present study and the study of other authors, it can be listed the following facts in connection to so-called ideal and real growth in roe deer for several hunting grounds in Vojvodina in 2013:

1. In 2013/14 hunting season (between the end of December 2013 and January 2014), 376 does were inspected and 244 embryos were found. These investigations established a large number of non-fertilized does (211). Average number of embryos per doe was 0.649 (rounded to 0.65). Average number of embryos per pregnant doe was 1.478 (rounded to 1.48).

2. Real growth or pre-hunting growth must be monitored and established every year at the end of August, during September and at the beginning of October, and especially after spring count, and also using data about shot accomplished.

3. Real growth of local roe deer population may considerably vary from year to year. For instance, at Novi Bečej hunting grounds it ranged between 0.42 and 0.91, with averagely 0.70 fawns per doe older than 2 years. In 2013, value for this hunting ground was 0.49, and for all four hunting grounds where trial was set average is 0.48. It is evident that the average is much smaller than established for conditions in our hunting grounds in previous period. Therefore it is recommended, based on given methodology, that hunting grounds management determine these parameters every year and to calculate this element which will be used in preparing the management plans.

4. Having in mind that roe deer is strictly territorial game species with small activity area – about 50 hectares for males and 35 hectares for females (Nikolandić and Degmečić, 2007) – ecological characteristics of certain biotopes have considerable effect on growth rate of local populations. In this case, these hunting grounds are representative for most Vojvodina hunting grounds and recommended coefficient of real growth may be used in planning for all other hunting grounds in Vojvodina.

5. Number and sex ratio of fawns observed during May and June may be used only as a general information about population growth, but not as data in establishing rate of real growth, since spring/summer mortality of fawns may be even up to 50%, and it also varies from year to year.

6. If during a five or more years large differences in planned and achieved growth are repeated, then coefficients of planned growth must be changed during revision of hunting grounds base document.

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UTVRĐIVANJE IDEALNOG I REALNOG PRIRASTA SRNA
(Capreolus Capreolus Linnaeus, 1758)
U LOVIŠTIMA VOJVODINE

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Izvod

Istraživanjima tzv. idealnog prirasta za uslove lovišta Vojvodine utvrđen je veliki broj neoplođenih srna. Prosečan broj fetusa-embriona po srni iznosio je 0,65 dok je ovaj prosek po bremenitoj - gravidnoj srni iznosio 1,48. Istraživanjima broja fetusa-embriona po garvidnoj srni u Engleskoj bavili su se: Barnilton et al. (1960) i dobili rezultat od 1,8; Short et al. (1966) - 1,8; Chapil et al. (1966), Prior (1968), Chapman et al. (1971) - 1,9; Holmes (1973) - 1,7; za Danske uslove lovišta Andersen (1953) je utvrdio - 1,9; u Švedskoj su utvrdili Essen (1966) - 2,3 i Borg (1970) - 2,2; za uslove mađarskih lovišta Fodor et al. (1979) - 2,0 i Szederjei et al. (1971) - 1,4, itd. Evidentno je da je ovaj prosek, dobijen na osnovu naših istraživanja, značajno odstupa, te sa ovakvim istraživanjima treba nastaviti još nekoliko godina. Ostvareni prirast, realni prirast ili prirast pred lov, treba utvrđivati svake godine posebno nakon prolećnog brojanja i na osnovu sopstvenih podataka o izvršenom odstrelu u tom lovištu. Ostvareni prirast lokalne populacije srna može značajno varirati od godine do godine, tako da za lovište Novi Bečej se kretao u dijapazonu od 0,42 do 0,91, sa prosekom za ovako dugačak period od 0,70 lanadi po srni starijoj od 2 godine. U 2013. godini, za ovo lovište ovaj parametar je iznosio 0,49, a za sva četiri lovišta, na kojima je bio postavljen ogled, prosek je iznosio 0,48. Ako se tokom pet ili više godina ponavljaju velike razlike u visini planiranog i ostvarenog prirasta, treba prilikom revizije izrade lovne osnove promeniti i koeficijente planiranog prirasta.

Ključne reči: srna, idealni prirast, realni prirast, embrion.

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ISOLATION OF KERATINOLYTIC FUNGI FROM SOIL SAMPLES OVERCAST BY PRESENCE OF CHICKEN

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*SUMMARY: Keratinophilic fungi are an important group of fungi that occurred in soil. The aim of this study was to isolate and identify keratinolytic fungi from the 10 soil samples overcast by presence of chicken from Slovakia. Isolation of the fungi was performed by hair bait technique. The isolated colonies were identified by morphologic feature of macro- and microconidia. The isolated keratinolytic fungi were classified into 10 species belonging to 6 genera. From the total of 123 isolates of keratinolytic fungi represents *Chrysosporium keratinophilum* 39 (31.7%), *Trichophyton ajelloi* (30.1%), *Myceliophthora vellerea* (10.6%), *Trichophyton terrestre* and *Chrysosporium evolceanui* (6.5%), *Chrysosporium fluviale* (5.7%), *Microsporum gypseum* (4.1%), *Arthroderma uncinatum* (2.4%), *Purpureocillium lilacinum* (1.6%), *Chrysosporium indicum* (0.8%). Most of the fungi (96 isolates) were isolated from the soils with the pH range of 7 to 8.*

Keywords: keratinolytic fungi, free-range chickens, *Chrysosporium*, *Trichophyton*.

INTRODUCTION

Keratins are valuable but unavailable fibrous animal proteins. They are components of a range of by-products occurring especially abundantly in slaughterhouses and meat and poultry plants: skin remains, bristle, animal hair, horns and hooves, feathers etc. (Korniłowicz-Kowalska and Bohacz, 2011). Keratins, which are among the hardest-to-degrade animal proteins, are the major component proteins in poultry feathers and are characterized by a tightly packed form in α -helixes and β sheets with a high degree of disulphide bonds (Yasushi et al., 2009). Keratinolytic fungi specialize in the decomposition of keratin, being the main component of keratinous substrata. Keratinophilic fungi associate keratinolytic fungi, utilizing non-protein components of the substrata or the products of keratin decomposition (Ulfig, 2005). Soil is rich in keratinous components

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which are most conducive for the growth and occurrence of keratinophilic fungi (Jain and Sharma, 2012). Rich keratinous materials in soil are the most reason of high incidence of keratinophilic fungi (Zarrin and Haghgoo, 2011). The prevalence of these fungi depends on different factors, such as the presence of creatinine in the soil, pH, and geographical location (Deshmukh and Verekar, 2006). The process of keratin decomposition has also been found to be very fast in soil and it plays a very important role in energy transformation and nutrient cycling in soil (Kushwaha, 2000).

The aim of this study was to isolate and identify keratinolytic fungi from the soil samples overcast by occurrence of chicken.

MATERIAL AND METHODS

Isolation of keratinolytic fungi from soils overcast by occurrence of chicken: A total of 10 samples were taken from different location from Slovakia (Table 1). Keratinophilic fungi were isolated by the hair-baiting method (Vanbreuseghem, 1952). For the isolation of keratinophilic fungi, only the superficial layer 5 cm of the humus horizon was used. Soil samples were poured into Petri dish (up to 10 subsample), 1 sample = 10 subsamples. Based on soil moisture, we applied 10 mL cykloheximid 500 mg/L + 50 mg/L chloramphenicol solutions. As bait were applied 10 fragments of sterilized horsehair on a Petri dish. Cultivation was carried out at 25±1°C for 2 – 3 months (every week check if any growth does occur on the fragments). The pH of each soil sample (25 g) was measured using a pH meter (Sentron), after dilution in sterile distilled water 125 ml with 10 minutes of agitation.

Table 1. Overview of studied soil samples

Soil samples	Locality	pH soil
1	Trnava 1	6.4
2	Trnava 2	8.1
3	Nitra 1	8.4
4	Nitra 2	6.7
5	Smolenice	7.5
6	Pezinok	8.6
7	Nové Zámky	6.4
8	Michalovce 1	7.7
9	Michalovce 2	7.1
10	Michalovce 3	7.7

Isolation of the keratinolytic fungi from colonized hair fragments: Potato Dextrose Agar (PDA) (Conda, Spain) was prepared and 100 µL of the antibiotic solution (chlortetracycline/chloramphenicol) was applied on the surface and evenly spread. Solution (chlortetracycline/chloramphenicol) was prepared: 100 mg/L + 100 mg/L

osmotic water, sterilization at 120°C for 15 min. The mycelium was transferred from a fragment colonized hair into the PDA plate with antibiotic solution. We did three / four lines on plate (scars) to be sure that the contaminating fungi will be well separated from the keratinophilic fungi. The cultivation temperature for PDA plates was 25±1°C, darkness, 4 – 6 days, until colonies appeared. Cultures were transferred to 2% Sabouraud Glucose Agar (SGA) (Conda, Spain) for identification. These plates were incubated at 25±1°C for 7 days and then were used for identification.

*Identification of keratinolytic fungi:*The identification of the resulting keratinophilic and keratinolytic fungi was based on their phenotypic characteristics according to De Hoog et al. (2000), Domsch et al. (1980) and Van Oorschot (1980).

RESULTS AND DISCUSSION

All off tested samples were positive on the keratinolytic fungi. Keratinolytic fungi belong to 10 species representing 6 different genera (Table 2). A total diversity of keratinolytic fungi recovered in this study accounted 123 isolates. The most common isolates were *Chrysosporium keratinophilum* (31.7 %), *Trichophyton ajelloi* (30.1 %) and *Myceliophthora vellerea*(10.6 %).

Table 2. Frequency of keratinolytic fungi isolated from soil samples in free-range chicken

Species	Isolates	
	n	%
<i>Arthroderma uncinatum</i>	3	2.4
<i>Chrysosporium evolceanui</i>	8	6.5
<i>Chrysosporium fluviale</i>	7	5.7
<i>Chrysosporium indicum</i>	1	0.8
<i>Chrysosporium keratinophilum</i>	39	31.7
<i>Microsporum gypseum</i>	5	4.1
<i>Myceliophthora vellerea</i>	13	10.6
<i>Purpureocillium lilacinum</i>	2	1.6
<i>Trichophyton ajelloi</i>	37	30.1
<i>Trichophyton terrestre</i>	8	6.5
Total	123	100

From the total 123 isolates of keratinophilic fungi represents *Chrysosporium keratinophilum* (39 isolates), which represent 31.7 %. Most *Chrysosporium* species are keratinophilic fungi, living on remains of hairs and feathers in soil (De Hoog et al., 2000). *Chrysosporium keratinophilum* and *Chrysosporium queenslandicum* are geophilic keratinolytic species (BSL-1). *Chrysosporium keratinophilum* was repeatedly isolated from onychomycoses and superficial infections and *Chrysosporium queenslandicum* was isolated from skin and nail infections (Reboux et al., 2005). *Chrysosporium* species are the most common keratinophilic fungi isolated from soil in many parts of the world (Shadzi et al., 2002; Deshmukh, 2002; Papini, 1998). Labuda et al. (2008) isolated (*Chrysosporium europae*, *Chrysosporium fluviale* and *Chrysosporium minutisporosum*) from the soil and children's sandpit samples in city park of Nitra and Nová Baňa (Slovakia). *Chrysosporium synchronum* was isolated by Labuda et al. (2009) as rediscovered in Slovakia. Microscopic fungi, namely *Chrysosporium zonatum*, *Malbranchea cinnamomea* and *Myceliophthora thermophila* were also isolated by Labuda et Kačínová (2007) from compost and sludge samples from two different locations in Slovakia.

Trichophyton ajelloi which represent 37 isolates (30.1%) is a geophilic fungus with a worldwide distribution. It cause infections in human and animals, but they are not often reported.

Microsporium gypseum which represent 5 isolates (4.1%) is geophilic fungus. This species is worldwide in distribution. The fungus also infects a variety of animals. The species produces *tinea corporis* and *tinea capitis* (Howard, 2003). *Microsporium gypseum* was isolated from park soils in Gorgan, north of Iran (Malek et al., 2013) and this species was more frequent than other keratinophilic fungi (22.96%).

Two species, *M. gypseum* and *T. ajelloi*, seem to possess the highest potential to digest keratinous materials regardless of the type of substrate (Blyskal, 2009). Many factors impact the degree of material biodeterioration, including microbial metabolic products, i.e., enzymes, acids, and pigments; chemical composition of the material and whether or not additional substances, such as dyes, are contained in the materials; moisture content and its accessibility to microorganisms; history of the material and its age; and local microclimate: availability of oxygen and light, temperature, and relative humidity (Blyskal, 2009).

Table 3. Keratinolytic fungi isolated from soil samples with different pH

Fungal genus	pH		
	6 – 7 (n)	7 – 8 (n)	8 – 9 (n)
<i>Arthroderma</i>	0	3	0
<i>Chrysosporium</i>	0	46	9
<i>Microsporium</i>	0	5	0
<i>Myceliophthora</i>	3	10	0
<i>Purpureocillium</i>	0	2	0
<i>Trichophyton</i>	12	30	3
Total	15	96	12

n= number isolates.

In this study, all 123 keratinolytic fungi were isolated from the soils with pH between 6 and 9 (Table 3). In the present study the *Chrysosporium* spp. (46isolates) and *Trichophyton* spp. (30isolates) were mostly detected in the soil samples with pH range of 7 to 8. We found 12 strains *Trichophyton* spp. from the soil samples with pH of 6 to 7. Asahi et al. (1985) demonstrated that keratinolytic enzymes were produced in pH of 6 to 9, and particularly the extracellular keratinase was active in pH = 9. The species *Arthroderma uncinatum*, *Microsporum gypseum* and *Purpureocillium lilacinum* were isolated from only the soil samples with pH of 7 to 8. Da Silvia Pontes and Oliveira (2008) also recorded that the keratinophilic fungi develop much better in alkaline pH. According to a research conducted by Jain and Sharma (2011), most of the keratinophilic fungi were isolated from pH 6.5 to 8.5.

CONCLUSION

This research reports the prevalence and distribution of keratinolytic fungi in soil samples in free-range chicken (*Gallus domesticus*) from the Slovakia. Keratinolytic activity has an important role in degradation of feather in natural environment.

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IZOLACIJA KERATINOLITIČKIH GLJIVA IZ UZORAKA ZEMLJIŠTA NA KOME SU BORAVILI PILIĆI

JAROSLAVA KAČINOVÁ, DANA TANČINOVÁ

Izvod

Keratinolitičke gljive su važna grupa gljiva, prisutna u zemljištu. Cilj ovog rada je bio da se izoluju i identifikuju keratinolitičke gljive iz 10 uzoraka zemljišta, na kome su boravili pilići u Slovačkoj. Na izolovanim kolonijama su identifikovana morfološke osobine makro i mikrokonidija. Izolovano je 10 vrsta i 6 rodova keratinolitičkih gljivica. Od ukupno 123 izolata, *Chrisosporium keratinophilum* je zastupljen sa 31.7%, *Trichophyton ajelloi* sa 30.1%, *Miceliophthora vellerea* sa 0.6%, *Trichophyton Terrestre* i *Chrisosporium evolceanui* sa 6.5%, *Chrisosporium Fluviale* sa 5.7%, *Microsporium gipseum* sa 4.1%, *Arthroderma uncinatum* sa 2.4%, *Purpureocillium lilacinum* sa 1.6% , a *Chrisosporium indicum* sa 0.8%. Većina gljivica je izolovana iz zemljišta u kome je vrednost pH iznosila 7 ili 8.

Ključne reči: keratinolitičke gljive, slobodno držanje, pilići, *Chrisosporium*, *Trichophyton*.

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IMPACT OF PLANTING DENSITY ON THE INCIDENCE RATE OF FRUITING BRANCHES OF LATE-RIPENING PLUM CULTIVARS INTENDED FOR PROCESSING*

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SUMMARY: The paper examines the incidence rate of fruiting branches of plum (mixed-type, long and short fruiting branches, thorn shoots and May blossoms), depending on the planting density in the newer plum cultivars, Krina and Mildora. A simultaneous study was conducted comparing the said cultivars intended for processing, with the standard cultivars of the same purpose, Čačanska rodna and Stanley. The study established that the differences in the quantitative share of the corresponding types of fruiting branches occur as a result of the conditions and systems of cultivation, as well as the cultivar traits and the development phase. May flowers are the most frequent type of fruiting branches in all of the examined cultivars, with a higher percentage share in the dense planting treatments; at the same time, the other types of fruiting branches recorded a higher incidence in the classical cultivation treatments, compared to the dense planting treatments.

Key words: plum, planting density, fruiting branches.

INTRODUCTION

Planting density, in combination with adequate cultivation forms and application of specific pomological measures, has a direct impact on the fruit set, amount of yield and quality of plum fruits. In dense-planting conditions, balance between the vegetative and generative potential is reached fairly quickly, thus enhancing an earlier fruit set and

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high yields (Miletić et al., 2011a; 2011b; Čmelik et al., 2006; Glišić et al., 2011). In dense formations, tree crowns are characterised by a specific structure of a fruit-bearing tree, dominated by short less vigorous fruiting branches and May blossoms (Mičić et al., 2006). Types of fruiting branches in plum have been described in detail by Mišić (1996) in the classical planting systems at standard distances 5.5-7.0 x 4.0-6.0 m, where the trees were grafted on the cherry plum (*Prunus cerasifera* Ehrh) seedling.

The share of the fruiting twigs of plum in the conditions of dense-cultivation formations was studied by Glišić and Milošević (2005). According to these authors, plum cultivars grafted on the cherry plum stem, planted in the second year of growth using the 4 x 2 m distance pattern, showed presence of different types of fruiting twigs. Veličković et al. (1997) and Mratinić et al. (2007a) report that the established differences in the quantity share of major types of fruiting twigs in the examined plum cultivars were primarily caused by the cultivar traits and the development phase of the plants.

The impact of the density of planting on the share of fruiting twigs was examined in with an aim to develop the growing technologies for new plum cultivars intended for processing, primarily drying (Mildora and Krina), as compared to the standard cultivars (Čačanska rodna and Stanley) of the same designation.

MATERIAL AND METHODS

The research was conducted during 2012 and 2013 at the Zdravljak facility of the Fruit Research Institute in Čačak. The plantation was set up in 2006, at an altitude of 550 m above the sea level, on a lot with a south-east exposition. The soil is of the brown-reddish albic luvisol type on limestone. The trial included the following cultivars: Čačanska rodna, Stanley, Mildora and Krina. The cultivars were grafted on the cherry plum seedlings (*Prunus cerasifera* Ehrh), with well-developed premature twigs. The selected cultivars were cultivated in five treatments:

- a) I treatment – 4.0 x 1.0 m (2500 trees/ha),
- b) II treatment – 4.0 x 1,5 m (1666.6 trees/ha),
- c) III treatment – 4.0 x 2.0 m (1250 trees/ha),
- d) IV treatment – 5.0 x 3.0 m (666.6 trees/ha),
- e) V treatment – 5.0 x 4.0 m (500 trees/ha).

In treatments I, II and III, by using corresponding pomo-technical measures, trees were given the shape of the modified planning form – the spindle bush, with changes in the layout, position and number of skeletal branches from the base, to the top of the crown. In this manner, it was possible to regulate the development of the vegetative mass in the higher sections of the crown, by developing shorter fruiting twigs and thorn shoots. In treatments IV and V, the adopted shape was the classical improved pyramid crown. In addition to the specific pomo-technical measures used in forming the cultivation form, measures of winter and summer pruning were also implemented, together with the cultivation of soil, fertilizing and protection against diseases and pests.

Determination of the share of fruiting twigs was performed in the flowering phase, when these are most easily spotted and recognised. The crowns of all the cultivars per planting

treatment (five trees) showed presence of mixed-type fruiting twigs, long fruiting twigs, short fruiting twigs, thorn shoots and May blossoms, as described by Mišić (1996).

The numerical values have been recalculated and shown as percentage values for each cultivar per cultivation treatment. The obtained two-yearly results have been statistically processed using the Fisher model of variance analysis, the ANOVA. The degree of significance of the differences between the presence of the same type of fruiting twigs per treatment was tested using the Duncan test for significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

In the past, during 19th and 20th centuries, Serbia was a leader in the field and enjoyed a worldwide reputation as a producer of prunes. However, more recently, Serbia's output and sales of prunes have been at a modest level (Mitrović, 2012). In order to increase the production and raise competitiveness in the global market, in addition to developing new technologies of cultivation and drying, Serbia will need to introduce in the production new cultivars with high quality features, which will also require a completely new approach to this issue. Modern plum-drying technologies require high quality fruits, with uniform size and mass (Mitrović et al., 2000). A high proportion of high quality fruits for drying are secured by using appropriate cultivation techniques, specific pruning and other pomological measures (Mratinić et al., 2007 b; Miletić et al., 2011b; Čmelik et al., 2006).

According to Mišić (1996), the plum has various types of fruiting branches. The mixed-type fruiting branches (usually 50 cm or longer) feature between two and four flower and vegetative buds at each node. The long fruiting branches (app. 40 cm long) and short fruiting branches (10 to 20 cm long) are studded on all sides with flower buds, with a vegetative bud at the top. The thorny, spear-shaped twigs represent short vegetative branches (between 0.5 and 10 cm long), most typically developed in domestic plum cultivars. These thorny Twigs bear good quality fruits. The May flowers (fruit blossoms) are short fruiting branches (2 to 4 cm long) with a large number of buds; while the middle section is occupied by vegetative buds, the remaining ones are flower buds. Similar descriptions and classifications of the fruit bearing branches in plum have been used by Tošić (1983), Bulatović and Mratinić (1996) and Milošević (2002). The results of the percentage share of the fruiting twigs in the examined cultivars are shown in Table 1.

Based on the above description, the percentage share of the mixed-type and long fruiting twigs in this trial was at a minimum value in the dense planting treatments, but it was radically increased in treatments IV and V. This tendency was marked in all of the tested cultivars, albeit in different ratios. In the Čačanska rodna cultivar, the percentage share of the mixed-type fruiting twigs in the dense planting formations was between 1.5 and 2.2%, as opposed to the 3.7 to 4.7% in the control group, while the share of other types of fruiting twigs ranged from 4.6 to 7.7%, i.e. from 9.1 to 10.9%.

In the Stanley cultivar, the mixed-type fruiting twigs were present in the dense planting treatments in the range from 4.1% to 7.7%, as opposed to the 9.1 to 10.4% in the control group, while the share of the long fruiting twigs ranged from 4.0 to 6.5%, i.e. 10.0 to 11.0%. Similar ratios were recorded in the new Krina cultivar in dense planting treatments, where the share

of the mixed fruiting branches ranged between 1.5 and 3.5%, as opposed to the 3.8 to 4.1% in the control group, whereas the share of the long fruiting branches ranged between 1.8% and 3.8%, i.e. between 5.5% and 5.9%. Compared to the Krina cultivar, the more vigorous Mildora cultivar grown in the dense planting treatments recorded a share of the mixed-type fruiting twigs in the range from 1.3 to 2.8 %, as opposed to the share ranging from 5.0 to 5.6% in the control group, while the share of the long fruiting twigs was in the range between 2.0 and 4.0%, i.e. 7.3 to 8.5%.

The obtained results indicate that all of the examined cultivars in the treatments of dense planting (I, II and III) recorded a high percentage share of short fruiting twigs and May blossoms. However, their share begins to decline with the increase of the planting distance (treatments IV and V). In the example of the non-vigorous Čačanska rodna cultivar, the share of the short fruiting twigs in the dense planting treatments ranged from 45.2 (treatment III) to 47.8% (treatment I), i.e. from 42.4 to 42.7%, whereas the share of the May blossoms was in the range from 44.7 (treatment III) to 45.9% (treatment I), i.e. from 41.6 to 44.6% in the control group. In contrast to this, the more vigorous Stanley cultivar recorded the percentage share of short fruiting twigs in the range between 31.6 (treatment III) and 36.9% (treatment I), as opposed to the control group, where this share ranged from 30.4 (treatment IV) to 31.6% (treatment V), whereas the share of May blossoms fell in the range from 46.4 (treatment II) to 48.8% (treatment I), i.e. from 40.1 (treatment V) to 48.8% (treatment I) in the control group.

Table 1. Share of different types of fruiting branches per cultivation treatment (%)

Cultivar	Treatment	Mixed-type fruiting twigs	Long fruiting twigs	Short fruiting twigs	Thorn shoots	May blossoms
Čačanska Rodna	I	1.5 d	4.6 e	47.9 a	0.1 b	45.9 a
	II	1.9 c	6.3 d	46.1 ab	0.1 b	45.6 a
	III	2.2 c	7.7 c	45.2 b	0.2 a	44.7 a
	IV	3.7 b	9.1 b	42.4 c	0.2 a	44.6 a
	V	4.7 a	10.9 a	42.7 c	0.1 b	41.6 b
Year (2012–2013)		*	*	*	*	*
Stanley	I	4.1 e	4.0 c	36.9 a	6.2 a	48.8 a
	II	6.3 d	5.5 b	35.1 a	6.7 a	46.4 ab
	III	7.7 c	6.5 b	31.6 b	6.9 a	47.3 a
	IV	9.1 b	10.0 a	30.4 b	6.3 a	44.2 b
	V	10.4 a	11.0 a	31.6 b	6.9 a	40.1 c
Year (2012–2013)		*	*	*	ns	*
Krina	I	1.5 a	1.8 d	42.8 a	1.1 b	52.8a
	II	3.0 a	3.1 c	40.9 ab	2.2 a	50.8b
	III	3.5 ab	3.8 b	40.8 ab	2.0 a	50.1b
	IV	3.8 b	5.5 a	40.4 b	2.0 a	48.3c
	V	4.1 c	5.9 a	39.8 b	1.9 a	48.3c
Year (2012–2013)		*	*	*	*	*

Mildora	I	1.3 d	2.0 e	43.1 a	0.6 d	53.0 ab
	II	1.5 d	3.1 d	41.6 a	0.9 cd	52.9 ab
	III	2.8 c	4.0 c	38.2 b	1.2 bc	53.8 a
	IV	5.0 b	7.3 b	34.5 c	1.4 ab	51.8 ab
	V	5.6 a	8.5 a	34.4 c	1.6 a	49.9 b
Year (2012–2013)		*	*	*	*	*

*Values within each column followed by the same small letter are not significantly different at the $p \leq 0.05$ by Duncan's test;
ns – non significant.

The Krina and Mildora cultivars belong to the category of vigorous cultivars. It is worth observing that when cultivated in the dense planting formations, Krina demonstrated a proportion of short fruiting branches (40.8 - 42.8%) similar to that recorded in the control treatments (39.8 - 40.4%), with the share of the May blossoms in the range between 50.1 and 52.8%, compared to 48.3% in the control group in both treatments. The Mildora cultivar recorded the share of the short fruiting branches in the range from 38.2 to 43.1%, compared to the share of 34.4 to 34.5% in the control group, whereas the May flowers reached the range from 52.9 to 53.8%, i.e. from 49.9 to 51.8% in the control group.

Milenković et al. (2006) state that the Krina and Mildora cultivars primarily bear fruits on the May blossoms and one-year fruiting branches, which corresponds to our results. Similar conclusions have been made about the Čačanska rodna and Stanley cultivars (Mišić, 1996).

Thorny shoots are a characteristic of the domestic plum cultivars (Mišić, 1996). Their presence in the crown, however, shows a declining tendency as the tree becomes older. Among the examined cultivars in the seventh, i.e. eighth year after planting, this trait was the least expressed in the Čačanska rodna cultivar where it ranged between 0.1% and 0.2%, while it was the strongest in the Stanley cultivar, ranging from 6.2 to 6.9%. As for the Krina and Mildora cultivars, the proportion of the thorny shoots fell in the range between 1.1 to 2.2% and 0.6 and 1.6%, respectively.

In general, the May blossoms are the most frequent type of fruiting twigs in all of the examined cultivars, with a higher percentage share recorded in the dense planting treatments (treatments I, II and III), and conversely – a lower share tendency recorded in the treatments implying larger planting distances (treatments IV and V).

The percentage share of the fruiting twigs is a specific trait of each cultivar. A higher incidence of short fruiting twigs and May blossoms occurs as a result of the applied pruning principles (winter and, especial, summer pruning) and bending of twigs. The formation of fruiting branches is also determined by the time of onset and increase in the yield. Yield has a direct impact on the vegetative growth of the crown, thus determining the percentage share of the fruiting branches.

The agro-ecological conditions and the implementation of care and pomological-technical measures have a direct impact on the form of the morphological traits of the crown, which is a specific trait of each individual cultivar. These conclusions are in accordance with the findings made by Veličković et al. (1997) and Mratinić et al. (2007a), who pointed out that the structure of the fruiting twigs depends on the cultivar and the development phase of the fruit. Hrotko (2004) states that in intensive plum plantations in Hungary containing 1400 – 2500 trees per hectare, implementation of suitable pomological-technical measures enables formation of a significant number of fruit-bearing trees in the very early years after planting, both on the bearing branches and on the central leader. Glišić and Milošević (2005) established that in the Čačanska rodna, Čačanska rana,

Čačanska lepotica and Stanley grafted on the cherry plum stock, planted at the 4 x 2 m distance, differences appeared in the presence of fruiting twigs in the second year after planting.

In the Čačanska rodna and Stanley cultivar, the dominant share was taken by the mixed-type fruiting twigs. While Čačanska rana recorded an even albeit very low proportion of all types of fruiting branches, in the Čačanska lepotica cultivar the dominant form were the short fruiting twigs (thorny shoots and blossom fruiting branches)

According to the authors, the highest number of the fruiting twigs per tree was recorded in the Čačanska lepotica cultivar, followed by the Čačanska rodna cultivar, while the smallest number of fruiting twigs was recorded in the Čačanska rana cultivar. This tendency was partly reflected in our trial as well, taking into account the age of the fruits, i.e. the time of the analysis of the fruiting twigs share.

The features of the fruiting twigs and their position in the crown have an impact on the size, mass and quality of the fruits (Mišić, 1996). The principles of standardisation and the increasingly demanding global and domestic markets insist on implementation of the comprehensive agro-technical and pomo-technical measures in order to secure high quality fruits. A high share of the fruits satisfying the quality requirements in the crown of each fruit tree also makes a positive impact on the higher economy of the fruit production and better financial indicators.

CONCLUSIONS

Based on the study of the impact made by the density of planting on the proportion of the various types of fruiting twigs (mixed-type, long and short fruiting twigs, thorny shoots and May blossoms) in the plum cultivars suitable for drying (Čačanska rodna, Stanley, Krina and Mildora), it is possible to make the following conclusion:

The differences in the quantitative share of the different types of the fruiting branches in the examined plum cultivars occurred primarily as a consequence of the prevailing conditions and the cultivation system applied, as well as the cultivar traits and the development phase.

The May blossoms are the most frequent type of the fruiting branches in all the examined cultivars. While their percentage share tended to be higher in the dense planting treatments, it also showed a declining tendency in the treatments utilising larger planting distances.

Other types of fruiting twigs recorded a higher percentage share in the classical cultivation treatments, when compared to the dense planting treatments.

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ZASTUPLJENOST RODNIH GRANČICA POZNIH SORTI ŠLJIVA NAMENJENIH PRERADI U ZAVISNOSTI OD GUSTINE SADNJE

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Izvod

U cilju razvoja tehnologije gajenja novijih sorti šljiva pogodnih za sušenje (Krina i Mildora u komparaciji sa standardnim sortama (Čačanska rodna i Stanley) iste namene, ispitvana je i zastupljenost rodni grančica (mešovite, duge i kratke rodne grančice, trnasti izraštaji i majski buketići) u zavisnosti od gustine sadnje. Razlike u kvantitativnoj zastupljenosti tipova rodni grančica u ispitivanih sorti šljive je pre svega posledica uslova i sistema gajenja, sortni karakteristika i stadijnog razvitka. Majski buketići su najzastupljeniji tip rodni grančica kod svih ispitivanih sorti, pri čemu je procentualna zastupljenost veća u tretmanima guste sadnje, sa tendencijom smanjenja u tretmanima sa većim rastojanjima sadnje. Ostali tipovi rodni grančica su procentualno više zastupljeni u tretmanima klasičnog uzgoja u odnosu na tretmane guste sadnje.

Ključne reči: šljiva, gustina sadnje, sorte za sušenje, rodne grančice.

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EFFECTS OF CHEMICAL THINNING OF SOUR CHERRY FLOWERS*

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SUMMARY: The paper presents the results of chemical thinning of sour cherry flowers of the Šumadinka and the Čačanski rubin cultivars. The treatments were performed in the full flowering phase, using 0.0025% concentration of the LG-GER-ATS (ammonium thiosulphate). Following the application of the LG-GER-ATS, the number of set fruits was reduced by 9.8% and 8.0% for the Šumadinka and Čačanski rubin cultivars respectively, compared to trees receiving no treatment. The thinning of the flowers had an impact on the increase of the fruit mass by 19.1% and 10.9% in the Šumadinka and the Čačanski rubin cultivars respectively. Trees treated with LG-GER-ATS recorded a higher yield in both years of trial – the yield in Šumadinka cultivar was higher by 20.7% per tree, while the yield of the Čačanski rubin cultivar was higher by 17.1% per tree.

Key words: sour cherry, ammonium thiosulphate, chemical thinning of flowers, fruit, yields.

INTRODUCTION

Chemical thinning of flowers or fruits in early embryonic phase is a regular pomotechnical measure for certain types of fruits. Chemical thinning is especially successfully performed in apples, peaches and apricots (Costa and Vizzotto, 2000; Keserović et al.; 2012, Lukić et al., 2010; 2012; Mratinić, 2012; Milatović, 2013). The thinning is performed in order to eliminate alternative productivity and obtain a higher average fruit mass, as well as a higher share of the larger-size fruits, better colouring and higher contents of sugar, acids, and other components (Link, 2000). According to Webster and Spencer (2000), excessive vigour of trees may result in a harvest of smaller-size fruits

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and fruits of lower quality, breaking of branches, exhaustion of trees and reduced frost resistance. Stopar (2002) points out that with non-thinned trees of the Golden Delicious apple cultivar, 83% of the harvested fruits were smaller than 70 mm, while at the same time the total yield in the treated trees was not larger than that of the non-treated trees, since the trees that produced larger-size fruits were at the same time the ones with a lower yield per tree. In recent years, there has been a growing use of thinning of flowers and fruits in the cherry production, with an aim of meeting the market demands for high-quality fruits (Whiting et al.; 2006; Schoedl et al.; 2007; Milić et al., 2012). Chemical thinning is insufficiently used in sour cherry production, mostly due to the fact that sour cherry fruits are mainly used in industrial processing.

The aim of the research was to evaluate the effect of the ammonium thiosulphate on the quality of fruit and the yield of sour cherry cultivars.

MATERIAL AND METHODS

The trial was conducted in the collection orchard of sour cherries in the Fruit Research Institute in Čačak, during 2011 and 2012. The orchard lies at an altitude of 242 m, on soil with the category of alluvial loam sediments. The sour cherries were planted at the distance of 4 x 3 m (833.3 trees/ha) and the trial included the Šumadinka and Čačanski rubin cultivars, both created at the Fruit Research Institute in Čačak.

The treatments were conducted at the phase of full bloom, in dry and warm weather conditions (22°C) using the LG-GER-ATS (ammonium thiosulphate) preparation, in the 0.0025% concentration (25 ml/10 l water). The orchard was treated with standard agro-technical and pomological measures, supported by the drip irrigation system. The trial was set up using the randomised block system (2 cultivars x 5 trees x 3 replications).

The ratio between percent fruit set and non-fertilised flowers was determined in the end-of-flowering and fruit set phase. Samples of fruits were taken every ten days during the period from the fruit set to the ripening of fruits. The fruit mass was measured in order to monitor the dynamics of growth of fruits, while the yield per tree and per unit of area was determined in the harvesting phase. Major pomological-technical traits of fruits were also determined.

The dimensions of fruits, stone and stalk were determined using a digital calliper with a 0.01 mm resolution, while the mass was determined using the 'Metler' technical scales, with a 0.01 g precision. The dry soluble matter was determined using the German 'KRUSS' digital refractometer.

The paper presents average results for the two-year period of the trial, statistically processed using the Analysis of Variance and Duncan's Test for significance threshold $P \leq 0,05$.

RESULTS AND DISCUSSION

The implementation of the ammonium thiosulphate caused a reduction in the number of set fruits in Šumadinka cultivar by 9.8% on average per year of trial, i.e. by an average 8.0% in Čačanski rubin cultivar. The results for the rate of non-fertilised flowers were

opposite to the results obtained for the rate of set fruits. There were highly significant differences that were determined between the treatments (Table 1).

Table 1. Effects of chemical thinning of flowers

Parametri	Year	Šumadinka		Čačanski rubin	
		treated	control	treated	control
Number of treated flowers	2011	747	984	877	728
	2012	783	726	723	742
	Mx	765	855	800	735
Set fruits (%)	2011	52,7 b	62,5 a	52,2 b	60,4 a
	2012	54.7 b	64.5 a	54.8 b	62.6 a
	Mx	53.7 b	63.5 a	53.5 b	61.5 a
Non-fertilised flowers (%)	2011	47,3 a	37,5 b	47,8 a	39,6 b
	2012	45.3 a	35.5 b	45.2 a	37.4 b
	Mx	46.3 a	36.5 b	46.5 a	38.5 b

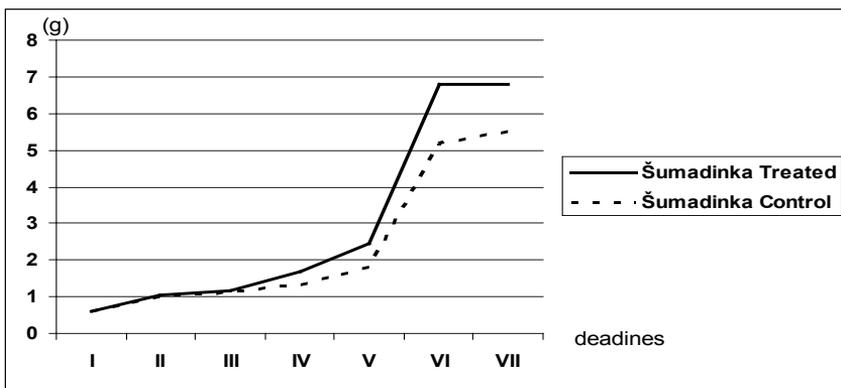
The reduction in the flowering and fruit-setting potential resulted in an increase in fruit size and mass among the treated trees. The fruit mass in the treated trees was on average higher by 1.3 g (19.1%) and 0.9 g (10.9%) in the Šumadinka and the Čačanski rubin cultivars, respectively, compared to the fruit mass of the non-treated trees. The differences detected in the fruit mass resulting from the different treatments were highly significant. The stone mass did not show any significant differences in different treatments so that consequently no statistical differences have been established for this parameter. The flesh ratio in treated Šumadinka cultivar was significantly higher for 2.5% than with untreated fruits, while it was only insignificantly (0.4%) higher in Čačanski rubin cultivar.

The stalk length is an important parameter for the speed and quality of sour cherry harvesting. The trial did not reveal any significant differences between the tested treatments, regarding the length of the stalk and the dry soluble matter contents. The obtained results indicate that the increased yield causes no disturbances in the content of the dry soluble matter, which is a basic indicator of the quality of fruits regarding determination of the proper time for harvesting and processing of fruits (Table 2).

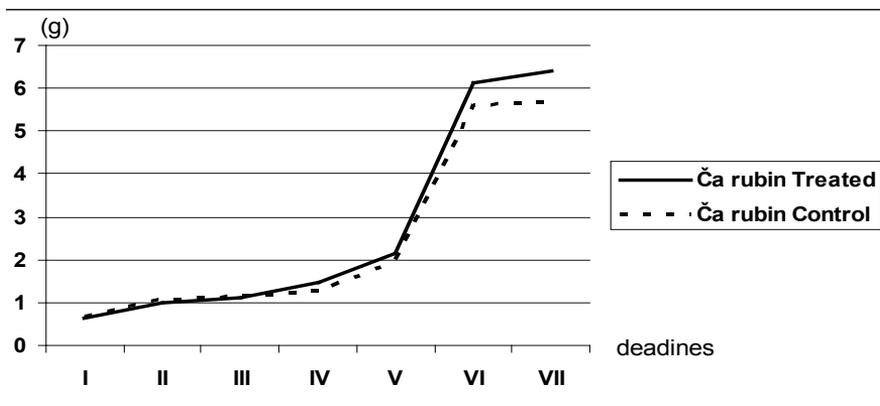
Table 2 Effects of thinning on fruit size and mass

Parameters <i>Parametri</i> treated		Šuma- dinka	Čačanski rubin		
		control	treated	control	
Fruit (mm)	Lenght	21.0 a	19.1 b	21.2 a	19.6 b
	Width	20.9 a	19.3 b	21.2 a	19.7 b
	Thickness	23.0 a	21.4 b	22.3 a	20.9 b
Stone (mm)	Lenght	11.7 a	11.1 a	11.5 a	11.4 a
	Width	11.8 a	9.9 b	10.1 a	10.2 a
	Thickness	7.9 a	7.6 a	7.8 a	7.7 a
Fruit mass (g)		6.8 a	5.5 b	6.4 a	5.7 b
Stone mass / <i>Masa koštice</i> (g)		0.45 a	0.50 a	0.54 a	0.50 a
Flesh ratio/ <i>Sadržaj mezokarpa</i> (%)		93.4 a	90.9 b	91.6 a	91.2 a
Fruit stalk-lenght / <i>Dužina peteljke</i> (mm)		35.3 a	36.8 a	37.4 a	38.2 a
Soluble solids / <i>Rastvorljive suve materije</i> (%)		13.4 a	13.7 a	12.8 a	12.5 a

Graphs 1 and 2 illustrate the growth dynamics of the fruits in the tested cultivars. The trial revealed that the fruits had a similar growth dynamics in the first growing phase (from the fruit-set until the beginning of stone formation), followed by an increasing difference in the fruit growth dynamics in the second phase (stone formation phase) and the significant increase in the fruit growth of treated trees, compared to the non-treated, occurring in the third phase (fruit-growth phase). These differences in the growth of fruits between the different treatments were especially noticeable in the Šumadinka cultivar.



Graph. 1. Dynamics of fruit growth of the Šumadinka cultivar (g)



Graph 2. Dynamics of fruit growth of the Čačanski rubin cultivar (g)

The best indicator of the positive effect of the flower-thinning in the tested sour cherry trees was the yield per tree and per unit of area. In the Šumadinka cultivar, the yield per tree in the first year was higher by 0.6 kg (8.1%), and in the second year by 6.6 kg (33.3%), i.e. the average increase in the yield for the two years of trial was 3.0 kg (20.7%). The increase in the yield in the Čačanski rubin cultivar in the first year was 0.7 kg (8.2%), and 3.9 kg (26.0%) in the second year, amounting to the average increase of 2.3 kg (17.1%) for the two trial years. Expressed in the yield per unit of area, the average increase in the Šumadinka cultivar yield was 3 t/ha (26.5%), while the average increase in the yield of the Čačanski rubin was 1.92 t/ha (19.6%). Highly significant differences have been detected between the treatments, in both yield per tree and yield per unit of area (Table 3).

Tab. 3. Effects of flower-thinning on the fruit yield

Parameters		Year treated	Šumadinka		Čačanski rubin	
			control	treated	control	treated
Yield	Kg / tree	I	7.4 a	6.8 a	8.5 a	7.8 a
		II	19.8 a	13.2 b	15.0 a	11.1 b
		Mx	13.3 a	10.3 b	11.75 a	9.45 b
	t/ha	I	6.17 a	5.67 a	7.08 a	6.49 b
		II	16.49 a	10.99 b	12.5 a	9.25 b
		Mx	11.33 a	8.33 b	9.79 a	7.87 b

Schoedl et al. (2007) report that chemical thinning of sour cherry cultivars using ammonium thiosulphate in two concentrations – one of 8.82 g/l and the other of 17.6 g/l – resulted in an increase in the fruit mass and the content of the dry soluble matter, without having a significant impact on the yield. In their treatment of the Bing cultivar raised on Gisela 6 rootstock, in addition to ammonium sulphate (2%), Whiting et al. (2006) were using fish oil (2%) + lime sulphur (2,5%) and a vegetable oil emulsion (200 gal/acre), achieving satisfactory results regarding fruit quality. Milić et al. (2012) were researching the effect that various preparations including ammonium thiosulphate had on thinning of flowers and size of fruits in sweet cherry cultivars, reaching a conclusion that thinning of flowers using ammonium thiosulphate resulted in a reduction of set fruits, accompanied by an increase in the fruit mass. The results achieved in sour cherries using the ammonium thiosulphate treatment indicate the possibility of a successful use of this compound in the thinning of sour cherry flowers.

Chemical thinning of sour cherry fruits is a practice rarely used in fruit production and apart from certain fragmentary and general declarations, no concrete research results exist in this area. The reason for this situation can be found in the fact that sour cherry fruits are primarily used for industrial processing. Mratinić (2002), Milenković et al. (2006) and Blagojević et al. (2012) state that fruits of the Šumadinka sour cherry cultivar can be used as table fruits, as well as in industrial processing, where they are converted into confitures, jams, juices and similar products requiring the use of larger-size fruits, while the fruits of the Čačanski rubin cultivar are primarily used in industrial processing. According to the results obtained by Rakićević et al. (2008), the Šumadinka cultivar is characterised by an average fruit mass ranging from 4.5 to 6.6 g, the flesh ratio of 83.5 to 84.0% and a yield per tree in the range of 10.8 to 12.6 kg, while Nenadić-Mratinić et al. (2006) state that the average fruit mass of the Čačanski rubin cultivar amounts to 5.61 g. This trial showed that the trees treated with ammonium thiosulphate produced fruits that were characterised by a larger mass than the ones found by the quoted authors, which points to the fact that chemical thinning of fruits is more justified, given the fact that it produces fruits of larger dimensions and mass. The yields per tree and unit of area recorded in the trial were also higher in trees undergoing the treatment than the results obtained by Rakićević et al. (2008), which can contribute to better financial results in sour cherry production. On the other hand, the increased yield did not have a significant impact on the dry soluble matter contents, i.e. quality of fruit, which provides further justification for a more widespread use of this treatment in future practice.

CONCLUSIONS

Based on the obtained results, it can be concluded that thinning of sour cherry flowers based on the use of ammonium thiosulphate has a positive impact on the fruit quality and yields.

The use of the LG-GER-ATS reduced the number of set fruits by 9.8% in the Šumadinka cultivar, i.e. by 8.0% in the Čačanski rubin cultivar, when compared to the non-treated trees. The thinning of the flowers led to an increase in the fruit mass of 19.1% in the Šumadinka cultivar, i.e. 10.9% in the Čačanski rubin cultivar, while at the same time the contents of the dry soluble matter in the fruits did not demonstrate any significant differences between the tested treatments. The trees treated with ammonium thiosulphate

recorded a higher yield in both trial years, with the actual increase in yield per tree of 20.7%, i.e. 26.5% increase in yield per unit of area in the Šumadinka cultivar, and 17.1% increase in yield per tree, i.e. 19.6% increase in yield per unit of area in the Čačanski rubin cultivar.

However, the success of the thinning treatment is highly dependent on the numerous external factors – primarily on the weather conditions during and following the treatment, the active substance of the preparation, its formulation and concentration, the time and method of application, and other factors. In order to be able to recommend the thinning of sour cherry flowers to be used in wider production practice as a necessary measure for improving the fruit quality and yield, it will be necessary to continue the research into various sour cherry cultivars, at various sites.

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EFEKTI HEMIJSKOG PROREĐIVANJA CVETOVA VIŠANJA

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Izvod

U radu su prikazani rezultati hemijskog proređivanja cvetova višanja, sorti Šumadinke i Čačanski rubin. Tretiranja su obavljena u fazi punog cvetanja preparatom LG-GER-ATS (amonijum tiosulfat) u koncentraciji od 0,25%. Primenom preparata LG-GER-ATS smanjen je broj zametnutih plodova za 9,8% kod sorte Šumadinka i za 8,0% kod sorte Čačanski rubin u odnosu na netretirana stabla. Proređivanje cvetova uticalo je na povećanje mase plodova za 19,1% kod sorte Šumadinka i za 10,9% kod sorte Čačanski rubin, dok se sadržaj rastvorljivih suvih materija u plodovima nije bitnije razlikovao između ispitivanih tretmana. Stabla tretirana amonijum tiosulfatom ostvarila su veće prinosi u obe godine ispitivanja za 20,7% po stablu ili 26,5% po jedinici površine kod sorte Šumadinka i za 17,1% po stablu ili 19,6% po jedinici površine kod sorte Čačanski rubin.

Cljučne reči: višnja, amonijum tiosulfat, hemijsko proređivanje cvetova, plod, prinosi.

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ANTIOXIDANT PROPERTIES OF SOYBEAN SEEDLINGS GROWN IN DIFFERENT SOIL TYPES*

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SUMMARY: The purpose of this work was to assess change in antioxidant ability and accumulation of antioxidants in soybean seedlings, as well as some morphological parameters (plant height, shoot and root mass) during abiotic stress. Activity of antioxidant enzymes [superoxide dismutase (SOD; EC 1.15.1.1) and peroxidases (GPX-guaiacol peroxidase and PPX-pyrogallol peroxidase, EC 1.11.1.7)], phenylalanine ammonia lyase (PAL; EC 4.3.1.5), as well as content of non-enzymatic antioxidants (total phenolics, tannins and flavonoids) were analyzed in leaves of soybean seedlings grown in different soil types [mollic vertisol (humic); calcic chernozem (calcic) and haplic fluvisol (calcaric)]. Seedlings grown in fluvisol had significantly higher activity of SOD (7.5-8.6%) and PAL (39.2-43.0%) in leaves, as well as total phenolics and tannins content (8.1-80.7%) in comparison to those grown in chernozem and vertisol. However, soybean grown in chernozem had the most favourable conditions for growth and the highest total flavonoids content (2.3 mg rutin g⁻¹ dry weight). Regardless the difference in response to higher and lower level of soil fertility, soybean seedlings grown in vertisol and fluvisol had similar morphological properties, which showed the great ability of their antioxidant systems to acclimate to change in soil quality.

Key words: *abiotic stress, antioxidants, Glycine max L., soil types*

Original scientific paper / *Originalni naučni rad*

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INTRODUCTION

The coordination between multiple pathways that reside in different cellular compartments is required due to acclimation of plants to changes in their environment, such as water and salt stresses, especially when the cell or the entire plant is exposed to a rapid decrease in water potential, or when additional environmental parameters are involved (Mittler et al., 2006). When different pathways are uncoupled, electrons that have a high-energy state are transferred to molecular oxygen (O_2) to form reactive oxygen species (ROS; Mittler, 2002). ROS, such as 1O_2 , H_2O_2 , O_2^- and $HO\cdot$, are toxic molecules capable of causing oxidative damage to proteins, DNA and lipids (Apel and Hirt, 2004). Antioxidants such as ascorbic acid and glutathione (GSH), as well as ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione peroxidase (GPX) are essential for ROS detoxification (Apel and Hirt, 2004; Mittler et al., 2004; Dietz et al., 2006). Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) is the entry-point enzyme into the phenylpropanoid pathway responsible for synthesis of plant phenylpropanoids or phenolics, many of which play important roles in plant defense and present important non-enzymatic antioxidants (Lawton and Lamb, 1987; Gerasimova et al., 2005; Lattanzio et al., 2006). Polyphenols and flavonoids suppress ROS formation by inhibiting enzymes, chelating trace elements involved in free-radical production, scavenging reactive species and upregulating or protecting antioxidant defenses (Choi et al., 2002; Grassmann et al., 2002).

In the northern Serbia, soybean, *Glycine max* L., represents one of the most frequently grown cultivar. It has been determined that variability of soil properties affects yield of soybean (Kravchenko and Bullock, 2000; Cox et al., 2003; Jiang and Thelen, 2004). The decline in soil moisture also results in a decrease in the diffusion rate of nutrients in the soil to the absorbing root surface (Hu and Schmidhalter, 2005). The purpose of this study was to investigate possible differences in activity of superoxide dismutase, peroxidases, phenylalanine ammonia lyase and contents of polyphenolics in leaves, as well as some morphological parameters of soybean seedlings grown in soils with different soil properties, with a view to assessing the ability of soybean antioxidant system to acclimate to change in soil quality without affecting the development at this stage of growth.

MATERIAL AND METHODS

Soil properties. The experiment was conducted on three different soil types (WRB, 2006): mollic vertisol (humic); calcic chernozem (calcic) and haplic fluvisol (calcaric). Soil samples were taken from chernozem, vertisol and fluvisol with soil probe from the surface horizon (0-30 cm) with three replicates approximately 10 m apart. Soil samples were air dried and sieved through a 2 mm sieve (0.2 mm for organic matter content analysis). Cylinders ($V=100\text{ cm}^3$) was applied to take undisturbed soil samples for measuring bulk density. Soil texture was determined by sieving using the pipette method, with sodium pyrophosphate as a dispersing agent. Soil water retention was measured using a porous plate and pressure membrane apparatus at matric potentials of -33 and -1500 kPa. Soil pH value is determined potentiometrically in a 1:2.5 soil-distilled water (pH- H_2O) and soil-1M KCl solution (pH-KCl). Scheibler apparatus was applied for gas-

volumetrically measuring of CaCO_3 content. Soil organic matter content was determined by a dichromate wet oxidation method.

Plant material. Seeds of soybean (*Glycine max* L.) cultivar Bečejka were obtained from the Institute of Field and Vegetable Crops, Novi Sad (Serbia). Seeds were sown at 10 cm depth. The experimental design was a randomized block with four replications. The sowing was done in an area of 34.5×16.5 m. The length of the experimental unit was 8 m and the width was 3.5 m. Plant material for biochemical and morphological analyses represented soy leaves ($n=40$ plants) sampled at the stage of the second trifoliolate (V2) (Fehr and Cavines, 1977).

Weather conditions of the experimental site. Average monthly air temperatures and precipitation on the experimental field at experimental sites in 2011 were presented in Figure 1. These results point to months of drought in this year.

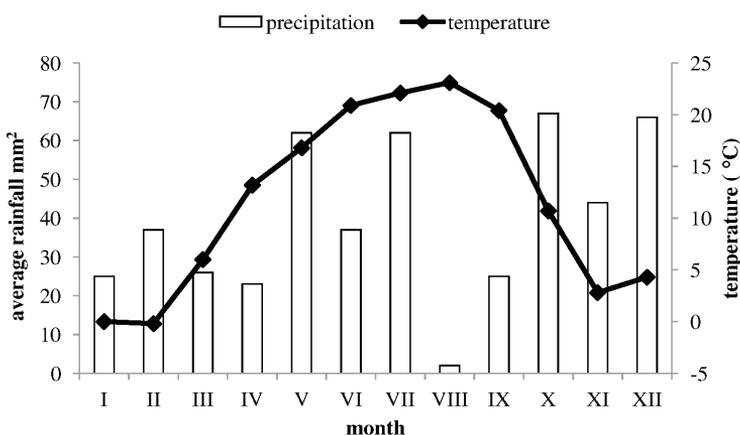


Figure 1. Average monthly air temperatures and precipitation (primary and secondary y-axis, respectively) in the experimental field (86 m above sea level).

Enzyme extracts. Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by monitoring the inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm (Mandal et al. 2008). Peroxidase (EC 1.11.1.7) activity was measured using guaiacol (guaiacol peroxidase; GPX) and pyrogallol (pyrogallol peroxidase; PPX) as substrates according to Morkunas and Gmerek (2007). Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) activity was performed according to Gerasimova et al. (2005). SOD, PPX, GPX, PAL and activities were expressed in U g^{-1} fresh weight (fw).

Polyphenols contents. Total polyphenols and tannins content of acetone extract of dry leaves and roots were determined by the Folin-Ciocalteu procedure (Hagerman et al., 2000) and expressed as $\text{mg catechin g}^{-1}$ dry weight (dw). The amount of flavonoids in methanolic extract of dry leaves and roots was performed by method described by Markham (1989) and expressed as mg rutine g^{-1} dw.

Statistical analyses. Assessed values of the biochemical (determinations made in triplicates) and morphological (measures obtained from 40 plants) analyses were expressed as means \pm standard error and tested by ANOVA followed by comparison of the means by Duncan's multiple range test ($P < 0.05$). Correlation between PAL activity and contents of total polyphenols, tannins and flavonoids was assessed by correlation analyses and expressed by coefficient of correlation (r). Data were analyzed using STATISTICA for Windows version 11.0.

RESULTS AND DISCUSSION

Soybean yield components are highly quantitative traits with low heritability, and influenced by differences arising from soil heterogeneity and environmental factors (Manavalan et al., 2010). Soil types used in this experiment have substantially different characteristics (Table 1).

Table 1. Basic properties of investigated soil types.

Soil type		Chernozem	Vertisol	Fluvisol
Elevation (m)		80	75	80
Location	N	45°20'31"	45°18'07"	45°14'50"
	E	19°51'26"	21°03'32"	19°51'02"
Depth (cm)		0-30	0-30	0-30
Texture (%)	CS	0	1.6	1.8
	FS	36.2	26.7	39.4
	S	30.2	31.6	36.7
	C	33.6	40.1	22.1
OM (%)		2.54	3.66	1.87
CaCO ₃ (%)		0.38	0.33	3.71
pH	KCl	6.01	5.69	7.17
	H ₂ O	7.02	6.48	8.01
N (%)		0.127	0.183	0.094
BD (g cm ⁻³)		1.57	1.22	1.43
Water retention (kPa)	-33	27.5	32.8	25.1
	-1500	15.3	16.3	11.8

(CS: Coarse sand; FS: Fine sand; S: Silt; C: Clay; OM: Organic matter; BD: Bulk density)

Chernozem is considered as moderate humic, slightly calcareous, neutral soil, with coarser texture than vertisol, while fluvisol showed coarser texture than chernozem, low humic content, medium calcareous and alkaline pH reaction. Observed vertisol is a fine-textured, very humic, non-calcareous and acidic soil. Chernozem and vertisol have more favourable soil structure than fluvisol (Ćirić et al., 2012), which provides better conditions for plant growth. Investigated vertisol and chernozem are developed at loess terraces, while fluvisol is formed at Danube river plain. Considering presented properties of soil we can emphasize that vertisol and chernozem has high level of fertility. Fluvisol has lower fertility due the low organic matter content, relatively high pH reaction and unfavourable structure.

Under optimal growth conditions, ROS are mainly produced at a low level in organelles such as chloroplasts, mitochondria and peroxisomes, while during stress, the rate of ROS production is dramatically elevated. ROS accumulation during stress greatly depends on the balance between ROS production and ROS scavenging (Mittler et al., 2004), that in turn depends on changes in growth conditions (light intensity, drought, salt and temperature stress, etc.), as well as the severity and the duration of the stress and the ability of the tissue to rapidly acclimate to the energy imbalance. Superoxide anion ($O_2^{\cdot-}$) is usually rapidly dismutated, either non-enzymatically or via SOD, to relatively stable H_2O_2 , which can produce $\cdot OH$ radical in the presence of divalent metal ions as Fe^{2+} (Delledonne, 2005). Activity of SOD was markedly increased in soybean leaves grown in fluvisol (Figure 2), contrary to that grown in chernozem and vertisol where peroxidases were significantly active (PPX and GPX, respectively). The $\cdot OH$ radical initiates chain reaction, including LP, enzyme inactivation and degradation of nucleic acids (Mehdy, 1994). Furthermore, plant peroxidases trigger conversion of H_2O_2 to water and oxygen, which gives them an important role in plant defense system (Almagro et al., 2009). PAL has been extensively studied in plants because of its decisive function in the biosynthesis of many secondary metabolites (Liu, 2006). Our results showed that PAL activity in leaves of soybean grown in fluvisol was significantly higher comparing to PAL activity in leaves of soybean grown in chernozem and vertisol (Figure 2).

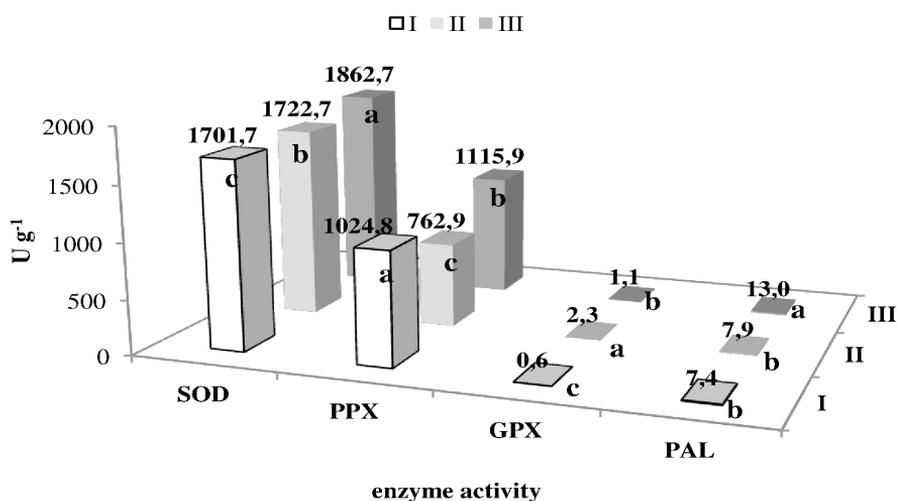


Figure 2. Activity of superoxide dismutase (SOD), peroxidases [pyrogallol (PPX) and guaiacol (GPX)] and phenylalanine ammonia lyase (PAL) in leaves of soybean seedlings grown in chernozem (I), vertisol (II) and fluvisol (III). Values marked with different letter differ significantly at $P < 0.05$ (Duncan's test).

Also, total polyphenolics and tannins content were higher in leaves of soybean seedlings grown in fluvisol and vertisol, in comparison to that grown in chernozem, but positive correlation between PAL activity and polyphenolics content (total polyphenolics $r=0.90$, total tannins $r=0.69$ and total flavonoids $r=0.72$) was determined only in plants grown in

vertisol (Figure 3). However, total flavonoids content assessed in leaves of soybean grown in chernozem, was also in a positive correlation with PAL activity ($r=0.86$) (Figure 3).

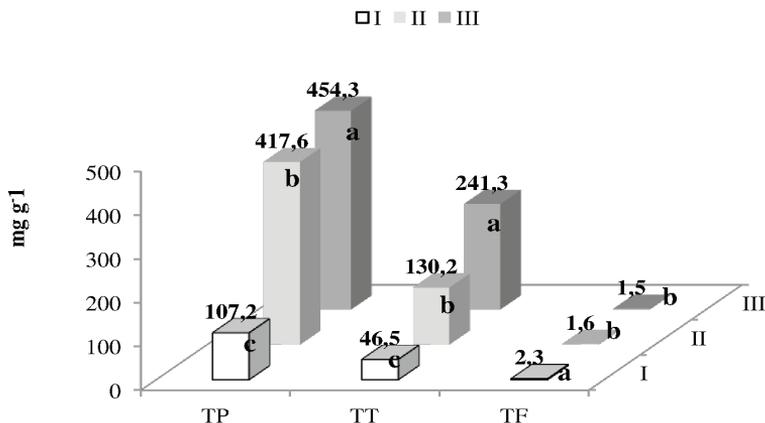


Figure 3. Total polyphenols (TP), tannins (TT) and flavonoids (TF) contents in leaves of soybean seedlings grown in chernozem (I), vertisol (II) and fluvisol (III). Values marked with different letter differ significantly at $P < 0.05$ (Duncan's test).

According to morphological analyses, soybean seedlings grown in chernozem had significantly higher values of shoot and root mass in comparison to those grown in vertisol and fluvisol (Table 2), yet different soil types did not changed the height of these plants (Table 2).

Table 2. Morphological properties of soybean seedlings grown in different soil types. Results represent a mean \pm standard error (SE) and coefficient of variation (CV) ($n=40$). Results marked with different letters differ significantly at $P < 0.05$ (Duncan's test).

	Chernozem			Vertisol			Fluvisol		
	<i>x</i>	SE	CV	<i>x</i>	SE	CV	<i>x</i>	SE	CV
Plant height (cm)	13,4 a	0,4	9,4	12,0 a	0,5	14,7	14,7 a	0,4	9,1
Shoot mass (g)	10,7 a	1,6	49,8	2,4 b	0,1	16,1	2,7 b	0,2	20,2
Root mass (g)	2,0 a	0,2	41,0	0,36 b	0,02	21,1	0,81 b	0,07	28,6

CONCLUSION

Leaves of soybean plants grown in soil with low level of fertility (fluvisol) had enhanced activity of SOD and PAL, while those grown in soil with higher fertility (chernozem and vertisol) had enhanced activity of peroxidases, which could point to different response of soybean antioxidant system to abiotic factors when grown in soils of different quality. Chernozem stimulated accumulation of flavonoids in soybean leaves and development

of soybean plants. Regardless the difference in response to higher and lower levels of soil fertility, soybean seedlings grown in vertisol and fluvisol had similar morphological properties, which showed the great ability of their antioxidant systems to acclimate to change in soil quality.

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ANTIOKSIDANTNE KARAKTERISTIKE KLIJANACA SOJE GAJENIH NA RAZLIČITIM TIPOVIMA ZEMLJIŠTA

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VLADIMIR ĆIRIĆ, MILAN POPOVIĆ

Izvod

Cilj ovog rada bio je da se utvrdi da li akumulacija antioksidanata i promene u antioksidantnoj sposobnosti u klijancima soje utiču na morfološke parametre (visinu biljke i masu nadzemnog i podzemnog dela biljke). Analizirane su promene u aktivnosti antioksidantnih enzima [superoksid-dismutaze (SOD; EC 1.15.1.1) i peroksidaza (GPX-gvajakol i PPX-pirogalol peroksidaze, EC 1.11.1.7), fenilalanin amonijum-lijaze (PAL; EC 4.3.1.5), kao i sadržaju neenzimskih antioksidanata (ukupnih fenola, tanina i flavonoida) u listu klijanaca soje gajene na različitim tipovima zemljišta [ritska crnica, černoze i fluvisol]. U listu klijanaca soje gajenih na fluvisolu zabeležena je značajno veća aktivnost superoksid-dismutaze (7.5-8.6%) i fenilalanin amonijum-lijaze (39.2-43.0%), kao i sadržaj ukupnih fenola i tanina (8.1-80.7%) u odnosu na klijance gajene na černoze i ritskoj crnici. Ipak, klijanci soje gajeni na černoze imali su najbolje uslove za rast i razvoj, kao i najveći sadržaj flavonoida (2.3 mg rutina g⁻¹ suve mase). Pored toga, morfološke karakteristike klijanaca soje gajenih na ritskoj crnici i fluvisolu se nisu značajno razlikovale, što navodi na zaključak na izuzetno izraženu adaptabilnost antioksidantnog sistema ovih biljaka na promene u kvalitetu zemljišta.

Ključne reči: abiotički stres, antioksidanti, *Glycine max* L., tipovi zemljišta

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COMPARISON OF OXIDATIVE STRESS PARAMETERS IN SOYBEAN SEEDLINGS INOCULATED WITH *MACROPHOMINA PHASEOLINA* FROM DIFFERENT ISOLATES*

ĐORĐE MALENČIĆ, BILJANA KIPROVSKI, VERA STOJŠIN,
DRAGANA BUDAKOV, GORANA POGANČEV¹

*SUMMARY: The research was focused on biotic stress caused by *Macrophomina phaseolina* (Tassi) Goid., a plant pathogen that causes charcoal rot by infecting the root and lower stem of soybean. Differences in pathogenicity of fungi cultures from five isolates (two from sugar beet, one from maize, one from soybean and one from sunflower) have been investigated in seedlings of two soybean cultivars (Meli and Balkan). Comparison of antioxidant systems of soybean seedlings showed different reactions of plants infected with *M. phaseolina* isolates of different origin. The highest pathogenicity showed isolate from sugar beet ŠR62/4 (2.80), while no significant difference was found for isolates ŠR55(3)/09 (2.58), MphSo (2.32) and MphSu (2.26). The isolate from maize, MphK, showed lowest pathogenicity (1.94). For the most of the biochemical parameters tested (lipid peroxidation, phenylalanine ammonium-lyase activity, total polyphenols, tannins and proanthocyanidins contents) the isolate from sugar beet (ŠR55(3)/09) induced the most significant reaction in soybean seedlings among all isolates investigated. Presence of this pathogen in the field conditions should be further monitored and the crop rotation should be seriously taken into account.*

Key words: *isolates, *Macrophomina phaseolina*, oxidative stress, pathogenicity, soybean.*

Original scientific paper / *Originalni naučni rad*

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INTRODUCTION

Development and life cycle of plants are affected by various external factors. Among these, two groups can be differentiated – abiotic factors such as the climate, or soil composition, and biotic factors such as influence of various pathogens and predators. A typical feature of resistance in plants is the induction of cell death at the site of attempted attack by the pathogen: the hypersensitive response (HR). This response occurs when a plant can specifically recognize the pathogen, during an incompatible interaction. One of the earliest biochemical changes and resistance responses observed after pathogen recognition is the oxidative burst, that is, the controlled release of superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) at the site of the pathogen ingress. The oxidative burst is thought to be required for several subsequent defense responses and is expressed in most if not all plant species (Malenčić et al., 2010). As a reaction to stress, antioxidant system is activated in plant cells. Antioxidant defence systems have to be part of aerobic metabolism to counteract oxidative damage from ROS. This includes antioxidant enzymes such as superoxide-dismutase (SOD, EC 1.15.1.1), catalase (Cat, EC 1.11.1.6) and different kinds of plant peroxidases, as well as some non-enzymatic antioxidants such as glutathione, tocopherols, carotenoids, ascorbate, flavonoids and other phenolic compounds (Malenčić et al., 2008).

This research focused on biotic stress caused by necrotrophic fungus *Macrophomina phaseolina* (Tassi) Goid., a plant pathogen that causes charcoal rot by infecting the root and lower stem of over 500 plant species. Although being described as typical pest of dry climate, it appears regularly in Serbia. Moreover, due to its ability to cause infection during all phenophases, it is considered as one of the economically most important pests on soybean, maize, sugar beet and sunflower.

The aim of this study was to compare biochemical parameters of oxidative stress induced by different *M. phaseolina* isolates and to examine relationship between the origin of isolates, their pathogenicity and soybean seedlings response to infection.

MATERIALS AND METHODS

In order to compare parameters of oxidative stress, both phytopathological and biochemical tests were applied. Morphological and breeding characteristics, as well as the pathogenicity test, were performed for five *M. phaseolina* isolates of different origin (host species). These included two isolates from sugar beet (ŠR55(3)/09 and ŠR62/4), one from maize (MphK), one from soybean (MphSo) and one from sunflower (MphSu). Seeds of two different soybean cultivars (Meli and Balkan) were obtained from the Institute of Field and Vegetable crops, from Novi Sad, Serbia. Seeds were inoculated using the method of artificial inoculation on filter paper in Petri dishes.

Seven-day-old seedlings were collected for the biochemical assays. One g of fresh plant material was homogenized with 10 ml 0.1 M K_2HPO_4 at pH 7.0. After centrifugation for 10 min at 4°C and 15 000 g the supernatant was used for enzyme activity measurements.

Superoxide-dismutase (SOD; EC 1.15.1.1) activity was measured by monitoring the inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm, according to method by Panda (2012), and expressed as $U\ g^{-1}$ fresh weight. Phenylalanine-ammonium lyase

(EC 4.3.1.5) activity was determined according to Gerasimova et al. (2005) and expressed in U g^{-1} fresh material (fr.m.). The lipid peroxidation intensity (LP) was measured as malondialdehyde (MDA) production at 532 nm with thiobarbituric acid (TBA). The total amount of TBARS (TBA-reactive substance) is given as nmol MDA g^{-1} fresh weight, as described by Moon and Shibamoto (2009).

Total phenols were extracted and determined from air-dried and pulverized plant material. Dry extracts have been made by mixing 0.2 g of previously dried and minced seedlings with 70% acetone (1/50, w/v). Their content was determined spectrophotometrically at 720 nm after reaction with Folin-Ciocalteu reagent using (+)-catechine for the standard curve. The total amount of soluble phenols was expressed as $\text{mg catechine g}^{-1}$ dry weight (d.w.) (Hagerman et al., 2000). Total tannins were determined by method of Makkar (2003), with addition of polyvinylpyrrolidone (PVPP), and the results are expressed as $\text{mg catechine g}^{-1}$ dry weight. Proanthocyanidins were determined according to the same method and expressed in $\text{mg leucoanthocyanidin g}^{-1}$ dry weight.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) can produce stable free radicals in ethanolic (EtOH) solution. Due to its odd electron, DPPH strongly absorbs at 517 nm. In order to evaluate the antioxidant potency through free radical scavenging by test samples, the change of optical density between test samples containing plant extracts and blank (without plant extracts) was assessed to evaluate the antioxidant potential of test samples (Lee et al., 1998).

Results were expressed as mean \pm standard error of mean (SEM) (the average of three experiments) and tested by ANOVA followed by comparison of the means by Duncan's multiple range test ($P < 0.05$). Data were analyzed using STATISTICA for Windows version 11.0.

RESULTS AND DISCUSSION

The highest pathogenicity in cultivar Meli showed isolate ŠR62/4 (2.80). Pathogenicity of ŠR 55(3)/09 (2.58), MphSo (2.32) and MphSu (2.26) isolates showed no significant difference. The isolate MphK showed statistically lowest pathogenicity (1.94). Rather similarly, the isolate ŠR62/4 proved to have the highest pathogenicity in cultivar Balkan, as well (2.47). Pathogenicity of ŠR55(3)/09 (2.31), MphSu (2.28), MphSo (2.24) and MphK (2.03) isolates did not differ in Balkan cultivar.

In addition to this, comparison of biochemical parameters showed differences between cultivars (Figure 1). Results obtained for intensity of lipid peroxidation (LP) proved lower MDA concentration in Meli cultivar. Control plants (uninfected plants) of cultivar Meli had higher values of MDA content than infected ones, however, Balkan plants infected with ŠR55(3)/09 and MphK isolates had MDA values higher than the control. These high concentrations indicate plants' inability to protect its cell membranes from ROS damage.

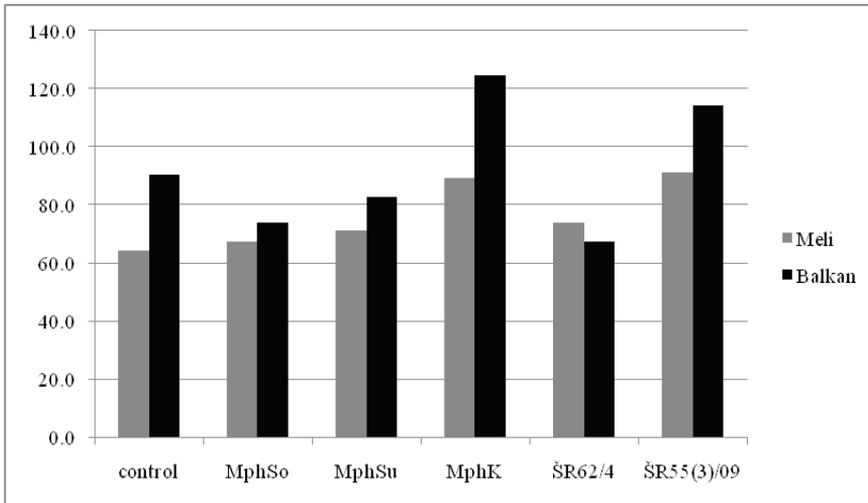


Figure 1. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on lipid peroxidation (nmol MDA g⁻¹ fr.w.)

The results for the superoxide-dismutase (SOD) activity showed no significant differences between control and infected plants after pathogen invasion (Figure 2). Isolate MphSo from soybean showed invariable values for SOD activity in infected and uninfected plants of both investigated cultivars, while isolate MphSu from sunflower caused an increase in SOD activity in cultivar Meli.

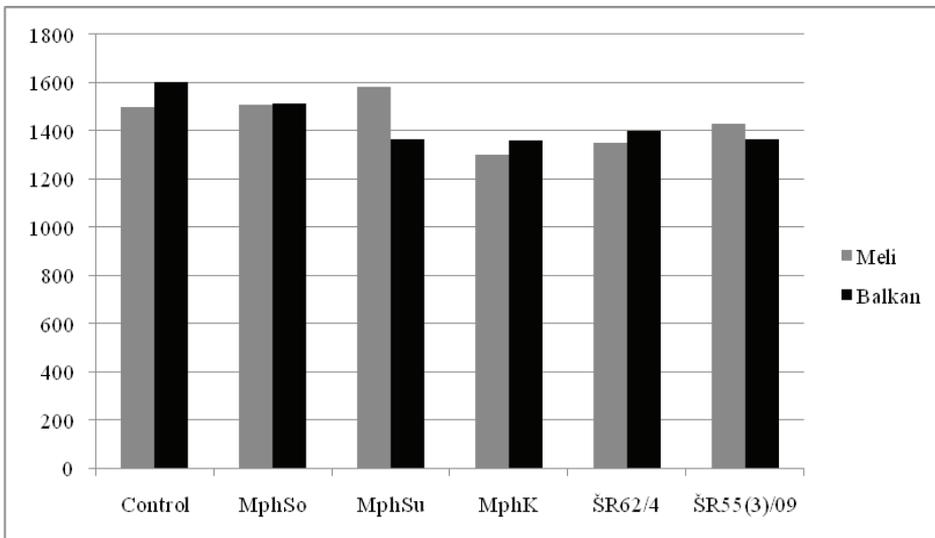


Figure 2. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on superoxide-dismutase activity (U g⁻¹ fr.w.)

Since one of the first responses observed in plants after pathogen recognition is the oxidative burst, it has been expected for infected plants to produce large amounts of O_2^- at the site of the attack. SOD is an inducible enzyme and its activity usually correlates with the O_2^- quantity. These results are not in agreement with our previous results which proved increase in SOD values after inoculation with *Sclerotinia sclerotiorum* as a response to biotic stress (Malenčić et al., 2010). Still, the scavenging system against reactive oxygen species seems to be less effective in soybean, and the activity of SOD is not enough to prevent oxidative damage, which is in agreement with some other findings (Malenčić et al., 2008; Baisak et al., 1994).

Similarly to our previous experiments on impact of necrotrophic fungi on phenylalanine ammonium-lyase (PAL) activity (Kiprovski et al., 2012), seedlings of Balkan cultivar infected with isolates MphSu, MphK and ŠR 55(3)/09, had markedly increased PAL activity in comparison to uninfected plants. The higher activity of the enzyme was detected in plants inoculated with isolates of lower pathogenicity. Contrary to that, all infected plants of cultivar Meli had decreased PAL activity in comparison to control (Figure 3).

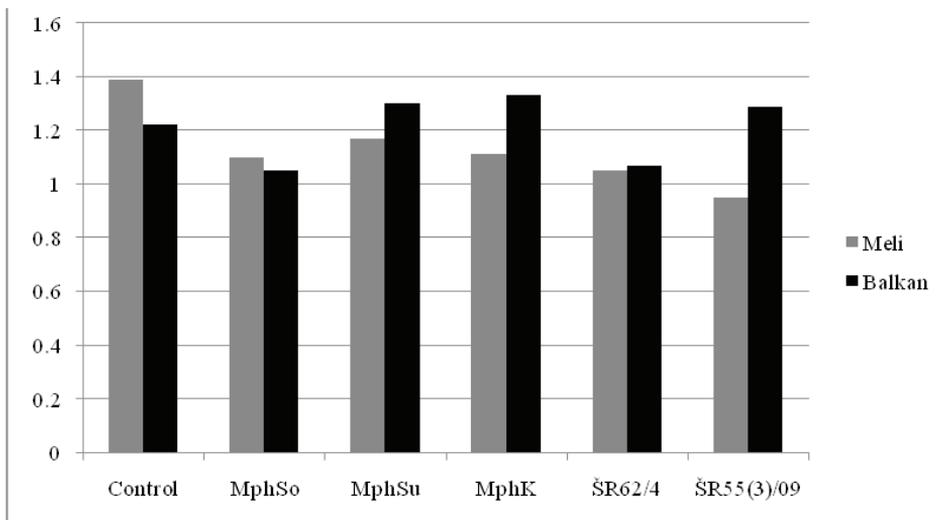


Figure 3. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on phenylalanine ammonium-lyase activity ($U\ g^{-1}\ fr.m.$)

It seems that both enzymes assayed, SOD and PAL, were heavily affected with the pathogen infection and similarly to some other diseases, such as *Sclerotinia sclerotiorum* or *Rhizoctonia solani*, soybean plants have no mechanisms to cope with the *Macrophomina phaseolina*, either. Plant specimens were harvested when infection occurred on 50% of treated plants, and it seems now that timing was too prolonged, i.e. the seedlings were already too affected. For this reason, we suggest in the future, in enzymatic studies, that seedlings/young plants of intolerant plant species should be collected earlier, not later than period when 30% of samples are showing full symptoms of the disease.

Taking into account that polyphenols play important role in plant immune system,

and results from previous research (Boudet et al. 1998; Kiprovski et al., 2014) we also focused on different polyphenol classes present in the plant tissues. The first parameter, describing total polyphenol content, showed effect of accumulation of these compounds in both cultivars investigated, with all isolates applied. Especially cv. Meli showed an increase in phenolics accumulation, ranging from 100% (for isolate MphSo) to 150% (for isolate ŠR55(3)/09. In cv. Balkan, same as in cv. Meli, the isolate ŠR 55(3)/09 induced the most pronounced plant response (Figure 4).

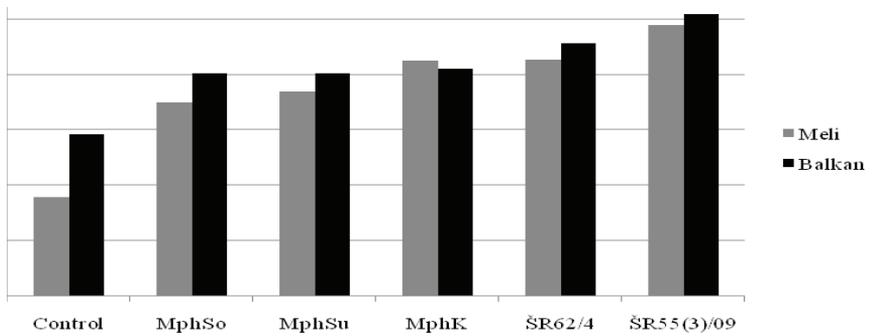


Figure 4. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on total polyphenols content (mg catechine g⁻¹ d.w.)

Total tannin content proved to be significantly higher in infected plants compared to control which was expected due to higher polyphenols content (Figure 5). Tannins comprise large amount of total phenols in plant cell and are known as good reactive oxygen scavengers. Thus, when attacked, plants produce additional tannins as a response to pathogen presence. Similarly to results for total polyphenols, the isolate ŠR55(3)/09 caused the highest tannin accumulation in both cultivars. The isolate with the lowest tannin accumulation induction, in both cultivars, was the isolate MphSu.

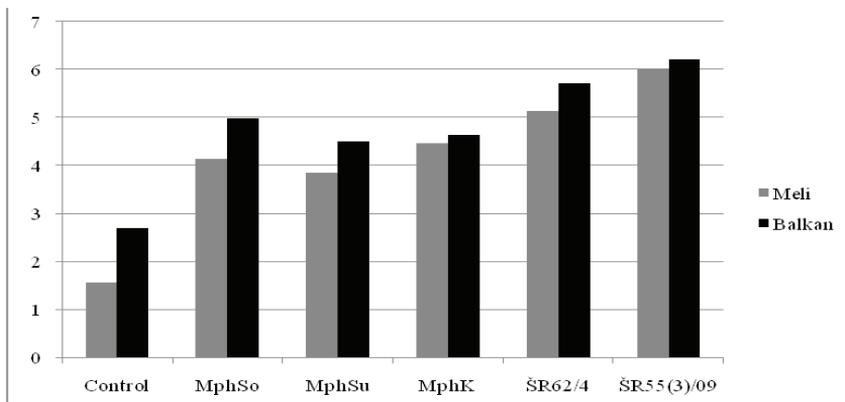


Figure 5. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on total tannins content (mg catechine g⁻¹ d.w.)

Proanthocyanidins content showed statistically significant differences between the cultivars. The constitutive level of these compounds in cv. Balkan was more than two-fold higher compared to cv. Meli. Still, cv. Meli responded to pathogen attack in uniform manner, i.e. all isolates used in the experiment provoked proanthocyanidins accumulation significantly higher than in uninfected plants. In cv. Balkan only isolates MphSo and ŠR55(3)/09 triggered elevated biosynthesis of these valuable antioxidant substances. Once again, isolate ŠR55(3)/09 induced the highest accumulation of proanthocyanidins in both cultivars. Comparison of proanthocyanidin contents among cultivars is presented in Figure 6.

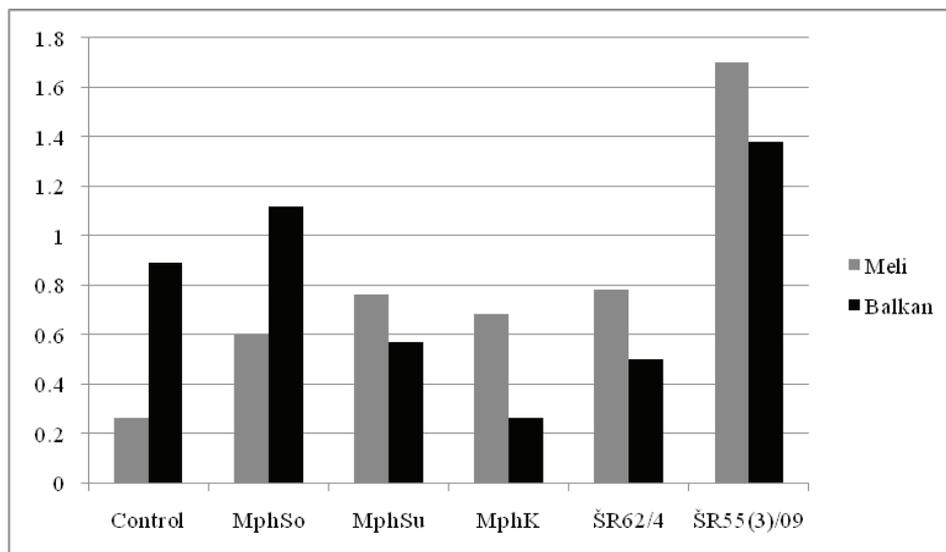


Figure 6. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on proanthocyanidins content (mg leucoanthocyanidin g⁻¹ d.w.)

DPPH radical scavenging activity is a measure of non-enzymatic antioxidant activity. Higher levels of DPPH activity have been correlated with tolerance to different stress conditions (Kang and Saltveit, 2002), but they also point out to the presence of biologically active biomolecules with pronounced antioxidant activity. In both cultivars the results showed that isolates MphSu, MphK and ŠR55(3)/09 had higher DPPH-scavenging values than the control and are in agreement with the results obtained for total polyphenols, tannin and proanthocyanidins contents. Lower values compared to uninfected plants obtained for isolates MphSo and ŠR62/4 imply that the infected plant lack natural plants antioxidants in concentration high enough to neutralize DPPH-free radicals. Results are expressed in % of neutralized DPPH-free radicals and presented in Figure 7.

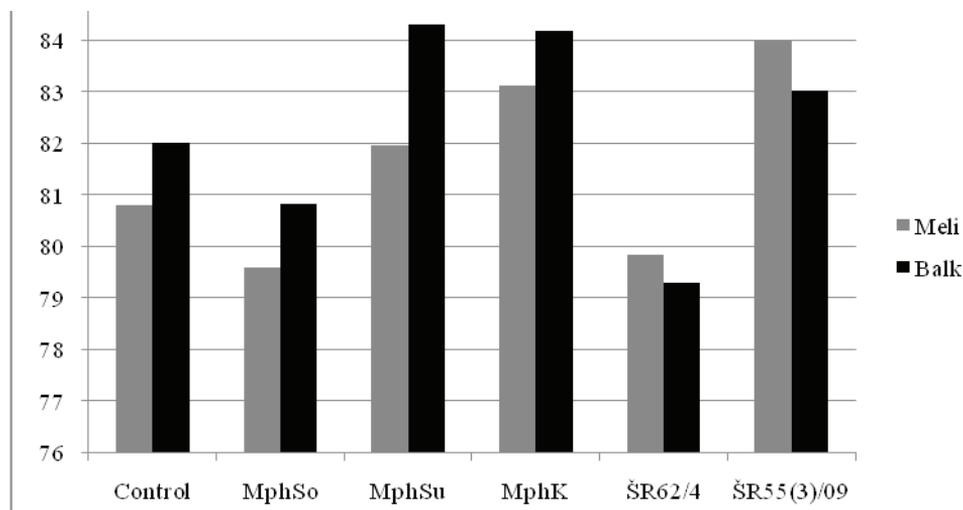


Figure 7. Effect of inoculation of soybean seedlings with *M. phaseolina* isolates on DPPH-radical scavenging activity (%)

CONCLUSION

Comparison of antioxidant systems in soybean plants showed different reactions between plants infected with *Macrophomina phaseolina* isolates of different origin. Overall pathogenicity was proven to be rather similar in both cultivars - Meli (2.07) and Balkan (2.00). Isolates provoked an intense reaction in infected plants, compared to each other; yet the highest pathogenicity showed isolate from sugar beet ŠR62/4 (2.80). Pathogenicity of ŠR55(3)/09 (2.58), MphSo (2.32) and MphSu (2.26) did not differ significantly in both investigated cultivars. The isolate from maize, MphK, showed lowest pathogenicity (1.94).

In Meli cultivar all of the isolates have induced increased lipid peroxidation intensity, whereas in Balkan cultivar the increase in MDA concentration was only recorded in plants infected with MphK and ŠR55(3)/09 isolates. Regarding activity of SOD it seems that scavenging system against reactive oxygen species was not effective in soybean seedlings, and the activity of SOD could not prevent oxidative damage. After inoculation with isolates of lower pathogenicity (MphSu, MphK and ŠR55(3)/09), PAL activity in cultivar Balkan increased, while in cultivar Meli the activity of this enzyme was invariable. Pathogen presence caused accumulation of plant total polyphenols and tannins in both cultivars. Accumulation of proanthocyanidins was the highest in both infected cultivars when isolate ŠR55(3)/09 was applied. Total non-enzymatic antioxidant capacity, presented as DPPH-test, was high in both cultivars, being around 80% of neutralized radicals. Overall, the *M. phaseolina* isolate coming from sugar beet - ŠR55(3)/09 showed the most significant changes for the most of the parameters tested in both cultivars and could be considered as the most detrimental to soybean cultivars. Its presence in the field conditions should be further monitored and the crop rotation should be seriously taken into account.

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**POREĐENJE PARAMETARA OKSIDATIVNOG STRESA U KLIJANCIMA
SOJE INOKULISANIM SA RAZLIČITIM IZOLATIMA *MACROPHOMINA
PHASEOLINA***

ĐORĐE MALENČIĆ, BILJANA KIPROVSKI, VERA STOJŠIN,
DRAGANA BUDAKOV, GORANA POGANČEV

Izvod

U radu je ispitivan biotički stres u klijancima soje izazvan gljivom *Macrophomina phaseolina* (Tassi Goid.), patogenom biljaka i uzročnikom ugljenaste truleži korena i donjeg dela stabla. Različite karakteristike izolata gljive (dobijenih sa šećerne repe (2), kukuruza (1), soje (1) i suncokreta (1)), ispitane su u dve sorte soje (Meli i Balkan). Poređenje antioksidantnih sistema u biljkama soje ukazalo je na različite odgovore između sorti soje inficiranih izolatima *M. phaseolina* različitog porekla. Izolati su izazvali intenzivnu reakciju u inficiranim klijancima: najveća patogenost zabeležena je kod izolata sa šećerne repe ŠR62/4 (2.80). Izolati ŠR55(3)/09 (2.58), MphSo (2.32) i MphSu (2.26) nisu pokazali statistički značajnu razliku u patogenosti. Izolat sa kukuruza, MphK, pokazao je najnižu patogenost (1.94). Za većinu ispitivanih biohemijskih parametara (lipidna peroksidacija, aktivnost fenilalanin amonijum-liaze, sadržaji ukupnih polifenola, tanina i proantocijanidina), izolat ŠR55(3)/09 sa šećerne repe, izazvao je najintenzivnije i najznačajnije reakcije u klijancima soje u poređenju sa svim ostalim ispitanim izolatima. Njegovo prisustvo u poljskim uslovima se mora podvrgnuti monitoringu, a o rotaciji useva na takvim oranicama se mora povesti više pažnje.

Ključne reči: izolati, *Macrophomina phaseolina*, oksidativni stres, patogenost, soja.

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TECHNOLOGICAL QUALITY OF DOMESTIC CABBAGE (*Brassica oleracea* var. *capitata* L.) POPULATIONS AND VARIETIES FROM THE VOJVODINA PROVINCE-SERBIA*

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ANAMARIJA STOJANOVIĆ, ALEKSANDRA SAVIĆ¹

SUMMARY: The research treated in this paper dealt with cabbage varieties and populations from the Vojvodina Province, Serbia. Those were two varieties, NS-Futoški and Orion, and two local populations, Čuruški and Deronjski. The common characteristics of these cabbages are late maturation, fresh use in the fall and the pickling of whole heads and sauerkraut. The tender leaves in the head make these cabbages suitable for the uses mentioned above. Since cabbage pickling is practiced widely in Serbia, our aim was to investigate the chemical composition of the local cabbages, i.e., the contents of total sugars, dry matter and minerals. It was found that the sugar content in the heads ranged from 5.27% (NS-Futoški) to 6.33% (Orion). The dry matter content ranged from 8.07% (NS-Futoški) to 11.03% (Orion). The contents of minerals ranged as follows: Magnesium – from 88.7 mg/kg (NS-Futoški) to 143 mg/kg (Orion), Potassium – from 3003mg/kg (Orion) to 3772 mg/kg (Čuruški), Calcium – from 417.1 mg/kg (Deronjski) to 519.9 mg/kg (Orion), and Sodium – from 152.7 mg/kg (Čuruški) to 210.9 mg/kg (Orion).

Keywords: pickling, sugar content, variability, cabbage.

INTRODUCTION

In the previous period two cabbage varieties were recognized: NS-Futoski, and Orion, recognized 2004 and 2007, respectively. The stated varieties in the region of Vojvodina are traditionally most often used for fresh consumption in autumn, but also for the pickling of whole heads and as sauerkraut. Besides, two local cabbage populations, Čuruški and Deronjski, from the same region have similar purpose, (Fig. 1).

Original scientific paper / *Originalni naučni rad*

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The tradition of growing cultivars and populations is primarily due to their intended use, which is fresh consumption, and sauerkraut making as well. In this way, local OP populations of cabbage or cultivars are specific to a particular cabbage-growing area (Červenski et al., 2011). Cabbage and cabbage products are interesting from both marketing and dietary points of view because cabbage has many beneficial effects on health. From a traditional point of view, cabbage in the form of sauerkraut is one of the best known traditional foods (Jevsnik et al., 2009). As in other Eastern European areas (Luczaj et al., 2007; Pieroni, 2008), in Vojvodina, most vegetables are preserved for consumption during the winter via lacto-fermentation. Cucumbers, cabbages, tomatoes, turnips, and sometimes egg plants are harvested, stored in barrels of salt water, and left to ferment. On the other hand, the people of Vojvodina have retained a few traditions surrounding their use of local plant resources. Although, the available local OP populations of cabbage are suitable for fresh consumption and sauerkraut making, local growers prefer to grow foreign cultivars (Červenski et al., 2010).

In the region of Vojvodina, cabbage is traditionally consumed in the autumn and winter mostly as sauerkraut, cabbage rolls and grated fresh cabbage. Locals in their households make many more other cabbage dishes. Therefore, local varieties and populations have an advantage over hybrids in the region, which makes it more attractive to consumers from the sensory point of view.

Geographic altitude, climate, relief and soil characteristics, classify Serbia among growing regions capable of continuous provision the market with quality goods during the whole season (Đilas et al., 2011).

Since cabbage pickling is practised widely in our country, aim of the study was to investigate the local cabbage varieties and populations chemical composition like total sugar content, dry matter content, and minerals content. In this way it would be possible to demonstrate good quality of the local cabbage varieties and populations for purposes of pickling and fermentation in the region of Vojvodina Province, Serbia.

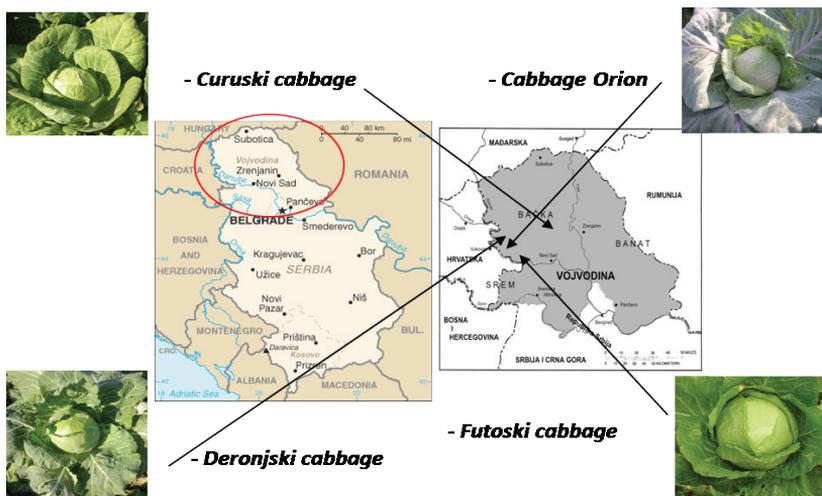


Figure 1. Local populations of white cabbage localize and collect in previous period (Červenski, et.al.2011)

MATERIALS AND METHODS

The studied material consisted of 2 local OP populations of cabbage: Čuruški cabbage and Deronjski cabbage and two varieties: NS-Futoški and Orion, which are suitable for fresh use late in the season as well as for pickling and which have a history of being grown in the country for more than 30 years now, (places are shown in Fig. 1).

Trials were carried out on chernozem at the Rimski Šancevi Experiment Field of the Institute of Field and Vegetable Crops in Novi Sad, Serbia, 45°19'N latitude and 19°50'E longitude, at altitude 79 m above mean sea level. The horizons in which most of the root system of cabbage develops are neutral in reaction and slightly calcareous. The soil has a medium supply of total nitrogen, an optimum supply of readily available phosphorus, and a high readily available potassium content (Vasin et al., 2002).

The trial was carried out over a one-year period (2011) using a randomized block design with three replications. Before the planting, the plots were fertilized prior to primary tillage using NPK (8:16:24) at 600 kg per hectare. During the season, the crops were top-dressed on two occasions, and they were protected from diseases and pests as well. After the transplantation, irrigation was applied. The crops were also irrigated in the course of the growing season on several occasions depending on the soil moisture status and plant water requirements. The time and degree of head maturity were determined based on visual observation and head compactness. Thirty plants were analysed per replicate and genotype.

We selected 50 cabbage heads of satisfactory firmness, hardness and uniform appearance from each cabbage variety and population. In the laboratory of the Institute of Food Technology in Novi Sad, percentage of dry matter, percentage of soluble dry matter (with refractometer) (Bylaw of taking samples 29/83), and total sugar content (using method developed by Luff Schoorl) in raw material were determined.

Chemical analysis of cabbage heads miner content was performed according to domestic legislations quality parameters of taking samples methods and chemical and physical analysis for quality control of fruit and vegetable products (Bylaw of taking samples 29/83).

RESULTS AND DISCUSSION

Brassica spp. have different edible portions that are sometimes separated for consumption. Water is the main component of *Brassica* spp. Water content varied between 76.09% and 92.5% in the samples studied. The sugar content is a basic parameter used in evaluating quality attributes of vegetables. Total soluble solids (TSS) can be used as an estimate of sugar content, (Martinez et al.,2010).

Winter cabbage varieties containing higher soluble dry matter and sugar content in heads are suitable for lactic acid fermentation. *Leuconostoc mesenteroides* is a bacterium associated with the sauerkraut and pickle fermentations. Sugar, added salt and temperature between 18 to 22 °C initiate growth of this organism in vegetables (4). It produces carbon dioxide that replaces the oxygen, making the environment anaerobic and suitable for the growth of subsequent species of *Lactobacillus*. In Vojvodina, varieties and populations NS-Futoški, Orion, and Deronjski Čuruški are cabbages most often used for pickling in the fall and early winter. Total sugar content in the heads of the examined varieties and

populations ranged from 5.27% to 6.33% in cv. NS Futoški and cv. Orion, respectively (Table 1). High sugar content makes those cabbages excellent for the purposes of fermentation and pickling.

Table 1. Results of chemical quality parameters of analysed cabbages

Parameters	Cabbages varieties and populations			
	NS-Futoški	Orion	Čuruški	Deronjski
Dry matter (%)	8,07	11,03	10,66	10,52
Soluble dry matter(%)	6,14	8,32	7,98	8,04
Total sugar content(%)*	5,27	6,33	6,11	6,13
Magnesium-Mg (mg/kg)	88,75	143,00	97,15	116,79
Potassium-K (mg/kg)	3500	3003	3772	3201
Calcium-Ca (mg/kg)	516,79	519,56	427,89	417,12
Sodium-Na (mg/kg)	165,52	210,92	152,76	157,89

*B y Luff-Schoolr method.

In the present study, dry matter content ranged from 8.07% to 11.03% and soluble dry matter content ranged from 6.14% to 8.32% in cv. NS Futoški and cv. Orion, respectively (Table 1). Dobričević et al. (2006) obtained results similar to ours.

Dobričević et al. (2006) reported that dry matter content in fresh cabbage heads ranged from 6.63 to 8.74%. These results are 2-3% less than the results we obtained. According to Martinez et al. (2010), variation in the composition of the plants is caused by many factors such as variety, growth conditions, time of harvest and maturity at harvest, post-harvest storage conditions and industrial processes. Hence, differences between our and the results obtained by Dobričević et al. (2006), could reasonably be attributed to different tissue strength and structure of cabbage varieties that were used in those two studies, as well as different growing conditions.

Chemical and sensory quality of raw material are affected by characteristics of production areas and climates. The variability reflects in the sustainability and quality of the final product. The dry matter content vary among years due to weather conditions and it is higher when there is less rainy days during the ripening and the harvest (Dobričević et al., 2006).

The study of Anunciação et al. (2010) involved 55 samples of cabbage, being 31 of the white species and 24 of the red species. The results expressed as milligrams of element per kilogram of sample demonstrated that the concentration ranges varied from 1603 to 4068 for potassium, 221,9 to 744,7 for calcium, 67,2 to 286 for magnesium, 27,2 to 591 for sodium. We obtained similar results. Mineral content in the heads of the examined varieties and populations ranged from 88.7 mg Mg/ kg to 143 mg Mg/kg in cv. NS-Futoški and Orion, respectively; 3003 mg K/kg to 3772 mg K/kg in cv. Orion

and cv. Čuruški, respectively; 417.1 mg Ca/ kg to 519.9 mg Ca/ kg in cv. Deronjski and cv. Orion, respectively; and 152.7 mg Na/ kg to 210.9 mg Na/ kg in cv. Čuruški and cv. Orion, respectively (Table 1).

Zahradnik et al. (2007), studied the effect of alternative organic fertilizers on the yield and nutritional value of cabbage. The authors reported that cabbage heads contained: 1865 to 1978 mg / kg of Potassium, 111 to 153 mg / kg of Magnesium, 222 to 276 mg / kg of Calcium, and 97 to 187 mg / kg of Sodium. Cabbage populations have been improved by farmers through mass selection for centuries. Their cultivation as a percentage of the entire cultivated area for cabbage is reducing. Compared with commercial hybrids, the local populations of cabbage are less productive and their heads lack uniformity and field durability, but they have thinner head leaves that are crisper and juicier. The type of use indicates that these are cabbages with thinner and juicier leaves, which predisposes their heads for fine rating and also makes their leaves readily bendable and easy to roll up when pickled. It is characteristics like those that give the local populations an advantage over hybrids (Koutsos et al., 2001).

CONCLUSION

In conclusion, fresh cabbage from the production area of Vojvodina is a good raw material for fresh use and biological fermentation. The climatic and pedological characteristics of the typical and traditional production area favor the production of late cabbage cultivars, suitable for this purpose. Cabbage populations have been improved by farmers through mass selection for centuries. Their cultivation as a percentage of the entire cultivated area for cabbage is reducing. Compared with commercial hybrids, the local populations of cabbage are less productive and their heads lack uniformity and field durability, but they have thinner head leaves that are crisper and juicier. The type of use indicates that these are cabbages with thinner and juicier leaves, which predisposes their heads for fine rating and also makes their leaves readily bendable and easy to roll up when pickled. It is characteristics like those that give the local populations an advantage over hybrids.

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TEHNOLOŠKI KVALITET DOMAĆIH SORATA I POPULACIJA KUPUSA (*Brassica oleracea* var. *capitata* L.) IZ AP VOJVODINE U SRBIJI

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ANAMARIJA STOJANOVIĆ, ALEKSANDRA SAVIĆ

Izvod

Istraživanja ovog rada su obuhvatila domaće sorte i populacije kupusa poreklom iz Autonomne pokrajine Vojvodine, u Srbiji. Dve domaće sorte su bile: NS-Futoški i Orion, a dve lokalne populacije su bile: Čuruški i Deronjski. Glavne i zajedničke karakteristike navedenih kupusa su: kasni način proizvodnje, sveža potrošnja te kišljenje celih glavicica ili ribanca. Upravo nežni listovi glavicice ovih kupusa doprinose navedenoj upotrebi. Pošto je kišljenje kupusa u Srbiji tradicija, cilj nam je bio da ispitamo hemijsku strukturu navedenih kupusa i to pre svega: sadržaj ukupnih šećera, suve materije i sadržaj mineralnih materija. Istraživanjem su dobijeni sledeći rezultati: sadržaj šećera u glavicama se kretao od 27% (NS-Futoški) do 6.33% (Orion). Sadržaj suve materije se kretao od 8.07% (NS-Futoški) to 11.03% (Orion). Sadržaj mineralnih materija je bio sledeći: Magnezijuma od 88.7 mg/kg (NS-Futoški) do 143 mg/kg (Orion), Kalijuma od 3003mg/kg (Orion) do 3772 mg/kg (Čuruški), Kalcijuma od 417.1 mg/kg (Deronjski) do 519.9 mg/kg (Orion) i Natrijuma od 152.7 mg/kg (Čuruški) do 210.9 mg/kg (Orion).

Ključne reči: kišljenje, sadržaj šećera, varijabilnost, kupus.

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EVALUATION OF SOME HAEMATOLOGICAL PARAMETERS IN PORCINE BLOOD AFTER EXPOSURE OF PATULIN *IN VITRO**

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SUMMARY: Patulin, a genotoxic mycotoxin produced by several species of Aspergillus, Penicillium and Byssochlamys is the most common mycotoxin in apples and apple-derived products. Patulin may be neurotoxic, immunotoxic, immunosuppressive, genotoxic, teratogenic and carcinogenic. The aim of the present study was to investigate the effect of patulin on chosen haematological parameters (red blood cells - RBC, white blood cells - WBC, platelets - PLT, haematocrit - HCT, haemoglobin - HGB) in porcine blood in vitro. Samples with patulin were incubated with patulin: 10 ng.ml⁻¹ in E1 group, 100 ng.ml⁻¹ in E2 group, and 1000 ng.ml⁻¹ in E3 group for 4 hours at 37°C. The group without any addition served as the control. Patulin caused significant decrease of WBC in all experimental groups when compared to the control. In case of RBC patulin significantly decreased the number of RBC in porcine blood followed by increase of hemolysis and decrease of HGB and HCT in all experimental groups when compared with the control group. The count of PLT was significantly decreased in all experimental groups against the control. Results of this study provide a foundation for further analysis and researches on mycotoxins impact on living cells and the system of possible protection against its effects as well as evaluation of various dose dependencies on haematological parameters.

Keywords: patulin, porcine blood, haematology.

Original scientific paper / *Originalni naučni rad*

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INTRODUCTION

Mycotoxins are secondary metabolites of molds that have adverse effects on humans, animals, and crops that result in illnesses and economic losses (Zain, 2011). Contamination of food and agricultural commodities by various types of toxigenic molds (fungi) is a serious and widely neglected problem (Bhat *et al.*, 2010). This contamination presented a hazard to human and animal health for decades (Gbore and Akele, 2010). Patulin, a genotoxic mycotoxin produced by several species of *Aspergillus*, *Penicillium* and *Byssochlamys* (Puel *et al.*, 2010; Ozsoy *et al.*, 2008) is the most common mycotoxin in apples and apple-derived products (Puel *et al.*, 2010). Patulin may be neurotoxic, immunotoxic, immunosuppressive, genotoxic, teratogenic and carcinogenic (González-Osnaya *et al.*, 2007). *In vivo* patulin caused severe damage in several organ systems like kidney, intestinal tissue (McKinley *et al.*, 1982; Speijers *et al.*, 1988) and immune system (Escoula *et al.*, 1988), if applied at a range between 2.5 and 41 mg.kg⁻¹ bw. Patulin has a strong affinity for sulfhydryl groups. Patulin adducts formed with cysteine are less toxic than the unmodified compound in acute toxicity, teratogenicity, and mutagenicity studies. Its affinity for SH-groups explains its inhibition of many enzymes (Puel *et al.*, 2010). Based on the provisional maximum tolerable daily intake (400 ng.kg⁻¹ bw/day), several countries have set legislations for the maximum amount of patulin in apples products. In the European Union, the limit is set to 50 µg.kg⁻¹ in apple juice and cider, 25 µg.kg⁻¹ in solid apple products (*e.g.* apple sauce) and 10 µg.kg⁻¹ in products for infants and young children (Commission of the European Communities, 2003). The aim of this study was to analyse the effect of patulin on haematological parameters of porcine blood *in vitro*.

MATERIAL AND METHODS

Animals and experimental design in vitro. Slovakian White gilts (n=24) at the age of 100-120 days were kept under standard conditions at the Experimental Station of the Animal Production Research Centre Nitra. Conditions of their care, manipulations and use corresponded to the instruction of EC no. 178/2002 and related EC documents, and they were approved by local ethics commission. Animals were slaughtered and blood samples were obtained.

Blood sampling and patulin treatment. Blood was collected into EDTA-treated tubes. Patulin (Sigma Aldrich, Saint Louis, USA) was added to blood samples at doses 10, 100 and 1000 ng.ml⁻¹ (Table 1). The blood samples without addition of patulin served as control group (C). The blood was incubated for 4 hours at 37°C.

Table 1: Application of patulin in to blood *in vitro*

Group	Patulin (ng.ml ⁻¹)
C	0
E1	10
E2	100
E3	1000

n=5 in each group; C- control group, E₁ - E₃ – experimental groups with various doses of patulin

Analysis of parameters. Haematological parameters (red blood cells - RBC, white blood cells - WBC, platelets - PLT, haemoglobin - HGB, haematocrit - HCT) were measured using haematology analyzer Abacus junior VET (Diatron®, Vienna, Austria).

Statistical analysis. Sigma Plot 11.0 (Jandel, Corte Madera, USA) was used to conduct statistical analyses. One-way ANOVA was used to calculate basic statistic characteristics and to determine significant differences among the experimental and the control groups. Data presented are given as mean and standard deviation (SD). Differences were compared for statistical significance at the level $P < 0.05$.

RESULTS

In this study selected haematological parameters were measured in blood samples after exposure of patulin *in vitro* for 4 hours at 37 °C. The results are shown in Figures 1-5. WBC count (Fig. 1) was significantly decreased ($P < 0.05$) in all experimental groups when compared with the control group. We did not observed any significant differences ($P > 0.05$) among the experimental groups. The values of WBC were similar in all experimental groups.

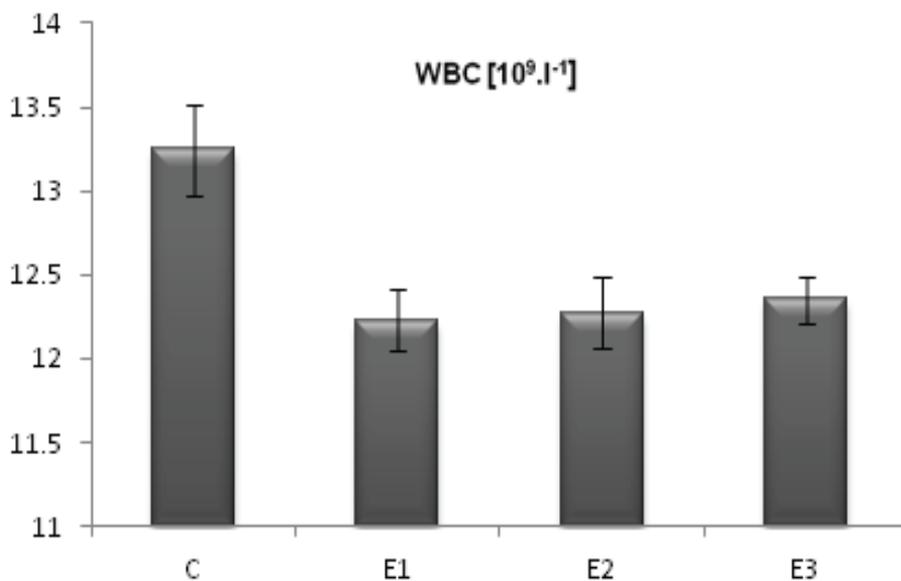


Figure 1. The effect of patulin on WBC in porcine blood *in vitro*
C - control, E1 – 10 ng.ml⁻¹, E2 100 ng.ml⁻¹, E3 - 1000 ng.ml⁻¹, WBC – white blood cells count, a,b – means significant differences ($P < 0.05$), one-way ANOVA

In case of RBC count (Fig 2), value of HGB (Fig 3) and HCT (Fig 4) we observed significant decrease ($P < 0.05$) of these parameters in all experimental groups when compared to the control. The lowest value of this three parameters were observed in E2 experimental group (100 ng.ml⁻¹ of patulin).

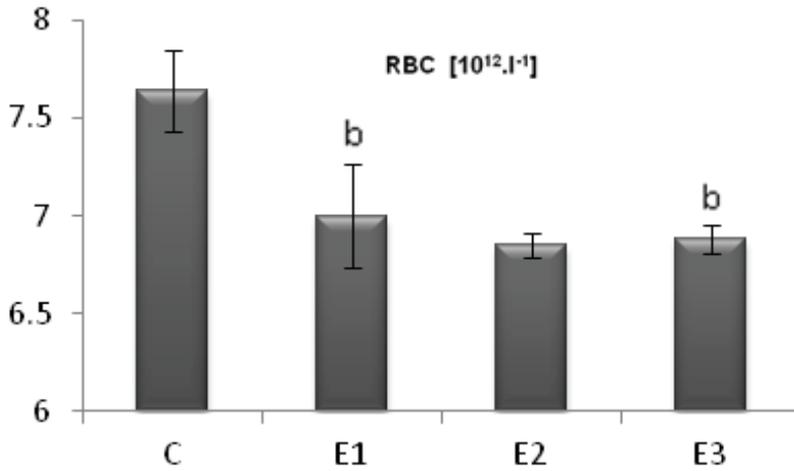


Figure 2. The effect of patulin on RBC in porcine blood *in vitro*
 C - control, E1 – 10 ng.ml⁻¹, E2 100 ng.ml⁻¹, E3 - 1000 ng.ml⁻¹, WBC – white blood cells count, a,b – means significant differences (P<0.05), one-way ANOVA

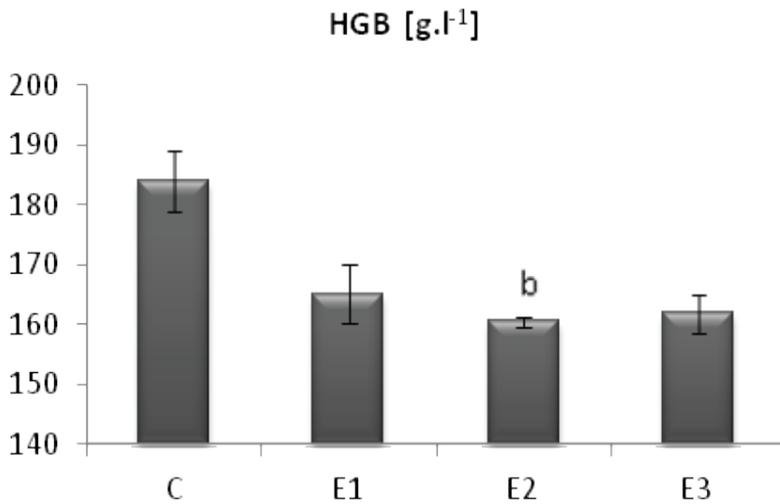


Figure 3. The effect of patulin on HGB in porcine blood *in vitro*
 C - control, E1 – 10 ng.ml⁻¹, E2 100 ng.ml⁻¹, E3 - 1000 ng.ml⁻¹, WBC – white blood cells count, a,b – means significant differences (P<0.05), one-way ANOVA

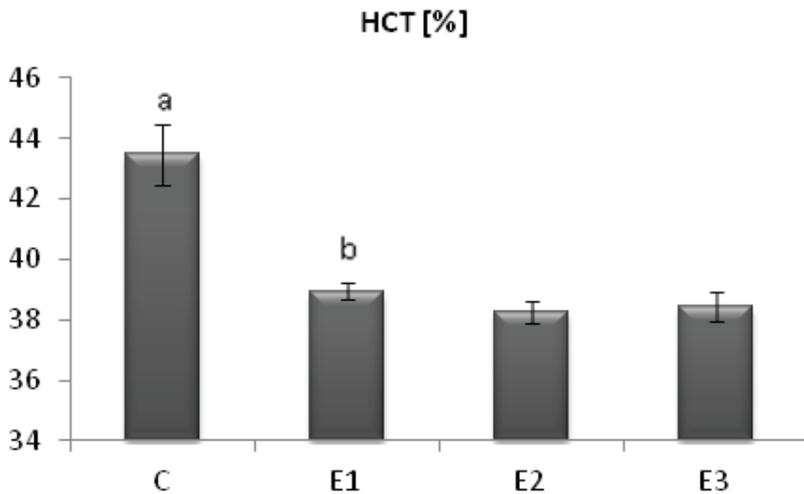


Figure 4. The effect of patulin on HCT in porcine blood *in vitro*
 C - control, E1 – 10 ng.ml⁻¹, E2 100 ng.ml⁻¹, E3 - 1000 ng.ml⁻¹, WBC – white blood cells count, a,b – means significant differences (P<0.05), one-way ANOVA

Significant decrease (P<0.05) of PLT count was found in all experimental groups against the control. The lowest count of PLT was observed in E1 group (the lowest amount of patulin).

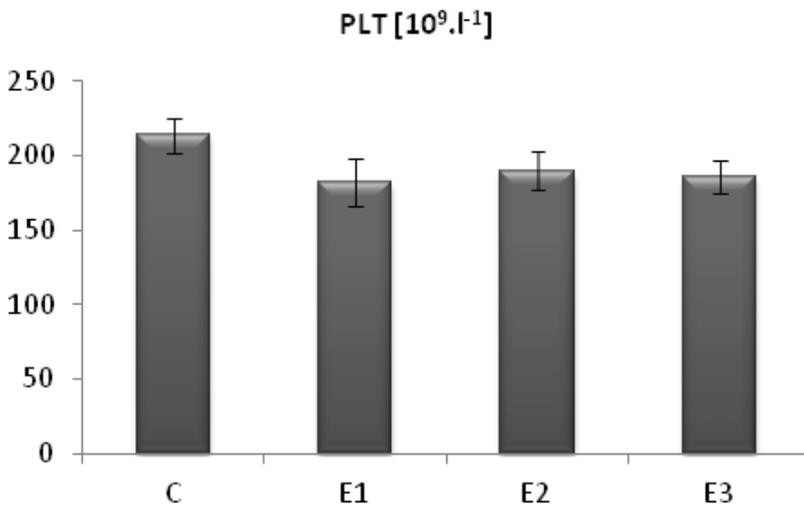


Figure 5. The effect of patulin on PLT in porcine blood *in vitro*
 C - control, E1 – 10 ng.ml⁻¹, E2 100 ng.ml⁻¹, E3 - 1000 ng.ml⁻¹, WBC – white blood cells count, a,b – means significant differences (P<0.05), one-way ANOVA

DISCUSSION

Current research was aimed at understanding the role of mycotoxin patulin on haematological parameters of porcine blood *in vitro*. Under normal physiological conditions, cells interact each other to synchronize their metabolic activity, gene expression, and other basic cellular processes (Capcarova *et al.*, 2013). Patulin has a strong affinity for sulfhydryl groups. Patulin adducts formed with cysteine are less toxic than the unmodified compound in acute toxicity, teratogenicity, and mutagenicity studies. Its affinity for SH-groups explains its inhibition of many enzymes (Puel *et al.*, 2010). This can be the reason why patulin affected each blood parameter in this study. Thus cells were not able to maintain the basic cellular processes. Decrease of observed parameters in our study may be caused also by the fact that it is *in vitro* study and patulin was not metabolised in organism. In another *in vitro* study, we observed slight decrease of WBC in porcine blood after treatment with deoxynivalenol (Zbyňovská *et al.*, 2013a). Significant decrease in WBC count was observed after exposure of zearalenone on porcine blood *in vitro* (Capcarova *et al.*, 2014). In *in vivo* study we observed insignificant ($P>0.05$) decrease of WBC after exposure of patulin in rabbits blood (Zbyňovská *et al.*, 2013b). After exposure of patulin *in vitro* we observed significant decrease of RBC count followed by decrease of HGB and HCT. It may be caused by haemolysis in samples of blood after short term exposure of this mycotoxin. Similar results observed Capcarova *et al.* (2014) in *in vitro* study after treatment with ZEN in porcine blood. In our study all doses of patulin caused intensive haemolysis. In the study with human erythrocytes (Jilani and Lang, 2013), the percentage of haemolysed erythrocytes increased slightly but significantly following exposure of erythrocytes for 48 h to zearalenone. Lupescu *et al.* (2013) observed similar effect adding mycotoxin patulin to human erythrocytes. Patulin stimulated Ca^{2+} entry into erythrocytes, an effect triggering suicidal erythrocyte death or eryptosis. Suicidal erythrocyte death what can lead to haemolysis of blood was observed after ochratoxin A (Jilani *et al.*, 2012) and zearalenone exposure (Jilani and Lang, 2013), characterized by cell membrane scrambling and cell shrinkage (Lang *et al.*, 2008). Circulating eryptotic erythrocytes are cleared from the blood, so after stimulation of eryptosis the percentage of eryptotic erythrocytes remains low *in vivo*. The accelerated loss of eryptotic erythrocytes following *in vivo* stimulation of erythrocytes may lead to anaemia (Lang *et al.*, 2008). Mycotoxins like DON also affected RBC *in vitro* in porcine blood. Their count was decreased in all experimental groups (Zbyňovská *et al.*, 2013a). In *in vivo* study we observed insignificant decrease ($P>0.05$) of RBC in rabbits blood after two weeks exposure of patulin (Zbyňovská *et al.*, 2013b). In different study Raju and Dewegoeda (2000) observed similar effect of aflatoxin, ochratoxin and T2-toxin alone or in combination on HGB. Ewuola and Egbunike (2008) found that after fumonisin B₁ administration in concentration 10 mg.kg⁻¹ in diet for growing rabbits haemoglobin concentration decreased, Gbole and Akele (2010) found that concentration of haemoglobin of female rabbits significantly decreased after fumonisin administration. In our *in vivo* study with intramuscular patulin administration to rabbits insignificant increase of PLT was observed (Zbyňovská *et al.*, 2013b). On the other hand, in present *in vitro* study significant decrease ($P<0.05$) of PLT in all experimental groups was found against the control. It is possible that in organism patulin activates certain mechanisms which helps cells defend from adverse effect of patulin. In *in vitro* study with ZEN on porcine blood,

Capcarova et al. (2014) found that exposure of this mycotoxin caused significant decrease ($P < 0.05$) of PLT. Results from our *in vitro* study with patulin are very similar with results of *in vitro* study with ZEA exposure (Capcarova et al., 2014). Similarly in another *in vitro* study significant decrease ($P < 0.05$) of PLT in porcine blood after exposure of DON was measured (Zbynovska et al., 2013a). In general, mycotoxins are able to modulate haematological parameters *in vitro*.

CONCLUSION

In the present study patulin treatment significantly affected haematological parameters. Results of this study provide a foundation for further analysis and researches on mycotoxins impact on living cells and the system of possible protection against its effects as well as evaluation of various dose dependencies on haematological parameters.

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UTVRĐIVANJE NEKIH HEMATOLOŠKIH PARAMETARA U KRVI SVINJA POSLE TRETMANA PATULINOM *IN VITRO*

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MARCELA CAPCAROVÁ

Izvod

Patulin, genotoksični mikotoksin, proizvod nekoliko vrsta roda *Aspergillus*, *Penicillium*, i *Bysochlamys* je mikotoksin koji se naj češće nalazi u jabukama i proizvodima od jabuka. Patulin može biti neurotoksičan, imunosupresivan, gernetoksičan, teratogen i karcinogen. Cilj ovog rada je da ispita uticaj patulina na neke hematološke parameter (eritrociti - EC, leukociti - LC, trombociti - TC, hematokrit - HTK i haemoglobin - HGB) u krvi svinja *in vitro*. Grupa koja nije primal patulin, služula je kao kontrola. Patulin je izazvao značajno smanjenje broja EC u svim grupama, u odnosu na kontrolu. Patulin je, takođe, izazvao značajno smanjenje broja EC, što je praćeno povećanjem hemolize i padom HGB i HTK, u svim tretmanskim grupama, u poređenju sa kontrolnom grupom. Ustanovljen je i značajan pad broja TC. Dobijeni rezultati čine dobru osnovu za dalja istraživanja i analize u vezi sa uticajm mikotoksina na žive ćelije i sisteme, kao i za iznalaženje mogućih načina zaštite od njihovog uticaja, kao i evaluaciju uticaja različitih doza mikotoksina na hematološke parameter.

Ključne reči: patulin, krv svinje, hematologija.

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UPDATING THE PREVALENCE OF CANINE DIROFILARIOSIS IN PET DOGS IN NOVI SAD, VOJVODINA, SERBIA*

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DUŠAN LALOŠEVIĆ², LJILJANA KURUCA¹, SANDRA NIKOLIĆ³,
DANIJELA NERAC³

*SUMMARY: The aim of this study is to update the prevalence of dirofilarial infections in pet dogs. From the year 2010 to the year 2014, a total of 143 blood samples were collected from privately owned pet dogs in Novi Sad. All samples were examined by wet blood smears, the modified Knott test and heartworm antigen test. Circulating microfilariae of both *Dirofilaria (D.) immitis* and *D. repens* were found in dogs. Prevalence values for *D. immitis* and *D. repens* were 16.78% (24/143) and 18.88% (27/143), respectively. We report mixed infection with both parasites for the first time. Results of this study, compared with results of previous investigations, shows increase of infection with *D. immitis* and increase of mixed infection with both *Dirofilaria* species. Further investigations are required with higher number of samples to confirm these findings.*

Key words: *D. immitis*, *D. repens*, prevalence, pet dogs.

INTRODUCTION

Nematodes of the genus *Dirofilaria (D.)* are currently considered emerging agents of parasitic zoonoses in Europe. Two main filarial infections occur in domestic and wild carnivores in Europe: *D. immitis*, the etiological agent of canine and feline heartworm disease, and *D. repens*, the main etiological agent of subcutaneous filarial infections. Climate changes, the existence of animal reservoirs (domestic and wild canides), and global movement of dogs have caused an increase in the spreading of these mosquito-borne nematodes (Genchi et al., 2011).

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Regarding dirofilariosis from the clinical point of view, heartworm disease is the most important. It can be a serious and potentially fatal disease in both cats and dogs (Ware, 2011; Atkins, 2009, 2014). *Dirofilaria repens* is a parasite of the subcutaneous connective tissues, mainly in dogs. Infection with *D. repens* is generally asymptomatic. Main clinical manifestations of *D. repens* infection are nodular multifocal dermatitis and pruriginous papule (Scott and Vaughn, 1987; Haliwell and Gorman, 1989). Both *Dirofilaria* species have zoonotic potential which expand the interests for these nematodes into human medicine (Simon et al., 2009, Simon et al., 2014).

First cases of canine dirofilariosis in Serbia were found in dogs during the autopsy (Milosavljević and Kulišić, 1989). During the last decade studies were performed in dogs on the subject of seroprevalence, diagnostic procedures, therapy and case report (Tasić et al 2008; Pajković et al, 2010; Spasojević Kosić et al 2011; Pavlović et al., 2012; Spasojević Kosić et al., 2012, 2014; Savić et al., 2014). Although data concerning prevalence of both *Dirofilaria* infections in Serbia have been published, it is of interest for human and veterinary medicine to follow up these infections among dogs. The aim of this study is to update the prevalence of dirofilarial infections in pet dogs.

MATERIAL AND METHODS

From the year 2010 to the year 2014 pet dogs from Novi Sad were tested for dirofilaria infections. This research was done in 143 privately owned pet dogs. At the moment of testing, dogs were at least 7 months old, exposed minimally to one mosquito season and without history of treatment with macrocyclic lactones. All dogs were clinically examined and blood samples were taken from all dogs with the purpose of their parasitological examination. The parasitological examination consisted of wet blood smears, the modified Knott test and antigen testing. Techniques for detecting circulating microfilariae include microscopic examination of fresh blood smears and modified Knott test. Detection and enumeration of circulating microfilariae (mf) of both *D. immitis* and *D. repens* were carried out by the modified Knott test (Bazzochi et al., 2008). Morphological characteristics of microfilariae, such as length, width, cephalic and caudal ends, were assessed in order to differentiate microfilariae of two *Dirofilaria* species (Genchi et al., 2007) (Figure 1.). Detection of circulating *D. immitis* antigens was carried out by commercial kit SNAP Heartworm RT Test (Idexx Laboratories) according to manufacturer's instruction.

In case of *D. repens* Knott test was sole diagnostic test for subcutaneous dirofilariosis. Concerning *D. immitis* infection, detection of either microfilaria or circulating antigen, or both microfilaria and antigen detection was diagnostic for heartworm infection.

RESULTS AND DISCUSSION

In this study we used recognized methods in the diagnosing of dirofilariosis (American Heartworm Society Canine Guidelines, 2014, ESCCAP 2012); we tested sensitive population of dogs, old enough to develop the adult form of the parasite, as well as dogs with clinical manifestations that may arise as a consequence of dirofilaria infections.

Among 143 examined dogs, 61 (42.66%) dogs had clinical signs (Table 1), while the rest of the dogs were asymptomatic. The most common clinical signs in dogs were cough and skin nodules. However, neither all dogs with the clinical signs were with the diagnosis of dirofilariosis nor all dogs with the diagnosis of dirofilariosis were with the clinical signs of diseases.

Table 1. Clinical signs in 61 symptomatic dogs

Clinical signs	Number of dogs
Cough	30
Dyspnoea	6
Fatigue	2
Weakness	2
Arrhythmias	2
Syncope	2
Cachexia	3
Skin nodules	7
Lameness	3
Ascites	1
Neurological signs	3

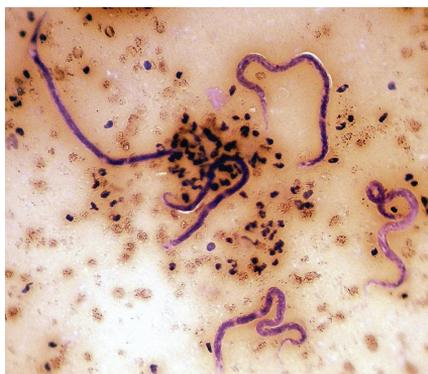


Figure 1. Microfilariae detected by modified Knott test (orig.)

Table 2. Number of infected dogs during study period

Year	Number of dogs	<i>D. repens</i>	<i>D. immitis</i>	Mixed infection
2010.	39	4	2	0
2011.	16	2	2	0
2012.	28	4	3	0
2013.	37	5	6	8
2014.	23	3	2	1
2010-2014	143	18	15	9

The number of tested dogs varied through time period. The number of dogs with *D. repens* was almost always higher in comparison with the number of dogs with *D. immitis*. Since 2013. we have observed the dogs with mixed infection, and according to our new data from this year, it is still present in 2014.

Circulating microfilariae of *D. repens* were found in 27 dogs, while circulating microfilariae of *D. immitis* were found in 17 dogs. The number of microfilariae vary from the minimum of 10 mf/ml to the maximum of 1750 mf/ml in case of *D. repens* infection. In dogs infected with *D. immitis*, minimum number of microfilariae was 40 mf/ml and maximum was 26900 mf/ml. Counting the number of microfilariae in blood is particularly important in assessing the efficacy of therapy (Grandi et al., 2010, Spasojević Kosić et al., 2014). In 22 dogs antigenemia related to *D. immitis* was detected, and in 7 of them no circulating microfilariae of *D. immitis* were found. In these dogs we perform Baermann test due to potential cross-reactions with *A. vasorum* in commercially available test kits for the detection of *D. immitis* antigen (Schnyder and Deplezes, 2012). The larvae of *A. vasorum* were not detected in these dogs. Opposite to these dogs with no circulating microfilariae, in 2 dogs with *D. immitis* infection no antigen was detected. Overall prevalence of *D. repens* in examined dogs was 18.88% (27/143), while the prevalence of *D. immitis* was 16.78% (24/143). Since mixed infection with both parasites was detected in 9 dogs (6.29%), the prevalence of heartworm as a single infection was 10.49% (15/143), while the prevalence of subcutaneous dirofilariosis as a single infection was 12.59% (18/143).

In order to compare the prevalence reported in this study with the prevalence of dirofilariosis in the previous studies it is necessary to take into consideration the methods used for the diagnosing of *D. immitis* and *D. repens* infection. By using both Knott test and antigen detection, studies of dirofilariosis in Novi Sad at first showed an infection only with *D. repens*, with the prevalence of 28.9%. At that time *D. immitis* was not diagnosed (Tasić et al., 2008). Among pet dogs in Novi Sad, prevalence of 5% (3/60) was determined by using only heartworm antigen testing (Pavlović et al., 2012). In the meantime, two studies were published on the subject of the prevalence of dirofilariosis. The first one reported the prevalence of dirofilariosis in military dogs of 14% (Pajković et al., 2010), but the authors did not differentiate microfilariae. The second one reported prevalence of

7% in the year 2004 and 11% in 2009 in pet dogs in Novi Sad (Savić et al., 2012), but in this study antibodies against *Dirofilaria* were detected. Compare to the study of Tasić et al. (2008), and our study from 2012 (Spasojević Kosić et al., 2012), which have provided full diagnostic protocol for dirofilarial infections, this study shows increase of infection with *D. immitis* and we report mixed infection in dogs with both parasites for the first time. Infection with *D. repens* in this, as well as in our previous study (Spasojević Kosić et al., 2012), is still lower than in first report of Tasić et al. (Tasić et al., 2008).

Due to the clinical importance of heartworm disease in veterinary medicine and zoonotic potential of *Dirofilaria* infection it is necessary to continue the study of prevalence among dogs and report data on periodic occasion.

CONCLUSION

This study reveals a 10.49% prevalence of *D. immitis* as single infection, 12.59% prevalence of *D. repens* as single infection, and 6.29% prevalence of mixed infections with both *Dirofilaria* species in pet dogs in Novi Sad.

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**AŽURIRANJE PREVALENCE DIROFILARIOZE
KOD PASA KUĆNIH LJUBIMACA U NOVOM SADU,
VOJVODINA, SRBIJA**

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DANIJELA NERAC

Izvod

Cilj ovog rada je da prikaže nove podatke o prevalenci infekcije dirofilarijama kod pasa kućnih ljubimaca. Od 2010. do 2014. godine pregledano je 143 uzoraka krvi prikupljenih od pasa iz Novog Sada. Uzorci krvi od svakog psa su pregledani nativnim krvnim razmazom, modifikovanim Knotovim testom i dokazivanjem postojanja antigena odraslog parazita. U krvi pasa su dijagnostikovane mikrofilarije i *D. immitis* i *D. repens*. Prevalenca infekcije *D. immitis* iznosila je 16,78% (24/143), dok je za *D. repens* prevalenca bila 18,88% (27/143). Ovo istraživanje prvi put otkriva mešanu infekciju sa oba parazita kod pasa. Rezultati ovog istraživanja, u odnosu na prethodna, pokazuju porast broja infekcija pasa sa *D. immitis* i povećanje broja mešanih infekcija sa oba parazita. Dalja ispitivanja, na većem broju pasa, su potrebna kako bi se potvrdile procene ovog istraživanja.

Ključne reči: *D. immitis*, *D. repens*, prevalenca, psi kućni ljubimci.

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ASSESSING THE AGRO-ECOSYSTEM PERFORMANCE IN A LONG-TERM WINTER WHEAT CROPPING*

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SUMMARY: The aim of this study was to access the impact of agricultural practices to the multiple agro-ecosystem function of winter wheat cropping systems. The data were obtained from a long-term experiment at the Rimski Šančevi experimental station of the Institute of Field and Vegetable crops. A simple performance-based index was used to discriminate among treatments based on the crop and soil parameters within the different agro-ecosystems. The following indices were used: food production, raw material production, nutrient cycling and greenhouse gas regulation. Continuous cropping resulted with decrease in the utilization of the agro-ecological potential. Our results indicate that the soil have lost productivity and quality, irrespective to the applied cropping practices. Accordingly, it was found that yield variation resulted mostly from unfavorable climate condition, thus fertilization had the essential effects on yield preservation. At the end of the investigated period 3-year rotation has highest utilization of the inherent soil productivity in comparison with investigated wheat-based cropping systems.

Key words: *agro-ecosystem performance, grain yield, winter wheat, cropping systems.*

INTRODUCTION

Agricultural practice determines the level of food production that imposes significant influence on the state of the global environment. The long-term environmental sustainability of the food and feed production is one of the major questions facing modern agriculture (Javadzadeh, 2014). Assessing the environmental sustainability of agricultural

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systems in a quantitative manner requires the identification and integration of diverse phenomena of ‘indicators’ of environmental effects, in a framework consistent with the evolving concept of sustainability (Sands and Podmore, 2000). Agro-ecosystems are highly complex and cropping systems perform multiple functions in their role as agro-ecosystems. Regarding agro-ecosystem performance as food and raw materials production, nutrient cycling, erosion control, greenhouse gas regulation, water regulation and waste treatment are main indices of agro-ecosystem management. Indices are tools for aggregating and simplifying information of a diverse nature into a useful and more advantageous form (Sands and Podmore, 2000). A simple performance-based index was developed to determine the relative sustainability of agricultural management system within the context of multiple agro-ecosystem functions and to give users a simple measure to assess sustainability of agricultural practices. Winter wheat cropping could provide a basis for evaluating the relationship between environment and yield performance in the investigated long-term trail as it has not undergone significant change over the years compared with other crops. In addition to that, winter wheat is a versatile crop that can be successfully grown under many environmental conditions with contrasting agronomical inputs as one of the most adapted crops (Chloupek et al., 2004). The aim of this study was to evaluate the winter wheat cropping with performance based index.

MATERIALS AND METHODS

The present study was performed on the long-term experiment (LTE) “Plodoredi” carried out at the Rimski Šančevi Experimental Field of the Institute of Field and Vegetable Crops in Novi Sad (45° 19’ N, 19° 50’ E, 84 m). The trial was established 1946/4 in order to improve production and conservation properties of chernozem, in 1970 different systems of crop production were introduced in order to examine their effect on yield of most important crops in Vojvodina (Milošev, 2000; Šeremešić et al., 2011).

The study treatments were:

1. 3-year crop rotation (maize-soybean-wheat), mineral fertilizer 100 kg N ha⁻¹+ crop residues (D3)
2. 2-year crop rotation (maize-wheat), mineral fertilizer 100 kg N ha⁻¹+crop residues (D2)
3. Monoculture (wheat-wheat), mineral fertilizer 100 kg N ha⁻¹+crop residues (MO)
4. 2-year rotation (maize-wheat), without fertilizers+crop residues (N2)
5. 3-year rotation (maize-soybean, soybean-wheat), without fertilizers+crop residues (N3)

For this study we used dataset of a treatments from the long-term experiment from 2000 to 2010 year. The counting of index is led by four basic steps: data grouping, calculation of averages, ranking and scoring treatments, and summing of scores within and across agro-ecosystem functions.

Step 1: Group Data within Agro-ecosystem Functions

Agro-ecosystem functions could be presented with appropriate indicators. The procedure is initiated by surveying the dataset for indicators that could be grouped within

agro-ecosystem functions (Liebig et al., 2001). According to Constanza (1997) for the purpose of analysis, ecosystem services are grouped into 17 major categories. The analysis considered the entire system, but we used four of them: food production, raw material production, nutrient cycling and greenhouse gas regulation. The food production as the first ecosystem service provides major portion of gross primary production extractable as food. The second ecosystem service is raw materials with ecosystem functions which includes that portion of gross primary production extractable as raw materials. The third ecosystem service is nutrient cycling with ecosystem functions-storage, internal cycling, processing and acquisition of nutrients. The fourth agro-ecosystem included in this paper is greenhouse gas regulation. Within each agro-ecosystem, indicators are selected to characterize the performance of that function (Liebig et al., 2001).

[**Agro-ecosystem performance** = f Food production $\times W_{fp} = f$ (grain yield, grain N content), Raw materials production $\times W_{rmp} = f$ (stover yield, stover N content), Nutrient cycling $\times W_{nc} = f$ (residual soil NO_3 , soil pH), Greenhouse gas regulation $\times W_{ggr} = f$ (soil organic C, early spring soil NO_3)]

Where W_{fp} , W_{rmp} , W_{nc} , W_{ggr} are the relative weights. Consequently, the relative weights may be adjusted for each function to account for differences in the number of indicators among functions (Liebig et al., 2001).

Step 2: Calculate Treatment Averages

This step contains calculation of treatments averages for each indicator. It is important to make a difference between indicators, because some of them are best evaluated over time, whereas the others are cumulative in their influence on agro-ecosystem functions, increasing or decreasing over time (Table 1.).

Table 1. Different cropping systems averages for indicators used to represent agro-system functions.

Agro-ecosystem indicators	Cropping system				
	D2	D3	MO	N2	N3
Food production					
Grain yield, kg/ha	5088	6086	4625	1102	1730
Grain N content, g/kg	98.82	118.61	90.18	19.21	31.19
Raw materials production					
Stover yield, kg/ha	5972	8699	6108.2	1340	2566
Stover N content, g/kg	9.8	11.8	9.01	1.9	3.1
Nutrient cycling					
Residual soil NO_3 -N, 0-90 kg/ha	116	120	100	45.5	48.5
Soil pH	7.17	7.43	7.11	7.50	7.60
Greenhouse gas regulation					
Soil organic C, kg/ha	60 200	63 850	64 750	50 820	51 320
Early spring soil NO_3 -N, kg/ha	24.9	22	20	11.5	15

Step 3: Rank and score treatments

According to Liebig (2001) treatment values are ranked for each indicator in ascending or descending order, depending on whether a higher value for the indicators are good or bad, or in simply terms good or bad criteria. After the treatment values are ranked, they are scored based on their relative differences from the optimal value. The most straightforward approach from data arranged in descending order it to assign a score of 1.0 to the highest treatment (Table 2.)

Table 2. Rank and score treatments for indicators within agro-ecosystem functions

Rank	Treatment	Score	Rank	Treatment	Score
Food production					
	Grain yield			Grain N content	
1	D3	1.00	1	D3	1.00
2	D2	0.84	2	D2	0.83
3	MO	0.76	3	MO	0.76
4	N3	0.28	4	N3	0.26
5	N2	0.18	5	N2	0.16
Raw materials production					
	Stover yield			Stover N content	
1	D3	1.00	1	D3	1.00
2	MO	0.70	2	D2	0.86
3	D2	0.68	3	MO	0.76
4	N3	0.29	4	N3	0.26
5	N2	0.15	5	N2	0.16
Nutrient cycling					
	Residual NO ₃ -N			Soil pH	
1	N2	1.00	1	N3	1.00
2	N3	0.94	2	N2	0.98
3	MO	0.45	3	D3	0.97
4	D2	0.39	4	D2	0.94
5	D3	0.37	5	MO	0.93
Green house regulation					
Soil organic C - Early spring soil NO ₃					
1	MO	1.00	1	N2	1.00
2	D3	0.97	2	N3	0.76
3	D2	0.91	3	MO	0.57
4	N3	0.78	4	D3	0.52
5	N2	0.77	5	D2	0.46

Step 4: Sum scores within and across agro-ecosystem functions

Upon summing scores within agro-ecosystem functions, the remaining step is to sum scores across functions. The final score would reflect a relative ranking of agro-ecosystem performance among treatments for functions included in the procedure (Table 3.).

RESULTS AND DISCUSSION

Quantifying the effects of management practices against agro-ecosystem functions is necessary to determine the sustainability of cropping systems (Liebig and Varvel, 2003). The overall agro-ecosystem performance score for the D2 is 84.42, for D3 is 97.57, as a highest, for MO 84.71, and for N3 62.43 and for N2 is 54.57 as the lowest. Based on the observed results crop yield as well as raw material production have higher difference of min. and max values, compared with the nutrient cycling and greenhouse gases regulation. Winter wheat monoculture has showed higher sustainability compared with N2, N3, D2 that indicated its positive contribution to a nutrient cycling and greenhouse gas regulation. However this can be described as cumulative effects of the long-term experiment. Increased productivity of D3 cropping system is based upon effects of 3-year rotation that in the long-term increases winter wheat yields. Raw material ($r = -0.88$) has significantly negative effects whereas nutrient cycling and greenhouse gases showed higher correlation with the agro-ecosystem performance (Table 3.). Also, food production (yield level) showed positive effects on agroecosystem performance. On the basis of the obtained results we anticipate that cropping systems with most effects on environmental condition are not those with the higher agro-ecosystems performance.

Table 3. Agro-ecosystem performance (AESP) scores for different winter wheat cropping systems

Agro-ecosystem function				Agro-ecosystem performance scores		
Treatments	Food production	Raw materials production	Nutrient cycling	Greenhouse gas regulation	Not scaled	Scaled to 100 (7=100%)
D2	1.67	1.54	1.33	1.37	5.91	84.42
D3	2.00	2.00	1.34	1.49	6.83	97.57
MO	1.52	1.46	1.38	1.57	5.93	84.71
N2	0.34	0.31	1.94	1.23	3.82	54.57
N3	0.54	0.55	1.98	1.30	4.87	62.43
Correlation to AESP	0.82	-0.88	0.96	0.97	-	-

CONSLUSIONS

This study showed that simple performance-based index is helpful tool in discriminating differences of agro-ecosystem performance between contrasting management systems in long-term winter wheat cropping system. Long-term cropping has resulted in differences

in soil organic carbon, soil pH and temporal soil NO₃. However, properly managed agro-ecosystems were capable to resist the pressure and responded with resilience to applied cropping technology over the years. Accordingly, it was found that yield decline resulted mostly from unfavorable climate condition and fertilization had the essential effects on yield preservation. At the end of investigated period three-year rotation (D3) had highest utilization of the inherent soil productivity compared with the unfertilized plots (N3).

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PROCENA PERFORMANSI AGROEKOSISTEMA U VIŠEGODIŠNJEM OGLEDU SA PŠENICOM

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Izvod

Cilj ovog istraživanja je bio da se utvrdi uticaj sistema gajenja pšenice na pokazatelje performansi agroekosistema: prinos, proizvodnja biomase, kruženje hraniva i regulacija gasova staklene bašte. Analizirani su višegodišnji eksperimenti na Rimskim Šančevima. Dugogodišnje korišćenje zemljišta u poljoprivredne svrhe uticalo je na promene u prinosu i pad produktivnosti zemljišta. Utvrđeno je da tropolje (kukuruz-soja-pšenica) ima najbolje korišćenje proizvodnih uslova i najmanje negativno delovanje na stanje životne sredine. Kvalitativni pokazatelj kao što je “analiza performansi” može uspešno da se koristi prilikom opisivanja funkcija agroekosistema u našim uslovima.

Ključne reči: performanse agroekosistema, prinos, ozima pšenica, sistemi ratarenja

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CONTROL OF *PORCINE CIRCOVIRUS* TYPE 2 INFECTIONS IN PIGS

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SUMMARY: Porcine circovirus type 2 (PCV2) is one of the leading viruses that impair the health status of pigs in the last decade and thus call into question the scope and efficiency of pig production. PCV2 infection control means control of all cofactors involved in the development of the disease and the so-called triggers with efforts for their eradication as well as immunoprophylaxis measures which are considered indispensable in controlling this disease. Today, 5 commercial vaccines are available on the European market, and are all based on the genotype of the PCV2 and show good efficacy. Basic issue regarding the successful vaccination is determining the optimal time for administration of the vaccine. Time of vaccination is best determined on the basis of the level of titer of colostrum antibodies in the blood serum of piglets and time of occurrence of PCV2 infections. At this moment eradication of this disease is farfetched, but vaccination is the main form of struggle against PCV2 infection.

Key words: PCV2, vaccine, control, piglets, immunity.

INTRODUCTION

Circovirus diseases belong to the group of new-generation diseases of pigs veterinary practice is confronted with in recent years and are considered to be the most

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controversial diseases in the pathology of these types of animals. *Porcine circovirus type 2* (PCV2) is a ubiquitous virus of domestic and wild pigs and is the primary cause of this disease in pigs. Increasing interest in circovirus infections began after the appearance of *Post weaning multisystemic wasting syndrome* (PMWS) in Canada in 1991, and retrospective studies have shown their existence in the late sixties. On the basis of new information about the pathology of the disease a new terminology was suggested, and now subclinical forms of circovirus infection are known in veterinary practice, then *Porcine circovirus systemic disease* (PCV-2-SD) (formerly PMWS), respiratory form of PCV2 disease (*Porcine circovirus lung disease*; PCV-2-LD), enteric form (*Porcine circovirus enteric disease*; PCV2-ED), an increasingly frequent reproductive form (*Porcine circovirus reproductive disease*; PCV2-RD), and *dermatitis nephrosis syndrome* (PDNS) (Segales, 2012). Today, these pathological entities are collectively referred to as Porcine circovirus associated diseases (PCVAD) (Segales et al., 2005; Segales et al., 2009). Which system will be affected, and thus which form of manifestation the disease will take, it is not possible to predict. But whatever form the disease takes, damage both direct and indirect is always very significant and threaten every rational production of pigs (Gagrčín, 2009). Regarding all of that PMWS is considered as economically the most important disease. It was found that direct and indirect costs in the EU amounted to about EUR 600 million per year (Armstrong and Bishop, 2004). In the US, this disease has cost manufacturers an average of 3-4 dollars per pig, with maximum losses ranging up to \$ 20 (Gillespie et al., 2009). This syndrome as a form of circovirus infection has expanded among the pig population in the Republic of Serbia and appears in pigs aged 6-16 weeks (Ivetić et al., 2004; Stojanac et al., 2013a). Control of these infections is very complex and difficult. The use of any medications was contraindicated (antibiotics, chemotherapy, etc.), which contributes to even greater complexity of control. However, immunoprophylaxis in terms of administration of specific vaccines has the most frequent application (Llorenc Grau-Roma et al., 2011). Today there are several types of vaccines that are used in the control of these infections in many countries with developed pig breeding (live, inactivated, subunit). The most successful vaccines are those based on the induction of an active immune response to the capsid protein of PCV2. This protein is designated as the immunogen, inducing the formation of protective antibodies (Kixmoller et al., 2008, Tacker et al., 2008). The successful immunization is the one that results in reduction of the clinical forms of the disease, and the prevention of tissue injury.

CONTROL OF CIRCOVIRUS INFECTIONS

Madec's plan. Before vaccines became commercially available, successful treatment and control of PCVAD diseases was primarily focused on providing good production practices which minimize stress, eliminate coinfections or reduce their effect, as well as removing potential factors that induce stimulation of the immune system and the progression of PCV2 infection. Today, Madec's plan with 20 items is used for control of PCV2 infection, which can be summed up in four golden rules which include: 1) limiting of contact between the pigs, 2) stress reduction, 3) good

hygiene, 4) good feed (Madec et al., 1999). At least 16 items from this plan should be applied in order to reduce mortality from 20% to single digits. One of the main items from the Madec's plan is to minimize the contact between pigs, given that direct contact is one of the most common routes of spreading infection in the herd. Establishing firm partitioning walls between the compartments and the adoption of *all in, all out* system throughout the farm is recommended in order to reduce the contact between pigs. Quarantine of newly acquired pigs is aimed at preventing the introduction of new infections (Rose et al., 2003).

Control of coinfections. An important element in the control of PCV2 infection is the suppression of certain diseases such as PPV, PRRS, enzootic pneumonia and swine flu, which intensify the severity of PCV2-induced lesions. Studies on risk factors for PCVAD on 62 farms in Spain indicated that vaccination of gilts against PRRS increases the chances of outbreak of circovirus diseases, while in contrast, vaccination of sows against atrophic rhinitis reduces the chances of developing these diseases (Lo'pez-Soria et al., 2005). Attempts to control PMWS by herd vaccination against PPV in the final fattening stage in the United States, with proven circulation of PPV were successful. However, these positive results have not been confirmed experimentally. The application of immunomodulators is not contested in any segment, but still, abandonment of the application of some vaccines and sanitation programs is too much of a risk. For this reason, and based on the available results, the producers of pigs in PCVAD-positive herds should take into account the determination of the approximate time of appearance of PCV2 infections with the aim to change the time of vaccination as a potential plan for minimizing the disease (Gagrčin, 2009).

Disinfection measures. The use of disinfectants in buildings and vehicles has proved to be effective and is recommended for control of PCV2 infections. *In vitro* reduction of a titer of virus was achieved by using sodium hydroxide, Virkon S, Roccal D Plus, Clorox bleach, 1-Stroke Environ, Fulsan and Tek-Trol under controlled laboratory conditions (Royer et al., 2001). The effectiveness of disinfectants in commercial terms is not known.

Hyperimmune serums therapy. Subcutaneous injection of PCV2 hyperimmune serum of suckling piglets and post-weaning piglets has proved to be effective in reducing mortality on farms with PMWS in France, Spain and the UK (Ferreira et al., 2001; Waddilove and Marco, 2002). However, the success of this procedure is variable, as in some of the farms it did not produce results. Moreover, recent studies have shown that there is no benefit from the application of hyperimmune serum (Hassing et al., 2006; Opriessnig et al., 2006). The mechanism of action of hyperimmune serums therapy is not sufficiently understood.

Nutritional factors. Partial control of PMWS on some farms in the UK was achieved by changes in the diet of diseased pigs (Donadeu et al., 2003). These changes include an increase in the density of feed for young pigs and supplementing with commercial additives, mainly with antioxidant effects. Some studies have shown that conjugated linoleic acid alleviates PCV2 experimental infections (Bassaganya-Riera et al., 2003). On the other hand it was suggested that the addition of vitamin E and/or selenium in feed can

be used on farms affected by PMWS (Baebko et al., 2004). Although these preliminary results, both experimental and field-tested, show that certain nutritional factors can mitigate the effects of PMWS, there is still not enough scientific information to determine the true effects of the diet on this disease.

IMMUNOPROPHYLAXIS OF CIRCOVIRUS INFECTIONS

The introduction of vaccines against PCV2 led to an enormous reduction of PCV2-SD and PDNS, and also one part of the economic justification refers to a significant reduction in PCV2-SI. Today, there are several commercial vaccines used for the prevention of PCVAD in pigs, and all are based on the PCV2 genotype. *Circovac*[®] (Merial) vaccine consists of an inactivated PCV2 virus and is used for vaccination of piglets older than 3 weeks as well as for vaccination of sows and gilts. The application of this vaccine in piglets is a one-time, while vaccination of sows and gilts requires two applications, one 3-4 weeks before insemination, and another 2-4 weeks before farrowing. *Ingelvac CircoFLEX*[®] (Boehringer Ingelheim), *Circumvent*[®] (Intervet/Merck), and *Porcilis*[®] PCV (Schering-Plough/Merck) are subunit vaccines based on a PCV2 capsid protein and are used on piglets 2 weeks old, except *Porcilis*[®] PCV (Schering-Plough/Merck) which is used on piglets older than 3 days. *FosteratM* PCV (Pfizer Animal Health Inc.) is a vaccine recently introduced to the market, now revised, and previously known as *Suvaxyn*[®] PCV2 One DoseTM (Fort Dodge Animal Health Inc.) (Nathan et al., 2012). The commercial vaccines are shown in table 1.

In Europe, examples of countries with high rates of vaccination (>80%) are Germany, United Kingdom, Ireland, Austria and Switzerland, while Russia, Denmark and Poland have low rates of vaccination (<30%). US, Canada, Mexico and Chile have a very high rate (80-98%), as well as Korea and Japan (70-90%), while China and Vietnam have a very low rate of vaccination (<5%).

The effectiveness of commercially available PCV2 vaccines has been extensively tested under controlled experimental conditions. Due to the limited cases of clinical diseases caused only by PCV2 virus, most studies on testing efficacy of PCV2 vaccines used model of co-infections with two or three causes. Using the model of coinfection has the advantage, given that it approximately corresponds to the field conditions in which certain pathogens of pigs may contribute to the development of the clinical forms of PCVAD. Co-infection with PRRS increases the severity of PCV2 infection in pigs, leading to increased secretion of circovirus through oronasal route and via faeces (Rovira et al., 2002; Sinha et al., 2011). Vaccination of piglets with Suvaxyn[®] PCV2 according to the PCV2/PRRS model, induces the generation of neutralizing antibodies with the simultaneous reduction of lung lesions and the quantity of the virus in faeces, serum, and lymph tissue, 28 days after inoculation (Opriessnig et al., 2008b). Several studies compared the efficacy of Suvaxyn[®] PCV2, Circumvent[®] and CircoFLEX[®] vaccines in various models of co-infections. Compared to the unvaccinated pigs, all vaccines induce formation of neutralizing antibodies and reduce quantity of virus in serum and lymph tissue (Opriessnig et al., 2009). Co-infection with *Mycoplasma hyopneumoniae* and

PCV2 also induces the development of clinical forms of PCVAD and was used as a model for testing PCV2 vaccines. The pigs vaccinated according to this model with Suvaxyn® and CircoFLEX® vaccines showed a reduction of PCV2 virus in the serum, reduction of the lymph lesions as well as a gain of body weight as compared to the control group (Kim et al., 2011). Various studies testing vaccine efficacy under controlled conditions clearly demonstrate the usefulness of these commercially available vaccines.

The potential protective effect of piglets after vaccination relies on the protective effect of PCV2 antibodies, either acquired passively (by vaccination of sows) or induced actively (vaccination of piglets). However, the low concentration or absence of antibodies after vaccination does not mean that the animal is not protected from PCV2 infection. Fenaux et al. (2004) found that, after vaccination with chimeric PCV1-2 virus, there was no seroconversion in all pigs, but also no clinical signs of disease were observed in these pigs after exposure to the PCV2 virus, nor PCV2 viremia was observed. These authors suggest the potential role of cell-mediated immunity in protection of pigs after vaccination. Protective immunity generated after vaccination reduces the possibility of an outbreak of PCV2 infection and is expected to protect the pigs in complex terrain conditions (Nathan et al., 2012). The duration of immunity following vaccination has not been well studied. Opriessnig et al. (2009) reported that NA are on a detectable level in piglets 3 months after an one-time vaccination, and that the vaccinated piglets are protected from circovirus infections compared to unvaccinated individuals. Martelli et al. (2011) monitored the serological response in piglets using the ELISA method, from the time of vaccination up to 35th week of life. Vaccinated and control groups had similar titers at the time of vaccination due to residual maternal antibodies, while significant increase in antibody titers in vaccinated piglets was observed two weeks after vaccination. The titer of the antibodies in the vaccinated animals was increased continuously reaching a peak between the 6th and 9th (12 to 13 log₂) week after vaccination, after which the level of total antibody level was slightly reduced, never going below 6 log₂.

The most successful vaccines are those based on the induction of an active immune response to the capsid protein (Cap) of circovirus type 2. This protein is designated as a major immunogen inducing protective antibodies, in contrast to the Rep protein which is poor immunogen (Kixmoller et al., 2008; Opriessnig et al., 2008b).

Current commercially available vaccines are based on the PCV2 genotype and all show good efficacy, although currently PCV2b genotype is a dominant type of infection worldwide. Subunit and inactivated vaccines have the advantage of stability and safety, but there are other vaccine technologies that are still in experimental stage which have been proven to stimulate anti-PCV2 immune response and prevent PCV2 infections. These are particularly the DNA-based vaccines, modified attenuated live vaccines, marker vaccines and vector vaccines. RNA-based anti-viral therapy and modified live vaccines are able to stimulate cellular and humoral immune responses in contrast to the subunit and inactivated vaccines (Beach et al., 2011; Nathan et al., 2012).

Table 1. Commercially available PCV2 vaccines in Europe

PCV2 Vaccines	<i>Ingelvac CIRCOFLEX®</i>	<i>Suvaxyn PCV2 One Dose</i>	<i>Porcilis PCV® Circumvent®</i>	<i>CIRCOVAC®</i>
Company	BOHRINGER INGELHEIM	FORT DOGE	INTERVET	MERIAL
Antigen	PCV2 Cap protein	Inactivated PCV1-2 chimera	PCV2 Cap protein	Inactivated PCV2
Licensed for:	Piglets (2 weeks old and older)	Piglets (4 weeks old and older)	Porcilis PCV®: Piglets 3 days old and older Circumvent®: (3 weeks old and older)	Sows, gilts
Dosage	One dosage of 1 ml IM	One dosage of 2 ml IM	2 ml IM Porcilis PCV®: Two dosages (3 days and 3 weeks) / One dosage (3 weeks of age) Circumvent®: Two dosage (3 and 6 weeks of age)	2 ml IM Primary vaccination: (3-4 weeks before insemination) Revaccination: 2-4 weeks before farrowing

Immunoprophylaxis in sows and gilts. Vaccination of sows is one of the strategies for prevention of PCVAD in piglets reducing viremia and increasing the amount of specific neutralizing antibodies in the colostrum. Passive immunity in the form of maternal antibodies has been proved to be at least partially protective against PCV2 infections. Under experimental conditions, piglets with high levels of maternal antibodies had lower incidence of PCV2 viremia compared to those with low levels of maternal antibodies, which proves that the protection against PCV2 infection depends on the amount of antibody titers. High titers generally provide solid protection against PCV2 infection, whereas lower titers do not protect against these infections (McKeown et al., 2005; Opriessnig et al., 2008a). Vaccination of pregnant sows on farms with acute PMWS increases the production performance of piglets, reduces the mortality of piglets before weaning and increases the body weight of their offspring (Pejsak et al., 2010). Also, vaccination of sows and gilts increases the number of piglets born alive and number of piglets per sow per year, and reduce the number mummified piglets (Tacker et al., 2008; Stojanac et al., 2013b; Stojanac et al., 2014). In clinical PCV2 infections vaccination boosts the immune response, average daily gain and decreases the fattening period of the

offspring (Kurmann et al., 2011). Vaccination of sows also reduces the quantity of viruses that is transmitted from sows to piglets during gestation and suckling period, but does not completely eliminate the virus excretion via the colostrum (Gerber et al., 2011).

Immunoprophylaxis in piglets. Vaccination of piglets in subclinical cases, leads to improved daily gain and feed conversion and reduces the mortality rate, and in clinical cases further reduces the number of rejects. Today, vaccines are commonly administered to weaned piglets and have been shown to enhance the production parameters in pigs naturally exposed to PCV2 (Stevančević et al., 2013). On farms affected with PMWS, vaccination with a Circovac® vaccine increased average daily gain and reduced the mortality rate of post-weaning piglets (Pejsak et al., 2010). A large number of studies demonstrate a significant reduction of mortality, duration of viremia, and increased daily gain after vaccination with CircoFLEX® vaccine (Fachinger et al., 2008; Kixmoller et al., 2008; Stevančević et al., 2013; Stevančević et al., 2014a). The time of vaccination of piglets is often questionable, considering possible interactions of maternal antibodies which protect the piglets from the development of PMWS (Stevančević et al., 2014b; Stevančević et al., 2014c). High titer of maternal antibodies interferes with the active seroconversion after vaccination, although the vaccine significantly reduces viral load and spread of the virus (Fort et al., 2009). In a study which used Ingelvac Circoflex vaccine, there was no difference in efficacy regardless of whether the pigs were vaccinated in the 3rd or the 6th week of age, indicating that maternal antibodies have no significant impact (Cline et al., 2008). Stevančević et al. (2014d) state that vaccination of piglets on 21st day of life had a certain advantage compared to vaccination on 15th day of life, with the observation that on the 15th day of life there is far greater influence of maternal antibodies on the creation and development of immune response in piglets following vaccination.

CONCLUSION

PCV2 infection control means control of all cofactors involved in the development of the disease and the so-called triggers with efforts for their eradication as well as immunoprophylaxis measures which are considered indispensable in controlling this disease. Basic issue regarding the successful vaccination is to determine the optimal time for administration of the vaccine. It is best to determine the time of vaccination based on the amount of colostrum antibodies titer in the blood serum of piglets and time of occurrence of PCV2 infections. Vaccination presents a measure of long-term protection of animals from weaning to the end of the fattening period and continuous administration of the vaccine in the herd leads to a reduction in infectious pressure in the herd and stabilization of the health status. At this moment eradication of this disease is farfetched, but vaccination is the main form of struggle against PCV2 infection.

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KONTROLA CIRKOVIRUSNIH INFEKCIJA (PCV2) SVINJA

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Izvod

Svinjski cirkovirus tip 2 (PCV2), jedan je od vodećih virusa koji narušavaju zdravstveno stanje svinja u poslednjoj deceniji i time dovode u pitanje obim i ekonomičnost proizvodnje svinja. Kontrola PCV2 infekcija podrazumeva kontrolu svih kofaktora u nastanku bolesti i takozvanih okidača uz napore za njihovo iskorenjivanje kao i mere imunoprofilakse koje se smatraju nezamenjivim u kontroli ove bolesti. Danas je na Evropskom tržištu dostupno 5 komercijalnih vakcina i sve su bazirane na PCV2a genotipu i pokazuju dobru efikasnost. Osnovno pitanje uspešne vakcinacije je odrediti optimalno vreme za aplikaciju vakcine. Vreme vakcinacije najbolje je odrediti se na osnovu visine titra kolostralnih antitela u krvnim serumima prasadi kao i vremenu nastanku PCV2 infekcija. U ovom trenutku iskorenjivanje ove bolesti je neostvarivo, pa vakcinacija predstavlja glavni vid borbe protiv PCV2 infekcija.

Ključne reči: PCV2, vakcina, kontrola, prasad, imunitet.

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THE IMPLEMENTATION OF A BIOSECURITY PLAN ON PIG FARMS BASED ON RISK ANALYSIS AND CONTROL OF CRITICAL POINTS (HACCP)*

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SUMMARY: Biosafety is a process on farm which aims to prevent the occurrence and spread of the disease on the farm. An effective biosecurity program consists of several elements and the need to make the whole program to ensure safety of products on the farm. This paper presents a model for the establishment of biosafety programs through the implementation of a system for ensuring product hazard analysis and critical control point (HACCP) at a pig farm. This work should be the basis for the implementation of the biosecurity program on farms, in order to develop its own program according to the situation on the farm.

Key words: biosecurity, pig farm, HACCP.

INTRODUCTION

The basic principle in the definition of “Biosafety“ is the desire to prevent the onset of disease (Dargatz et al., 2002). Biosecurity on pig farm represents all the measures taken with the aim of preventing the entry of infectious agents in the farm and control the spread of infection within the farm. The aim of biosafety program is to prevent exposure to animal pathogens that are not on the farm, and to minimize the impact of endemic pathogens. Biosecurity pig farm can be defined as the planning and implementation of programs in order to minimize the different types of risks that have a detrimental effect on the farm and the animals on it. Procedures within the biosecurity are related to improving the health and productivity of pigs. Biosecurity programs can be viewed from multiple viewpoints. Frequently, biosafety is referred to as the obligation that the state implements

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its policies in order to protect their resources and protect the health of consumers (Hueston and Taylor, 2002, Ušćebrka et al, 2006). General framework that is used to protect the health of consumers is adherence to the principles adopted by the Codex Alimentarius Commission (CAC, 2002). These principles are defined in Good hygienic practices in meat production (CAC, 2005). This document is required to manage these risks before slaughter and during the slaughter process, with the application of the HACCP system (CAC, 2003).

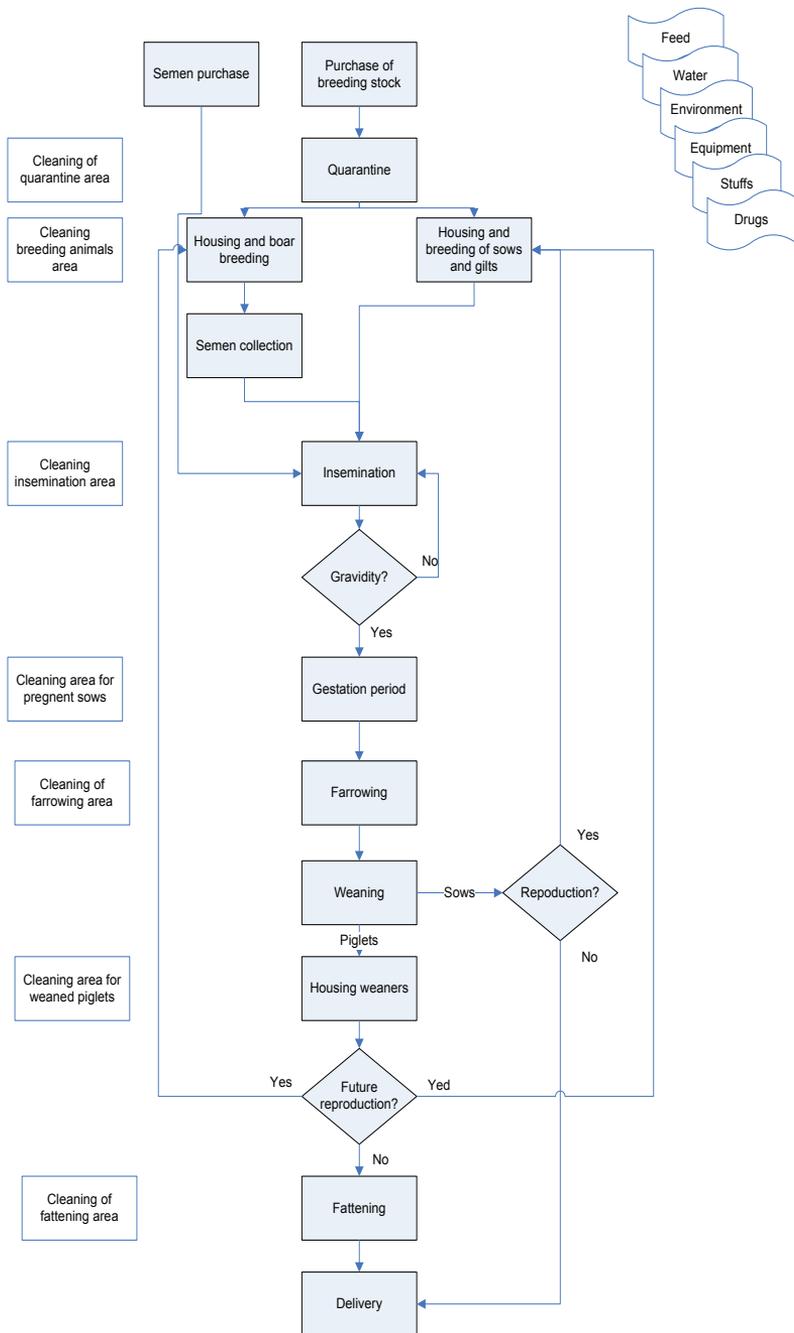
The HACCP system is based on hazard analysis and critical control point. Hazards can be divided into physical, chemical and biological. Unlike chemical hazards, which usually represent the residue in meat substances that are harmful to human health, which are clearly defined by certain rules, biological hazards, which is mainly the pathogenic microorganisms, are not clearly identified in the appropriate regulations. Therefore, not clear or control these hazards by independent auditors (Horcher and Pointon, 2011). A number of factors are involved in the development and maintenance of an effective program for ensuring food safety on the farm (Ušćebrka et al, 2012a) and biosecurity program (Ušćebrka et al, 2012b). All factors can be seen as a link in the joint chain, a biosafety program is only as strong as its weakest link (Levis and Becker, 2011; Ušćebrka et al, 2012c; Žikić et al, 2012). Application of the Hazard Analysis and Critical Control Points (HACCP) and sanitation protocols are important measures in controlling the entry and spread of infectious in herds agents (Stanković et al, 2008; Ušćebrka et al, 2009; Bojkovski et al, 2011).

It is now known that the biosecurity, good manufacturing practices and HACCP are very important elements in intensive pig production, where a large number of animals kept at a relatively small area (Bojkovski et al, 2010a; Bojkovski et al, 2010b). However, for a well-developed HACCP model, it is necessary to establish and maintain prerequisite programs, such as good manufacturing practices, good hygienic practices, good laboratory practice (Kanački et al, 2008; Ušćebrka et al, 2008; Ušćebrka et al, 2011, Kanački et al, 2013). In addition to HACCP models, as well as methods at the farm level may indicate the necessary elements of biosafety program, and other models can be successfully applied in the assessment of biological risks (Ušćebrka et al, 2012d).

The aim of this paper is to present the biosecurity plan on the pig farm that was made based on the analysis Risk and Critical Control Points (HACCP).

STEPS IN THE IMPLEMENTATION OF BIO-SAFETY PLAN BASED ON HACCP

Step 1: Establish of the HACCP team. In order to develop a HACCP plan, it is necessary to appoint a HACCP team which should find people who have adequate knowledge of HACCP, veterinary medicine, microbiology, epidemiology of food borne diseases, pig production. Team members should be selected based on their experience in the development of HACCP systems, implementation of preventive procedures, participation in risk assessment, access to research in the field of food safety training in HACCP systems, as well as knowledge of pig production.



Scheme 2 Example flowchart commercial pig farms.

Step 2 and 3 Establishment of scale, product descriptions and intended use. The scope of the HACCP study should be based on the existing production process, and if the farm has a closed production cycle or participate in certain parts of the production process (breeding, production of breeding material, production of pigs, ...). The largest number of commercial farms in Serbia have the entire production chain and the end product is fattened that comes slaughterhouse and is intended for consumption by consumers

Step 4 and 5 Defining flowchart and its verification. In accordance with the manufacturing processes in the pig farms flowcharts are formed. The flowchart containing all the processes and all inputs and outputs from these processes. Example of flowchart is shown in Scheme 2 and it is desirable to create a table with the given process and all activities related to these processes. After creating the flowchart, it is necessary on the farm to do its verification.

Step 6 Identification of risk –principle 1. Principle 1 includes identification of potential hazards in swine production and will be the basis for determining the critical control points (CCP). Identification of potential microbiological hazards is the most important part of HACCP study. Thus, for each farm must be made to identify potential microbial hazards on the basis of the epizootic status of the farm, the occurrence frequency of certain pathogens and other relevant information. Based on the identified microbiological hazards are evaluated risks to animal health and consumer. In assessing the risk taken, the probability of occurrence and severity of the health of the people / animals when danger appears. Risk assessment is used to Table 1, the values are calculated according to the formula $Risk = O \times S$, where O - the probability of occurrence and S - seriousness.

Values for probability of occurrence are:

- 1 practically impossible
- 2 low probability
- 3 can be complex but not so far happened
- 4 happened on our farm
- economic loss can lead
- 5 commonplace

Values for seriousness are:

- 1 not important
- 2 can disrupt the delivery of products
- 3 can lead to economic loss
- 4 can lead to disease or significant
- 5 closure of production

Table 1. Significance of risk on the base of probability of occurrence and seriousness

		Seriousness (S)				
		1	2	3	4	5
Probability of occurrence	1	1	2	3	4	5
	2	2	4	6	8	10
	3	3	6	9	12	15
	4	4	8	12	16	20
	5	5	10	15	20	25

All values of 8 to 25 are significant risks and must be taken into account when establishing CCPs. CCP is determined through a decision tree. HACCP team should assess and establish appropriate control measures in order for the hazards which are significant further reduce risks. Potential hazards and diseases in swine production, mode of spread and control measures used in the prevention of these diseases are given in Table 2.

Table 2. Potential diseases on the pig farm, means of spread and control measures (modification of Horcher and Pointon, 2011)

Disease	Means of spread	Control measures
Aujeszky's disease (AD)	Direct contact, semen, aerosol, contaminated food dead rats, mice and cats.	Control the movement of pigs and semen. Filters in places where grain food enters the silo.
Vesicular stomatitis (VS)	Insect bites, infected objects and equipment (catheters, needles, clothing, boots, ...).	Control the movement of pigs and semen.
Swine vesicular disease (SVD)	Direct contact between pigs, semen, meat products, food swill , infected objects.	Control movement of pigs, semen, people and objects from different farms and different animals. Ban on use of swill.
Foot-and-mouth disease (FMD)	Direct contact between pigs, semen, meat products, food, swill, through people or heavily contaminated water.	Control of animal movements, semen, people and objects from other farms and within farms. Ban on use of swill. Chlorination of water or the prevention of direct access to animal source of drinking water.
African swine fever (ASF)	Direct contact between pigs, semen, meat products, swill feeding, infected items and equipment.	control movement of animals, semen people and objects from other farms and within farms. Prohibition of use of swill feeding.
Classical swine fever (CSF)	Direct contact between pigs, semen, meat products, feeding swill through infected objects and equipment (catheters, needles, clothing, boots, ...)	Control the movement of pigs, semen and people. Ban on feeding of swill.
Rabies (B)	Dog or bat bites.	Enclosure in order to prevent the arrival of dogs.
Trichinosis (T)	Contact with infected rats or swill.	controls the movement of pigs and meat products.
Swine flu (SI)	Direct contact between pigs. The theoretical danger that people can transmit.	Control the movement of pigs. Disease control policy employed on the farms.
Porcine respiratory coronavirus (PRCV)	Direct contact between pigs.	Control the movement of pigs.
Pigs reproductive and respiratory syndrome (PRRS)	Direct contact between pigs, semen, aerosol.	Control the movement of pigs and semen.
Transmissible gastroenteritis (TGE)	Direct contact between pigs.	Control the movement of pigs.

Results of significance assessment of risk in accordance with Table 1 are shown in Table 3, these are only approximate values, responsible person, based on their experience and the situation on the farm to determine the probability of occurrence and severity.

Table 3. Potential diseases, probability of occurrence (O), seriousness (S) and risk.

Disease	Probability of occurrence	Seriousness	Risk(OxS)
Aujeszky's disease (AD)	3	5	15
Vesicular stomatitis (VS)	1	5	5
Swine vesicular disease (SVD)	3	5	15
Foot-and-mouth disease (FMD)	3	5	15
African swine fever (ASF)	3	4	12
Classical swine fever (CSF)	3	4	12
Rabies (B)	2	4	8
Trichinosis (T)	2	4	8
Swine flu (SI)	4	3	12
Pig respiratory coronavirus (PRCV)	3	3	9
Pigs reproductive and respiratory syndrome (PRRS)	3	4	12
Transmissible gastroenteritis (TGE)	3	4	12

In accordance with the results shown in Table 3 only vesicular stomatitis not to be taken in a further analysis of the threat and determining the CCP. But for all the potential hazards in accordance with the flow chart, it should make a table of hazard analysis, for all inputs in the individual processes (Table 4).

Table 4. Hazards, mode of entry in the heard and control measures (modification of Horcher and Pointon, 2011)

Entry	Hazards	Control measures
Animals supplying	AD, VS, SVD, FMD, ASF, CSF, B, T, SI, PRCV, PRRS, TGE	Animals supplying from proven sources. Quarantine and validated by trained personnel.
Sperm	AD, VS, SVD, FMD, ASF, CSF, PRRS	Getting semen from proven suppliers
Food	AD	Grilles to prevent contamination of food with dead animals (rats,)
Water	SVD, FMD, ASF, CSF	Preventing contact of wild or domestic animals with a source of water to be used by the animals on the farm, chlorination.
Staff and visitors	AD, VS, SVD, FMD, ASF, CSF, B, SI, PRCV, PRRS, TGE	Privacy visits, entry control, the use of boots and protective clothing equipment.
Equipment	SVD, FMD, ASF, CSF	Only approved and / or disinfected equipment can be used.
Litter	FMD, SVD	Litter procured from farms where there is no doubt that animals infected with contagious diseases.
Vehicles	FMD, SVD, VS, AD, ASF, CSF, B, SI, PRCV, PRRS, TGE	Disinfection of vehicles. Drivers do not leave the vehicle or use special footwear and clothing.
Pests and other animals	AD, VS, SVD, FMD, ASF, CSF, B, T, SI, PRCV, PRRS, TGE	Disinfection of vehicles. Drivers do not leave the vehicle or use special footwear and clothing.
Aerosol	AD, SVD, FMD, SI, PRRS	Farm is geographically isolated.

Step 7 Determination of critical control points (CCP) - principle 2. A critical control point is a stage process in which the application of control measures necessary to prevent, eliminate or reduce risk to an acceptable level of risk. CCP is the place where control measures must be applied. Based on Table 4, which show the hazards of depending on the input in the process, forming a list of processes in which there is hazards exist, control points that are controlled in these processes and critical limits (Table 5).

Table 5. Processes, hazards, critical control points (CCP) and critical limits (modification of Horcher and Pointon, 2011)

Process	Hazards	CCP	Critical limits
The introduction of pigs to the farm	AD, VS, SVD, FMD, AKS, CSF, SI, PRCV, TGE, PRRS	Pigs order. Check on arrival by trained personnel.	Purchase only from trusted suppliers. Only qualified personnel may decide to enter the pigs on the farm. Only healthy animals without signs of the disease may enter the pig farm. Inputs to the farm with the prior use of quarantine.
Semen supplying	AD, VS, SVD, FMD, AKS, CSF, SI, PRCV, PRRS	Semen ordering	Ordering semen only from farms that are available on the above mentioned diseases and have developed system of biosafety.
Entering of equipment on farm	AD, VS, SVD, FMD, AKS, CSF, SI, PRCV, TGE, PRRS	When entering	Equipment visual clean and disinfected appropriately.
Entry of employees and visitors	AD, VS, SVD, FMD, AKS, CSF, SI, PRCV, TGE, PRRS	When entering	When entering only healthy employees and visitors were allowed to enter. They must be provided with a clean farm boots and clothes to be washed and sanitized hands on the entry
Transport of pigs	AD, VS, SVD, FMD, AKS, CSF, SI, PRCV, TGE, PRRS	Order of means of transport and arrival at the place of loading.	Only selected carriers can sort shipping. Transport means visually clean and with a certificate of disinfection.
Litter entrance	FMD	Litter order	Only selected vendors, visual inspection of litter.

Step 8 Establish critical limits - principle 3. Critical limits are established for each control measure and represent the limits that are acceptable for the control measure (Table 5 and Table 6).

Table 6. HACCP Plan (modification of Horcher and Pointon (2011))

Hazards	C o n t r o l measure	Critical limits	Monitoring	Correc-tive actions	Records
AD, VS, SVD, FMD, ASF, CSF, SI, PRCV, PRRS, TGE	Supply of pigs tested settings from herds with known health status	The supplier shall make available to the veterinarian for consultation	What: pigs Where: at the herd How: questionnaire When: when ordering Who: trained staff	Refusal of orders.	A list of selected suppliers.
AD, VS, SVD, FMD, ASF, CSF, SI, PRCV, PRRS, TGE	Checking pigs by trained staff	Pigs must be healthy	What: pigs Where: at the entrance How to: check and questionnaire When: at arrival Who: trained staff	Refusal of pigs.	Date of checks, copies of documents and a copy of the action taken
AD, VS, SVD, FMD, ASF, CSF, SI, PRCV, PRRS, TGE	Getting sperm from selected suppliers	Supplier of sperm works in accordance with high standards of biosecurity	What: supplier of sperm Where: at the entrance How: questionnaire When: when ordering Who: trained staff	Refusal of semen.	Date of checks, copies of relevant documents, the action taken
AD, VS, SVD, FMD, ASF, CSF, SI, PRCV, PRRS, TGE	Only previously announced Visitors necessary for the operation of the farm can enter the farm	For previously announced visitors provided clean boots and clothing. Visitors to wash their hands before entering	What: visitors Where: at the entrance How: by filling records When: on arrival Who: trained staff	No entry	Diary of visits; activities undertaken
AD, VS, SVD, FMD, ASF, CSF, SI, PRCV, PRRS, TGE	Means of transport must be clean and disinfected	The selected carrier that operates in accordance with good practice Vehicles must be visually pure certificate of cleaning and disinfection	What: vehicle Where: at the entrance How to: Verify a certificate, a questionnaire and a list of approved carriers When: on arrival Who: trained staff	No entry	A list of approved carriers, date checks, certificates of disinfection and activities undertaken
...

Step 9 Establish a monitoring system - principle 4. Observation or monitoring of critical limits is a check that the threat under control at this stage of the process. For each critical point, it is necessary to answer the five questions and the what, how, where, when and by whom. These questions and answers are entered in the HACCP plan, which is shown in Table 6.

Step 10: Defining corrective action - principle 5. Corrective measures represent actions to be taken when it is determined that the critical limits are not respected, and that the hazards is not under control. The goal is to apply corrective measures correcting a mistake at this stage and tried to prevent in the future. Corrective measures are entered in the table HACCP plan (Table 6). The table entries are HACCP plan and records should document the fulfillment of requirements in terms of achieving and maintaining critical limits.

In this way the HACCP plan is also unique biosafety document. Applying all entries HACCP plan establishes an efficient and effective biosecurity plan.

Step 11: Establish verification procedures - principle 6. HACCP plan that contains all the elements of biosecurity should be verified and the verification can be done in one of these ways:

- independent verification of control measures by the management of the farm or independent vocational entities;
- independent review of critical limits for all CCPs by management or independent professional person;
- checking by a third party that will require a detailed review of the documentation on the functioning of the various elements of the HACCP plan.

Step 12 Establish a system of documentation and record keeping - Principle 7. For the successful functioning HACCP system is one of the most important activities is the proper documentation. Accordingly, it is necessary to develop procedures for maintaining documents and records.

INSTEAD OF A CONCLUSION

From the above it can be concluded that biosecurity is one of the most important activities on the farm. Biosafety is impossible to separate from the integrated system for ensuring food safety and is an important part of his. Since the program biosecurity prevent, eliminate or reduce to an acceptable level only biological hazard and to those that cause animal diseases and / or consumers, food safety assurance system at the farm is a broad term. However, for an efficient and effective biosecurity plan is necessary to do all the steps necessary to establish a system for ensuring food safety.

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IMPLEMENTACIJA PLANA BIOSIGURNOSTI NA OSNOVU ANALIZE RIZIKA I KONTOLE KRITIČNIH TAČAKA (HACCP) NA FARMI SVINJA

**GORDANA UŠĆEBRKA, DRAGAN ŽIKIĆ,
ZDENKO KANAČKI, SLOBODAN STOJANOVIĆ**

Izvod

Biosigurnost je proces na farmi koji ima za cilj da spreči pojavu i širenje bolesti na farmi. Efikasan program biosigurnosti sastoji se iz više elemenata i treba da čini celinu sa programima za obezbeđenje bezbednosti proizvoda na farmi. U ovom radu biće prikazan model uspostavljanja programa biosigurnosti kroz primenu sistema za obezbeđenje proizvoda analizom opasnosti i kontrolom kritičnih tačaka (HACCP) na farmi svinja. Ovaj rad treba da bude osnova za implementaciju programa biosigurnosti na farmama, sa ciljem razvoja sopstvenog programa u skladu sa situacijom na samoj farmi.

Ključne reči: biosigurnost, farma svinja, HACCP.

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TAST, A.: Endocrinological basis of seasonal infertility in pigs. PhD Thesis, Faculty of Veterinary Medicine, Finland, Helsinki, 2002.

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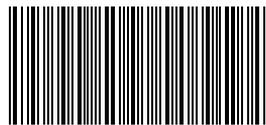
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