



University of Novi Sad- Univerzitet u Novom Sadu

Faculty of Agriculture- Poljoprivredni fakultet



CONTEMPORARY AGRICULTURE SAVREMENA POLJOPRIVREDA

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AFLATOXINS – MAIZE SAFETY ECONOMY CHALLENGE IN 2012.

IGOR JAJIĆ, BILJANA PERIŠIĆ, SAŠA KRSTOVIĆ, JOVANA KOS¹

SUMMARY: The aim of this study was to investigate the presence of aflatoxins in maize, and their safety and economic impact. As our country is among the largest exporters of corn, the consequences which infected corn leaves certainly are not negligible. Monitoring implementation is the only solution that will stop and prevent further aflatoxins contamination. In this paper, 44 commercial maize samples were analyzed for aflatoxins presence. It was found that 63.6% of samples infected. The level of aflatoxins in infected samples ranged from 5-367 ppb, while the average value of 74.5 ppb, with a standard deviation of 79.5.

Key words: aflatoxins, maize, Serbia, export.

INTRODUCTION

Aflatoxin is persistent mycotoxin produced by several *Aspergillus* fungi, primarily *Aspergillus flavus*, under certain conditions when it grows in ears or on grain. They are commonly found in peanuts, cottonseed, corn and their products. In wheat, rice, soybeans oats, and sorghum aflatoxins only rarely are noted (Sweets and Wrather, 2009).

Infection of corn by *Aspergillus spp.* occurs through the silk. The spores germinate, and the fungus colonizes the silks in hot conditions. The fungus can then grow down the silk channel and around the developing ear. Yellow-brown silks that are still moist are most susceptible to colonization and invasion down the silk channel. Fresh, not pollinated silks are relatively resistant, and brown, dry silks can be colonized, but growth of the fungus down the silk channel is limited (Vincelli, 1995). The fungus appears to grow from the ear tip toward the base by colonizing the silk first, then the glumes and the kernel surface. After the silks die, the growth of the fungus is rapid in hot weather. If conditions are favorable for the production of aflatoxin, it is much more difficult to prevent infection from *Aspergillus spp.* and subsequent aflatoxin production (Anderson, 1983; Gardisser, 1989).

Original scientific paper / Originalni naučni rad

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Another means of infection is kernel injury from insects or birds provides that are easily colonized by the fungus. Even if kernels are uninfected at harvest, the presence of *Aspergillus flavus* spores on kernel surfaces sets the stage for post harvest contamination with aflatoxin. When temperature and moisture conditions permit, *Aspergillus flavus* spores can germinate and infect injured or broken kernels within a day or two of harvest (Vincelli, 1995). According to some studies, *Aspergillus* observed somewhat greater representation during the summer period (Škrinjar et al, 2008). Aflatoxin production by the fungus is most active at 25-32°C, but development of the fungus usually stops when the temperature is below 13°C and grain moisture is 15% or less (Herman and Trigo-Stockli, 2002).

Corn (*Zea mays* L.) is a major crop in Serbia, where it plays an important role in the economy, in animal feed production, alcohol fermentation and direct human consumption. Although aflatoxin contamination in corn is uncommon in Serbia, occasional incidents do occur and can create significant economic losses for individual producers. Our limited survey data and general experience indicate that the incidence of aflatoxin contamination in Serbia is similar to that of other European countries. Furthermore, one or more cases of contamination occur when shelled corn is not promptly dried or properly stored. Rapid detection of contaminated corn is important because aflatoxin normally survives processing and may be concentrated in products or processed fractions (Payne, 1992; Abbas et al., 2002).

The economic impact of aflatoxins derive directly from crop and livestock losses as well as indirectly from the cost of regulatory programs designed to reduce risks to animal and human health. Throughout the world there are many advisory bodies concerned with food safety, including the World Health Organization (WHO), Codex Alimentarius and the European Food Safety Authority (EFSA), and they regularly assess the risk from mycotoxins and advise on controls to reduce consumer exposure. According to the Commission Regulation (EC) No 1881/2006 maximum level for certain contaminants regarding to aflatoxin in maize for human consumption is 10 ppb (µg/kg). In Serbia, maximum permissible level of aflatoxin is 50 ppb (µg/kg) in feed (Službeni glasnik, 4/2010). Although maximum levels for aflatoxins and some other mycotoxins are included in Serbian legislation, there is not enough information about the results of mycotoxicological food and feed control (Jakšić et al., 2011).

Aflatoxins losses to livestock and poultry producers from aflatoxin-contaminated feeds include death and the more subtle effects of immune system suppression, reduced growth rates, and losses in feed efficiency. Other adverse economic effects of aflatoxins include lower yields for food and fiber crops (Anon, 1989). At present, economically disastrous events like the widespread aflatoxins contamination in region are unpredictable even in the short term. However, it will be necessary to study the effect of heat stress on mycotoxin level in corn over a longer time period to establish the significance of maize safety.

The aim of this study was to examine the content of aflatoxin in maize samples, and inquire the effect of infection on maize export from Serbia.

MATERIAL AND METHOD OF THE STUDY

Samples: Samples of maize from the 2012 harvest were collected from different locations in the Republic of Serbia. Samples were taken immediately after the harvest. There were 44 samples of maize. Immediately after sampling, 1000 g of each sample were prepared by grinding in a laboratory mill in such a way that >93% passed through a sieve with pore diameter of 0.8 mm. After that, the sample was homogenized by mixing. Samples thus prepared were packed in plastic bags and stored in a freezer at -20°C until analysis. Prior to each analysis, the samples were allowed to reach room temperature.

Analysis: 20 g of samples were extracted with 100 ml of 70% methanol and filtered through the filter paper. The immunochemical analysis was performed using the Veratox, Aflatoxin (Total), Quantitative Test Kit (Neogen, Lansing, MI, USA) with four calibration standard solutions (0, 5, 15 and 50 ppb). In the mixing well were placed the conjugate (100 µl) and the standard or sample (100 µl). To make a homogeneous mixture, the liquid was drawn up and dispensed three times with the aid of a micropipette. A volume of the mixed solution (100 µl) was transferred to the well with antibodies and incubated at room temperature for 2 minutes. The solution was then removed and wells were rinsed 5 times by distilled water. After that, the substrate (100 µl) was added and three-minute incubation was stopped by adding "stop" reagent (100 µl). Optical densities on the basis of which aflatoxins content was calculated were read using the reader of microtitration plates with a 630 nm filter. According to the manufacturer the limit of determination was 5 µg aflatoxins/kg sample, and range of quantitation 5-50 ppb.

RESULTS

In this paper there were 44 samples of commercial maize processed, from different producers. As it shown in Table 1, for 63.6% samples presences of aflatoxins were detected. Results are represented in ppb (µg/kg).

Table 1. Analyzed samples of corn for aflatoxins content
Tabela 1. Analizirani uzorci kukuruza na sadržaj aflatoxina

Number of analyzed samples / <i>Broj analiziranih uzoraka</i>	44
% of infected samples / <i>% kontaminiranih uzoraka</i>	63.6
Range AFs (ppb) / <i>Opseg AFa (ppb)</i>	5-367
Average for positive samples (ppb) / <i>Prosečna vrednost pozitivnih uzoraka (ppb)</i>	74.5
SD / <i>SD</i>	79.5
Number of samples that exceeded the ML set by the EU regulative (%) / <i>Broj uzoraka koji premašuju MDK prema EU (%)</i>	20 (45.5)
Number of samples that exceeded the ML set by Serbian regulative (%) / <i>Broj uzoraka koji premašuju MDK prema srpskoj regulativi (%)</i>	13 (29.6)

Aflatoxins content in positive samples ranged from 5 to 367 ppb. The average value of aflatoxins in contaminated samples was 75.5 and standard deviations of these samples were 79.5. Level of aflatoxins exceeded in 45.5% analyzed samples, according to EU Regulative. In aforementioned samples, 29.6% exceeded level of 50 ppb set by Serbian regulation.

DISCUSSION

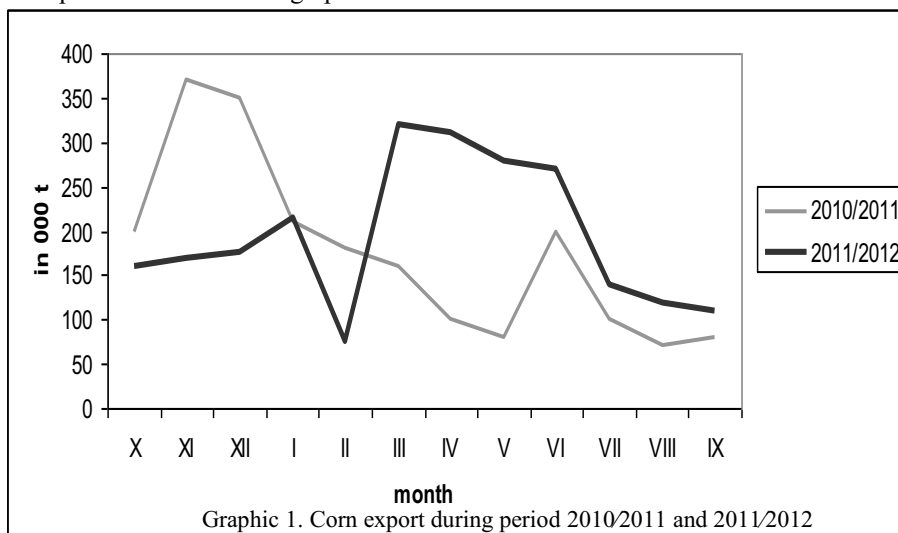
In September 2012, harvest area of maize was 1,275,888 ha with expected yield/ha of 2.79 t, which is 45.9% yield/ha less than the year before, and 52.4% less than in 2010, although the harvest area in 2012 was greater than in previous years. Expected production of maize in 2012, was reduced for 45.5% compared to previous year, and 51.0% lower in comparison to 2010. These results are represented in Table 2.

Table 2. Maize production from 2010. to 2012.

Tabela 2. Proizvodnja kukuruza od 2010 do 2012.

REPUBLIC OF SERBIA, Maize/ Year <i>REPUBLIKA SRBIJA, Kukuruz/ Godina</i>	2010	2011	2012
Harvest area, ha <i>Žetvena površina, ha</i>	1,262,000	1,258,167	1,275,888
Production, t <i>Proizvodnja, t</i>	7,207,191	6,479,564	3,532,602
Yield per ha, t <i>Prinos po ha, t</i>	5.86	5.15	2.79

In period from March to June, maize grain export was up to 52% higher than the season before (2010/2011). Summary, in 2011/2012 exports of corn was achieved economic record of 2,317,303 t, compared to the year 2010/11 exports increased for 16% (Statistical office of the Republic of Serbia, 2012). Corn export during these two periods has shown in graphic 1.



Increased frequency of controls related to aflatoxins: Most notifications on aflatoxins are related to product/ country of origin combinations for which imposed increased frequencies of controls at import are in force. As such, the number of notifications is enhanced by the increased frequency of control which resulted from the problem identified (RASFF, 2012). Report for feed product, from October 2012, has shown in Table 3. It represents alert for Serbian corn which is released from Italy. Aflatoxins content in corn was exceeded from permitted value, with 126.5, 161.1, 57.1 and 80.8 ppb, thus distribution status for Serbia was restricted to notifying country (RASFF, 2012).

Table 3. Reports feed Week 41 (10/08/2012 - 14/10/2012)

Tabela 3. Izveštaj za hranu za životinje, nedelja 41 (10/08/2012 - 14/10/2012)

Date	Ref.	Notification Type	Notification reason	Notified By	Topic subject	Undertaken actions
10/10 2012	2012 .1427	Information for Attention	Border control Consignment released	Italy	Aflatoxins (B1 = 126.5 / B1 = 161.1 / B1 = 57.1 / B1 = 80.8 µg/kg - ppb) in corn from Serbia, via Croatia	Official detention

CONCLUSION

In addition to high percentage of aflatoxins presence in analyzed samples (63.6%), it is devastating fact that even 45.5% and 29.6% of samples exceeded the maximum tolerable level of aflatoxins, given in the EU, and Serbian regulations, respectively. From the safety point of view, these figures are extremely worrying, also there are crucial for the export of this culture.

For these reasons it is necessary to carry out monitoring in order to prevent occurrence of aflatoxins and other mycotoxins and applied all actions to prevent or decrease the occurrence of these hazardous contaminants to of humans and animals health. Also it is necessary to take care of economic parameters.

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AFLATOKSIN – BEZBEDNOST KUKURUZA, EKONOMSKI IZAZOV U 2012. GODINI

IGOR JAJIĆ, BILJANA PERIŠIĆ, SAŠA KRSTOVIĆ, JOVANA KOS

Izvod

Cilj ovog rada je bio ispitati kukuruz na prisustvo aflatoksina, i na koji način će ova informacija uticati, pre svega na bezbednost ovog hraniva, ali i na ekonomski parametar kada je u pitanju izvoz kukuruza. Naša zemlja spada među najveće izvoznike kukuruza, tako da prisutnost aflatoksina u kukuruzu ima značajne posledice po ekonomske parametre trgovanja ovim hranivom. Ispitana su 44 komercijalna uzorka kukuruza, pri čemu je utvrđeno da je 63,6% ispitanih uzoraka kontaminirano aflatoksinom. Nivo aflatoksina u zaraženim uzorcima se kretao od 5-367 ppb, dok je prosečna vrednost iznosila 74,5 ppb.

Ključne reči: aflatoksin, kukuruz, Srbija, izvoz.

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THE EFFECT OF BOVINE SERUM ALBUMIN IN CULTURE MEDIA ON BOVINE SPERMATOZOA MOTILITY PARAMETERS *IN VITRO**

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SUMMARY: The aim of this study was to analyze the influence of bovine serum albumin (BSA) used in different culture media on motility and viability of bovine spermatozoa during a 24-hour in vitro cultivation. We compared native samples (N) and samples cultivated in a commercial egg yolk medium (R) with samples cultivated in several experimental media containing 10% or 20% BSA. The analysis was carried out during three time periods (time 0, time 1 hour and time 24 hours) using Sperm VisionTM CASA system. Our study shows an obvious time-dependent decrease of the spermatozoa viability parameters in all experimental groups cultured for 24 hours. The lowest spermatozoa motility parameters were detected in native sample. Culture media with 10% BSA content showed only average quality and unsatisfactory results. Overall best motility parameters were detected when applying 20% BSA, especially in the medium D containing triladyl, 20% BSA and 5% glucose. Comparing results detected in this group with the results from the commercial egg yolk medium significant ($P<0.01$) differences for progressive motility, as well as for distance average path ($P<0.001$), velocity average path ($P<0.001$) and amplitude of lateral head displacement ($P<0.001$) were detected. This study demonstrates that BSA could be a good protein supplement for long-term bovine spermatozoa cultivation and further processing.

Key words: BSA, spermatozoa cultivation, protein supplementation, bulls, CASA.

Original scientific paper / *Originalni naučni rad*

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INTRODUCTION

Artificial insemination (AI) has become one of the most important pillars in animal biotechnology. Especially in the cattle AI industry, bull semen quality is highly important to ensure a good biological material for breeding as well as a certain biodiversity protection (Ibrahim et al., 2000; Lukáč et al., 2007). Before preservation and distribution for usage in AI, several laboratory tests are routinely conducted in an attempt to predict the fertilization potential of the processed semen. Such tests include but are not limited to sperm motility or viability, total sperm output, and morphology (Ibrahim et al., 2000). Working with semen samples in laboratory environment requires a provision of optimal conditions for their *in vitro* cultivation.

In vitro cultivation of spermatozoa is a relatively complicated process, since the sperm cells are extremely sensible to *ex vivo* conditions. Therefore, preparing a culture medium, which ensures high spermatozoa motility and viability during extended periods of cultivation, is complicated (Balaban et al., 1999). Semen cultivation media usually contain glucose or fructose as an energetic substrate, egg yolk as a protein supply and glycerol (McPhie et al., 2000; Matsuoka et al., 2006). However, preparation of a uniform semen cultivation medium may vary because of the quality of the egg yolk. Also, extended cultivation changes the structure of egg yolk and decreases its quality, what complicates the obtention of relevant results. For this reason, investigations have been conducted to find an alternative protein substrate for spermatozoa cultivation (Muller-Schlosser et al., 1995).

Bovine serum albumin (BSA) has been recently used as a protein alternative to egg yolk. It is a large globular protein (66 000 Da) with a good essential amino acid profile (Peters et al., 1977), which has been added to culture media of various mammalian cells to mimic some growth enhancing effects of serum. Previous studies have reported that the role of albumin appears to be protective as a result of its general capacity and ability to trap toxic substances in the culture media (Yamane et al., 1976) and lipid binding properties (Fox and Kelly, 2003). It may play a role in mediating lipid oxidation, since BSA has been shown to protect lipids against phenolic induced oxidation *in vitro* (Smith et al., 1992). Investigations using semen from various mammalian species have indicated that BSA stimulates sperm motility by an unknown mechanism (Harrison et al., 1982; Klem et al., 1986).

Most of the experiments have been designed to look at the function of BSA on sperm capacitation, fertilization and cryoconservation hence there is a lack of information about the effect of BSA as a culture medium component on the general spermatozoa *in vitro* viability.

Extended spermatozoa cultivation is essential for studies related with questions of male infertility origin. The aim of such analysis is to see how spermatozoa viability or fertilization capacity is changed by administration of a certain substance (i.e. heavy metals, antioxidants or growth factors; Lovášová et al., 2002) or under the influence of a specific factor (i.e. UV radiation, nutrition; Formicki et al., 2010). Therefore, results of such experiments could be helpful in further prevention and possible treatment of infertility.

This study was designed to test the effects of two BSA concentrations (10% and 20%) on the bovine spermatozoa motility parameters during a 24 hour *in vitro* cultivation.

MATERIAL AND METHODS

Bovine semen samples were obtained from 15 adult breeding bulls (Slovak Biological Services, Nitra, Slovak republic). The samples had to accomplish the basic criteria given for the corresponding breed. Semen was obtained on a regular collection schedule using an artificial vagina.

After collecting the samples were stored in the laboratory at room temperature (22-25°C) and basic measurements were performed – volume (ml), pH and concentration ($\times 10^9/\text{ml}$). Basic characteristics of the samples are shown in Table 1.

Table 1. Basic characteristics of bull semen samples used in the experiment

No	Breed	Volume [ml]	pH	Concentration [$\times 10^9 \text{ ml}^{-1}$]
1	Limousine	7.0	6.48	5.20
2	Pinzgau	8.0	6.44	5.70
3	Holstein	4.5	6.35	2.80
4	Fleckvieh	6.5	6.59	2.10
5	Fleckvieh	5.5	6.40	1.90
6	Fleckvieh	5.0	6.14	1.02
7	Holstein	8.0	6.30	2.50
8	Holstein	5.0	6.44	2.50
9	Holstein	8.0	6.50	4.90
10	Holstein	6.0	6.32	2.20
11	Holstein	6.0	6.52	2.20
12	Holstein	7.0	6.18	2.30
13	Holstein	6.0	6.21	1.30
14	Holstein	7.0	6.24	5.30
15	Holstein	10.0	6.15	2.60

Six cultivation media with a different composition (Table 2) were prepared, five of them containing BSA (Fluka, Sigma-Aldrich, USA). Fresh semen was added to each medium in a dilution ratio 1:40 and subsequently cultivated in an incubator (37°C) for 24 hours. Semen diluted in physiological saline solution (sodium chloride 0.9% w/v, Bieffe Medital, Italia) was used as native sample.

Table 2. Culture media used in the experiment

Group	Composition
N - Native group	Native sample diluted in physiological saline solution.
R - Commercial medium	Triladyl (MiniTüb, Tiefenbach, Germany), egg yolk and redistilled water.
A	Triladyl, 10% BSA (Sigma Aldrich, St. Louis, USA), 5% glucose (D-glukosa monohydrat p.a; Penta, Chrudim, Czech republic) and distilled water.
B	Triladyl, 10% BSA, 5% glucose, 1% trehalose (D(+)-trehalose; Sigma-Aldrich, USA) and distilled water.
C	Triladyl, 20% BSA and distilled water.
D	Triladyl, 20% BSA, 5% glucose and distilled water.
E	Triladyl, 20% BSA, 5% glucose, 1% trehalose) and distilled water.

Motility analysis was carried out using the CASA – SpermVision (MiniTüb, Tiefenbach, Germany) system with Olympus BX 51 phase microscope (Olympus, Japan) at cultivation times 0 hour, 1 hour and 24 hours. Each sample was placed into Makler Counting Chamber (depth 10 μm , Sefi-Medical Instruments, Izrael) and the following parameters were evaluated: percentage of motile spermatozoa (motility > 5 $\mu\text{m/s}$), percentage of progressive motile spermatozoa (motility > 20 $\mu\text{m/s}$), distance average path (DAP, μm), velocity average path (VAP, $\mu\text{m/s}$) and amplitude of lateral head displacement (ALH, μm).

Statistical analysis was carried out using the statistical program GraphPad Prism 3.02 (Graphpad Software incorporated, San Diego, California, USA). Results are quoted as arithmetic mean \pm standard deviation (SD). Comparison of semen parameters in groups was evaluated with the Dunnett's Post Test. The level of significance was set at A ($P<0.001$); B ($P<0.01$); C ($P<0.05$).

RESULTS AND DISCUSSION

In vitro effects of different BSA concentrations on bovine spermatozoa motility parameters were analyzed in three time periods (Time 0 hours, 1 hour, 24 hours). Evaluation of the percentage of motile spermatozoa showed decreased values in all experimental groups cultured for 24 hours (Table 3). Initial (time 0) spermatozoa motility was significantly ($P<0.001$) high in the group D containing 20% BSA, and 5% glucose, compared to group R containing the egg yolk commercial medium. This observation could be explained by an initially high concentration of energetic and protein substrate in the medium. The highest inhibitory effect of spermatozoa motility after 24 hours was detected in the group N (28.86%; $P<0.001$), followed by group B (42.89%; $P<0.001$) containing 10% BSA, 5% glucose and 1% trehalose. The lowest motility inhibition was found in group D (66.46%) and group R (62.81%).

Table 3. Spermatozoa motility (%) in groups and time periods (mean±SD)

24 h	0 h	1 h	
N/MOT	87.31±5.16	85.66±2.35	28.86±9.09 ^A
R/MOT	83.86±6.57	87.23±5.28	62.81±8.04
A/MOT	89.99±2.96	91.07±4.12	52.28±16.12 ^B
B/MOT	92.62±3.37	83.25±5.81	42.89±13.17 ^A
C/MOT	96.15±1.49	84.43±13.15	55.41±13.96
D/MOT	97.39±2.03 ^A	91.29±3.77	66.46±9.99
E/MOT	87.46±3.67	89.17±4.39	57.75±12.87

x – mean, S.D. – standard deviation

^A P<0.001, ^B P<0.01, ^C P<0.05

Different observations were made in the percentage of progressive spermatozoa motility (Table 4). The initial progressive motility was significantly ($P<0.001$) high in group D compared to group R. The parameter decreased slowly in all groups after 1 hour but a significant ($P<0.01$) difference between groups A (10% BSA and 5% glucose) and D compared to group R was detected. A radical spermatozoa progressive motility inhibition was observed after 24 hours in all of the groups. Comparing experimental medias with the commercial medium we recorded a significant ($P<0.01$) difference between group D and group R. Group A, which appeared to have good progressive motility parameters after 1 hour (87.18%), showed just an average progressive motility (37.79%) after 24 hours. The lowest progressive spermatozoa motility was detected in the group N (14.67%). The highest parameters were found in the group D (55.67%; $P<0.001$) - it was indeed the only group presenting a progressive motility over 50 %.

Table 4. Spermatozoa progressive motility (%) in groups and time periods (mean±SD)

Group	0 h	1 h	24 h
N/PROG	86.65±5.41	77.97±4.15	14.67±5.968 ^A
R/ PROG	80.99±5.54	78.55±6.47	35.33±11.44
A/ PROG	87.65±1.59	87.18±4.42 ^B	37.79±12.31
B/ PROG	89.71±2.95	79.52±8.12	26.43±10.33
C/ PROG	93.20±2.13	79.18±13.59	37.33±16.12
D/ PROG	93.85±2.05 ^A	89.08±3.99 ^B	55.76±12.06 ^A
E/ PROG	86.79±3.54	83.41±4.96	45.27±11.58 ^B

x – mean, S.D. – standard deviation

^A P<0.001, ^B P<0.01, ^C P<0.05

The DAP analysis revealed significant differences ($P<0.001$) between groups C (20% BSA) and D as compared to the R group at time 1 as well as at time 24 (39.66 and 34.24 μm versus 27.43 μm , 20.66 and 20.31 μm versus 14.24 μm , respectively). Distance average path after 24 hours was the shortest in the group R and the longest in the group C (Table 5).

Table 5. Spermatozoa distance average path (μm) in groups and time periods (mean \pm SD)

Group	0 h	1 h	24 h
N/DAP	45.59 \pm 3.91	27.43 \pm 0.61	14.24 \pm 1.36
R/DAP	38.36 \pm 11.86	28.70 \pm 1.56	14.88 \pm 1.92
A/DAP	43.37 \pm 1.25	37.06 \pm 3.39	14.63 \pm 3.49
B/DAP	42.50 \pm 2.25	35.98 \pm 4.29	14.86 \pm 1.88
C/DAP	37.12 \pm 2.11	39.66 \pm 3.48 ^A	20.66 \pm 2.86 ^A
D/DAP	31.22 \pm 0.63	34.24 \pm 1.51 ^A	20.31 \pm 2.62 ^A
E/DAP	40.82 \pm 6.71	32.62 \pm 2.41	16.85 \pm 2.13

x – mean, S.D. – standard deviation

^A P<0.001, ^B P<0.01, ^C P<0.05

Measurement of spermatozoa VAP (Table 6) after 1 hour demonstrated significantly (P<0.001) higher values in groups A, B and especially in group C when compared to group R (83.80, 81.28 and 90.70 $\mu\text{m/s}$ respectively versus 62.88 $\mu\text{m/s}$). After 24 hours of cultivation, VAP decreased in all of the groups but significant differences were found in groups C (45.04 $\mu\text{m/s}$) and D (43.45 $\mu\text{m/s}$) with the highest values of VAP compared to the commercial medium group with the lowest value (31.25 $\mu\text{m/s}$).

Table 6. Spermatozoa velocity average path ($\mu\text{m/s}$) in groups and time periods (mean \pm SD)

Group	0 h	1 h	24 h
N/VAP	104.40 \pm 9.38 ^A	62.88 \pm 2.17	31.25 \pm 3.55
R/VAP	86.45 \pm 27.00	66.97 \pm 4.12	32.30 \pm 4.65
A/VAP	101.90 \pm 2.23 ^A	83.80 \pm 8.21 ^A	32.51 \pm 9.05
B/VAP	100.00 \pm 4.46 ^A	81.28 \pm 11.11 ^B	31.86 \pm 3.79
C/VAP	88.12 \pm 4.68	90.70 \pm 6.77 ^A	45.04 \pm 6.57 ^A
D/VAP	72.92 \pm 1.83 ^B	77.66 \pm 4.18	43.45 \pm 5.73 ^A
E/VAP	91.98 \pm 15.45	77.51 \pm 5.64	36.93 \pm 3.99

x – mean, S.D. – standard deviation

^A P<0.001, ^B P<0.01, ^C P<0.05

Important statistical differences were observed in the amplitude of lateral displacement (Table 7). ALH was significantly (P<0.001) higher in all experimental groups compared to the commercial medium. Similar results were observed after 1 hour with the exception of group N. Significant (P<0.001) differences were observed in groups B, C, D (with the highest value of 3.616 μm) and E compared to R group with the lowest value of 2.264 μm . The decrease of ALH in the group N slowed down, revealing significantly (P<0.05) higher values compared to the R group after 24 hours.

Table 7. Spermatozoa amplitude of lateral displacement (μm) in groups and time periods (mean \pm SD)

Group	0 h	1 h	24 h
N/ALH	3.05 \pm 0.41	4.23 \pm 0.16	2.88 \pm 0.21 ^C
R/ALH	3.13 \pm 0.76	4.04 \pm 0.15	2.26 \pm 0.21
A/ALH	5.39 \pm 0.11 ^A	5.14 \pm 0.51 ^A	2.39 \pm 0.43
B/ALH	5.03 \pm 0.20 ^A	5.20 \pm 0.58 ^A	3.20 \pm 0.29 ^A
C/ALH	5.68 \pm 0.34 ^A	5.85 \pm 0.54 ^A	3.53 \pm 0.67 ^A
D/ALH	5.76 \pm 0.18 ^A	5.10 \pm 0.38 ^A	3.62 \pm 0.56 ^A
E/ALH	3.97 \pm 0.56 ^A	5.92 \pm 0.37 ^A	3.56 \pm 0.28 ^A

x – mean, S.D. – standard deviation

^A P<0.001, ^B P<0.01, ^C P<0.05

The results demonstrate that spermatozoa samples cultivated in media containing BSA had better viability parameters when compared to the commercial medium based on egg yolk. Overall best viability parameters were observed in the medium consisting of triladyl, 20% BSA, 5% glucose and distilled water.

Proteins have been extensively employed as a supplement for culture media used to handle and cultivate spermatozoa, gametes and embryos. Protein molecules provide nutrients and protection to the cell, behave as colloids in solution and contribute to the osmotic pressure of fluids (Gebauer et al., 1970; Correa-Pérez et al., 2003).

The discovery that egg yolk has a beneficial effect on fertility led to its widespread use in bull semen extenders (Pace and Graham, 1974). Additionally it has been widely regarded as an essential ingredient for freezing diluents of bovine semen, apparently providing protection to spermatozoa membranes against cold shock and damage during cooling, freezing and thawing (Foulkes et al., 1980). Still, it is difficult to produce egg yolk semen diluents of uniform quality, because of individual quality differences of the yolk. Egg yolk is also relatively unstable for extended periods of time because of high content of fatty acids sensitive to degradation. Sperm cells usually form clusters making it almost impossible to perform an adequate analysis. Therefore, it seems that removal of chicken egg yolk from semen diluents offers several advantages, such as consistency improvement and elimination of various pathogens (Muller-Schlosser et al., 1995; Matsuoka et al., 2006).

BSA could be a good protein alternative because of its stability, nutritional quality and protective functions (Bakst and Cecil, 1992). There are several studies focusing on the possible effects of BSA on spermatozoa viability in different animal species.

Harrison et al. (1982) washed and diluted rabbit and ram spermatozoa in media containing various protein substances. BSA was found to be better than polyvinylpyrrolidone, ovalbumin, or alpha-lactalbumin at stimulating and maintaining motility levels as well as reducing the tendency of washed spermatozoa to stick to glass. According to Matsuoka et al. (2006) the rates of progressive motility in post-thawn ram semen were significantly higher in media containing BSA than in the Tris-fructose-egg yolk control group. Also, the spermatozoa viability was significantly improved and the rates of intact acrosomes were higher. Yamashiro, et al. (2006) found that collection of goat semen into tubes containing BSA resulted in improvement of the sperm freezability, higher sperm motility and intact acrosomes. Moreover, a field trial by Fukui, et al. (2008) revealed that a semi defined semen extender containing BSA provides a fertility potential after intrauterine insemination higher to that achieved with semen extender containing

egg yolk in sheep. On the contrary, Serniene et al. (2001) state that the addition of BSA was not significant for boar spermatozoa motility, vigor rate and number of viable and non-damaged spermatozoa per ejaculation.

Our results confirm the stimulating effects of BSA towards selected measurable characteristics describing bovine spermatozoa motility. As media containing 20% BSA presented the overall best viability results, we can assume that this could be the optimal BSA concentration for bovine spermatozoa preservation. Lower BSA concentration (10%) did not ensure enough protection for spermatozoa during cultivation with a very similar effectiveness to the egg yolk control. There is no detailed information on the BSA concentration in semen diluents for any species, which would allow us a direct comparison with our data. Our results agree with the conclusion of Uysal and Bucak suggesting that 20 mg/ml BSA was the best concentration improving post-taw motility and viability of ram spermatozoa. According to the authors, concentrations lower than 20 mg/ml of BSA (e.g. 10 mg/ml and 5 mg/ml) were not sufficient to preserve the quality of frozen-thawed semen. On the other hand, El-Kon (2011) recorded better Egyptian Buffalo spermatozoa motility and viability parameters even with 10 or 15% BSA when compared to the control. 10% and 15% BSA in ram and goat semen extenders proved to be optimal according to the findings of Matsuoka et al. (2006) and Yamashiro et al. (2008). It is however difficult to contrapolate our results with these studies, as the maximum concentration of BSA described, was 15%. Experiments using 20% BSA were not performed in any of the studies. However it is concluded that diluents containing a low concentration of BSA result in sperm characteristics similar to a commercial diluent. On the other hand, the experiments of Bankst and Cecil (1992) studying motility characteristics of turkey spermatozoa before and after storage for 24 h in diluents with and without BSA showed that even 1% BSA significantly increased the percentage of motile spermatozoa and sperm velocity, linearity, lateral head displacement and beat frequency, even the overall fertility potential remained unchanged.

The worst results from our study were obtained from the commercial egg yolk medium therefore we assume that the egg yolk medium may be appropriate for a short-term storage of spermatozoa only. It is not ideal for 24 hour cultivation because of the egg yolk unstableness and cell cluster formation.

CONCLUSION

This study demonstrates that BSA has beneficial effects on bovine spermatozoa motility and viability characteristics, which are essential for further processing of semen and an overall fertility potential. According to our results, BSA could be a good protein supplement for long-term bull spermatozoa cultivation especially when using higher concentrations. We suggest that the optimal concentration of BSA for bovine spermatozoa cultivation is 20%, since experimental media containing 20% BSA presented the overall best viability results. Nevertheless, we have to be aware on the fact that a proper protein supplementation is not the only factor crucial for a satisfactory *in vitro* spermatozoa motility and viability. An appropriate energetic substrate and minerals, as well as optimal laboratory conditions are equally important for a successful *in vitro* spermatozoa cultivation.

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**UTICAJ ALBUMINA GOVEĐEG SERUMA U KULTIVACIONOM
MEDIUMU
NA PARAMETRE POKRETLJIVOSTI SPERMATOZOIDA BIKA *IN VITRO***

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Izvod

Cilj rada je da se ustanovi uticaj albumina goveđeg seruma (BSA) dodatog u različite kultivacione medijume, na pokretljivost i preživljavanje spermatozoida bika, tokom 24h kultivacije *in vitro*. Ovi parametri su upoređivani sa nativnim uzorcima sperme kultivisanim u komercijalnom žumančano-jajčanom mediumu, sa eksperimentalnim mediumima, u koje je dodato 10% ili 20% BSA. Uzorci su analizirani posle 0h, 1h i 24h kultivacije, upotrebom sistema Sperm Vision™ CASA. Rezultati pokazuju progresivno opadanje stepena preživljavanja spermatozoida u svim eksperimentalnim mediumima, sa produžavanjem vremena kultivacije. Naj bolji parametri pokretljivosti spermatozoida su postignuti u mediumu sa dodatkom 20% BSA. Dobijeni rezultati pokazuju da BSA može biti dobar proteinski dodatak u medijume za dugotrajnu kultivaciju spermatozoida bika.

Cljučne reči: BSA, kultivacija spermatozoida, dodavanje proteina, CASA, bik.

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EUROPEAN MYCOTOXIN CONTAMINATION IN 2012: INCREASING CONCERN

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SUMMARY: *Mycotoxin contamination of crops represents a widespread problem in the animal feed industry. The major classes of mycotoxins are aflatoxins (Afla), deoxynivalenol (DON), fumonisins (FUM), zearalenone (ZEN) and ochratoxin A (OTA). The aim of this study is to obtain information on the occurrence of 5 major mycotoxins in various animal feeds and ingredients samples from different European regions. A total of 4,023 samples sourced worldwide (from January to December 2012), 1,654 sample from Europe were analysed for the most important mycotoxins in terms of agriculture and animal production – Afla, ZEN, DON, FUM and OTA. Samples were analysed by high performance liquid chromatography (HPLC) and Enzyme-Linked Immunosorbent Assay (ELISA). Only single commodities were analysed by ELISA. More complex matrixes which could interfere with the ELISA method such as Dried Destillers Grains with Solubles and finished feed were analysed by HPLC. For each toxin, results below the quantification limits were expressed as non-detected. All five major mycotoxins were highly prevalent in European countries and only 21% of all sampled were tested negative for the presence of the analysed mycotoxins. In 40% of animal feed and ingredient samples, more than one different mycotoxin was found. Out of all 4,023 samples, Afla were present in 41%, ZEN in 50%, DON in 67%, FUM in 58% and OTA in 56%. Similar to previous years, DON and FUM present the most prevalent group of mycotoxins with average contamination of 496 and 409 ppb; however average detected contamination levels were lower this year in comparison to 2011 (DON: 673 ppb and FUM 539 ppb). Survey results presented above clearly demonstrate that mycotoxins are a topic of concern in animal feed. An effective mycotoxin risk management program is a key factor for reaching optimum performance in animal husbandry.*

Key words: *mycotoxin, analyses, contamination, Europe.*

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INTRODUCTION

Mycotoxins and pesticide residues are among the top food and feed safety concerns related with an altering climate (Miraglia et al., 2009). Mycotoxins depend on climatic conditions as the ability of fungi to produce them is mainly influenced by temperature, relative humidity, insect attacks and the general stress conditions of plants. The complex topic of climate change involves not only temperature rise but also predicted variations in relative humidity, carbon dioxide levels, inter-relationships between different fungal genera, and different crops in different geographical locations (Bryden, 2012; Miller, 2008). Temperature and water availability are the two main factors which affect the lifecycle of all organisms. It is suggested that slightly elevated CO₂ concentrations and interactions with temperature and water availability may stimulate growth of some mycotoxigenic fungi, especially under water stress (Magan et al., 2011).

A factual example of erratic climate patterns and their impact on mycotoxin occurrence is the series of floods which occurred in Australia, mainly in the state of Queensland in December 2010, and less than one month later in the state of Victoria. As a result of that, and unlike what had been registered for the country in the previous years, wheat sourced in Australia in 2011 hit skyrocketing levels and presented the highest zearalenone and deoxynivalenol levels recorded worldwide (Rodrigues and Naehrer, 20112).

The 2012 draught in the US raised the mycotoxin awareness all over the world, but instead of in addition to the the US we faced the also true challenge in Europe: The aflatoxin scandal started in Serbia and was exported to Germany and the Netherlands. The origin of contaminated corn was mainly southeastern Europe, like Bulgaria, Greece, Romania, Ukraine, Hungary, Serbia and Italy. Up to 40 times higher concentrations of aflatoxin B₁, than the regulatory limit of 5 ppb in compound feed for dairy cattle was found in corn samples (e.g. 204 ppb aflatoxin B₁ in corn from Serbia). Fungal growth and the ability to produce mycotoxins like aflatoxins, are dependent on climatic conditions. Heat and drought stress are known to favour the growth of *Aspergillus flavus* and *Aspergillus parasiticus*, the fungi producing aflatoxins. Due to changing weather patterns, even well planned crops in usually aflatoxin free areas may become exposed to conditions favorable for contamination. After Serbia's harvest was already reduced by 45 % due to the severe drought in 2012, an increased awareness towards the predictable mycotoxin problem is essential for farmers. BIOMIN, a pioneer in mycotoxin risk management, has been conducting over the years a mycotoxin survey which allows feed and animal producers to assess the risks of using certain feedstuffs/feeds from different regions.

MATERIALS AND METHODS

From January to December 2012, a total of 4,023 samples collected worldwide were analyzed for the presence of mycotoxins. In total, 14,468 analyses were carried out for the most important mycotoxins in terms of agriculture and animal production – aflatoxins (Afla), zearalenone (ZEN), deoxynivalenol (DON), fumonisins (FUM) and ochratoxin A (OTA). For the purpose of data analysis, non-detection levels were

based on the quantification limits of the test method for each mycotoxin. For more details regarding the analytical procedure, please contact the authors.

RESULTS

Worldwide 25%, 46%, 64%, 56% and 31% of all the samples surveyed tested positive for contamination with Afla, ZEN, DON, FUM and OTA, respectively. Compared with data from the previous year 2011 (Table 1), an increase in the occurrence of fusariotoxins (ZEN, DON, FUM and ZEN) was observed together with a slight decrease in Afla.

Table 1. Regional content of mycotoxin in Europe in feedstuffs and feed
Tabela 1. Sadržaj mikotoksina u hranivima i stočnoj hrani u Evropi po regionima

North Europe (Norway, Finland, Sweden, Demark) <i>Severna Evropa (Norveška, Finska, Švedska, Danska)</i>						
North Europe/ <i>Sev. Evropa</i>	Afla	ZEN	DON	FUM	OTA	T-2
Number of tests/ <i>br.uzoraka</i>	4	103	103	0	6	103
Percent positive (%) / <i>procenat pozitivnih (%)</i>	0	71	88	-	67	29
Average of positive (µg/kg)/ <i>prosečna vrednost pozitivnih(µg/kg)</i>	-	41	1564	-	1	106
Maximum (µg/kg) / <i>maksimum (µg/kg)</i>	0	861	21540	0	2	273
Central Europe (Austria, Belgium, Czech Republic, Germany, France, Hungary, Romania, Slovakia, Slovenia and Poland) <i>Centralna Evropa (Austrija, Belgija, Češka, Nemačka, Francuska, Mađarska, Rumunija, Slovačka, Slovenija, Poljska)</i>						
Central Europe/ <i>Centralna Evropa</i>	Afla	ZEN	DON	FUM	OTA	T-2
Number of tests/ <i>br.uzoraka</i>	119	829	1158	111	135	232
Percent positive (%) / <i>procenat pozitivnih (%)</i>	19	50	68	23	23	16
Average of positive (µg/kg)/ <i>prosečna vrednost pozitivnih(µg/kg)</i>	7	41	744	113	10	31
Maximum (µg/kg) / <i>maksimum (µg/kg)</i>	36	675	12000	493	71	137
South Europe (Italy, Greece, Portugal, Spain, Croatia, Bulgaria and Turkey) <i>Južna Evropa (Italija, Grčka, Portugal, Španija, Hrvatska, Bugarska, Turska)</i>						
South Europe/ <i>Južna Evropa</i>	Afla	ZEN	DON	FUM	OTA	T-2
Number of tests/ <i>br.uzoraka</i>	173	193	215	166	168	192
Percent positive (%) / <i>procenat pozitivnih (%)</i>	44	43	51	84	70	8
Average of positive (µg/kg)/ <i>prosečna vrednost pozitivnih(µg/kg)</i>	6	35	330	879	4	258
Maximum (µg/kg) / <i>maksimum (µg/kg)</i>	87	604	10455	13457	64	3051
East Europe (Ukraine, Belarus, Lithuania, Estonia, Latvia and Russia) <i>Istočna Evropa (Ukrajina, Belorusija, Litvanija, Estonija, Latvija, Rusija)</i>						
East Europe/ <i>Istočna Evropa</i>	Afla	ZEN	DON	FUM	OTA	T-2
Number of tests/ <i>br.uzoraka</i>	73	74	104	81	91	94
Percent positive (%) / <i>procenat pozitivnih (%)</i>	71	42	67	54	79	56
Average of positive (µg/kg)/ <i>prosečna vrednost pozitivnih(µg/kg)</i>	5	32	285	485	6	70
Maximum (µg/kg) / <i>maksimum (µg/kg)</i>	10	340	960	1930	50	200

More than 1,600 samples were sourced in different European regions (Table 1). Due to the information gathered from previous years, samples originating from Northern Europe were mainly analysed for ZEN and DON. As expected, these were the major contaminants of commodities and feeds sourced from this region. Especially in terms of DON contamination levels were found to be fairly high. In Central Europe, DON remained the most commonly occurring mycotoxin followed by ZEN. In Southern Europe, similar to previous years, FUM was the most prevalent mycotoxin, followed by OTA.

CONCLUSIONS

In view of the results shown for the more than 1,600 samples from Europe analyzed in the year 2012, it is clear that the majority of commodities and feed used in animal nutrition is contaminated with at least one mycotoxin. More frequently than not, more than one mycotoxin will be present in the same ingredient or feed. Prevention of the negative effects of these hazardous substances in animal health and performance is crucial.

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KONTAMINACIJA MIKOTOKSINIMA U EVROPI U 2012: SVE VEĆI RAZLOG ZA ZABRINUTOST

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Izvod

Kontaminacija useva mikotoksinima je rasprostranjen problem u industriji hrane za životinje. Glavne vrste mikotoksina su aflatoksini (AFLA), deoksinivalenol (DON), fumonizin (FUM), zearalenon (ZEN) i ohratoksin A (OTA). Cilj ove studije je da se dobiju informacije o prisutnosti 5 najznačajnijih mikotoksina u uzorcima hrane za životinje i komponentama iz različitih evropskih regiona. Ukupno 4023 uzoraka iz sveta (od januara do decembra 2012.) analizirani su na sledeće

mikotoksine - AFLA, ZEN, DON, FUM, i OTA. Uzorci su analizirani tečnom hromatografijom (HPLC) i *enzyme-linked immunosorbent* testom (ELISA). Pojedina hraniva analizirani su samo sa ELISA testom. Složenije matrice koje mogu ometati ELISA metodu, kao što su DDGS i gotova hrana su analizirani tečnom hromatografijom. Kod svih toksina, rezultati koji su se nalazili ispod granice kvantifikacije su izraženi kao ne detektovani. Svih pet mikotoksina bili su veoma rasprostranjeni u evropskim zemljama, a samo 21% testiranih uzorka su bili negativni na prisustvo toksina. U 40% stočne hrane i hraniva, utvrđeno je više od jednog mikotoksina. Od analiziranih 4023 uzoraka, Afla su prisutni u 41%, ZEN u 50%, DON u 67%, FUM u 58% i OTA u 56%. Kao i prethodnih godina, DON i FUM predstavljaju najčešću grupu mikotoksina sa prosekom kontaminacije od 496 i 409 ppb. Prosečna kontaminiranost uzoraka je bila niža ove godine u odnosu na 2011. (DON 673 ppb i FUM 539 ppb). Rezultati istraživanja jasno pokazuju da su mikotoksini tema za brigu u industriji hrane za životinje. Efikasan program za upravljanje rizikom od mikotoksina je ključni faktor za postizanje optimalnih rezultata u stočarstvu.

Ključne reči: mikotoksin, analize, kontaminacija, Evropa.

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SOWS FERTILITY AFTER INTRACERVICAL OR POSTCERVICAL INSEMINATION WITH REDUCED SPERMATOZOA NUMBER IN AI DOSE*

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SUMMARY: The aim of this study was to determine whether the application of intrauterine (postcervical) AI, with twice reduced volume (50 ml) and the number of sperm (2×10^9) doses can increase the sows fertility, compared to the classical intracervical insemination (dose volume 100 ml with 4×10^9 spermatozoa). After classical intracervical insemination, farrowing rate was significantly lower (67.5%) only after classical intracervical AI, with reduced spermatozoa number per AI dose ($P < 0.01$). Using new intrauterine (postcervikalne) AI technology, the farrowing rate were not significantly different ($P > 0.05$), depending on the number of spermatozoa number in AI dose (83.3% and 79.2%). The results show that the use of intrauterine insemination, with doses twice reduced in volume and sperm count, can significantly increase the reproductive efficiency of the AI boars.

Key words: Intracervical, postcervical, AI, fertility, sow.

INTRODUCTION

In the classical technology of artificial insemination, used diluted liquid semen doses, volume 100 ml, with 3 to 5×10^9 progressively motile sperm (Almin et al., 2006; Stančić et al., 2009). From a single ejaculate, it can be obtained on average 21 insemination doses, or about 2100 doses per boar per year (Singleton, 2001). Insemination dose is usually preserved 1 to 2 days at 17°C, before using for insemination (Johnson et al., 2000). However, in the intensive pig production these number of AI doses is not enough for effective reproductive exploitation of genetically superior boars. The formation of double insemination doses number per ejaculate, with reduced dose volume (50 ml) and sperm number (2×10^9), could be the solution of this problem. On this way, a sufficient number of insemination doses per one ejaculate, can be obtained from one boar. Such reduced dose is possible to

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use with intrauterine insemination technology, while the achieved level of sows fertility is similar to that obtained using classical intracervical insemination (doses of 100 ml volume, with 4×10^9 spermatozoa) (Vansickle, 2002; Roseboom et al., 2004; Mesalira et al., 2005; Serret et al., 2005; Stančić et al., 2006; Stančić et al., 2007; Stančić et al., 2008; Stančić et al., 2010).

The aim of this study was to determine whether intrauterine insemination with doses of twice reduced volume and sperm count, increase the sows fertility compared with classical intracervical insemination.

MATERIALS AND METHODS

Classical, intracervical insemination and intrauterine (postcervical) insemination was performed with dose volume of 50 ml, contained 4×10^9 or 2×10^9 progressively motile sperm. Insemination was performed during the one year. The total of 480 sows, second to the fifth farrowing parity, (60 per each group) was inseminated by each insemination procedure and dose sperm number ($60 \times 4 \times 2 = 480$). Insemination where performed in the estrus detected at day 5 after weaning. Lactation lasted 28 days. The first insemination was carried out 12h, and second 36h after standing estrus detection. For conventional insemination were used sterile disposable catheters (Foamtip safe blue[®]), and for intrauterine insemination were used sterile disposable catheters Foamtip "Verona"[®] (Minitübe, Germany). Semen were diluted with BTS1, for short-term storage of liquid diluted boar semen (Minitübe, Germany). Value for farrowing rate and litter size were recorded. For the statistical analysis, "Statistica 9" software were used.

RESULTS

Using classical (intracervical) insemination, by both 4×10^9 or 2×10^9 progressively motile sperm per AI dose, achieved farrowing rate was significant ($P < 0.01$) different (81.7% and 67.5%). However, farrowing rate was not significant ($P > 0.05$) different (83.3% and 79.2%) after intrauterine insemination with 4×10^9 or 2×10^9 progressively motile sperm per AI dose (Table 1).

Table 1. Effect of insemination method and sperm number i dose on farrowing rate

Method of insemination	Spermatozoa number per AI dose	
	4×10^9	2×10^9
Classic intracervical	81.7% ^{AX} (98/120)	67.5% ^{BX} (81/120)
New Intrauterine	83.3% ^{AX} (100/120)	79.2% ^{AY} (95/120)

Different capital letters ($P < 0.01$); ^{AB} Within the rows;
^{XY} Within the columns. In parenthesis: (No. farrowed/No. inseminated).

The average number of live born piglets per litter, after classical intracervical insemination, was not significant different ($P > 0.05$) after AI with 4×10^9 (10.08) or with 2×10^9 spermatozoa per dose (10.14).

After intrauterine insemination, the average number of live born piglets did not differ depending on the dose sperm number (10.48 and 10.58), but these values were significantly ($P < 0.01$) higher than those obtained after intracervical insemination (Table 2).

Table 2. Average litter size at farrowing

Method of insemination	Litter size (n)	Spermatozoa number per AI dose	
		4×10^9	2×10^9
Classic intracervical	Live	10.08 ^{Ax}	10.14 ^{Ax}
	Dead	0.46	0.54
	Total	10.54	11.68
New Intrauterine	Live	10.48 ^{Ay}	10.58 ^{Ay}
	Dead	0.46	0.48
	Total	10.94	11.06

Different capital letters ($P < 0.01$); The small letters ($P < 0.05$).

^{AB} Within the rows; ^{xy} Within the columns.

DISCUSSION

Our results clearly show that the intrauterine insemination, with double reduced dose volume (50 ml) and sperm number (2×10^9), result with statistically significant ($P < 0.01$) higher farrowing rate (79.2%), compared to the intracervical insemination (67.5%). The average number of live born piglets per litter was significantly ($P < 0.01$) higher after intrauterine insemination with reduced dose sperm number (10.48 and 10.58), compared to the intracervical insemination (10.08 and 10.14) by insemination with 4×10^9 or 2×10^9 spermatozoa in AI dose.

Using intrauterine (postcervical) insemination with different doses of volume (100, 85, 50, 30 and 20ml) and different sperm numbers (4, 3, 1.5 and 1×10^9), result with 78 and 96% farrowing rate and 9 to 12 live born piglets per litter (Vansickle, 2002; Roseboom et al., 2004; Mesalira et al., 2005; Serret et al., 2005; Stančić et al., 2006; Stančić et al., 2007; Stančić et al., 2008; Stančić et al., 2010). By the sperm deposition in the cranial parts of the female reproductive tract (the body of the uterus, uterine horns, uterotubal junction or fallopian tubes), the volume of insemination dose and sperm number per dose can be radically reduced, with the same or higher fertility of inseminated sows, compared with the classical intracervical insemination (Mezalira et al., 2005; Stančić et al., 2007). Numerous studies show that the optimal value of the sows fertility has been achieved when insemination is performed approximately 24 hours before ovulation, with doses contained 2×10^9 spermatozoa. Increasing the sperm number per dose does not affect sows fertility, while reducing the number of sperm under the 2×10^9 leads to a decrease in sows fertility parameters (Knox, 2004; Stančić et al., 2007; Stančić et al., 2010).

The formation of twice more doses number from the same ejaculate, requires twice reduction of the sperm number of in a dose, and twice level of ejaculate dilution proportion. However, using a twice smaller dose volume (50 ml) and sperm cells number (2×10^9), it is not necessary to double the degree of ejaculate dilution. Adding large amounts of artificial extender in native semen, leads to a reduction in sperm progressive motility and agglutination (Harrison et al., 1978). This is due to reduction in amount of native protein and natural antioxidants, and other natural ingredients of seminal plasma, which are essential for the normal function integrity

and of sperm cell membrane (Kommisrud et al., 2002; Boe-Hansen et al., 2005). In addition, the sperm plasma has a significant impact on the process of sperm transport in the female reproductive tract (Rath et al., 1989) and is a significant factor in the regulation time of ovulation (Weitz et al., 1990b). On the other hand, it was found that the semen of a large number of boar does not tolerate the increasing degree of dilution. Namely, the results of numerous studies indicate that semen in only 20 to 30% of boars retained $\geq 65\%$ progressive motility during 72h of storage, on $+17^{\circ}\text{C}$, in dilution rate 1:4 (Weitz, 1990; Stančić et al. 2003).

Practical contribution to the results of our research consists in the fact that twice a smaller dose volume and sperm count can be used in the application of postcervikalne (intrauterine) insemination technology, without significant decrease in sows fertility. Thus it is possible to significant increase the reproductive exploitation of genetically superior boars in the modern pig production.

CONCLUSION

Based on these results, it can be concluded:

1. Farrowing rate was significantly lower using classical (intracervical), compared with intrauterine (postcervical) insemination of sows.
2. Using postcervical insemination AI doses with twice reduced volume and sperm count, it is possible to significant increase the boars reproductive exploitation.

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FERTILITET KRMAČA POSLE INTRACERVIKALNOG ILI POSTCERVIKALNOG VO U TOPLOJ I HLADNOJ SEZONI GODINE

IVAN STANČIĆ, BLAGOJE STANČIĆ, SAŠA DRAGIN, IVAN RADOVIĆ,
ALEKSANDAR BOŽIĆ

Izvod

Smanjen fertilitet nerastova i krmača, tokom toplijeg perioda godine, značajno smanjuje reproduktivnu efikasnost u intenzivnoj proizvodnji svinja. Cilj rada je bio da se ustanovi da li je primenom nove tehnologije intrauterine (postcervikalne) inseminacije, dozama duplo redukovano volumena (50ml) i broja spermatozoida (2×10^9), u odnosu na klasičnu intracervikalnu inseminaciju (4×10^9), moguće postići sličan fertilitet krmača. Posle klasične intracervikalne inseminacije, dozama sa redukovanim brojem spermatozoida (2×10^9), vrednost prašenja je bila znatno niža (67,6%), u odnosu na intrauteriono osemenjavanje, kako primenom doza sa 4×10^9 spermatozoida (83,3%), tako i primenom doza sa 2×10^9 spermatozoida (79,2%). Primenom nove tehnologije intrauterine (postcervikalne) inseminacije, postignute vrednosti prašenja nisu bile statistički značajno različite, u zavisnosti od broja spermatozoida u dozi. Dobijeni rezultati pokazuju da je primenom intrauterine inseminacije, dozama duplo redukovano volumena i broja spermatozoida, moguće značajno povećati stepen reproduktivnog iskorištavanja nerastova, bez smanjenja fertiliteta osemenjenih krmača.

Ključne reči: Intracervikalno, postcervikalno, VO, fertilitet, krmača.

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PRODUCTION OF CASCADE CHRYSANTHEMUMS IN PUBLIC UTILITY COMPANY "GRADSKO ZELENILLO" NOVI SAD

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SUMMARY: *The study was conducted in a semi-controlled greenhouse conditions in the nursery of the Public Utility Company "Gradsko zelenilo" in Novi Sad. In order to broaden the range of potted chrysanthemum, in addition to multi-flowered ones, four new varieties suitable for getting cascade chrysanthemums are introduced experimentally in the manufacturing, whose production required special breeding technology. Testing decorative features included measurement of cascades' length as well as number of flowers per dm² of a plant, and measurement parameters are presented in tables and charts. There are many possibilities in their use and some of the solutions are shown in the photographs.*

Key words: Chrysanthemum, cascade, production.

INTRODUCTION

Chrysanthemums are perennial or annual plant species of the family *Compositae* from China and now widespread in the Mediterranean, the Middle East, East Asia and parts of South Africa (Potkonjak et al., 2006; Welch, 2008; Wolff et al., 1995). The most important hybrid is *Chrysanthemum x morifolium* sin. (*C. grandiflorum*) originating from *C. indicum*. (Bradley et al., 1997; Herrington, 2008; Chen et al., 2010). The demand for chrysanthemum production, encompassing cut-flower, garden, potted plants and ground-cover types, is increasing worldwide. (Zhang et al., 2011).

There are a number of classifications and one of the most important divisions is in the garden and exhibition chrysanthemums (Salter, 2009). Garden chrysanthemums are able to survive winter in the open in most northern countries, they tolerate wind and rain and form a multitude of tiny flowers without mechanical aid. Group of exhibition chrysanthemum includes many varieties and has a very complex classification system (Cooke, 2007). During their growth they need a mechanical support and they survive winter in greenhouse conditions (Smith, 2000). The most common classification is according to a flower form, its blossoming time and whether you manually pruned buds or not. Chrysanthemums which do not need to

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be pruned include the following groups: spray, pompons, bushy, charm and cascade, which are categorized according to the shape and size of a plant (Norris-Brenzel , 2001).

Cascade chrysanthemum is a type of exhibition chrysanthemum and, while it is very popular and appreciated in the global market because of its attractive and unusual appearance, it is not widespread among domestic manufacturers. This form requires special manufacturing technology because its type is not genetically determined (Anderson, 2007; Silverman, 2004). Varieties suitable for obtaining a cascade characterized by considerable growth potential in length and width and a soft stem, are formed through special growing technology. Beside cascades, so-called railings, pillars and pyramids can be produced from the identical varieties in a similar manner (Norris-Brenzel , 2001). Form of growing cascade chrysanthemum started in Japan and it is called *kengai*, which is a traditional expression for waterfall, symbolizing the resemblance. Flat Korean variety is mostly used to obtain the cascade that can reach the length up to 3m (Dey, 2002). The largest exhibitions of chrysanthemum in the world are organized in the United States, England and Japan during "National Holiday of Chrysanthemum" (Singh, 2006). In addition to the remarkable cascade chrysanthemum, there are also bonsai forms, as well as hanging baskets (Maisano et al., 1971).

Because of their drop-down looks and different range of colors and flower shapes, which are offered by a large number of varieties, cascade chrysanthemums can be applied to open spaces (terraces, balconies, street lights and planters) for a short period of time. As short-day plants they may suffer from early autumn frosts. Exposed within the interior of the bus and train stations, airports, major shopping centers and similar spaces, they may provide a much longer flowering effect.

The aim of this study was to investigate the possibility of growing four varieties of chrysanthemum suitable for cascade growing and show the great potential of this type of chrysanthemum in horticultural production and landscape architecture.

MATERIALS AND METHODS

The surveys were conducted in 2012. in experiments performed in semi-controlled greenhouse conditions in the nursery of the Public Utility Company "Gradsko zelenilo" in Novi Sad. In that nursery, the production of potted tiny-flowered varieties, so called multi-flowered chrysanthemums, has been carried out for many years, and the end of production is scheduled for 1st November, the Day of the Dead.

To expand the range of potted chrysanthemum, authors of the paper started the experimental production of cascading chrysanthemums after developing the detailed plan of production by using the guidelines of the National Society of Chrysanthemum USA. Four French varieties were taken as research subjects: *Alba Tourly*, *Berruyere Cuivre Tourly*, *Armoricainte Tourly*, *Petite Maman Pichery*.

The experiment started 22.07.2012. by planting rooted cuttings in accordance with biological requirements of species, which were provided by the supplier DOO POMEX Sremski Karlovci. 50 rooted cuttings of each variety were planted in pots with a diameter of 19 cm. Flower pots were filled up to 2cm below the top with substrate manufactured by REKYVA, which made milled peat obtained by special technology of drying and processing. Then 9g of fertilizer OSMOCOTE

formulations (15:9:12) is added to each pot. Cuttings were planted at a depth of 3 cm in the middle of the pot, placed at a distance of 40 cm from each other. In order to prevent lodging of seedlings caused by *Pythium spp.* plants were watered 0.15% with a product PREVICUR ENERGY, and then preventively treated with a mixture of medicines DECIS 2.5 EC 0.05% + ALERT S 0.08% + TREND 0.02% for preventing fungal diseases and insects. Pots were placed on the parapets from where it was possible to carry out irrigation by a process of subirrigation and also to recharge with water soluble fertilizer. First pruning of 3-4 leaves was done in early August 2012 and growth of all branches was stopped, except for the main one, in order to stimulate lateral branching (Swithinbank et al., 2006). After two days, the plants were fed with water soluble NPK fertilizer by FERTICARE KEMIRA formulations (10:5:26) at a concentration of 0.1% once a week. After nine days a wire grid, support of the edge of the pot, was set at an angle of 40 degrees to the edge. Ellipsoidal bracket tied main stems of plants in order to reverse direction of growth of the main stem towards the ground instead of the height. Tying to the bracket was performed with a soft flexible wire in a hook shape. In late August, the second pruning of the sprouts was performed on 3-4 leaves of lateral sprouts in order to stimulate branching and to get a flat, thick and long, in other words, peltate shape (Swithinbank et al., 2006). In mid-September, when days became shorter, the process of budding began so any further pruning was halted. After the red mites *Paonychus ulmi* were observed on plants, the mixture of following products was applied: ACTARA 25WG 0.05% + DECIS 2.5 EC 0.05% + NISSORUN 10EC 0.05% + TILT 250EC 0.05% + TREND 0.02%. The treatment was repeated the next day with acaricides NISSORUN 0.05% + TREND 0.02%.

Also, in order to encourage flowering process, it was subjected to the new formulation of the same fertilizer in the process of feeding (15:30:15). Supplemental feeding was intensified twice a week. During the last part of October, out of four varieties, the first blossomed *Berruyere Cuivre Tourly*, after two days *Petite Maman Pichery*, and all the other varieties three days after that one.

RESULTS

On a sample of 200 pots, all four varieties in 50 pots, which were treated in an identical agro-technical conditions, the number of flowers and the length of the cascade as indicators of decorative properties of the varieties were tested.

Parameters of descriptive statistics presented in Table 1 were calculated based on the values in a program "STATISTICA".

Table 1. The descriptive statistic parameters of decorative properties of tested varieties

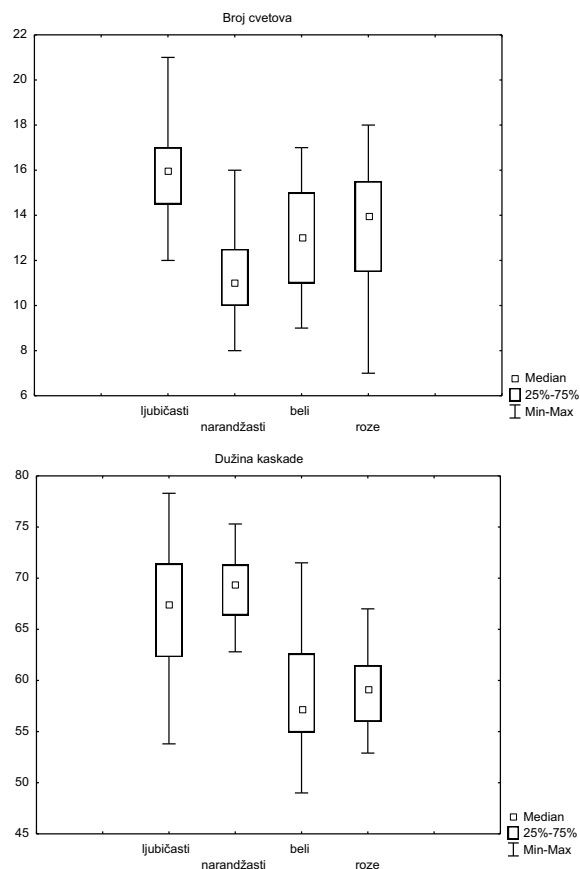
Variety		Arithm etic mean	Med ian	Mod us	Minim um	Maxi mum	Varia nce	Stand ard deviat ion	Coeffic ient of variatio n in %
Armorica inte Tourly Purple flower	Flow er num ber	16,13	16,00	15,00	12,00	21,00	5,65	2,38	14,74
	Casc ade length t	67,09	67,40	71,40	53,80	78,30	38,78	6,23	9,28
Berruyer e Cuivre Tourly Orange flower	Flow er num ber	11,30	11,00	12,00	8,00	16,00	3,81	1,95	17,26
	Casc ade length t	69,09	69,35	66,00	62,80	75,30	10,68	3,27	4,73
Alba Tourly White flower	Flow er num ber	12,90	13,00	12,00	9,00	17,00	5,12	2,26	17,54
	Casc ade length t	58,52	57,15	55,30	49,00	71,50	33,67	5,80	9,91
Petite Maman Pichery Pink flower	Flow er num ber	13,23	14,00	14,00	7,00	18,00	8,28	2,88	21,76
	Casc ade length t	58,83	59,10	Multi ple	52,90	67,00	10,10	3,18	5,40

Based on these indicators we can conclude that, on average, *Berruyere Cuivre Tourly* variety had the lowest number of flowers, 11.3 flowers per dm², and the biggest variety *Armoricainte Tourly* with 16.13 flowers per dm², recorded the absolute maximum number of flowers, 21 flower per dm². When it comes to the average length of the cascade the poorest results were given by the variety of *Albatourly*, 58.52 cm, and the best *Berruyere Cuivre Tourly* variety 69.09 cm. The minimum length of a cascade of only 49.0 cm was recorded with *Alba Tourly* variety.

In most of the pots of *Armoricainte Tourly* variety was 15 flowers per dm² and the most common length of the cascade was 71.40 cm. The largest number of pots of *Berruyere Cuivre Tourly* and *Alba Tourly* had 12 flowers per dm², with a cascade

length of 66.0 cm and 55.30 cm. Variety of *Petite Maman Pichery* gave bimodal result in measuring the length of cascades (55 cm and 56.8 cm), while the most common number of flowers per dm² was 14.

The greatest variability in the number of flowers came from the variety with pink flowers *Petite Maman Pichery* CV = 21.76%, and in the length of the cascade from *Alba Tourly* CV = 9.91%. Graphical representation of the calculated parameters of decorative properties of the varieties is presented in the Box - Whiskers diagrams. (Figure 1)



Graph. 1. Box - Whiskers diagrams of calculated parameters of decorative properties of the varieties

The key difference in the production of cascade chrysanthemums compared to previously produced potted multi-flowered chrysanthemums is that they do not have the possibility of genetic formation of cascade shape, but the preferred form of the cascade is produced by appropriate, strictly defined technology. This means that cascade chrysanthemums require a greater commitment of staff working on their production, as well as a number of operations. Despite that, numerous opportunities in the application, as well as decorative features of varieties, justify a more complex production.



Fig. 1. Cascade and multi-flowered chrysanthemums set in planters, Liberty Square, Novi Sad, November 2012.



Fig. 2. Cascading chrysanthemums on street lamps, Liberty Square, Novi Sad, November 2012.

CONCLUSION

Observing the values of all tested decorative components, it can be said that the *Armoricainte Tourly* variety emerged as the most suitable genotype for the formation of cascade shape, and that all four tested varieties have already met a catalogue selected standard of color and shape of flowers. Authors' recommendation for the next growing season is to start the production earlier. This way, with the extension of the period of production, and with a bigger number of completed pruning, longer, more dense and compact cascades would be produced.

In the development of horticultural production of the Republic of Serbia, increase of flower production and expansion of flower assortment for the greater competitiveness on both domestic and international markets is expected. Cascade production development would contribute to the efforts of the industry of our country. Conducted experiment shows that the production of cascade chrysanthemums is feasible, but it is necessary to conduct further research on the economic efficiency and profitability of the production.

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PROIZVODNJA KASKADNIH HRIZANTEMA U JAVNOM KOMUNALNOM PREDUZEĆU „GRADSKO ZELENILU“ NOVI SAD

GORDANA KOPILOVIĆ, DUŠANKA GAŠIĆ

Izvod

Istraživanja su sprovedena u polukontrolisanim plasteničkim uslovima u rasadniku Javnog komunalnog preduzeća "Gradsko zelenilo" Novi Sad. U cilju proširenja asortimana saksijskih hrizantema, pored do sada proizvedenih multifloralnih, u proizvodnju se eksperimentalno uvode četiri nove sorte pogodne za dobijanje kaskadnih hrizantema, za čiju proizvodnju je bilo neophodno primeniti posebnu tehnologiju uzgoja. Ispitivanje dekorativnih svojstava obuhvatilo je merenje dužine kaskade kao i broja cvetova po dm² biljke, a parametri merenja prikazani su tabelarno i grafički. Postoje brojne mogućnosti u njihovoj primeni a neka od rešenja prikazana su na fotografijama.

Ključne reči: Hrizantema, kaskadna, proizvodnja.

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EFFECT OF WATER STRESS ON THE YIELD OF COWPEA (*Vigna unguiculata* L. Walp.) IN TEMPERATE CLIMATIC CONDITIONS

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SUMMARY: Today, cowpea has remained a rather neglected and underutilized crop in Serbian agriculture. Due to its multipurpose nature, cowpea could be reintroduced into the agriculture of Serbia and other southeast European regions. To get more information about that possibility an experiment was carried out in field conditions under both rainfed (non-irrigated) and irrigated (well-watered) conditions in Novi Sad (45°20' N latitude, 19°51'E longitude, 84 m above sea level) in the years of 2011 and 2012. The experiments were arranged in completely randomized design with three replications. Overhead sprinkler irrigation system was used. Two different genotypes of cowpea (G_1 and G_2) were studied. To estimate the sensitivity of cowpea to water stress drought tolerance indices were estimated. The plant seed yield of cowpea was significantly higher in irrigation (10.33 g plant⁻¹) than in rainfed (4.35 g plant⁻¹) conditions. Determined drought tolerance indexes revealed that genotype G_1 was more tolerant to drought stress than genotype G_2 . To obtain more information on behavior of these two cowpea genotypes under stress conditions, comprehensive and comparative studies, including new cowpea genotypes should be carried out. Obtained results could be used in breeding programs to make cultivars which will enable this crop to be grown in large area in the region of Serbia.

Key words: cowpea, water stress, yield.

INTRODUCTION

Cowpea (*Vigna unguiculata* [L.] Walp.) is an important source of protein and one of the most drought-resistant food legumes that widely grown in the semiarid regions where drought is a major production constraint (Ehlers and Hall, 1997,

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Dadson et al., 2005). In Serbia cowpea occupies rather neglected area (Mikić, et al, 2010).

Prevailing climate in the region is continental with four marked season. Drought is a regular phenomenon. It appears almost every year influencing the yield of growing plants. In dry years, yields are reduced by 52-76% in relation to the average yields obtained in the region. It means that in the variable climatic conditions of Serbia, in which summers are semi-arid to semi-humid, high and stable yields of growing plants can reliably be obtained only by supplementing crop water requirement through irrigation. Only optimum conditions permit the plants to use water according to their needs (Bošnjak, 2001).

Drought tolerance is defined as the ability of plants to live, grows, and yields satisfactorily with limited soil water supply or under periodic water deficiencies (Ashley, 1993). In cowpea cultivation, the most sensitive stages to water deficit or water stress are just prior to and during bloom (Davis et al., 1991), seed filling stage (Cordeiro et al., 1998) and vegetative stage, followed by the flowering and fruiting stages (Carvalho et al., 2000). To develop specific cowpea genotypes with special focus on irrigated conditions for different environments and social conditions is necessary (Santos et al., 2000). There is a need for cowpea cultivars that more tolerant to water deficit or more efficient in water use (Anyia and Herzog, 2004).

The aim of research was to obtain initial results on the possibility of cowpea growing in climatic conditions of Serbia region. Results of the effect of water stress on yield of cowpea will be used in breeding programs to develop cultivars resistant to stressful environmental conditions, as well as more efficient in wat

MATERIAL AND METHODS

The experiments were conducted at Rimski Šančevi experiment field of Institute of Field and Vegetable Crops in Novi Sad (N 45°20', E 19°51') on the calcareous chernozem soil on the loess terrace, during 2011-2012 years. The experiment included irrigated (well-watered) and non-irrigated (rainfed) treatment. The experiments were established in completely randomized design with three replications and adapted to technical specifications of the sprinkling irrigation system. Two different genotypes of cowpea (G_1 and G_2) were grown. The size of the experimental unit was 3 m². The row spacing between and within the rows were 0.5 and 0.05 m respectively. Seed sowing was performed by hand on May 20, 2011 and May 9, 2012, respectively. Genotypes G_1 and G_2 were harvested on August 22 and 28, 2011 and on August 20 and 29, 2012 respectively. Irrigation was scheduled on the basis on water balance method and every day calculation of the status of readily available water in the soil layer of 0.5 m. Daily water used on evapotranspiration was calculated using hydrophytothermic index which had been estimated at 0.16 for soybean in the climate of Vojvodina (Bošnjak, 1983). Hydrophytothermic index of 0.16 for soybean was used because the value for cowpea has not been determined yet, as well as cowpea is similar to soybean. To estimate the sensitivity of cowpea genotypes to water stress, drought tolerance indices such as Stress Tolerance (TOL), Mean Productivity (MP), Geometric Mean Productivity (GMP), Stress Susceptibility Index (SSI), Stress Index (SI), Stress Tolerance Index (STI), Harmonic Mean (HM), Yield Index (YI) and Yield Stability Index (YSI) were estimated for each cowpea genotype based on seed yield (g plant⁻¹) under stress and non-stress environment.

$$\begin{aligned}
\text{TOL} &= Y_p - Y_s \text{ (Rosielle and Hamblin, 1981)} \\
\text{MP} &= (Y_p + Y_s) / 2 \text{ (Rosielle and Hamblin, 1981)} \\
\text{GMP} &= (Y_p * Y_s)^{1/2} \text{ (Fernandez, 1992)} \\
\text{SSI} &= [(1 - (Y_s / Y_p))] \text{ (Fischer and Maurer, 1978)} \\
\text{SI} &= 1 - (\bar{Y}_s / \bar{Y}_p) \text{ (Fischer and Maurer, 1978)} \\
\text{STI} &= (Y_p * Y_s) / (\bar{Y}_p)^2 \text{ (Fernandez, 1992)} \\
\text{HAM} &= [2 * (Y_p * Y_s)] / (y_p + Y_s) \text{ (Kristin et al., 1997)} \\
\text{YI} &= Y_s / \bar{Y}_s \text{ (Lin et al., 1986)} \\
\text{YSI} &= Y_s / Y_p \text{ (Bousslama and Schapaugh, 1984)}
\end{aligned}$$

Y_p and Y_s : Seed yield of each genotype under non-stress and stress conditions, respectively.

\bar{Y}_p and \bar{Y}_s : Mean seed yield of all genotypes under non-stress and stress conditions, respectively

Data reported for yield of cowpea were assessed by analyses of variance (ANOVA) and Fisher's LSD test was used for any significant differences at the $P < 0.05$ and $P < 0.01$ levels between the means. All the analyses were conducted using software package statistics 8.0 series 608c (StatSoft Inc. USA).

RESULTS AND DISCUSSION

Monthly values of water stress during growing season of cowpea were calculated by water balance method (Tab. 1 and 2).

Table 1. Water balance of cowpea – 2011

	May	June	July	August	Growing season
t	20.6	21.0	22.3	22.9	21.7
hfti	0.11	0.17	0.18	0.17	0.16
ETm (mm)	25	107	124	86-109	G ₁ 342- G ₂ 365
P (mm)	22	32	61	2	117
Δ	-3	-57	0	0	
r (mm)	60	57	0	0	
ETa (mm)	25	89	61	2	177
m (mm)	0	18	63	84-107	165-188
v (mm)	0	0	0	0	0

Table 2. Water balance of cowpea – 2012

	May	June	July	August	Growing season
t	16.2	23.1	25.3	23.9-24.9	23.5
hfti	0.11	0.17	0.18	0.17	0.16
ETm (mm)	40	118	140	84-123	G ₁ 382- G ₂ 421
P (mm)	45	22	31	0	98
Δ	+5	-60	0	0	
r (mm)	60	60	0	0	
ETa (mm)	40	82	31	0	153
m (mm)	0	36	109	84-123	229-268
v (mm)	5	0	0	0	0

t – mean monthly air temperature (°C); hfti – hydrophithothermic coefficient (mm/°C); ETm - the maximum evapotranspiration – irrigated (mm); P – monthly rainfall sum (mm); $\Delta \pm$ – difference in rainfall (P) and ETm represents deficit or suficit after consuming or filling the reserve of readil available water; ETa - the actual evapotranspiration - rainfed (mm); d – deficit of readily available water (mm); s – suficit (mm).

The period under study (2011-2012) had varying weather conditions. This was especially true of the amount and distribution of precipitation. The growing seasons (May/August) of 2011 and 2012 had the rainfall amounts of 117 mm and 98 mm respectively, which are 155.8 and 174.0 mm less than the long term average (272.8 mm) (Fig. 1, Tab. 1 and 2). Both years were very droughty and unfavorable for plant production. In both years drought stress started in June and lasted to the end of the season. Less favorable year for cowpea production was 2012 with total deficit of readily available water from 229 - 268 mm for G₁ and G₂ respectively (Tab. 2). In 2011 total deficit of readily available water was 165 - 188 mm for G₁ and G₂ respectively (Tab. 1).

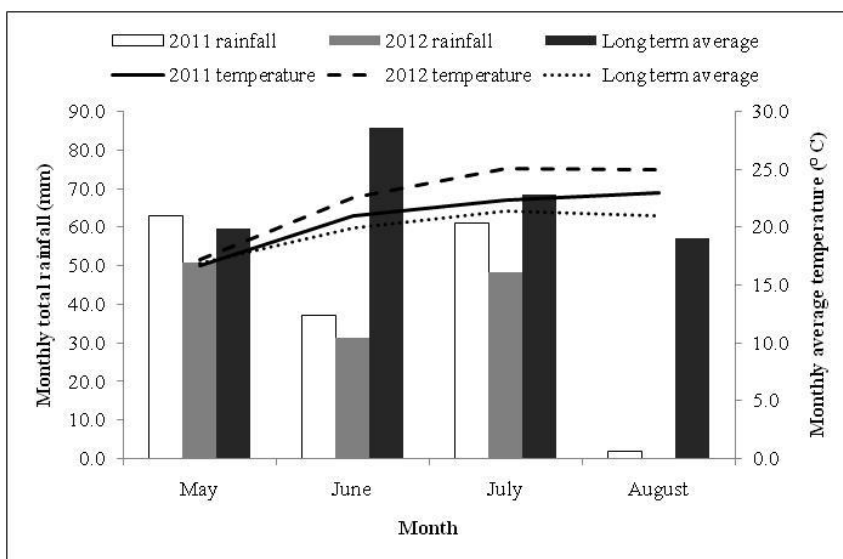


Fig. 1. Monthly average air temperatures (°C), monthly total rainfall (mm) in both years (2011 and 2012) in the cowpea growing season and long term average (1963-2010) (Rimski Šančevi, 1963-2009)

Not just a small amount of rainfall, but also their uneven distribution (Fig. 1) caused the need for irrigation. It was added 90 mm and 180 mm of water by irrigation in 2011 and 2012 respectively (Tab. 1). Given data indicate that climatic patterns in Serbia are changeable and long-term predictions of precipitation are not possible. That confirm supplementary character of irrigation in the region, i.e. rainfall can affect the soil water regime and irrigation schedule of growing plants (Pejić et al., 2012).

Table 3. Irrigation schedules and irrigation water applied

Year	Irrigation water applied (mm)			Irrigation water applied in the season (mm)
	Month			
	June	July	August	
2011	30 mm – 30 June	60 mm – 24 July	-	90
2012	30 mm – 18 June	60 mm – 11 July	30 mm – 4 August	180
	60 mm – 25 June			

Several studies conducted for a wide range of environments have demonstrated that cowpea yield increases with irrigation (Peksen, 2007, Abayomy and Abidoye, 2009). In the study period, on average, the yield of cowpea was significantly higher in irrigated ($10.33 \text{ g plant}^{-1}$) than in rainfed conditions ($4.35 \text{ g plant}^{-1}$) (Table 3). The average yield decrease of cowpea due to water stress was in average $5.98 \text{ g plant}^{-1}$, ranging from $9.74 \text{ g plant}^{-1}$ in year 2012 which was unfavorable for cowpea production to $5.95 \text{ g plant}^{-1}$ in 2011 which had slightly better conditions for cowpea production. Yield of cowpea (g plant^{-1}) obtained in the study both for irrigated and rainfed conditions are consistent with results reported by Peksen (2013) who found the yield of cowpea (Cv. Karagoz-86, Samsun, Turkey) for irrigated and rainfed conditions of 9.73 and $4.64 \text{ g plant}^{-1}$ respectively.

Table 3. Yield of cowpea (g plant⁻¹) in irrigated and rainfed conditions

Genotype (C)	Irrigated	Year (A)		Average	
	Rainfed (B)	2011	2012	(BC)	(B)
G ₁	I	9.72	15.28	12.5	10.33
	R	3.77	5.54	4.65	4.35
Average (AC)		6.74	10.41		
G ₂	I	7.56	8.75	8.16	
	R	3.15	4.93	4.04	
Average (AC)		5.36	6.84	Average (C)	
Average (AB)	I	8.64	12.01	8.58	
	R	3.46	5.23	6.1	
Average (A)		6.05	8.62		
				7.34	

LSD	A	B	C	AB	AC	BC	ABC
0.01	0.5333	0.6237	1.038	1.0703	1.7812	1.7812	3.9691
0.05	0.3805	0.4449	0.7404	0.7065	1.1758	1.1758	2.1623

Water is vital for plant growth, development and productivity. Permanent or temporary water deficit stress limits the growth and the performance of the cultivated plants more than any other environmental factor (Lobato et al., 2008, Shao et al., 2009). Aranús et al. (2003) reported that, among the environmental factors affecting crops, the water input, expressed as the sum of rainfall and irrigation during the growing period, explained the large part of the yield variability.

Cowpea is usually better adapted to drought, high temperatures and other biotic stresses compared with other crop plant species. Because of that it is primarily grown in drier regions of the world where is one of the most drought-resistant food legumes (Dadson et al, 2005). Cowpeas are grown under both irrigated and rainfed conditions. The crop responds positively to irrigation, but will also produce well under dry land conditions. The effect of drought stress on the yield of cowpea depends on genotype, intensity and duration of stress and the growth stage exposed to water stress. In both years the yield of cowpea was significantly influenced by water stress (Tab. 3) which clearly indicates that high and stable production of this plant in the region is only possible by supplementing crop water requirement through irrigation.

Determined drought tolerance indexes (Tab. 4) revealed that G₁ genotype was more tolerant to drought stress than that G₂ genotype. To obtain more information on behavior of these two genotypes under different stress conditions, comprehensive studies, should be carried out. Results of the study should be used in breeding

programs to develop cultivars that suit to temperate climatic conditions of the Serbia region.

Table 4 Drought tolerance indices over two years in cowpea under irrigated and rainfed conditions

Year	Genotype	Y _{irr}	Y _d	TOL	MP	GMP	SSI	STI	HAM	YI	YSI
2011	G ₁	9.72	3.77	5.95	6.74	18.87	0.61	0.49	5.43	1.08	0.39
	G ₂	7.56	3.15	4.41	5.36	7.56	0.58	0.32	4.44	0.91	0.42
2012	G ₁	15.28	5.54	10.41	9.17	9.2	0.64	0.58	8.13	1.05	0.36
	G ₂	8.75	4.93	6.84	6.14	6.57	0.44	0.3	6.31	0.94	0.56
Average	G ₁	12.5	4.66	8.18	7.96	14.04	0.62	0.54	6.78	1.06	0.39
	G ₂	8.16	4.04	5.62	5.75	7.07	0.51	0.44	5.38	0.92	0.49

CONCLUSION

Very high seed yield losses due to water deficit or drought stress may occur in cowpea cultivation under rainfed conditions. Study results revealed that high and stable production of cowpea in the region is only possible by supplementing crop water requirement through irrigation. Nevertheless, G₁ genotype was more tolerant to drought stress than that G₂ genotype, some more cowpea genotypes should be investigated in order to get cultivars resistant to stressful environmental conditions, as well as more efficient in water use.

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**EFEKAT VODNOG STRESA NA PRINOS VIGNE (*Vigna unguiculata* L.
Walp.)
U UMERENIM KLIMATSKIM USLOVIMA**

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Izvod

U poljoprivredi Srbije vigna se gaji na zanemarljivo malim površinama. Zbog višenamenske upotrebe vigna bi mogla da zauzme značajnije površine u poljoprivredi Srbije i području jugoistočne Evrope. Za dobijanje više informacija o mogućnosti gajenja vigne na ovim prostorima obavljena su eksperimentalna istraživanja u poljskim uslovima sa i bez navodnjavanja (Novi Sad, 45°20' N, 19°51'E, 84 m nadmorske visine) u periodu 2011/2012 godine. Ogled je postavljen po randomiziranom rasporedu parcela u tri ponavljanja. Navodnjavanje je obavljeno sistemom za navodnjavanje kišenjem. U ogledu su bila zastupljena dva genotipa (G_1 and G_2). Osetljivost genotipova vigne na vodni stres utvrđena je obračunom indeksa suše. Prinos vigne je bio signifikantno veći u uslovima navodnjavanja (10,33 g biljci⁻¹) u odnosu na varijantu bez navodnjavanja (4,35 g biljci⁻¹). Utvrđeni indeksi suše ukazuju veću otpornost na vodni stres genotipa G_1 u odnosu na genotip G_2 . Za dobijanje više informacija o osetljivosti ispitivanih genotipova na vodni stres, opsežnija istraživanja, uključujući i druge genotipove su neophodna. Dobijeni rezultati će biti korišćeni u oplemenjivačkim programima za dobijanje sorata koje će omogućiti da ova biljna vrsta zauzme veće površine u Srbiji.

Ključne reči: vigna, vodni stres, prinos.

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THE INFLUENCE OF THE METHOD OF PRODUCTION ON THE CONTENT OF MINERAL SUBSTANCES AND NITRATES IN LETTUCE

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JELENA¹

SUMMARY: The lettuce is a very good and inexpensive source of minerals (N, P, K), but in the winter it is prone to accumulation of toxic substances (nitrates). The main goal of our research is to examine the impact of new technologies of production (land cover) on the quality of lettuce produced in the winter. Mineral content varied depending on the mode of production. The values of nitrogen content in lettuce tend to rise from bare ground (2.89-4.58%) to covered (3.09-4.85%) soil. The type of material that covers the land also has an impact on the content of potassium. The applied versions of the cover did not significantly affect the phosphorus content. There is a trend of nitrate increase when applying different methods of production.

Key words: salad, quality, cultural practices.

INTRODUCTION

Lettuce is a very valuable vegetable variety because of its dietetic characteristics, its vitamin values, the possibilities of production, and its presence on the markets during the whole year. It belongs to the sort of green and yellow vegetables that have a special significance in the diet because of a high content of scarce micronutrients such as copper, zinc, iron and magnesium, and especially in the period (spring-autumn) when there is a lack of these micronutrients (Miskovic et al., 2001; Lazic et al., 2001).

Biochemical content of the edible parts of the vegetables depends on the variety, and on the applied agricultural techniques (Lazic, et al., 2000). According to the research of the aforesaid authors, the aim of using new technologies in vegetable production is profitability along with a rational use of all the inputs directed towards the quality and environmental protection. There are also some significant results (Škorić M. 1996, Bošnjak Đ. 2005) indicating that the increased use of drop-by-drop irrigation system, along with covering of land with a suitable foil and use of nutritive elements, contribute to making a quality final product.

Original professional paper / *Originalni stručni rad*

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The quality of lettuce largely depends on the content of mineral substances (N; P; K), as well as on the content of nitrate, which in larger quantities can be harmful for human consumption. The basic aim of our researches is to test the influence of new production technologies (land covering) on the chemical composition of lettuce.

MATERIAL AND METHOD

During the two-year period (2009-2010), these tests were carried out on the lettuce varieties Archimedes RZ, Santoro RZ and Kibou RZ in a greenhouse without additional heating on the experimental field that belongs to the Faculty of Agriculture in East Sarajevo.

The trial was set in a randomized block system with four repetitions on the experimental plot sized 2.4m² (0.3x8m). There were three rows in this experimental plot, and each row represented a new variety. Planting of the substrate Klasmann for production of the planting material was done in containers, without nosedive in the first decade of September. The planting material, which was 25 days old, was planted at the distance of 20 cm within the row, and at the distance of 30 cm between the rows, so the complex of 150,000 plants/ha was achieved. As for the irrigation system, we used a drop-by-drop one, which was set together with the covering of land. We used four variants of land covering in the trial: mulching before planting with black polyethilen foil; agrotexile covering of plants after planting with agrotexile (17 grams); combination of mulching and agrotexile.

Picking of lettuce was done in the phase of its technological maturity. The chemical analysis of the planting material, i.e. the content of nitrogen, potassium, phosphorus and nitrates was done at the Chemical and Technology Faculty in Novi Sad by applying standard methods used by many scientific institutions.

RESULTS AND DISCUSSION

While analyzing the two-year research results, it was determined that lettuce contained between 3.14 % - 4.8 % of nitrogen. The content of nitrogen in lettuce depended on the applied variants of covering. The average content in all the applied variants of covering was 3.14%.

The highest content of nitrogen (3.34%) was found when the agrotexile variant was applied, and the lowest one was found in the control variant (2.89%).

The differences in the content of nitrogen in the applied variants of covering, compared to the control one, were estimated at the threshold of significance of 1% (Tab. 1).

Depending on the selected varieties, the content of nitrogen differed within the range 2.84% - 3.50%. The determined differences in nitrogen content of the variety Archimedes (3.50%) compared to the varieties Santoro (2.84%) and Kibou (3.8%) were at the level of significance of $P < 0.01$.

Tab.1. The means of nitrogen content (%) in lettuce in 2009
 Tab.1 Srednje vrijednosti sadržaja azota (%) kod salate u 2009.godini

Covering land/pokrivanje zemljišta (A)	Variety /Sorta (B)			Average /Prosjeck
	Archimed	Santoro	Kibou	
Control/kontrola	3.11	2.62	2.95	2.89
covering of black PE foil/pokrivanje sa crnom polietilenskom folijom	3.40	2.81	3.07	3.09
agrotexile/agrotekstil	3.87	3.07	3.23	3.34
black PE foil + agrotexile/crna polietil.folija + agrotekstil	3.62	2.84	3.14	3.23
Average/Prosjeck	3.50	2.84	3.08	3.14
LSD	A	B	AxB	
0.05	0.04	0.03	0.07	
0.01	0.05	0.05	0.10	

The average content of nitrogen in the second year of researches was 4.68%. This content is by 1.54% higher than the one from the first year of the trial. The influence of different variants of covering on the content of nitrogen in the leaves of lettuce was prominent in the second year of the research, too. The control variant resulted in the lowest content of nitrogen (4.58%) while the polyetilen foil variant resulted in the highest content of nitrogen (4.85%).

The varieties do not show more significant differences in the nitrogen content. The highest nitrogen content was determined in the Archimedes variety (4.90%), while the other two tested varieties had approximately the same nitrate content – Santoro (4.56%) and Kibou (4.60%).

By analyzing the interaction effect between the covering and the variety, it is interesting to conclude that the differences in the nitrogen content among the varieties tested on the fourth variant of covering were not statistically justified.

Tab.2. The means of nitrogen content (%) in lettuce in 2010
 Tab.2. Srednje vrijednosti sadržaja azota (%) kod salate u 2010.godini

Covering land/pokrivanje zemljišta (A)	Variety /Sorta (B)			Average /Prosjeck
	Archimed	Santoro	Kibou	
Control/kontrola	4.96	4.33	4.44	4.58
covering of black PE foil/pokrivanje sa crnom polietilenskom folijom	5.16	4.72	4.69	4.85
agrotexile/agrotekstil	4.82	4.58	4.52	4.64
black PE foil + agrotexile/crna polietil.folija + agrotekstil	4.67	4.62	4.73	4.67
Average/Prosjeck	4.90	4.56	4.60	4.68
LSD	A	B	AxB	
0.05	0.11	0.10	0.20	
0.01	0.15	0.13	0.27	

The phosphorus content.

Human nutritive needs for phosphorus range from 550 to 650 mg on a daily basis (Novaković et al. 2002). Phosphorus is important for all vital functions of our body. It is a component of bones, teeth, RNA and DNA, of phospholipids, every membrane of every cell, of the basic energy unit of ATP, and a certain number of enzymes and coenzymes.

The average phosphorus content in all the variants of covering applied regardless of the variety itself was 0.82%. The differences in phosphorus content among the variants of covering applied did not have statistical validation. The differences in the content of Phosphorus in the varieties Santoro and Kibou compared to the variety Archimedes, were estimated at the level of 1%

Tab.3. Means of phosphorus content (%) in lettuce in 2009.year
Tab.3. Srednje vrijednosti sadržaja fosfora (%) kod salate u 2009.godini

Covering land/ <i>pokriivanje zemljišta (A)</i>	Variety / <i>Sorta (B)</i>			Average /Prosjeck
	Archimed	Santoro	Kibou	
Control/ <i>kontrola</i>	0.76	0.93	0.82	0.84
covering of black PE foil/ <i>pokriivanje sa crnom polietilenskom folijom</i>	0.79	0.77	0.90	0.82
agrotexile/ <i>agrotekstil</i>	0.74	0.88	0.89	0.84
black PE foil + agrotexile/ <i>crna polietil.folija + agrotekstil</i>	0.72	0.81	0.91	0.81
Average/ <i>Prosjeck</i>	0.75	0.85	0.88	0.82
LSD	A	B	AxB	
0.05	0.04	0.03	0.07	
0.01	0.05	0.04	0.09	

The phosphorus content in the second year of research ranged from 1.33 to 1.59%. The average content of all the variants was 1.49%, which is by 1.11% more than in the previous year. The varieties tested had approximately the same phosphorus content in leaves. The only significant differences in phosphorus content were observed between varieties Santoro (1.53%) and Kibou (1.44%).

The highest content of phosphorus (1.54%) was found in the combination of mulching + agrotexile, while the lowest content was found in the controls (1.43%).

Tab.4. the means of phosphorus content (%) in lettuce in 2010
 Tab.4. Srednje vrijednosti sadržaja fosfora (%) kod salate u 2010.godini

Covering land/ <i>pokriivanje zemljišta (A)</i>	Variety / <i>Sorta (B)</i>			Average /Prosjeck
	Archimed	Santoro	Kibou	
Control/ <i>kontrola</i>	1.52	1.43	1.33	1.43
covering of black PE foil/ <i>pokriivanje sa crnom polietilenskom folijom</i>	1.56	1.57	1.44	1.52
agrotexile/ <i>agrotekstil</i>	1.47	1.53	1.50	1.50
black PE foil + agrotexile/ <i>crna polietil.folija + agrotekstil</i>	1.56	1.59	1.48	1.54
Average/ <i>Prosjeck</i>	1.52	1.53	1.44	1.49
LSD	A	B	AxB	
0.05	0.10	0.17	0.17	
0.01	0.13	0.11	0.23	

The potassium content.

The major physiological role of K is regulation in maintaining water balance in the human body. It is considered that the daily need for K is 0.8 to 1.3 g. According to Novakovic et al. (2002), people who frequently eat "fast food" and do not take fresh fruit and vegetables may have a low potassium intake.

The two-year research results showed that potassium content ranged from 3.81% to 4.81%, depending on the year, the variety and the covering.

Tab.5. The means of potassium content (%) in lettuce in 2009
 Tab.5. Srednje vrijednosti sadržaja kalijuma (%) kod salate u 2009.godini

Covering land/ <i>pokriivanje zemljišta (A)</i>	Variety / <i>Sorta (B)</i>			Average /Prosjeck
	Archimed	Santoro	Kibou	
Control/ <i>kontrola</i>	4.46	3.88	4.15	4.16
covering of black PE foil/ <i>pokriivanje sa crnom polietilenskom folijom</i>	3.81	4.10	4.61	4.49
agrotexile/ <i>agrotekstil</i>	5.01	4.75	4.86	4.87
black PE foil + agrotexile/ <i>crna polietil.folija + agrotekstil</i>	4.73	4.54	4.65	4.64
Average/ <i>Prosjeck</i>	4.74	4.31	4.57	4.54
LSD	A	B	AxB	
0.05	0.06	0.05	0.11	
0.01	0.08	0.07	0.15	

The differences in the variants of covering are evaluated at the level of significance of $P < 0.01$, which indicates that the type of material used for covering of the soil affects the content of potassium in lettuce leaves. The gained results are in agreement with Balalić's reports (2004) which say the different methods of production (without mulching, mulching, mulching + agrotexile) show very significant differences in K content in lettuce.

The differences are also noticed in the potassium content and in different varieties. The highest content is recorded in the case of the variety Archimedes (4.74%). The variety Kibou had (4.57%), while the lowest content was recorded in the case of the variety Santoro (4.31%).

The differences among the varieties are highly significant, indicating that the varieties affect the potassium content in lettuce leaves. The results of the research confirmed that the potassium content depends on the variety (Koudela and Petrikova, 2008)

The highest content of potassium is found in all varieties in the case of the variant agrotexile Archimedes (5.01%), Santoro (4.75%), Kibou (4.86%).

The average content of potassium in the second year of research, regardless of the treatments tested, was 4.47%, which is similar to the previous research year.

Tab.6. Mean values of potassium content (%) in lettuce in 2010

Tab.6. Srednje vrijednosti sadržaja kalijuma (%) kod salate u 2010.godini

Covering land/ <i>pokrivanje zemljišta (A)</i>	Variety / <i>Sorta (B)</i>			Average / <i>Prosjeck</i>
	Archimed	Santoro	Kibou	
Control/ <i>kontrola</i>	4.44	3.85	4.12	3.89
covering of black PE foil/ <i>pokrivanje sa crnom polietilenskom folijom</i>	4.74	4.10	4.58	4.45
agrotexile/ <i>agrotekstil</i>	5.01	4.74	4.86	4.87
black PE foil + agrotexile/ <i>crna polietil.folija + agrotekstil</i>	4.72	4.53	4.80	4.68
Average/ <i>Prosjeck</i>	4.72	4.28	4.59	4.47
LSD	A	B	AxB	
0.05	0.10	0.17	0.17	
0.01	0.13	0.11	0.23	

The variants of covering applied affected the potassium content in the second year of research, too. The differences between the variants of covering were significant at the level of 1%. The values of potassium content ranged between 3.89% (control) and 4.87% (agrotexile). Balalic (2004), in his work, also reports about the results similar to ours.

In this year of research the differences in the content of potassium per variety were noticeable, too. The differences in potassium content between varieties Archimedes and Kibou were significant at the level of 1%, while the same differences between varieties Archimedes and Santoro i.e. Santoro and Kibou had significance at the level of 5%. The analysis of the interaction effect between a variety and a covering shows that it is highly significant in this year of research, too.

The nitrate content in lettuce depends on many factors. There is more nitrate in vegetables cultivated with higher doses of nitrogen and organic fertilizers at low relative air humidity, under drought conditions, low light intensity, during the short day, and temperatures above 25 °C degrees. According to the research by Kastori and Petrovic (2003), the interaction of temperature and light intensity is very significant for accumulation of nitrate. The cited authors point out that the accumulation of nitrate favors high substrate temperature and low light intensity.

The research results show that the four types of covering showed different nitrate contents. The highest content of nitrate (3192.25 mg/kg) was recorded in the application of agrotextile variant, and the lowest nitrate content (2597.83 mg / kg) in the control variant. The nitrate content ranged from 2606.12 mg / kg to 3464.56 mg/kg depending on the variety.

Tab.7. The mean values of nitrate (mg/kg) in lettuce in 2009
Tab.7. Srednje vrijednosti sadržaja nitrata (mg/kg) u salati u 2009.godini

Covering land/ <i>pokrivanje zemljišta (A)</i>	Variety / <i>Sorta (B)</i>			Average / <i>Prosjek</i>
	Archimed	Santoro	Kibou	
Control/ <i>kontrola</i>	3313.00	2290.00	2190.50	2597.83
covering of black PE foil/ <i>pokrivanje sa crnom polietilenskom folijom</i>	3491.50	2403.00	2523.00	2805.83
agrotextile/ <i>agrotekstil</i>	3652.50	3253.00	2681.00	3192.25
black PE foil + agrotextile/ <i>crna polietil.folija + agrotekstil</i>	3411.25	2782.25	3029.75	3074.41
Average/ <i>Prosjek</i>	3464.56	2682.06	2606.12	2917.58
LSD				
0.05	97.04	84.03		168.08
0.01	129.70	112.32		224.66

In the second year of research, the average value of nitrate content ranged from 2197.25 mg/kg (control) to 2526.25 mg/kg (agrotextile).

The differences determined in the average values of nitrate content at different variants of covering are rated at the threshold of significance of 1%, only the differences stated between the third and fourth variant of covering were not statistically justified.

Tab.8. The mean values of nitrate (mg/kg) in lettuce in 2010
 Tab.8. Srednje vrijednosti sadržaja nitrata (mg/kg) u salati u 2010.godini

Covering land/ <i>pokriivanje zemljišta (A)</i>	Variety / <i>Sorta (B)</i>			Average Prosjeck
	Archimed	Santoro	Kibou	
Control/ <i>kontrola</i>	2727.25	2001.50	1863.00	2197.25
covering of black PE foil/ <i>pokriivanje sa crnom polietilenskom folijom</i>	2831.75	2211.00	2136.50	2393.08
agrotexile/ <i>agrotekstil</i>	2889.70	2524.00	2165.00	2526.25
black PE foil + agrotexile/ <i>crna polietil.folija + agrotekstil</i>	2923.00	2148.50	2487.00	2519.50
Average/ <i>Prosjeck</i>	2842.90	2221.25	2162.87	2409.02
LSD				
0.05	61.61	53.34	213.47	
0.01	82.35	71.30	285.33	

In the second year of research, the order of the varieties studied, in terms of this trait, was identical to the one in the previous year. Archimedes variety had the highest average value of nitrate content (2842.9 mg/kg), and Kibou variety had the lowest one (2162.87 mg/kg). The research by Ekonomakis and Saed (2002) showed that nitrate accumulation depends on the variety. Similar results are found in the works of Lazic et al. (2002). According to their research, the nitrate content is a varietal characteristics and leafy lettuce has the highest level (350.30 mg/kg fresh weight) and Roman salad the lowest of nitrate content (310, 90 mg/kg fresh mass).

CONCLUSION

Based on the results of the two-year studies of the effect of production methods on the mineral substances and nitrate content in lettuce, the following conclusions can be made:

- The content of mineral substances varied depending on the way of production. The nitrogen content was significantly higher in the covered soil. The values of nitrogen content in lettuce tend to rise from bare ground (2.89-4,58%) to a covered (3,09-4,85%) soil. The type of material for covering soil also has an effect on the content of potassium. The highest potassium content of 4.87% was evident in the variant of covering soil with agrotexile, which is 18% more compared to the control variant.
- The covering variants applied did not significantly affect the phosphorus content.
- The nitrate content estimated as a harmful substance for human consumption depended on the method of production. There is an emphasized trend of increasing

nitrate with the application of various methods of production. The highest nitrate content (2526.25-3192.25 mg/kg) was in the variant of covering soil with agrotexile, which is 16% more compared to the control variant (2197.25-2597.83 mg/kg).

•The values of the maximum nitrate content in our trial were below the acceptable standard (4500 mg/kg for lettuce grown in the protected space) as provided by the European Commission EC.

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UTICAJ METODE PROIZVODNJE NA SADRŽAJ MINERALNIH MATERIJA I NITRATA U SALATI

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Izvod

Salata je jako dobar i jeftin izvor mineralnih materija (N, P, K) međutim u zimskom periodu sklona je i nakupljanju štetnih materija (nitrata). Osnovni cilj naših istraživanja je ispitati uticaj novih tehnologija proizvodnje (pokriivanje zemljišta) na kvalitet salate proizvedene u zimskim uslovima. Sadržaj mineralnih materija varirao je u zavisnosti od načina proizvodnje. Vrijednosti sadržaja azota imaju tendenciju porasta od nepokrivenog zemljišta (7.47%) ka pokrivenom (7.98%) zemljištu. Vrsta materijala za pokrivanje zemljišta također ima uticaja i na sadržaj kalijuma. Primjenjene varijante pokrivanja nisu značajno uticale na sadržaj fosfora. Izražena je tendencija povećanja nitrata sa primjenom različitih načina proizvodnje.

Ključne riječi: salata, kvalitet, agrotehničke mjere.

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POMOLOGICAL CHARACTERISTICS OF WILD CHERRY GENOTYPES (*PRUNUS AVIUM* L. /MOENCH/) FROM POTKOZARJE POPULATION*

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SUMMARY: *This paper presents the results of the investigation of wild cherry (*Prunus avium* L. /Moench/) population in the area of Potkozarje. The studied genotypes grow in association with sweet chestnut (*Castanea sativa* Mill.) on acidic soil. Indicators of tree growth of wild cherry genotypes and pomological characteristics show a high variability inside the population. The selected specimens are an excellent source of reproductive material for the nursery production.*

Key words: *wild cherry, soil, pomological analysis, morphological properties.*

INTRODUCTION

Wild cherry trees (*Prunus avium* L. /Moench/) were selected from the population which belongs to forest communities of the chestnut and sessile oak (*Quercus Castanetum sativae* Wrab.) in the area of Potkozarje. The site is located in the northwest part of the Republic of Srpska on the territory of Gradina and the altitude of 290 to 390 meters. Gradina is a hilly area above the village Dragočaj on the Banja Luka-Prijedor road, about twenty kilometres from Banja Luka. Gradina is a private property and the trees in the forest are of generative origin.

The relief of the sites has a western exposure with a greater inclination and a north one with lower. The flattened ridge at the highest ground is dominated by several plateaus. The bedrock consists of brown acid rocks on which acid brown soil (district cambisol) and illimerised soil (luvisol) have developed.

The selected wild cherry trees on the site Gradina are of different classes of age, indicating the diversity of the population. The study of the diversity of the genus *Prunus*, including the wild cherry (*Prunus avium* L. / Moench /), was done by

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Pandey et al. (2008) in the temperate regions of India and Mratinić et al. (2012) in the south-eastern part of Serbia. The pomological characteristics of the cherry fruit cultivars with different ripening stages in the Belgrade Danube basin were investigated by Milatović and Đurović (2010).

MATERIAL AND METHODS

Within natural populations of the chestnut, oak and beech, the wild cheery is present as an associated species. The site and tree selection and subsequent fruit harvesting was carried out during the 2011-2012 season. Two soil profiles and three soil semi-profiles were selected for analysis. For the profile number 1, where the observed populations were examined, morphological, physical and chemical properties were investigated, while the soil analysis was carried out in the laboratory of Agropedology at the Agricultural Institute in Banja Luka. The type of soil was determined based on the morphology profile studies and the soil maps were provided by the Agropedology Institute of Sarajevo. The wild cherry trees were isolated at the edge of the forest community.

The biometrical basis on ten selected trees of wild cherry (*Prunus avium* L. /Moench/) was determined and the inter-population variability of morphological characteristics of fruits was tested. Moreover, 30 fruits were randomly selected for the pomological analysis. Measurements of the fruit length and width as well as stem length were taken using a micrometre of 0.01 mm precision. Fruit and stone weight measurements were taken on an analytical scale (precision 0.01 g). The collected data was processed using STATISTICA 10 SOFTWARE application (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

The study area has a temperate continental climate characterized by cold winters and warm dry summers. According to data from the meteorological station of Banja Luka, in 2011 and 2012, the average annual temperature was 12.1 °C, which is in accordance with the mean monthly temperature of 12.2 °C, measured during the flowering of the wild cherry. At the time of fructification, the monthly average temperature was 17.2 °C and 20.4 °C in May and June, respectively. The overall annual precipitation was 588.2 mm, with 37.7 mm measured in April, 62.6 mm in May and 37.0 mm in June.

The above precipitation distribution is optimal for wild cherry populations, as it enables the development of the most significant pomological characteristics. Moreover, the overall annual precipitation is comparable to the values measured at similar sites across the wider area of Southeast Europe.

In the study population of wild cherry in Potkožarje area, Gradina locality, the pedological profile was opened on the November 6th, 2011, at 370 m alt. The topology of the analysed locality is mountainous, with south-western exposure and forest cover. The profile structure is: Aoh – A(B) – B(C) – C.

Table 1. Mechanical composition of the studied district cambisol from locality Gradina
Tabela 1. Mehanički sastav ispitivanog distričnog kambisola sa lokaliteta Gradina

Depth(cm)/ <i>Dubina (cm)</i>	The percentage share of fractions/ <i>Procentualni udeo frakcija</i>					Textural markings by Gračanin/ <i>Teksturna oznaka po Gračaninu</i>	Degree of colloid by Gračanin/ <i>Stepen koloidnosti po Gračaninu</i>
	Sand/ <i>Pesak</i>	Powder/ <i>Prah</i>	Physical clay/ <i>Fizička glina</i>	Colloid clay/ <i>Koloidna glina</i>			
	2.0- 0.05mm	0.05- 0.01mm	<0.01mm	<0.002 mm u NaOH	<0.002 mm u H ₂ O		
0-4	24.40	27.60	48.00	16.20	6.52	Clay loam/ <i>Glinovita ilovača</i>	Moderate colloidal/ <i>Umereno koloidno</i>
4-20	34.48	9.40	46.60	29.20	15.60	Clay loam/ <i>Glinovita ilovača</i>	Very colloidal/ <i>Jako koloidno</i>
20-40	44.00	7.12	58.40	47.00	25.20	Sandy clay/ <i>Peskovita glina</i>	Extremely colloidal/ <i>Vrlo jako koloidno</i>

Table 1 indicates that the sand content increases with depth, making the soil mostly water-permeable, without prolonged stagnation. Moreover, the powder content decreases with depth. The soil particles less than 0.002 mm in diameter, containing NaOH, indicate that rough colloidal clay predominates at this site. Considering their percentage participation in the overall composition, the analysed district cambisol is moderately to highly colloidal. The percentage of clay increases from 48% to 58.40%, with increasing depth and precipitation filtration through the profile is high.

Soil pH reaction is an important property, as it affects many physical and chemical processes, as well as the vital functions of plants the soil supports.

Table 2. The pH reaction of the soil solution in the pedological profile from locality Gradina

Tabela 2. pH reakcija zemljišnog rastvora u pedološkom profile sa lokaliteta Gradina

Depth (cm)/ Dubina (cm)	pH reaction in/ <i>pH reakcija u</i>		Hydrolytic acidity (me/100g)/ <i>Hidrolitička kiselost (me/100g)</i>
	H ₂ O	KCl	
0-4	5.10	3.70	35.50
4-20	4.82	3.60	33.13
20-40	5.00	3.50	20.50

The soil acidity was investigated, as the height of the active acidity in soil is primarily affected by carbonic acid, which leads to the soil acidification. The data reported in Table 2 indicate that the value of the active soil acidity slightly decreases

or remains unchanged (5.10-5.00 pH). Substitution acidity ranges from 3.70 in the first investigated horizon to 3.50 at the highest depth, i.e. the acidity increases with depth. According to the classification provided by Scheffer-Schachtschabel (1998), the studied district cambisole belongs to the category of very acidic soils.

Rodrigues et al. (2008) studied the sweet and sour cherry cultivars in germplasm bank in Portugal, with the aim of identifying the variability between different genotypes. The soil and agroecological conditions under which their study was conducted are similar to those found in this research area. The results Rodrigues et al. reported indicate presence of variability between pomological fruit properties.

Finally, it should be noted that the content of humus in the soil regulates fertility. Humus is a complex dynamic compound, created during degradation and humification of organic residues in the soil and should thus be investigated.

Table 3. Content of humus and nutrients of studied district cambisol from locality Gradina

Tabela 3. Sadržaj humusa i hraniva u ispitivanom distričnom kambisolu sa lokaliteta Gradina

Depth (cm)/ <i>Dubina (cm)</i>	Humus (%)	CaCO ₂	Accessible nutrients (mg/100g of soil)/ <i>Pristupačna hraniva (mg/100g of soil)</i>	
			P ₂ O ₅	K ₂ O
0-4	5.00	-	1.60	14.70
4-20	1.96	-	-	6.50
20-40	0.64	-	-	7.50

The humus content in the analysed soil in the surface horizon is 5.00% (Table 3), and belongs to humic soil (Scheffer-Schachtschabel, 1998). Moreover, the percentage of humus decreases rapidly with depth. Although the wild cherry tends to favour deep and neutral pH soils, the wild cherry trees at the investigated site have adapted to the environmental conditions and soil type. The wild cherry trees observed in the study area were in good condition and vitality – the properties affecting the flowering and fructification, the variability of which was examined in this study.

The results of bioecological characteristics of the investigated wild cherry trees (Table 4) indicate that the tree height ranged from 3.00 m (G9) to 25.00 m (G3), which points to high variability (CV = 80.34%). Moreover, very high variability is observed in the values of trunk volume and diameter, with the coefficients of variation of 82.91% and 84.24%, respectively. The crown width of the investigated trees ranged from 4.15 m (G9) to 15.25 m (G3)—with the latter measured for the most developed specimen. The coefficient of variation for the crown width, as well as the trunk height, was slightly lower compared to other properties. Table 4 indicates that the crown width, measured at four exposure sites, varies depending on the tree position and the presence of neighbouring trees.

Table 4. Indicators of tree growth of wild cherry genotypes from locality Gradina
Tabela 4. Pokazatelji rasta genotipova divlje trešnje sa lokaliteta Gradina

Genotype / Genotip	Tree height (m)/ Visina stabla (m)	Trunk height (m)/ Visina debla (m)	Trunk volume (m ³)/ Obim debla (m ³)	Diameter at breast height (m)/ Prsni prečnik (m)	Crown width (m)/ Širina krošnje (m)				Mean value of crown width/ Sr. vrednost širine krošnje
					East/ Istok	West/ Zapad	North/ Sever	South/ Jug	
G1	6	1.05	0.61	0.1940	1.5	3.2	2.4	2.3	4,70
G2	14	2.80	0.80	0.2540	3.7	3.3	3.8	3.9	7,35
G3	25	3.30	2.02	0.6430	5.3	8.1	9.1	8.0	15,25
G4	5	1.75	0.32	0.1019	5.1	0	3.8	0	4,45
G5	6.5	3.25	0.46	0.1460	2.4	2.0	1.1	3.0	4,25
G6	7	1.20	0.62	0.1974	4.8	1.2	1.1	3.9	5,50
G7	7.5	2.70	0.62	0.1970	6.7	8.0	2.1	4.2	10,50
G8	3.5	1.25	0.18	0.0574	2.8	1.5	2.3	2.0	4,30
G9	3	1.80	0.17	0.0541	2.5	1.3	3.0	1.5	4,15
G10	5	1.80	1.70	0.5414	3.5	2.3	3.5	2.3	5,80
X	8.25	2.09	0.75	0.2374	3.8	3.1	3.2	3.1	6,60
SD	6.62	0.85	0.62	0.1999	1.61	2.78	2.28	2.13	3.61
Cv (%)	80.3	40.8			42.0	90.2	71.0	68.7	
	4	1	82.91	84.24	8	0	7	7	54.53

The correlation analysis included five measured parameters (Table 5) and the results indicate a high correlation between tree height and other parameters. The interrelation of the trunk volume, trunk diameter and crown width was also statistically significant for the analysed correlation coefficients. These results are in accordance with those reported by Faust and Zagaja (1984) and Khadivi-Khub et al. (2011). Moreover, these authors suggest that the correlation between the tree height and the trunk diameter (measured at breast height) of wild cherry specimens of different height and age found in natural populations of Iran was positive.

Table 5. Correlation analysis of wild cherry genotypes morphometrical characteristics from locality Gradina.

Tabela 5. Korelaciona analiza morfometrijskih karakteristika genotipova divlje trešnje sa lokaliteta Gradina

	Trunk height/ Visina debla	Trunk volume/ Obim debla	diameter at breast height/ Prsni prečnik	Crown width/ Širina krošnje
Tree height/ Visina stabla	0.646*	0.718*	0.718*	0.879*
Trunk height/ Visina debla		0.404	0.403	0.621
Trunk volume/ Obim stabla			1.000*	0.715*
Diameter at breast height/ Prsni prečnik				0.715

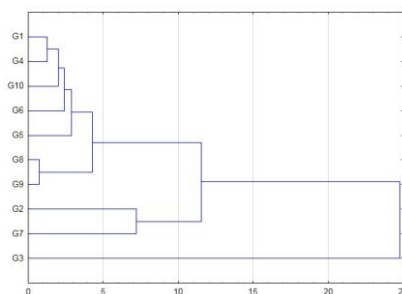
- marked values represent statistically significant correlation coefficients

- vrednosti označene zvezdicom predstavljaju statistički značajne koeficijente korelacije

Hjalmarsson and Ortiz (2000) studied the variability of wild cherries in different habitats in three Scandinavian countries in order to investigate potential differences in populations. Their findings suggest existence of different wild cherry ecotypes, which vary in fruit quality and resistance to low temperatures.

The cluster analysis based on quantitative morphological characteristics indicates two major groups of genotypes with five subclusters (Figure 1). The first cluster consists of all genotypes of the wild cherry except genotype G3, which has extremely high values of tree height, trunk diameter at breast height and crown width.

The first subcluster consists of genotypes G2 and G7, with approximately uniform values of trunk height. The next group consists of wild cherry genotypes G1, G4, G5, G6, G8, G9 and G10. In this subcluster, genotypes G1 and G4 were grouped based on their tree height values. Genotypes G8 and G9 also stand out based on their uniform tree height, trunk volume and diameter (at breast height), as well as the average value of the crown width. In the next subcluster, genotype G5 stands out due to the lower values of trunk diameter at breast height and average crown width. Genotypes G6 and G10 are in the fifth subcluster, based on similar crown width measurements.



Graph. 1. Cluster analysis of wild cherry genotypes growth parameters from locality Gradina.

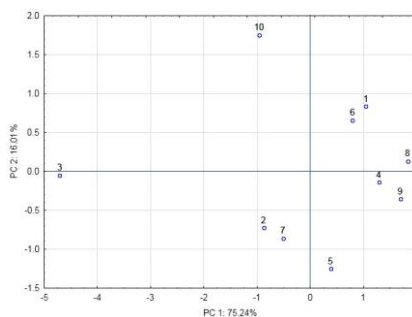
Graf. 1. Klaster analiza pokazatelja rasta genotipova divlje trešnje sa lokaliteta Gradina

According to the multivariate principal component analysis, the most variable among examined parameters were tree height, crown width, and the trunk circumference and diameter. These parameters loaded on the first principal component axis and contributed to the total variation by 75.24%. The second principal component axis accounted for a further 16.01% of the total variance and was defined by the trunk height (Table 6). Principal component values were not significant for the third and fourth axis. Scatterplot showed separation of genotype G3 from all other genotypes, along the first axis, as well as separation of genotype G10 on the second axis. Several groups of genotypes could be recognized. First group comprised genotypes G1 and G6, second included G4, G8 and G9, while the third was constituted of genotypes G2, G5 and G7. As the cluster analysis results were fully confirmed by those of the principle component analysis, outliers G3 and G10 can be considered significantly different from other investigated genotypes (Figure 2).

Table 6. Multivariate principal component analysis of wild cherry genotypes growth parameters, from locality Gradina.

Tabela 6. Analiza glavnih komponenta pokazatelja rasta ispitivanih genotipova divlje trešnje sa lokaliteta Gradina

	PC 1	PC 2	PC 3	PC 4
Tree height/ <i>Visina stabla</i>	-0.918*	-0.166	-0.256	0.253
Trunk height/ <i>Visina debla</i>	-0.683	-0.691*	0.330	-0.017
diameter at breast height/ <i>Prsni prečnik</i>	-0.900*	0.404	0.160	-0.005
Trunk volume/ <i>Obim debla</i>	-0.900*	0.405	0.158	-0.003
Crown width/ <i>Širina krošnje</i>	-0.912*	-0.145	-0.304	-0.235
Eigen value/ <i>Svojstvena vrednost</i>	3,761	0,801	0,317	0,119
Total variance explained (%)/ <i>Ukupna varijansa (%)</i>	75,24	16,01	6,35	2,40
Cumulative variance explained (%)/ <i>Kumulativna varijansa (%)</i>	75,24	91,25	97,60	100,0



Graph. 2. Multivariate principal component analysis scatterplot based on morphological parameters of wild cherry genotypes from locality Gradina.

Graf 2. Dijagram rasporeda genotipova divlje trešnje sa lokaliteta Gradina zasnovan na morfološkim parametrima pokazatelja rasta

In addition to the investigation of morphological growth parameters of wild cherry trees, a pomological investigation was also performed (Table 7). The average fruit length and width values were 12.60 mm and 13.60 mm, respectively. Fruit stalk length varied from 33.45 mm (G9) to 49.20 mm (G8). The average fruit weight was 1.38 g, while the coefficient of variation for this characteristic was 29.79%. The greatest value of fruit weight was measured for genotype G9 (1.85 g), while the lowest was found for genotype G3 (0.81 g). The genotype labelled G3 had greatest vigour, according to morphological parameters, highest yield, lowest fruit weight, and highest flesh ratio. The most variable parameter was fruit stone weight—ranging from 0.20 g (G3) to 0.30 g (G10)—with the coefficient of variation of 39.42%. Stone/fruit ratio was also calculated and was in the range 12.97 % (G9) to 28.26% (G4).

The differences between the investigated wild cherry genotypes were determined by Duncan multiple range tests, which indicate that genotypes G3, G2

and G9 were significantly different from other genotypes, according to fruit width. High value of fruit stalk length differentiated genotype G8 from others. Based on fruit weight, differences were also determined among genotypes G1, G6 and G8.

Table 7. Pomological characteristics of investigated wild cherry genotypes from locality Gradina.

Tabela 7. Pomološke karakteristike ispitivanih genotipova divlje trešnje sa lokaliteta Gradina

Genotype/ <i>Genotip</i>	Fruit length(mm)/ <i>Dužina ploda</i>	Fruit width (mm)/ <i>Širina ploda</i>	Fruit stalk length (mm)/ <i>Dužina peteljke</i>	Fruit weight (g)/ <i>Masa ploda</i>	Stone weight (g)/ <i>Masa koštice</i>	Yield (%)/ <i>Randm an</i>
G1	12.15 ^{cd}	12.69 ^{de}	45.27 ^b	1.13 ^e	0.26 ^{ab}	23.01
G2	13.18 ^{ab}	13.34 ^d	40.45 ^c	1.39 ^{cd}	0.29 ^{ab}	20.86
G3	12.47 ^{abc}	11.08 ^f	35.73 ^{de}	0.81 ^f	0.20 ^c	24.70
G4	11.13 ^d	12.02 ^e	41.01 ^c	0.92 ^f	0.26 ^{ab}	28.26
G5	12.74 ^{abc}	13.34 ^d	34.64 ^e	1.47 ^c	0.28 ^{ab}	19.04
G6	13.30 ^{ab}	14.31 ^c	44.95 ^b	1.63 ^b	0.25 ^{abc}	15.33
G7	11.65 ^{cd}	14.24 ^c	37.76 ^d	1.50 ^c	0.25 ^{abc}	16.66
G8	12.19 ^{cd}	13.41 ^d	49.20 ^a	1.26 ^d	0.25 ^{abc}	19.84
G9	13.43 ^{ab}	16.19 ^a	33.45 ^e	1.85 ^a	0.24 ^{bc}	12.97
G10	13.72 ^a	15.40 ^b	45.30 ^b	1.84 ^a	0.30 ^a	16.30
Average	12.60	13.60	40.78	1.38	0.26	19.70
SD	2.56	2.07	6.60	0.41	0.10	4.67
Cv(%)	20.30	15.24	16.19	29.79	39.42	23.70

The correlation analysis of wild cherry pomological characteristics revealed statistically significant relationships between the investigated features (Table 8). The most statistically significant correlation coefficients were determined for fruit length and width, followed fruit length and weight, fruit width and weight, and finally fruit and stone weight.

Table 8. Correlation analysis of wild cherry genotypes pomological characteristics from locality Gradina.

Tabela 8. Korelaciona analiza pomoloških karakteristika ispitivanih genotipova divlje trešnje sa lokaliteta Gradina

	Fruit width/ <i>Širina ploda</i>	Fruit stalk length/ <i>Dužina peteljke</i>	Fruit weight/ <i>Masa ploda</i>	Stone weight/ <i>Masa koštice</i>
Fruit length/ <i>Dužina ploda</i>	0.198*	-0.032	0.296*	0.051
Fruit width/ <i>Širina ploda</i>	1.000	-0.030	0.707*	0.107
Fruitstalk length/ <i>Dužina peteljke</i>		1.000	-0.027	0.041
Fruit weight/ <i>Masa ploda</i>			1.000	0.173*
Stoneweight/ <i>Masa koštice</i>				1.000

-statistically significant correlation coefficients, $p \leq 0.01$

- statistički značajni koeficijenti korelacije, $p \leq 0.01$

The discriminant analysis of pomological characteristics determined those which carry most of the variability of the sample (Table 9). Of the five investigated characteristics (values ≥ 0.700), the most significant was fruit weight, which separated genotypes along the first axis. The second axis was determined by fruit petiole length. As the discriminant analysis indicated that the first two variables explain over 95% of total variability of the sample, the subsequent interpretation includes only the first and second axis (DA1 and DA2). The first principal component contributes to the variability by 59.90%, the second explains 35.8% (Figure 3), whereas the remainder is explained by successive components of declining significance.

The discriminant analysis results indicate significant sample heterogeneity. However, we cannot group all the genotypes, as only G2, G5 and G7 as well as G6 and G10 formed their respective groups, based on similar values of the investigated pomological characteristics. The lowest fruit weight separated genotype G3 on the second axis (DA2) and the values of fruit stalk length separated genotypes G8 and G9, as these showed significant differences.

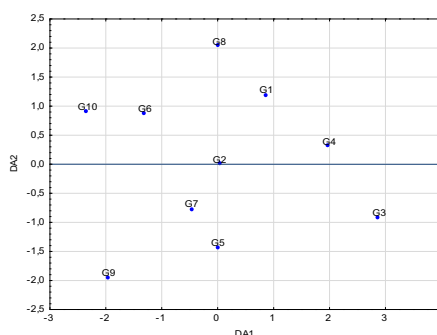
Table 9. Discriminant analysis of pomological characteristics of the wild cherry genotypes, from locality Gradina

Tabela 9. Diskriminantna analiza pomoloških karakteristika ispitivanih genotipova divlje trešnje sa lokaliteta Gradina

	DA1	DA2	DA3	DA4	DA5
Fruit length/Dužina ploda	0.04303	0.04439	0.45914	0.67009	0.62072
Fruit width/Širina ploda	0.32575	0.01294	0.77618*	0.09455	0.67158
Fruit stalk length/ Dužina peteljke	0.20891	1.00624*	0.03000	0.05966	0.03439
Fruit weight/Masa ploda	0.88794*	0.12032	0.39878	0.02964	0.58878
Stone weight/Masa koštice	0.00314	0.10425	0.47836	0.75711*	0.44892
Eigen value/ Svojevrednost	2.50184	1.49642	0.09008	0.06383	0.02372
Cumulative variance explained/Kumulativna proporcija (%)	0.59911	0.95746	0.97903	0.99431	1.00000

-marked values are significant to the axis of the discriminant analysis

-vrednosti označene zvezdicom su značajne za osu diskriminantne analize



Graph. 3. Diagram of pomological characteristics of wild cherry genotypes from locality Gradina based on the first two axes of discriminant analysis

Graf. 3. Dijagram rasporeda pomoloških karakteristika genotipova divlje trešnje sa lokaliteta Gradina na osnovu prve dve ose diskriminantne analize

The pomological characteristics indicate great variability in the population of the selected genotypes of wild cherry in the area of Potkozarje.

CONCLUSION

The area Potkozarje, Gradina locality, is characterized by the district cambisol or brown acid soil type with the Aoh – A(B) – B(C) – C profile. The first two studied horizons are of clay loam, and the third horizon is of sandy clay type. These soils are water-permeable and well aerated. Their chemical properties indicate strong acidity while their base content is low. The soil also lacks physiologically active

phosphorus, while potassium content ranges from poor to moderate. The wild cherry is well adapted to the conditions of habitat.

The results reported here indicate great variability in the development of wild cherry specimens. The height of the investigated wild cherry trees in the population of Potkozarje ranged from 3.00 m (G9) to 25.00 m (G3), indicating high variability (CV = 80.34%). Very high variability was also observed for the trunk volume and diameter measured at breast height, with the variation coefficients of 82.91% and 84.24%, respectively. Crown width of the investigated trees ranged from 4.15 m (G9) to 15.25 m (G3).

The pomological characteristics indicate that the average fruit length and width values were 12.60 mm and 13.60 mm. Fruit petiole length varied from 33.45 mm (G9) to 49.20 mm (G8). The average fruit weight was 1.38 g, while the coefficient of variation for this property was 29.79%. The highest weight was measured in genotype G9 (1.85 g), and the lowest in genotype G3 (0.81 g). The most variable feature is the stone weight, with the coefficient of variation of 39.42% and the measured values ranging from 0.20 g (G3) to 0.30 g (G10). Finally, flash ration ranged from 12.97% (G9) to 28.26% (G4).

The study population of the wild cherry in habitats of Southeast Europe is important for selection of trees that have adapted to existing environmental conditions. The selected specimens are an excellent source of reproductive material for the nursery production of rootstocks, and will thus be used for grafting cherry cultivars.

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**POMOLOŠKE KARAKTERISTIKE GENOTIPOVA DIVLJE TREŠNJE
(*PRUNUS AVIUM* L. /MOENCH/) IZ POPULACIJE SA POTKOZARJA**

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TODOROVIĆ, DRAGAN TODOROVIĆ

Izvod

U radu su prikazani rezultati ispitivanja populacija divlje trešnje (*Prunus avium* L. /Moench/) na lokalitetu Potkozarja. Ispitivani genotipovi rastu u sastojini sa pitomim kestenom (*Castanea sativa* Mill.) na zemljištu kisele hemijske reakcije. Pokazatelji rasta stabala kao i pomološke karakteristike plodova divlje trešnje pokazuju veliku varijabilnost unutar populacije. Izabrani genotipovi predstavljaju izvor reprodukcionog materijala za rasadničku proizvodnju.

Ključne reči: divlja trešnja, zemljište, pomološka analiza, morfološke osobine.

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HARMFUL PLANTS IN GRASSLAND VEGETATION ASS. *TRIFOLIO-AGROSTIETUM STOLONIFERAE* MARKOVIĆ 1973 IN VOJVODINA*

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SUMMARY: Grassland phytocenosis stands Trifolio-Agrostietum stoloniferae in Vojvodina comprise 157 plant species. Floristic composition analysis of ass. Trifolio-Agrostietum stoloniferae stands revealed the presence of 22 poisonous species (14.01%), of which 4 (2.55%) were highly poisonous and 18 (11.46%) mildly poisonous. In addition, the worthless weed plant group comprises of 67 taxa (42.68%). The studied stands include 13 invasive species.

Key words: *Trifolio-Agrostietum stoloniferae, pasture vegetation, wet meadows, poisonous plants, weeds.*

INTRODUCTION

Meadows and pastures are extremely important natural resources characterizing the vegetation in Serbia. In addition to their phytocenological, phytogeographic and syntaxonomic value, grasslands are of great economic importance (Kojić et al., 2004). Natural and semi-natural grasslands in Serbia cover about 1.4 million hectares of land (Aćić et al., 2013).

The vegetation of meadows and pastures forms herbaceous communities of a predominantly closed-system character, consisting of multi mesophytes. The value of plant species varies greatly, from very harmful and toxic to those of high quality (Mrfat-Vukelić et al., 2003). Grassland productivity is particularly positively affected by increased biodiversity. This apparent correlation has been attributed mainly to niche complementarity and facilitation among species, as well as to the “sampling effect”, i.e. an increased probability of including a highly productive or highly competitive species at the higher diversity levels (Assaf et al., 2011).

The productivity and biodiversity of meadows and pastures are also affected by the weed species. As weeds in these ecosystems are generally of low nutritional value and have unpleasant taste and odor, their presence adversely affects the

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ecosystem quality. Until the 1930s, with the exception of the species of the Poaceae and Fabaceae families that are considered desirable and useful, all others were categorized as worthless and harmful plants. Typically, the weeds found on natural meadows and pastures are classified into the following three categories: highly poisonous plants, mildly poisonous (or harmful) plants, and worthless weed (Mrfat-Vukelić et al., 1997). It should be noted that very adaptive and aggressive invasive species—by competing for space, light, water and nutrients, thus hindering the growth of desirable forage plants—directly affect pasture biodiversity and biomass.

In Vojvodina, pasture vegetation ass. *Trifolio-Agrostietum stoloniferae* Marković 1973 developed as a semi-ruderal phytocoenosis of a secondary character. It belongs to *Agropyro-Rumicion* Nordh. 1940 alliance, *Agrostietalia stoloniferae* Oberd 1967 formation, comprising mesophilic grassland (pasture) phytocenoses formed in Vojvodina at stream valley floors, as well as surfaces close to settlements and roads. More broadly, this vegetation belongs to mesophilic meadow community *Molinio-Arrhenatheretea* Tx. 1937 (Kojić et al., 2004).

The aim of this paper is to analyze the presence of harmful (highly poisonous, mildly poisonous, and worthless) weeds in stands of pasture association *Trifolio-Agrostietum stoloniferae* in Vojvodina.

MATERIJAL I METOD RADA

Phytocenological studies of ass. *Trifolio-Agrostietum stoloniferae* stands in Vojvodina pertain to the areas surrounding the streams: Borkovac, Zovalj, Rovača, Mandelos, Kudoš, Jegrička, Tamiš, Nera, Begej and Brzava, Kikinski kanal, Podunavlje and Potisje.

Weed species of natural meadows and pastures are categorized as highly poisonous plants, mildly poisonous (or harmful) plants and worthless weeds, according to Mrfat-Vukelić et al. (1997).

RESULTS AND DISCUSSION

Stands of the ass. *Trifolio-Agrostietum stoloniferae* in Vojvodina comprise 157 plant species (Tab. 1), which is considered as high plant diversity (Lazić, 1995; Džigurski and Nikolić, 2012). According to Butorac (2004), this association in Eastern Slavonia and Baranja includes 122 taxa, with 80 taxa found in Srem region. This great floristic wealth characterizing the stands in Vojvodina probably stems from the diverse ecological conditions prevailing in periodically flooded and thus wetter areas. Stands of the sub-association *Trifolio-Agrostietum stoloniferae* subass. *agrostetosum albae* Marković 1973 developed here, as well as at surfaces of much stronger xerophilic character, where a stand *Trifolio-Agrostietum stoloniferae* subass. *cynodontetosum* Marković 1973 formed.

Floristic composition analysis indicates that families Asteraceae (29 taxa), Poaceae (23 taxa) and Fabaceae (19 taxa) are the most abundant, followed by Lamiaceae and Cyperaceae (with 9 taxa each) and Scrophulariaceae (7 taxa) families.

Table 1. Poisonous plants in the floristic composition of the ass. *Trifolio-Agrostietum stoloniferae* in Vojvodina (HPP – highly poisonous plant, MPP – mildly poisonous plant, WS – worthless species)

Tab. 1. Štetne biljke u florističkom sastavu sastojina ass. *Trifolio-Agrostietum stoloniferae* u Vojvodini (HPP - vrlo otrovna biljka, MPP - slabo otrovna biljka, WS - loša i bezvredna vrsta)

Fam. Equisetaceae		Fam. Scrophulariaceae	
<i>Equisetum arvense</i> L.	HPP	<i>Gratiola officinalis</i> L.	MPP
Fam. Ranunculaceae		<i>Kickxia elatine</i> (L.) Dum.	
<i>Ranunculus polyanthemus</i> L.	MPP	<i>Linaria vulgaris</i> Mill.	
<i>Ranunculus repens</i> L.	MPP	<i>Odontites rubra</i> Gilib.	
<i>Ranunculus sardous</i> Cr.	MPP	<i>Rhinanthus rumelicus</i> Vel.	MPP
Fam. Amaranthaceae		<i>Veronica arvensis</i> L.	WS
<i>Atriplex litoralis</i> L.		<i>Veronica chamaedrys</i> L.	WS
Fam. Brassicaceae		Fam. Verbenaceae	
<i>Capsella bursa-pastoris</i> (L.) Med.	MPP	<i>Verbena officinalis</i> L.	
<i>Rorippa austriaca</i> (Cr.) Bess.	WS	Fam. Lamiaceae	
<i>Rorippa sylvestris</i> (L.) Bes.		<i>Calamintha vulgaris</i> (L.) Druce.	WS
<i>Sinapis arvensis</i> L.	WS	<i>Glechoma hederacea</i> L.	
Fam. Caryophyllaceae		<i>Lycopus europaeus</i> L.	
<i>Cerastium caespitosum</i> Gilib.	WS	<i>Lycopus exaltatus</i> L.	
<i>Cerastium dubium</i> (Bast.) Schw.	WS	<i>Mentha aquatica</i> L.	MPP
<i>Stellaria graminea</i> L.	WS	<i>Mentha longifolia</i> (L.) Nath.	MPP
Fam. Apiaceae		<i>Mentha pulegium</i> L.	MPP
<i>Daucus carota</i> L.		<i>Mentha verticillata</i> L.	
<i>Eryngium campestre</i> L.	WS	<i>Prunella vulgaris</i> L.	WS
<i>Pastinaca sativa</i> L.		Fam. Asteraceae	
<i>Sium latifolium</i> L.		<i>Achillea millefolium</i> L.	
<i>Torilis anthriscus</i> (L.) Gmel.	WS	<i>Ambrosia artemisiifolia</i> L.	WS
<i>Torilis arvensis</i> (Huds.) Link.	WS	<i>Artemisia vulgaris</i> L.	WS
Fam. Chenopodiaceae		<i>Artemisia maritima</i> L.	
<i>Chenopodium album</i> L.		<i>Bellis perennis</i> L.	WS
Fam. Convolvulaceae		<i>Bidens tripartitus</i> L.	WS
<i>Convolvulus arvensis</i> L.		<i>Carduus acanthoides</i> L.	MPP, WS
<i>Calystegia sepium</i> (L.) Br.	WS	<i>Carduus nutans</i> L.	MPP, WS
Fam. Oenotheraceae		<i>Cichorium intybus</i> L.	WS
<i>Epilobium adnatum</i> Gris.		<i>Cirsium arvense</i> (L.) Scop.	MPP, WS
Fam. Euphorbiaceae		<i>Cirsium lanceolatum</i> (L.) Scop.	MPP, WS
<i>Euphorbia cyparissias</i> L.	HPP	<i>Crepis setosa</i> Hall.	WS
<i>Euphorbia esula</i> L.	HPP	<i>Erigeron canadensis</i> L.	WS
<i>Euphorbia platyphyllos</i> L.	HPP	<i>Eupatorium cannabinum</i> L.	WS
Fam. Rubiaceae		<i>Helminthia echinoides</i> Gaertn.	
<i>Galium aparine</i> L.		<i>Inula britannica</i> L.	
<i>Galium mollugo</i> L.	WS	<i>Matricaria chamomilla</i> L.	
<i>Galium verum</i> L.	WS	<i>Matricaria inodora</i> L.	WS
<i>Sherardia arvensis</i> L.	WS	<i>Picris hieracioides</i> L.	WS
Fam. Geraniaceae		<i>Pulicaria dysenterica</i> (L.) Bernh.	
<i>Geranium dissectum</i> Jusl.	WS	<i>Senecio erucifolius</i> L.	MPP
<i>Geranium pusillum</i> Burm.	WS	<i>Sonchus arvensis</i> L.	WS
Fam. Primulaceae		<i>Sonchus asper</i> (L.) Mill.	
<i>Lysimachia nummularia</i> L.	WS	<i>Stenactis annua</i> (L.) Nees.	
Fam. Malvaceae		<i>Taraxacum officinale</i> Web.	
<i>Althea officinalis</i> L.		<i>Xanthium italicum</i> Mor.	
<i>Malva sylvestris</i> L.	WS	<i>Xanthium spinosum</i> L.	WS
Fam. Lythraceae		<i>Xanthium strumarium</i> L.	WS
<i>Lythrum salicaria</i> L.	WS	<i>Xeranthemum annuum</i> L.	WS

<i>Lythrum virgatum</i> L.	WS	Fam. Alismataceae	
Fam. Fabaceae		<i>Alisma plantago-aquatica</i> L.	WS
<i>Galega officinalis</i> L.	MPP	Fam. Juncaceae	
<i>Lotus angustissimus</i> L.		<i>Juncus articulatus</i> L.	WS
<i>Lotus corniculatus</i> L.		<i>Juncus compressus</i> Jacq.	WS
<i>Lotus tenuis</i> W. et K.		<i>Juncus gerardii</i> Lois.	
<i>Medicago falcata</i> L.		<i>Juncus inflexus</i> L.	WS
<i>Medicago lupulina</i> L.		Fam. Cyperaceae	
<i>Medicago sativa</i> L.		<i>Bolboschoenus maritimus</i> (L.) Palla	WS
<i>Ononis arvensis</i> L.	WS	<i>Carex distans</i> L.	WS
<i>Ononis spinosa</i> L.	WS	<i>Carex divulsa</i> Good.	WS
<i>Trifolium angulatum</i> W. et K.		<i>Carex hirta</i> L.	WS
<i>Trifolium campestre</i> Schreb		<i>Carex praecox</i> Schreb.	WS
<i>Trifolium filiforme</i> L.		<i>Carex spicata</i> Huds.	WS
<i>Trifolium fragiferum</i> L.		<i>Carex vesicaria</i> L.	
<i>Trifolium hybridum</i> L.		<i>Carex vulpina</i> L.	WS
<i>Trifolium pratense</i> L.		<i>Heleocharis palustris</i> (L.) R. Br.	WS
<i>Trifolium repens</i> L.		Fam. Poaceae	
<i>Trifolium striatum</i> L.		<i>Agropyrum repens</i> (L.) P. B.	
<i>Vicia angustifolia</i> Gruf.		<i>Agrostis alba</i> L.	
<i>Vicia hirsuta</i> (L.) S. F. Gray.		<i>Agrostis verticillata</i> Vill.	
Fam. Plantaginaceae		<i>Alopecurus pratensis</i> L.	
<i>Plantago lanceolata</i> L.		<i>Andropogon ischaemum</i> L.	WS
<i>Plantago major</i> L.	WS	<i>Bromus arvensis</i> L.	WS
<i>Plantago media</i> L.	WS	<i>Bromus commutatus</i> Schr.	
Fam. Polygonaceae		<i>Bromus mollis</i> L.	WS
<i>Polygonum aviculare</i> L.	WS	<i>Bromus tectorum</i> L.	
<i>Rumex crispus</i> L.	MPP	<i>Calamagrostis epigeios</i> (L.) Roth.	WS
<i>Rumex hydrolapathum</i> Huds.	MPP	<i>Cynodon dactylon</i> (L.) Pers.	
<i>Rumex pulcher</i> L.	MPP	<i>Dactylis glomerata</i> L.	
Fam. Rosaceae		<i>Eleusine indica</i> (L.) Gaertn.	
<i>Agrimonia eupatoria</i> L.	WS	<i>Festuca arundinacea</i> Schreb.	
<i>Crataegus monogyna</i> Jacq.	WS	<i>Festuca pratensis</i> Huds.	
<i>Potentilla anserina</i> L.	WS	<i>Festuca pseudovina</i> Hack.	
<i>Potentilla argentea</i> L.	WS	<i>Hordeum maritimum</i> With.	
<i>Potentilla reptans</i> L.	WS	<i>Hordeum murinum</i> L.	
Fam. Sambucaceae		<i>Lolium perenne</i> L.	
<i>Sambucus ebulus</i> L.	WS	<i>Phragmites communis</i> Trin.	WS
Fam. Plumbaginaceae		<i>Poa pratensis</i> L.	
<i>Statice gmelinii</i> Willd.		<i>Poa trivialis</i> L.	
Fam. Urticaceae		<i>Setaria glauca</i> (L.) P. B.	WS
<i>Urtica dioica</i> L.	WS	Fam. Typhaceae	
Fam. Dipsacaceae		<i>Typha angustifolia</i> L.	
<i>Dipsacus laciniatus</i> L.	WS	<i>Typha latifolia</i> L.	
<i>Dipsacus sylvestris</i> Huds.			

Prior studies on ass. *Trifolio-Agrostietum stoloniferae* in Vojvodina (Lazić, 1995; Stojanović et al., 1996; Džigurski and Nikolić, 2012) revealed the presence of 107 (68.15%) weed species, indicating the need for more comprehensive weed flora analysis, aimed at determining its composition and effect on biodiversity and productivity of these stands. Mrfat-Vukelić et al. (1997) also point out high participation of weed species in the vegetation of Serbian meadows and pastures (36.0-75.6% of all species), indicating significant floristic diversity.

Floristic composition analysis of ass. *Trifolio-Agrostietum stoloniferae* stands in Vojvodina indicated the presence of 22 toxic plants (14.01%), of which 4 (2.55%) were highly poisonous and 18 (11.46%) mildly poisonous. All the identified plants

belonging to families Euphorbiaceae (*Euphorbia cyparissias*, *E. esula* and *E. platyphyllos*) and Equisetaceae (*Equisetum arvense*) are classified as highly poisonous. Majority of the mildly poisonous plants belong to Asteraceae (*Carduus acanthoides*, *C. nutans*, *Cirsium arvense*, *C. lanceolatum* and *Senecio erucifolius*), Ranunculaceae (*Ranunculus polyanthemus*, *R. repens* and *R. sardous*), Polygonaceae (*Rumex crispus*, *R. hydrolapathum* and *R. pulcher*) and Lamiaceae (*Mentha aquatica*, *M. longifolia* and *M. pulegium*) families. The worthless plant group comprises 67 taxa (42.68%). The findings of this analysis are in line with the composition of ass. *Trifolio-Agrostietum stoloniferae* in the region of Serbia, where Mrfat-Vukelić et al. (2003) noted the presence of 17.7% poisonous species, of which 3.8% were highly poisonous and 13.9% mildly poisonous. In general, the authors determined that the mean participation of harmful species in the communities of *Molinio-Arrhenetheretea* class in Serbia, in addition to *Festuco-Brometea* class, is the greatest, when compared to other grassland phytocoenoses classes. Somewhat lower percentage of poisonous plants in the stands of the analyzed phytocenoses in Vojvodina stems from greater floristic wealth of these stands, confirming the positive effect of biodiversity on the quality of pasture vegetation.

The floristic composition of the analyzed stands includes 13 invasive plant species. Of these, *Ambrosia artemisiifolia*, *Bellis perennis*, *Carduus nutans*, *Cirsium arvense*, *Euphorbia esula*, *Eupatorium cannabinum*, *Linaria vulgaris*, *Lotus corniculatus*, *Lythrum salicaria*, *Rumex crispus*, and *Trifolium repens* are recognized as invasive for European region (Global Invasive Species Database). In addition, *Ambrosia artemisiifolia*, *Eleusine indica* and *Xanthium spinosum* are on the List of Invasive Species on the Territory of Vojvodina (IASV). With the exception of *Trifolium repens*, *Ambrosia artemisiifolia* and *Cirsium arvense*—characterized by abundance and coverage scores of 2-5 in the studied stands—the abundance and coverage scores for the remaining invasive species are relatively low.

CONCLUSION

Grassland phytocenosis stands *Trifolio-Agrostietum stoloniferae* in Vojvodina comprise 157 plant species. Stands of sub-association *Trifolio-Agrostietum stoloniferae* subass. *agrostetosum albae* Marković 1973 form in periodically flooded and wetter habitats, whereas stands *Trifolio-Agrostietum stoloniferae* subass. *cynodontetosum* Marković 1973 are typically found in much more arid habitats.

The most diverse families are Asteraceae (29 taxa), Poaceae (23 taxa) and Fabaceae (19 taxa).

Floristic composition analysis of ass. *Trifolio-Agrostietum stoloniferae* stands revealed the presence of 22 poisonous species (14.01%), of which 4 (2.55%) were highly poisonous and 18 (11.46%) mildly poisonous. In addition, the worthless weed plant group comprises of 67 taxa (42.68%).

The studied stands include 13 invasive species that – due to their high biological potential, competitive ability, adaptability and aggressiveness – contribute to the reduction of biodiversity, quality and productivity of grassland ecosystems. Population monitoring of the listed invasive species is thus necessary in order to prevent further structural destruction and impoverishment of floristic composition.

Powerful anthropo-zoogenic effects on pasture ecosystems lead to substantial changes in the floristic composition of these ecosystems. Thus, negative flora selection is also affected by excessive eutrophication and soil compaction, which

further accelerate the decline in biodiversity and reduce the productivity of pasture ecosystems. Better habitat management is thus recommended, as a prevention of their further degradation, ensuring existence of pasture vegetation in the future.

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**ŠTETNE BILJKE U PAŠNJAČKOJ ASS. *TRIFOLIO-AGROSTIETUM*
STOLONIFERAE MARKOVIĆ 1973 U VOJVODINI**

DEJANA DŽIGURSKI, LJILJANA NIKOLIĆ

Izvod

Sastojine ass. *Trifolio-Agrostietum stoloniferae* u Vojvodini čini 157 biljnih vrsta. Zbog prisustva čak 68,15% korovskih vrsta u florističkom sastavu i njihovog uticaja na biodiverzitet i produktivnost ove pašnjačke zajednice, urađena je analiza korovske flore sa posebnim osvrtom na prisustvo otrovnih, loših i bezvrednih biljaka. Tako je konstatovano je 14,01% otrovnih biljaka (vrlo otrovnih je 2,55% i slabo otrovnih 11,46%) i 42,68% loših i bezvrednih korovskih biljaka. U istraživanoj pašnjačkoj vegetaciji zastupljeno je i je 13 invazivnih vrsta.

Ključne reči: *Trifolio-Agrostietum stoloniferae*, pašnjačka vegetacija, vlažne livade, otrovne biljke, korovi.

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SEROPREVALNCE OF INFECTIOUS CAUSES OF ABORTION IN SOWS*

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SUMMARY: The objective of the present study was to investigate the seroprevalence of potential agents among aborted sows in a commercial swine farm in Serbia. Infectious agents may be specific and non-specific pathogens. Specific viruses, bacteria and protozoa, which come to the fetus through the blood or through the vaginal opening. Non-specific pathogenic, mainly bacteria, causing the systemic infection and they can cause a miscarriage and the death of the fetus of action in the uterus (endometritis) or direct cytolytic effect on the fetus. Almost all the fertility parameters in sows (reproductive and productive) may be affected by different infectious diseases. Changes in reproductive parameters may also occur without the appearance of appreciable pathological findings or with clinical signs often overlapping or similar to different diseases or pathogens. Since the reproductive efficiency is one of the main factors in the pig production, the activity should be focused on reducing the abortion, particularly in farm with intensive way of keeping the pigs where the occurrence of abortion should be reduced to sporadic cases. All the clinical aspects and the pathological findings should be taken into account to address a tentative diagnosis with the support of laboratory findings. Regular serological monitoring is the basis of health care of pigs, and therefore the control and prevention of reproductive disorders.

Key words: abortion, infection, reproductive parameters, sows.

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INTRODUCTION

There is a constant need in pig production in order to increase reproductive efficiency measured by the number of piglets produced. This value depends on a number of genetic and paragenetic factors and directly is affected to total production of pigs, both, in nudity and in economic terms (Stančić et al., 2012). Modern pig breeds with high genetic potential of the individual parameters of reproductive performance, which can result in the production of more than 30 weaned piglets per sow per year. However, different disorders of reproductive functions are one of the main reasons why this value is very difficult to achieve in practical terms (Gagrčin et al., 2001). The main objective of the ventures is to obtain a greater number of piglets per sow, reducing their mortality and achieving better production results. There are numerous factors that lead to a reduction in reproductive efficiency, which leads to the continuity, scope and increased cost of production (Gagrčin et al., 2007). One of the leading factors of reproductive efficiency are abortions in sows (Stojanac et al., 2011).

Infectious agents that cause abortions may be specific and non-specific pathogens. Specific are viruses, bacteria and protozoa, which come to the fetus through the blood or through the vaginal opening. Non-specific pathogens are mainly bacteria which cause systemic infection and connected to the miscarriage and death of the fetus with affecting the uterus (endometritis) or having direct cytolytic effect on fetus (Martinez et al., 2008).

The aim of this study was to investigate the serological status of sows that had a miscarriage. Serum was checked for the presence of antibodies to *Brucella* spp., *Leptospira* spp., *Listeria Monocytogenes*, *Salmonella* spp., Porcine reproductive and respiratory syndrome virus, Classical swine fever, Porcine circovirus diseases, Aujeszky's disease and Porcine parvovirus.

MATERIALS AND METHODS

Farms

The test was performed on the pig farm with of 2000 sows from January 2011 to December 2012 year. The farm is of closed type with the intensive way of keeping pigs. Lactation lasts for 28 days. After weaning, sows are placed in insemination room in group boxes (10 sow per a box) until entering into estrus, when they are moved to incarceration where they are inseminated and remain until 28th day of pregnancy. Following a positive ultrasound control sows are placed in the waiting area in group boxes (10 sows) and remain there until 110 day of pregnancy. Sows are fed installment, twice a day, and water intake is at their will. Microbiological and mycological control of raw materials for feed preparation and prepared food is conducted regularly.

Immunoprophylaxis

Seven days before weaning sows are vaccinated against classical swine fever and Aujeszky's disease. Vaccination and revaccination of sows against colibacillosis and necrotic enteritis was done on 80th and 100th day of pregnancy. Vaccination of pigs against *Mycoplasma* infection and circovirus infections was conducted on the studied farm.

Sample collection and laboratory analysis

There were 164 abortions in the experimental period. Within 24 hours of miscarriage, blood was taken from sows by the puncture in brachiocephalic plexus. Testing was conducted at reference laboratory. The blood serum of sows who had abortions was examined on the presence of antibodies specific for: *Brucella* spp. using rapid agglutination method, *Leptospira* spp. using the microscopic agglutination test, *Listeria monocytogenes* using agglutination method, and *Salmonella* spp. by indirect ELISA. The presence of antibodies against the viruses was also determined for: respiratory and reproductive syndrome swine (PRRS) by ELISA test, classical swine fever (CSF) by ELISA test, and circovirus infections (PCV-2) using indirect ELISA (Nawagitgul et al., 2002). The titer of antibodies specific for the Aujeszky's disease (AD) was determined by serum neutralization and to swine parvovirus (PPV) using hemagglutination inhibition.

Data analysis

Data were entered into an Excel spreadsheet (Microsoft Excel 2007) and imported into Stata (Stata 8 Intercooled for Windows 9x) in which data were analyzed. Descriptive analysis was done in MiniTab version 14 (MiniTabR14b) and Excel (Microsoft Excel 2007).

RESULTS AND DISCUSSION

On the test farm in a period of two years (2011-2012) there were 10789 farrowing, which makes 98.48% of all pregnant sows in this period. The rest of the pregnant sows, 164 (1.52%) had early partus or abortion (Table 1). The incidence of abortion was 1.52%, which is above the level (0.8-1%), which many researchers consider to be the limit for good, quality reproduction (Alborali and Pozzi, 2012).

Table 1. Number of abortions in the studied farm (2011-2012)
Tabela 1. Broj pobačaja na ispitivanoj farmi (2011-2012)

	Farrowing	Abortion	TOTAL
Number	10789	164	12122
%	98,48	1,52	100

Infectious factors, as main causes of reduced reproductive efficiency can lead to miscarriage and death of the fetus where the fetus by inflammation of the endometrium, or the direct influence on the fetus or sows. Table 2 shows the results of serological tests in aborted sows on the presence of specific antibodies for potential causes of miscarriage.

On the tested farm a infectious agents of brucellosis, leptospirosis and listeriosis are not identified. *Salmonella* seroprevalence of 72% is similar to what was found by other researchers (Funk, 2008; Baptista et al., 2009; Shishak et al., 2011; Hernandez et al., 2013).

Out of 164 aborted sows, 92 were positive for PRRS. This result is slightly lower than the result reached by the Tummaruk et al., 2009.

Seven tested sows were seronegative on CSF. Bearing in mind that the farm implements regular vaccination against CSF, the reason why seroconversion did not take place in these cases should be found in the failure of vaccine application.

The presence of antibodies specific for PCV-2 in 147 sows, tells about the fact that circovirus infection of pigs is widespread throughout the world and are a potential threat to cause abortion (Perreul et al., 2010).

Table 2. The presence of antibodies in the blood serum of sows

Tabela 2. Prisustvo antitela u krvnom serumu krmača

	Positive	Negativ	TOTAL
<i>Brucella</i> spp.	0	164	164
<i>Leptospira</i> spp.	0	164	164
<i>Listeria monocytigenes</i>	0	164	164
<i>Salmonella</i> spp.	118	46	164
PRRS	92	72	164
KKS	157	7	164
PCV-2	147	17	164

Parvovirus infection is widespread in clinically healthy pigs worldwide and has enzootic character. It is the constant presence of parvoviral infection on the farm, that caused all sows to be seropositive, established a high titer of antibodies, which protect the piglets from intrauterine infection (Stojanac et al., 2012).

Regular vaccination of sows against Aujeszky's disease can explain why none of the aborted sows was seronegative (Table 3).

Table 3. The titer of antibodies to PPV and AD

Tabela 3. Titar antitela za PPV i AD

	Mean	Interval of variation
PPV	3786	1024-16364
AD	12,7	2-32

CONCLUSION

Based on the analyzed and processed data it could be concluded that abortions have an important impact on the reproductive efficiency of pigs. For the improvement of inter-reproductive efficiency and reduction of the prevalence of abortion it is necessary to conduct regular serological monitoring of the entire herd. In this way we would have an insight into the health status of the herd, which will serve for the proper implementation imunoprophylactic measures, or, therefore, this reduce the number of abortions.

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SEROPREVALENCA INFEKTIVNIH UZROČNIKA POBAČAJA KOD KRMAČA

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LJUBOJEVIĆ, SLOBODAN KRAGIĆ

Izvod

Cilj ovog ispitivanja je bio da se ispita seroprevalenca potencijalnih uzročnika pobačaja kod krmača na komercijalnim farmama u Srbiji. Infektivni uzročnici mogu biti specifični i nespecifični patogeni. Specifični su virusi, bakterije i protozoe, koji dolaze do ploda putem krvi ili kroz vaginalni otvor. Nespecifični patogeni su uglavnom bakterije koje su uzročnici sisitemske infekcije i oni mogu izazvati pobačaj i uginuće plodova dejstvom na matericu (endometritis) ili direktinim citolitičkim dejstvom na plod. Skoro na sve parametre plodnosti krmača mogu da utiču različite infektivne bolesti. Do promena u reproduktivnim parametrima može doći i bez spoljašnjih promena ili se klinički znaci bolesti često preklapaju ili su isti kod različitih uzročnika. Imajući u vidu da je reproduktivna efikasnost jedan od vodećih faktora u proizvodnji svinja, treba da se radi na smanjenju broja pobačaja, naročito u farmama sa intezivnim načinom držanja svinja, gde bi se prevalenca pobačaja trebala svesti na sporadične pojave. Sve kliničke simptome i patološke nalaze treba uzeti u obzir kod postavljanja dijagnoze sa posebnom potporom laboratoriskih nalaza plotkinja koje su pobacile. Redovan serološki monitoring predstavlja osnov zdravstvene zaštite svinja, a samim tim kontrolu i preventivu reproduktivnih poremećaja.

Ključne reči: pobačaj, infekcija, reproduktivni parametri, krmače.

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SEROPREVALENCE OF *SALMONELLA ENTERICA* IN SWINE FARMS IN SERBIA*

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SUMMARY: Salmonellosis in humans, originating from pork, is an important zoonosis and the subject of this study was to investigate the seroprevalence of Salmonella enterica in swine herds. From each of the 10 farms 30 fattener pigs were examined. Selection of farms was based on the willingness of farmers to cooperate. The immune status of pigs was determined using the indirect ELISA test. The results showed a high level of seroprevalence of Salmonella enterica (33%) on the studied farms. General seroprevalence was 90% on the tested farms. Serological surveillance of Salmonella infections in herds of fatteners provides detection of high-risk herds and enables evaluation of the effectiveness of control measures implemented in primary production, and all of this in order to reduce contamination of pig carcasses.

Key words: pig, seroprevalence, Salmonella, antibodies.

INTRODUCTION

Salmonella infections can cause clinical signs of disease in various species, but in pigs these usually manifest subclinical or result in only mild transient diarrheas (Davies, 2001). In animals' food, *Salmonella enterica* prevalence estimation can serve for multiple purposes: to estimate the on-farm prevalence for risk factor analysis, intervention assessment and producer feedback, and to predict the food safety risk of products entering the food chain. In pork production, the more *Salmonella enterica* that is carried into the plant, via the pigs, the greater the risk of equipment contamination and final product contamination (Dahl and Sorensen,

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2001). Positive *Salmonella* status in fattener pigs on the farm, confirmed either by serological or bacteriological testing, increases the risk of transmission of *Salmonella* from asymptomatic intestinal presence to carcasses in the abattoir (Beloeil et al., 2004). Although it is impossible to determine the true extent of salmonellosis, pork is considered one of the main sources of salmonellosis in humans.

The main factors affecting the contamination of fattener pigs with *Salmonella*, according to the reported studies, are: (1) hygiene: washing hands, (2) management of the herd: herd size, the continuous production of pigs, and holding (type of floor and separation by walls), (3) the practice of feeding: granulation and pH of food and feeding type (all or moist), (4) health disorders: infections by parasites, antibiotic use and health status of the herd (Kranker et al., 2001). In the literature there are many factors that can enhance infection with *Salmonella*, such as *Lawsonia intracellularis* and porcine reproductive and respiratory syndrome virus (PRRSV) (Kranker et al., 2001; Farzan et al., 2006; Stojanac et al., 2013). A better knowledge of the epidemiology of *Salmonella* in swine herds is needed in order to identify effective control measures and implement a control program. Information on *Salmonella* infections can be acquired by using a variety of sources, such as the presence of antibodies in the blood or meat juice, or the presence of *Salmonella* in faecal samples or mesenteric lymph nodes (Bonde and Sørensen, 2012). Studies indicate a link between high seroprevalence and presence of *Salmonella* in faecal samples from the herd or abattoir (Stege et al., 2000; Sørensen et al., 2004), while serological and bacteriological test may be less correlated at the individual level (Casey et al., 2004; Nollet et al., 2005).

The aim of this study was to describe the seroprevalence of *Salmonella enterica* on pig farms in Serbia.

MATERIALS AND METHODS

Sampling and laboratory analysis

The study involved 10 farrow-to-finish pig farms, located all over Serbia. The farms involved in the study had to be confined farrow-to-finish operations of the intensive type and managed according to the batch-farrowing system (weaning on the same day of a group of piglets born the same week and age-segregated rearing) and an all-in/all-out hygiene policy for farrowing, post-weaning and fattening sections. Farm selection also was based on the farmers' willingness to cooperate.

The blood serum samples were collected during 2011 and 2012 from farrow-to-finish herds. Blood was taken by the puncture of the brachiocephalic plexus of the pigs. A blood serum sample from each pigs was frozen, and blood serum (harvested after thawing) was examined for specific anti-bodies against *Salmonella enterica* using an indirect ELISA (Nielsen et al., 1998). Samples with an OD% > 10 were considered seropositive. From each farm, 30 blood serum samples were taken.

Data analysis

Data were entered into an Excel spreadsheet (Microsoft Excel 2010) and imported into Stata (Stata 8 Intercooled for Windows 9x) in which data were analyzed. Descriptive analysis was done in MiniTab version 14 (MiniTabR14b) and Excel (Microsoft Excel 2010).

RESULTS AND DISCUSSION

A serological survey within this study supplies us with information on the epidemiology of *Salmonella* infections on pig farms in Serbia. *Salmonella enterica* was found in 9 out of 10 investigated farms. Out of the 300 blood serum samples 67% were negative and 33% positive for the presence of antibodies specific for *Salmonella enterica* (Table 1). Herd seroprevalence was 90%, and the average seroprevalence within herd was 33% (minimum 0; maximum 63.33), 70% of a herd had a seroprevalence between 30% and 63.33% for an OD-value of 10%. The literature states that the seroprevalence of *Salmonella* is 93% in Germany, 59% in Denmark, 79% in Greece and 72% in Sweden, evaluated at the test cut-off of OD₄₅₀ > 10, and herd cut-off of 1 or more seropositive animals. Average seroprevalence within the herd is 24% in Germany, 9% in Denmark, 14% in Greece and 10% in Sweden (Lo Fo Wong, 2001). In this study, the average seroprevalence was 33%, which indicates a higher level compared to the results of other authors (Nowak et al., 2007; Bonde and Sørensen, 2012).

Specific antibodies to *Salmonella enterica* have been found in 99 out of 300 blood sera examined. On one farm no antibodies specific for *Salmonella* were found. This farm implements the liquid feeding system. The connection between the liquid-feeding and the lower prevalence of *Salmonella* in pigs has been confirmed in several cases (van der Wolf et al., 1999; der Wolf et al., 2001; van Winsen et al., 2002; Lo FoWong et al. 2004). On-farm intervention to reduce the prevalence of *Salmonella* is difficult to perform; nevertheless, this is important in reducing the risk of this pathogen's presence on pig skins and consequently pork carcasses at abattoirs (Blagojevic et al., 2011). This study preliminary suggests a high level of prevalence of *Salmonella* in pigs on farms in Serbia. The results clearly indicate that the reduction of infection in pigs is one of the key points in the strategy of pork safety.

Table 1. *Salmonella enterica* seroprevalence estimates provided by blood serum sample collected from swine farms in Serbia

Tabela 1. Seroprevalencija *Salmonella enterica* u krvnim serumima svinja na farmama u Srbiji

Farm	Numberd tested	Number positive	% positive
I	30	4	13,33
II	30	9	30,00
III	30	19	63,33
IV	30	11	36,67
V	30	17	56,67
VI	30	9	30,00
VII	30	0	0,00
VIII	30	12	40,00

IX	30	10	33,33
X	30	8	26,67
Total	300	99	33,00

CONCLUSION

Serological monitoring of *Salmonella* infections in swine herds enables detection of high risk and evaluation of the control measures effectiveness in primary production, which is a prerequisite for reducing a meat contamination in the abattoir. The results of this study confirmed the literature reports that it is necessary to improve biosecurity and hygiene measures on farms. Good Agricultural Practices should be presented in specific guide to help farmers to control *Salmonella* on the farms.

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SEROPREVALENCIJA *SALMONELLA ENTERICA* NA FARMAMA SVINJA U SRBIJI

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Izvod

Salmoneloza ljudi poreklom od svinjskog mesa je važna zoonoza i predmet ovog istraživanja je bio da se ispita seroprevalencija *Salmonella enterica* u zapatima svinja. Sa svake od 10 farmi, ispitano je po 30 tovljenika. Izbor farmi je zasnovan na spremnosti farmera na saradnju. Imunološki status svinja je izvršen korišćenjem indirektnog ELISA testa. Rezultati prikazuju visok nivo seroprevalencije *Salmonella enterica* na ispitivanim farmama. Opšta seroprevalencija je bila 90% na ispitivanim farmama. Serološki nadzor infekcija salmonelama u zapatima tovljenika omogućava otkrivanje visokog rizika zapata i procenu efikasnosti kontrolnih mera implementiranih u primarnoj proizvodnji, a sve i cilju smanjenja kontaminacije trupova svinja.

Ključne reči: svinje, seroprevalencija, Salmonela, antitela.

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HORMONAL AND METABOLIC PROFILE IN SIMMENTAL DAIRY COWS DURING DRY PERIOD, EARLY AND MID LACTATION*

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SUMMARY: The aim of this study was to investigate endocrine and metabolic characteristic of Simmental cows in dry period, early and mid lactation. Experiment was included 45 Simmental cows: 15 in dry period (2 weeks before calving, period 1), 15 in early lactation (in first week after calving, period 2) and 15 in mid lactation (4-5 month after calving, period 3). Frequency distribution analysis shows that most of the parameters takes a normal distribution of distribution, unless the value of the total lipids (early lactation), urea (early lactation), T3 (dry period and mid lactation), T4 (early lactation) and insulin (dry period). The moment of blood collection has a substantial impact on the value of hormones and metabolites, except for total protein, T4 and insulin. Significant F value and posthoc LSD were significant for: glucose (F=25.44; LSD 1:2, 1:3, significantly higher concentrations of glucose the dry period), cholesterol (F=15.62; LSD 1:3, 2:3, significantly higher concentrations of cholesterol in the middle of lactation), triglycerides (F= 50.42 ; LSD 1:2, 1:3, significantly higher concentrations in the dry period), total lipids (F =27.89; LSD 1:3 , 2:3, significantly higher concentration in the middle of lactation), NEFA (F=10.74; LSD 1:2, 1:3, significantly higher concentrations in early lactation), BHB (F=24.39; LSD 1:2, 1:3, 2:3, significantly higher concentrations in early lactation), albumin (F=9.2, LSD 1:2, 1:3, significantly higher concentrations in the dry period), urea (F=11.61; LSD 1:2, 2:3, significantly reduced concentration in early lactation), T3 (F=3.31; LSD 1:3, 2:3, significantly lower concentrations in dry period and early lactation), growth hormone (F=4.17; LSD 1:2, 2:3, higher concentrations in early lactation). Concentrations of hormones and metabolites in Simmental cows depend on whether the blood was taken during the dry period, early lactation and mid lactation. Differences in metabolite values indicate the existence of reduced food intake, a negative energy balance, the mobilization of lipids and altered functional status of hepatocytes

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in early lactation. Most of the studied parameters showed a normal frequency distribution. The absence of normal frequency distribution for some parameters, particularly during early lactation, may indicate high heterogeneity of cows in the process of adaptation to the periparturient period.

Key words: *simental cows, endocrine status, metabolic profile, lactation, dry period.*

INTRODUCTION

Parturition and lactogenesis are accompanied by many physiological changes that facilitate the maintenance of homeostasis (Bauman and Currie, 1980). Adaptation of the endocrine system during the transitional period is the key factor in maintaining metabolic balance (Aceves et al, 1985). Homeorhesis induces intense lipid mobilization and ketogenesis, and the liver is adapted to metabolic changes in dairy cows. Intensive postpartal lipid mobilization and ketogenesis are sufficient for a series of compensatory metabolic processes with changes in blood metabolic profile during lactation in healthy cows (Bell, 1995; Drackley, 1999; Remppis et al, 2011; Cincović et al., 2012). In addition to these, the period around calving is characterized by numerous metabolic and hematological changes such as decreased glycemia, decreased cholesterol concentrations, low concentrations of urea, elevated bilirubin concentration, reduced calcemia (Cincović et al., 2011; Đoković et al., 2013). Interdependent changes occur in the GH/insulin/IGF-I/glucose signaling pathway in early lactation (Lucy, 2001). Insulin plays a role in the adaptation of organic matter metabolism in dairy cows during the transitional period and during lactation, particularly in terms of nutrient redistribution and partitioning towards the mammary gland (insulin resistance) (Butler et al., 2003; Balogh et al., 2008). A decrease in thyroid hormone levels (hypothyroidism) occurs in the blood of peripartal cows, particularly during early lactation, when body reserves are mobilized for the production of high amounts of milk (Tiirats, 1997; Huszenicza et al., 2002).

The aim of this study was to investigate endocrine and metabolic characteristics of Simmental cows in dry period, early and mid lactation.

MATERIAL AND METHODS

Experiment was included 45 Simental cows: 15 in dry period (2 weeks before calving, period 1), 15 in early lactation (in first week after calving, period 2) and 15 in mid lactation (4-5 month after calving, period 3). Blood samples were obtained by venepuncture of v.jugularis, 3-4 hours after morning feeding. Serum epruvettes with vacutainer system were used. Samples were transported to the laboratory immediately after venepuncture. Concentration of T3, T4, insulin and growth hormone were determined using a standard ELISA kit (Endocrine technologies inc., USA) and measured by Humareader device. Biochemistry parameters (glucose, NEFA, cholesterol, total lipids, triglycerides, total proteins, albumin and urea) were determined using a Biosistem, Elitech or Randox kit and measured by CobasMira+ spectrophotometer.

Statistics: Characteristics of frequency distribution were analysed by calculation of standard skewness standard kurtosis and Shapiro-Wilk test of

normality. Difference in concentration of parameters in three periods was analysed using a ANOVA analysis with posthock LSD test.

RESULTS AND DISCUSSION

Frequency distribution analysis shows that most of the parameters takes a normal distribution of distribution, unless the value of the total lipids (early lactation), urea (early lactation), T3 (dry period and mid lactation), T4 (early lactation) and insulin (dry period). When you look at the symmetry of the distribution (skewness), it is for most parameters identical for all three test periods. However, there are some disparities when it comes to the distribution of NEFA, BHB and urea. For values of NEFA and BHB in early lactation was found negative skewness value (left turn), indicating the dominance above-average values of NEFA and BHB. In contrast, the frequency distribution of urea in early lactation had a high positive value of skewness, which indicates the dominance of below-average values. All these results are shown in Table 1.

The moment of blood collection has a substantial impact on the value of hormones and metabolites, except for total protein, T4 and insulin (Table 2, Figure 1-13). Significant F value and posthock LSD were significant for: glucose (F = 25.44 ; LSD 1:2, 1:3 , significantly higher concentrations of glucose the dry period), cholesterol (F= 15.62; LSD 1:3, 2:3, significantly higher concentrations of cholesterol in the middle of lactation), triglycerides (F= 50.42 ; LSD 1:2, 1:3, significantly higher concentrations in the dry period), total lipids (F = 27.89 ; LSD 1:3 , 2:3, significantly higher concentration in the middle of lactation), NEFA (F= 10.74; LSD 1:2, 1:3, significantly higher concentrations in early lactation), BHB (F = 24.39; LSD 1:2, 1:3, 2:3, significantly higher concentrations in early lactation), albumin (F= 9.2 ; LSD 1:2, 1:3, significantly higher concentrations in the dry period), urea (F= 11.61; LSD 1:2, 2:3, significantly reduced concentration in early lactation), T3 (F= 3.31; LSD 1:3, 2:3, significantly lower concentrations in dry period and early lactation), growth hormone (F = 4,17; LSD 1:2, 2:3, higher concentrations in early lactation).

It is found that concentration of many metabolites are depend of energy balance (Đoković et al, 2013). The concentration of glucose is significantly lower in the first week postpartum, because of the reduced food intake and increased utilization of glucose for milk production (Doepel et al., 2002). NEFA concentration increases in the week after calving as a result of energy deficit and changes in hormonal status of cows (Drackley et al., 2005, Bertoni et al., 1998). NEFA is used for energy purposes and is a precursor for the synthesis of BHB in hepatocytes (Guo et al., 2008). The concentration of BHB is a significant indicator of the health and productivity of cows. Cows with high values of BHB have lower reproductive capacity, significantly lose body condition, produce a small amount of milk and suffer from extreme metabolic changes (Kessel et al., 2008, Huszenicza et al., 2006). Serum levels of glucose, triglycerides, total cholesterol, total protein, albumin and urea are indicators of hepatic function and decreases in their concentration may imply fat infiltration in the liver (Sevinc et al., 2003; Wathes et al., 2007; Đoković et al., 2007; Đoković et al., 2011). The concentration of urea depends on the intake of food, and energy balance (Rastani et al., 2006). Reduced concentration of proteins may occur as a result of increased use of amino acids in processes of gluconeogenesis in the period around calving. In favor of this assumption there is an

elevated concentration of AST, which is a significant indicator of catabolism of proteins and their use for gluconeogenesis (Seal and Reynolds, 1993). Lower insulin concentration in periparturient period compared to mid lactation is consequence of insulin resistance when decreased insulin concentration provide higher lipolysis and glucose utilisation in udder (DeKoster and Opsomer, 2013). The hormonal activity of thyroid gland has an important role in the transitional period for determining the cell metabolism intensity, metabolism of lipids and carbohydrates and lactation course itself by its thyroid hormones. Under the conditions of a negative energy balance and of high lipomobilization, the concentrations of thyroidea hormone in the blood were significantly lower in transitional period, with a markedly declined triiodothyronine in the blood shortly before and after calving (Đoković et al., 2010).

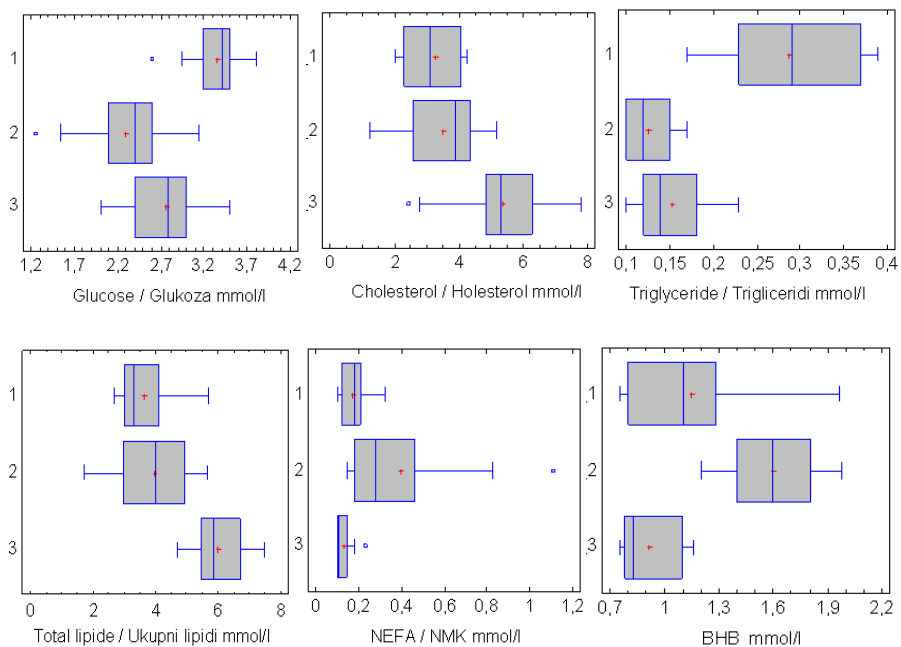
Table 1. Frequency distribution characteristics of endocrine and metabolic parameters in three different periods

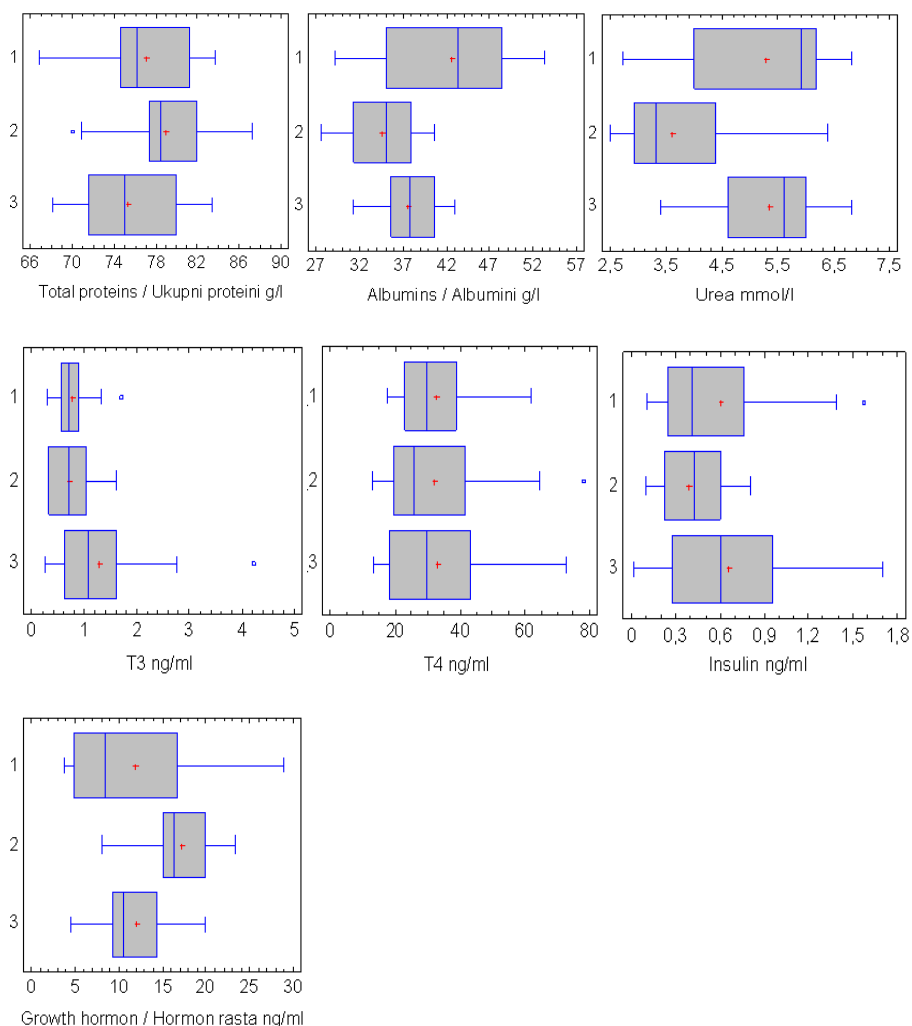
Parametar	Dry period			Early lactation			Mid lactation		
	Skew.	Kurt.	Normal. distrib.	Skew.	Kurt.	Normal. distrib.	Skew.	Kurt.	Normal. distrib.
Glucose mmol/l	-1,44	1,16	Yes	-0,9	0,42	Yes	-0,35	-0,15	Yes
Cholesterol mmol/l	-0,38	-1,11	Yes	-0,62	-0,33	Yes	-0,7	0,57	Yes
Triglycerides mmol/l	0,33	-0,57	Yes	0,88	-0,63	Yes	1,2	0,03	Yes
Total lipide mmol/l	1,7	1,7	Yes	3,1	2,6	No	0,65	-0,76	Yes
NEFA mmol/l	1,5	0,68	Yes	-1,1	0,79	Yes	1,84	1,55	Yes
BHB mmol/l	1,35	0,36	Yes	-0,1	-0,97	Yes	0,77	-1,3	Yes
Total proteins g/l	-1,01	0,44	Yes	-0,1	0,04	Yes	0,28	-0,54	Yes
Albumin g/l	-0,55	-0,51	Yes	-0,46	-0,27	Yes	-0,38	-0,22	Yes
Urea mmol/l	-1,21	-0,61	Yes	2,23	1,68	No	-0,75	-0,29	Yes
T3 ng/ml	2,01	1,5	No	1,17	0,33	Yes	3,32	3,84	No
T4 ng/ml	1,91	0,8	Yes	2,5	1,65	No	1,47	0,41	Yes
Insulin ng/ml	2,36	1,34	No	0,97	0,15	Yes	0,97	0,15	Yes
Growth hormon ng/ml	1,31	-0,26	Yes	0,55	-0,21	Yes	0,55	-0,21	Yes

Table 2. ANOVA and LSD test – Influence of blood sampling period to hormone and metabolite concentration

Parametar	F	LSD
Glucose mmol/l	25,44**	1:2, 1:3
Cholesterol mmol/l	15,62**	1:3, 2:3
Triglycerides mmol/l	50,42**	1:2, 1:3
Total lipide mmol/l	27,89**	1:3, 2:3
NEFA mmol/l	10,74**	1:2, 1:3
BHB mmol/l	24,39**	1:2, 1:3, 2:3
Total proteins g/l	2,26	/
Albumin g/l	9,2**	1:2, 1:3
Urea mmol/l	11,61**	1:2, 2:3
T3 ng/ml	3,31*	1:3, 2:3
T4 ng/ml	0,04	/
Insulin ng/ml	1,17	/
Growth hormon ng/ml	4,17**	1:2, 2:3

*p<0,05; **p<0,01





Graph 1-13. Hormone and metabolite concentration in dry period (1), early (2) and mid (3) lactation.

CONCLUSION

Concentrations of hormones and metabolites in Simmental cows depend on whether the blood was taken during the dry period, early lactation and mid lactation. Differences in metabolite values indicate the existence of reduced food intake, a negative energy balance, the mobilization of lipids and altered functional status of hepatocytes in early lactation. Most of the studied parameters showed a normal frequency distribution. The absence of normal frequency distribution for some parameters, particularly during early lactation, may indicate high heterogeneity of cows in the process of adaptation to the periparturient period.

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HORMONALNI STATUS I METABOLIČKI PROFIL KRAVA SIMENTALSKE RASE U PERIODU ZASUŠENJA, TOKOM RANE LAKTACIJE I SREDINE LAKTACIJE

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Izvod

Cilj ovog rada je da se ispituju endokrine i metaboličke karakteristike krava simentalске rase u različitim periodima laktacije i zasušenju. U ogled je uključeno 45 krava: 15 krava u zasušenju (2 nedelje pre teljenja, period 1), 15 krava u ranoj laktaciji (prva nedelja posle teljenja, period 2) i 15 krava u sredini laktacije (4-5 meseci posle teljenja, period 3). Analiza distribucije frekvencije pokazuje da većina parametara zauzima normalnu raspodelu distribucije, osim vrednosti ukupnih lipida (rana laktacija), uree (rana laktacija), T3 (period zasušenje i sredina laktacije), T4 (rana laktacija) i insulin (zasušenje). Ispitivanjem koncentracije hormona i metabolita u različitim periodima laktacije i zasušenju, zaključujemo da je uticaj momenta uzimanja krvi na vrednost metabolita značajna za sve ispitivane parametre, osim za ukupne proteine, T4 i insulin (Tabela 2, Grafik 1-13). Statistički značajna vrednost F testa i posthock LSD razlike iznose: glukoza (F=25,44; LSD 1:2, 1:3, značajno viša koncentracija glukoze u zasušenom periodu); holesterol (F=15,62, LSD 1:3, 2:3, značajno viša koncentracija holesterola u sredini laktacije); trigliceridi (F=50,42; LSD 1:2, 1:3, značajno viša koncentracija u periodu zasušenja); total lipide (F=27,89; LSD 1:3, 2:3, značajno viša koncentracija u sredini laktacije); NEFA (F=10,74; LSD 1:2, 1:3, značajno viša koncentracija u ranoj laktaciji); BHB (F=24,39; LSD 1:2, 1:3, 2:3, značajno viša koncentracija u ranoj laktaciji); albumini (F=9,2; LSD 1:2, 1:3, značajno viša koncentracija u periodu zasušenja); urea (F=11,61; LSD 1:2, 2:3, snižena koncentracija u ranoj laktaciji); T3 (F=3,31; LSD 1:3, 2:3, niža koncentracija u periodu zasušenja i ranoj laktaciji); growth hormon (F=4,17; LSD 1:2, 2:3, viša koncentracija u ranoj laktaciji). Koncentracija hormona i metabolita kod krava Simentalske rase zavisi od toga da li je krv uzeta u periodu zasušenja, rane laktacije ili sredine laktacije. Razlike u vrednosti metabolita ukazuju na postojanje smanjenog unosa hrane, negativnog energetskog bilansa, sa mobilizacijom lipida i izmenjenim funkcionalnim statusom hepatocita u ranoj laktaciji. Većina ispitivanih parametara pokazuje normalnu distribuciju frekvencije. Izostajanje normalne distribucije za pojedine parametre, posebno u periodu rane laktacije ukazuje na veliku heterogenost krava u procesu adaptacije na peripartalni period.

Ključne reči: simentalске krave, endokrini status, metabolički profil, laktacija, zasušenje.

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INFLUENCE OF PROPYLENE GLYCOL TO ENDOCRINE AND METABOLIC CHARACTERISTIC IN COWS DURING PERIPARTURIENT PERIOD*

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SUMMARY: *During periparturient period must be provided extra energy. Propylene glycol can rapidly supply transition dairy cows with energy and can be converted to propionic acid in the rumen and transport to liver, where it is converted in glucose. The aim of this study is to estimate influence of propylene glycol to metabolic and endocrine profile in periparturient period. We hypothesized that influence of propylene glycol in protection of energy metabolism in early lactation should be proven trough analysis of the relationship between metabolic and endocrine parameters with NEFA (level of lipolysis) and BHB (level of ketogenesis). Experiment involved 50 healthy holstein-frisian cows (25 – group supplemented with propylene glycol 100g per meal, twice daily, three weeks before calving and three week after calving and 25 – control group). Blood samples were collected one week before expected calving, at calving day and one week after calving by venipuncture of v.coccigea. Concentration of NEFA, BHB, insulin, IGF-I, glucose and bilirubin was measured. The results show that cows that received propylene glycol are less burdened with metabolic stress. Thus, cows treated with propylene glycol have higher concentrations of glucose, insulin, IGF-I and lower concentrations of NEFA, BHB and bilirubine. Post-hock analysis it can be concluded that cows in early lactation show these differences, but the differences in metabolite concentrations before calving were not significant. Cows in which was applied propylene glycol are less burdened by metabolic stress, which is reflected in lower proportion of cows with signs of metabolic stress. Efficacy of propylene*

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glycol may be seen in the fact that propylene glycol is changing relationships and strength of links between metabolites. In the first week after calving, when there is most pronounced metabolic load was found a significant negative correlation between plasma NEFA or BHB with insulin, IGF-I and glucose concentration and positive correlation with bilirubin concentration. Correlation between metabolite was significantly lower or was absent in the group of cows that received propylene glycol. We performed the analysis of the regression model and tested the difference between the b parameters in cows that received propylene and control groups. For each unit of NEFA or BHB was found significantly lower decrease in the concentration of insulin, IGF-I and glucose and significantly lower increase in the concentration of bilirubin in relation to the test value of the control group. Energy added by propylene glycol changes metabolic characteristics of cows characterized by reduced lipid mobilization and ketogenesis with their reduced relationship with other metabolic parameters.

Key words: dairy cows, propylene glycol, metabolic profile.

INTRODUCTION

Negative energy balance in dairy cows during transition period (21 days before and 21 days after calving) leads to many metabolic changes. These metabolic changes occur as a result of the entry of the mammary gland in the metabolic processes. Negative energy balance is the result of decreased food intake, higher consumption of glucose in the udder and decrease insulin sensitivity. The consequence of mentioned metabolic changes is higher lipid mobilization from body depot with increased concentration of NEFA (nonesterified fatty acid). Higher NEFA mobilization in liver leads to higher concentration of ketone body in blood, especially BHB (beta-hydroxybutyrate) (Ingvarsen and Andersen, 2000). Lipomobilisation and ketogenesis burden hepatocytes with lipide infiltration as consequence. Cows with liver lipidosis show higher bilirubine and lower IGF-I concentration (Bobe et al, 2004). The ability of the hepatocytes to use NEFA and BHB for energy purpose is depends of propionate from rumen. But, decreased dry mater intake in transition period reduces propionate concentration. Therefore during periparturient period must be provided extra energy. Propylene glycol can rapidly supply transition dairy cows with energy and can be converted to propionic acid in the rumen and transport to liver, where it is converted in glucose (Nielsen and Ingvarsen, 2004). In many experimental study cows supplemented with propylene-glycol showed decreased lipide mobilization (lower NEFA concentration) and ketogenesis (lower BHB concentration) with change in hormonal status, milk production and reproductive efficiency (Studer et al., 1993; Formigoni et al., 1996; Rizos et al, 2008; Hoedemaker et al, 2004).

The aim of this study is to estimate influence of propylene glycol to metabolic and endocrine profile in periparturient period. We hypothesized that influence of propylene glycol in protection of energy metabolism in early lactation should be proven trough analysis of the relationship between metabolic and endocrine parameters with NEFA (level of lipolysis) and BHB (level of ketogenesis).

MATERIAL AND METHODS

Experiment involved 50 healthy holstein-frisian cows. Cows were divided in two groups with 25 cows: group supplemented with propylene glycol (100g per meal, twice daily, three weeks before calving and three week after calving) and control group. Blood samples were collected one week before expected calving, at calving day and one week after calving by venipuncture of v.coccigea. Concentration of insulin and IGF-I was measured using a standard ELISA procedures (Cusabio). Concentration of glucose, NEFA, BHB and bilirubin were measured using a standard kit (Randox, UK). The samples were analyzed immediately after the taking by automatically analyzer (Rayto).

Statistical procedures included t-test for difference between mean value in experimental and control group for each examined parameters in blood. Reduction of metabolic stress by propylene glycol was examined using a t-test for difference in proportion of cow burden with metabolic stress in experimental and control group. Criteria for metabolic stress included: insulin <0.5 ng/ml, IGF-I <25 ng/ml, glucose <2mmol/l, NEFA >0.6mmol/l, BHB >0.9mmol/l and bilirubin >9 μ mol/l. Correlation between NEFA and BHB and insulin, glucose, IGF-I and bilirubin in first week after calving was analysed using a t-test for Pearson correlation coefficient separately in experimental and control group. Change in metabolic profile in relation to value of NEFA and BHB in first week after calving was detected using a test for b parameters in regression analysis, separately in experimental and control group.

RESULTS AND DISCUSSION

The results show that cows that received propylene glycol are less burdened with metabolic stress. Thus, cows treated with propylene glycol have higher concentrations of glucose, insulin, IGF-I and lower concentrations of NEFA, BHB and TBIL (Table 1). Post-hock analysis it can be concluded that cows in early lactation show these differences, but the differences in metabolite concentrations before calving were not significant. Bojković-Kovacević et al. (2011) in their observation concluded that the application of propylene glycol leads to an increase in glucose concentration above the normal range, while the concentration of BHB and TBIL was significantly lower. Our findings are in the spirit of these results. The higher the concentration of insulin during the application of propylene was found in the work of Lien et al. (2010), but it did not exist at any time of sampling, but only in the first days after parturition (4-6), where we also performed blood sampling. Prepartum application of propylene glycol from 21 d before calving to the d of parturition reduced prepartum plasma concentrations of NEFA and BHBA and increased prepartum plasma concentrations of glucose and insulin (Juchem et al., 2004; Miyoshi et al., 2001; Pickett et al., 2003; Stokes and Goff, 2001). Also, peripartum application of propylene glycol leads to decrease blood concentrations of NEFA and BHBA and increases blood concentrations of glucose, insulin and cholesterol (Butler et al., 2006; Formigoni et al., 1996; Hoedemaker et al., 2004). Our results are consisted with previously results. The application of propylene glycol leads to an increase in IGF-I in the weeks before and the first week after calving (Hoedemaker et al, 2004), which was confirmed by our results. In one experiment on a large group of cows has been shown that the application of propylene glycol has no effect on the levels of insulin, glucose, IGF-I and NEFA

(Lomander et al., 2012). However, in this study, the propylene was administered only postpartum, with a significant effect of the herd of cows which are encouraged, and we conclude that the significant effect of propylene is absent as a result of a heterogeneous group. Lower serum bilirubin levels and lower activity of liver enzymes may be related to reduce triglyceride accumulation in hepatocytes, which was found in cows that received propylene glycol (Studer et al, 1993). Also, the application of propylene glycol has a positive effect on cholesterol (Formigoni et al, 1996). It is known that a higher value implies higher cholesterol VLDL lipoproteins that transport fats from hepatocytes. Cows in which was applied propylene glycol are less burdened by metabolic stress, which is reflected in lower proportion of cows with signs of metabolic stress (Table 2).

Efficacy of propylene glycol may be seen in the fact that propylene glycol is changing relationships and strength of links between metabolites. In the first week after calving, when there is most pronounced metabolic load was found a significant negative correlation between plasma NEFA or BHB with insulin, IGF-I and glucose concentration and positive correlation with bilirubin concentration. The existence of a correlation is consistent with previously obtained results (Wathes et al, 2007). Correlation between metabolite was significantly lower or was absent in the group of cows that received propylene glycol (Table 3). We performed the analysis of the regression model and tested the difference between the b parameters in cows that received propylene and control groups. For each unit of NEFA or BHB was found significantly lower decrease in the concentration of insulin, IGF-I and glucose and significantly lower increase in the concentration of bilirubin in relation to the test value of the control group (Table 4). The above results indicate that application of propylene glycol reduces the impact of lipid mobilization and ketogenesis (NEFA and BHB) on metabolic parameters. Lipid mobilization and ketogenesis are the most important consequences of negative energy balance, which could affect many aspects of metabolic and functional status of hepatocytes in cows (González et al, 2011; Cincović et al, 2012; Đoković et al, 2013). High concentrations of NEFA and BHB in cows imply high risk of periparturient disease (Ospina et al., 2010; Cincović et al, 2012a) and our early obtained results show a significant strong negative correlation between NEFA and insulin levels in diseased cows (Cincović et al, 2012b).

Table 1: Prepartum and postpartum metabolic profile in control and cows supplemented with propylene glycol

	Control			Propylene			SEM	Week	Propylene
	-1	0	+1	-1	0	+1			
Insulin <i>ng/ml</i>	0,72	0,42	0,55	0,89	0,67	0,71	0,11	<0,01	<0,05
IGF-I <i>ng/ml</i>	51,2	30,1	29,5	62,6	49,9	38,8	5,5	<0,05	<0,01
Glu <i>mmol/l</i>	2,71	2,19	2,9	2,94	2,69	3,32	0,14	<0,05	<0,05
NEFA <i>mmol/l</i>	0,69	0,84	0,43	0,41	0,54	0,28	0,11	<0,01	<0,05
BHB <i>mmol/l</i>	0,59	0,99	0,56	0,51	0,64	0,33	0,21	<0,01	<0,05
BIL <i>μmol/l</i>	8,2	10,1	7,05	6,5	7,23	5,89	1,13	<0,05	<0,05

Table 2: Proportion of cows with suboptimal metabolic status

	Control			Propylene			Week	Propylene
	-1	0	+1	-1	0	+1		
Insulin <i>ng/ml</i>	4	12	8	4	8	4	<0,05	<0,05
IGF-I <i>ng/ml</i>	4	16	12	4	8	4	<0,01	<0,01
Glu <i>mmol/l</i>	8	24	12	2	16	4	<0,01	<0,01
NEFA <i>mmol/l</i>	4	20	16	0	12	12	<0,01	<0,05
BHB <i>mmol/l</i>	4	32	8	4	12	4	<0,01	<0,01
BIL <i>μmol/l</i>	8	20	16	0	16	8	<0,01	<0,05

Tabela 3: Influence of propylene glycol supplementation to relationship between NEFA, BHB and other metabolites in first week after calving

	NEFA		P Difference	BHB		P Difference
	Propylene	Control		Propylene	Control	
Insulin <i>ng/ml</i>	-0.28 <0.05	-0.49 <0.01	<0.05	0.30 <0.05	0.56 <0.01	<0.05
IGF-I <i>ng/ml</i>	-0.14 NS	-0.31 <0.05	<0.05	0.33 <0.05	0.47 <0.01	NS
Glu <i>mmol/l</i>	-0.22 NS	-0.51 <0.01	<0.01	0.24 NS	0.55 <0.01	<0.05
BIL <i>μmol/l</i>	0.26 NS	0.32 <0.05	NS	0.33 <0.05	0.52 <0.01	<0.05

Table 4: Regression analysis – testing of b parameters which means change in metabolic value for each unit of NEFA or BHB in control group and group supplemented with propylene glycol in first week after calving

	NEFA		P Difference	BHB		P Difference
	Propylene	Control		Propylene	Control	
Insulin	- 0.05±0.008	- 0.09±0.007	<0.01	- 0.07±0.01	- 0.09±0.013	<0.05
IGF-I	- 0.32±0.11	- 0.44±0.09	<0.05	- 0.37±0.09	- 0.51±0.10	<0.01
Glu	- 0.08±0.02	- 0.11±0.03	<0.01	0.11±0.09	0.17±0.11	NS
Bil	0.52±0.27	0.63±0.31	NS	0.47±0.12	0.6±0.15	<0.05

CONCLUSION

Cows treated with propylene glycol have higher concentrations of glucose, insulin, IGF-I and lower concentrations of NEFA, BHB and TBIL, especially in early lactation. These cows are less burdened by metabolic stress, which is reflected in lower proportion of cows with signs of metabolic stress. Energy added by propylene glycol changes metabolic characteristics of cows characterized by reduced lipid mobilization and ketogenesis with their reduced relationship with other metabolic parameters.

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UTICAJ PROPILLEN GLIKOLA NA ENDOKRINE I METABOLIČKE KARAKTERISTIKE KRAVA U PERIPARTALNOM PERIODU

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Izvod

Tokom peripartalnog perioda kravama se mora obezbediti dodatna energija za početak laktacije. Propilen glikol može veoma brzo dati energiju koja je potrebna kravama tako što biva konvertovan u propionsku kiselinu u buragu, koja biva transportovana u jetru gde se dalje konvertuje u glukozu. Cilj ovog istraživanja je da se ispita uticaj dodavanja propilen glikola u hrani na metabolički i endokrini profil krava u peripartalnom periodu. Predpostavka je da se zaštitni efekat propilen glikola može dokazati analizom korelacije između metaboličkih i endokrinih parametara sa koncentracijom NEFA (neesterifikovane masne kiseline, pokazatelj lipolize, u vezi sa insulinskom rezistencijom) i BHB (beta-hidroksibutirat, pokazatelj ketogeneze, u vezi sa masnom jetom). U eksperiment je uključeno 50 krava holštajn-frizijske rase: 25 krava je dobijalo propilen glikol 100g/obrok/krava, dok je 25 krava bila kontrolna grupa i nije dobijala ovaj energetski suplement. Krv je uzorkovana u nedelji pre teljenja, nedelji u kojoj se krava otelila i u nedelji potom venepunkcijom *v.coccigea*. Merena je koncentracija NEFA, BHB, insulina, IGF-I (insulinu sličan faktor rasta I) glukoze i bilirubina. Dobijeni rezultati pokazuju da su krave koje su dobijale propilen glikol mnogo manje opterećene metaboličkim stresom u ispitivanom periodu. Tako, ove krave pokazuju višu koncentraciju glukoze, insulina, IGF-I i nižu koncentraciju NEFA, BHB i bilirubina. Ove razlike su postojale u nedeljama posle teljenja, ali ne i pre teljenja. Manje opterećenje metaboličkim stresom kod krava koje su dobijale propilen glikol odlikuje se i značajno manjom proporcijom krava koje pokazuju znake metaboličkog stresa (indikator metaboličkog stresa: insulin <0.5 ng/ml, IGF-I <25 ng/ml, glukoza <2mmol/l, NEFA >0.6mmol/l, BHB >0.9mmol/l i bilirubin >9μmol/l). Efikasnost propilen glikola može da se objasni činjenicom da aplikacija ovog energetskog prekursora menja relacije između metabolita. U prvoj nedelji posle teljenja, kada je metaboličko opterećenje krava najveće, postoji značajna negativna korelacija koncentracije NEFA ili BHB sa insulinom, IGF-I i glukozom i pozitivna korelacija sa koncentracijom bilirubina. Kod krava koje su dobijale propilen glikol ove korelacije izostaju. Regresionom analizom je pokazano da za svaku jedinicu NEFA odnosno BHB postoji značajno manji pad vrednosti insulina, IGF-I i glukoze i značajno slabiji porast koncentracije bilirubina u grupi krava koje su dobijale propilen glikol u odnosu na kontrolnu grupu. Energija dobijena iz propilen glikola smanjuje stepen lipidne mobilizacije i ketogeneze i smanjuje njihov uticaj na metaboličku adaptaciju koja se ogleda u kretanju vrednosti ostalih ispitanih metaboličkih parametara.

Ključne reči: mlečne krave, propilen glikol, metabolički profil.

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METABOLIC CHARACTERISTICS OF COWS ON FARMS WITH DIFFERENT WELFARE SCORE*

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SUMMARY: Animal welfare is an important factor influencing the productivity and health. Modern research in the area of animal welfare includes an analysis of parameters obtained from the animals themselves on farms. The aim of this study is to investigate influence metabolic characteristic of cows on farms with different welfare score. Measuring the welfare on cow farms was conducted according to the protocol of Welfare Quality® scoring system. Experiment was included 9 farms. Each of four principles of welfare was analyzed. According to the scores of welfare, farms were divided into two groups: a group of cows with low scores (score 0-50), a group of cows with high scores (score 51-100). Difference between mean value of metabolic parameters of cows originating from farm with low and high welfare scores were analyzed by a Student t-test. Cows from farm with lower welfare score according to principle of good housing showed a tendency to lower glucose and calcium concentration in mid lactation period. Cortisol concentration was higher in early ($p < 0.1$) and mid lactation ($p < 0.05$). Metabolic adaptation of cows in relation to welfare principle of good feeding showed that cows from farm with low scores showed: higher NEFA, BHB, bilirubine and cortisol concentration in early lactation. Metabolic changes in mid lactation showed tendency to higher BHB, and lower glucose and urea concentration. Cows from farm with lower welfare score according to principle of good health showed higher NEFA, BHB and cortisol and lower glucose concentration. The metabolic characteristics of cows depend on providing the principle of welfare on farms. Cows from farm with lower welfare scores showed higher cortisol concentration and signs of metabolic stress, such as higher NEFA, BHB and bilirubin concentration and lower concentration of glucose and urea. The most important principles, which significantly affects these characteristics, are the principles of good health and good feeding. These results show that it is possible to estimate the welfare of cows based on metabolic status, which requires further investigation.

Key words: cows, welfare, metabolic stress, cortisol.

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INTRODUCTION

Animal welfare is an important factor influencing the productivity and health. It is defined as the ability of animals to adapt to different environmental conditions (Hristov et al., 2007). The sustainable production and stability in animal production depends from provide of all welfare criteria and principles as well as eliminate stressors (Belić and Cincović, 2010). Assessment of well-being can be done by analyzing environmental conditions in which the animals live. Modern research in the area of animal welfare includes an analysis of parameters obtained from the animals themselves on farms (Whay et al., 2003b). Our previous result (Cincović et al., 2012) showed that the health and productive characteristics of cows depend on providing the principle of welfare on farms. The most important principle, which significantly affects these characteristics, is the principle of good health. Milk production and reproductive characteristics depend on the principles of good nutrition, good housing and good health. Good production is determined by many factors on the farm, so ensuring a quality veterinary supervision (the principle of health) must be a priority.

The aim of this study is to investigate influence metabolic characteristic of cows on farms with different welfare score.

MATERIAL AND METHODS

Measuring the welfare on cow farms was conducted according to the protocol of Welfare Quality® scoring system. This system provides the assessment of the welfare of cows on the basis of four principles alongside the associated criteria, the principle of good housing, good nutrition principle, the principle of good health and the principle of good behavior of cows. Evaluation of farms was performed based on four principles of welfare. We used statistical software that has been formed based on standards set out in the Welfare Quality® Assessment Protocol for Cattle, 2009, ISBN/EAN978-90-78240-04-4. According to the scores of welfare, farms were divided into two groups: a group of cows with low scores (score 0-50), a group of cows with high scores (score 51-100). Score was calculated for each of the four principles. Nine dairy farm on the territory of Serbia participated in this experiment. At each farm blood was sampled from 7 cows in early and 7 cows mid lactation. Total number of cows included in experiment was 126. Metabolic status included concentration of NEFA, BHB, glucose, urea, Ca, total bilirubin and cortisol. For this purpose was used kit manufactured by Randox (UK) and ELISA kit for cortisol (Uscn Life Science). All measurements were performed on Rayto spectrophotometers.

Statistics: Difference between mean value of metabolic parameters of cows originating from farm with low and high welfare scores were analyzed by a Student t-test.

RESULTS AND DISCUSSION

Cows from farm with lower welfare score according to principle of good housing showed a tendency to lower glucose and calcium concentration in mid lactation period. Cortisol concentration was higher in early ($p<0.1$) and mid lactation ($p<0.05$). Results are presented in Table 1. Metabolic adaptation of cows in relation to welfare principle of good feeding showed that cows from farm with low scores showed: higher NEFA, BHB, bilirubine and cortisol concentration in early lactation. Metabolic changes in mid lactation showed tendency to higher BHB, and lower glucose and urea concentration. Results are presented in Table 2. Cows from farm with lower welfare score according to principle of good health showed higher NEFA, BHB and cortisol and lower glucose concentration. Results are presented in Table 3.

Cortisol is hormone of stress (Hristov i Bešlin, 1991). The role of this hormone is generally positive, at least immediately, improving fitness by energy mobilization (Sapolsky et al., 2000). Higher concentration of cortisol in our results agree with previous general conclusions that stressed cows had higher cortisol concentration (Mendoza et al., 2000). Level of cortisolemia is highly influenced by usual and unusual manipulation with cows (Bertoni et al., 2005). Good housing Other metabolic characteristic of cows from farm with lower welfare score are indicative for metabolic stress. Stressors and poor nutritional management causing reduction in voluntary DMI will result in large increases in NEFA. The main blood indicators of lipomobilization in ruminants are BHB, the most important and abundant ketone body, and NEFA. NEFA are preferentially and greatly accumulated as TG in the liver, primarily because of a decrease in the very low density lipoproteins synthesis by hepatocytes, and consequently bilirubine concentration increase (Cincović et al., 2011; Cincović et al., 2012a; Đoković et al., 2013). Good feeding and good health principles of welfare are in relation with body condition score, lameness, skin lesion and other. Body condition score is in relation with energy balance, food energy and metabolic characteristic (suffering decreased food intake with consequently metabolic changes), and changes as lameness and skin lesion are very painful (which increases cortisol concentration), so it is clear why there are certain metabolic and hormonal adaptations in dairy cows according to welfare score. These results show that it is possible to estimate the benefit of cows based on metabolic status, which requires further investigation.

Table 1: Concentration of metabolic parameters between cows originating from farm with low and high welfare scores according to principle of good housing

Metabolic parameters	Period of lactation	Welfare score- good housing		p
		0-50 (4 farms)	51-100 (5 farms)	
NEFA mmol/l	Early	0.65±0.17	0.55±0.16	NS
	Mid	0.34±0.11	0.29±0.09	NS
BHB mmol/l	Early	0.86±0.19	0.71±0.14	NS
	Mid	0.41±0.11	0.33±0.14	NS
Glucose mmol/l	Early	2.78±0.8	2.66±0.5	NS
	Mid	3.01±0.5	3.55±0.32	<0.1
Urea mmol/l	Early	3.3±0.44	3.5±0.37	NS
	Mid	5.2±0.6	5.8±0.4	NS
Ca mmol/l	Early	2.2±0.4	2.3±0.3	NS
	Mid	2.3±0.4	2.8±0.3	<0.1
Bilirubin µmol/l	Early	8.8±1.4	8.1±1.12	NS
	Mid	5.5±0.9	5.1±1.1	NS
Cortisol ng/ml	Early	18.6±3.1	15.5±2.2	<0.1
	Mid	12.81±1.8	10.1±2.1	<0.05

Table 2: Concentration of metabolic parameters between cows originating from farm with low and high welfare scores according to principle of good feeding

Metabolites and hormone	Period of lactation	Welfare score- good feeding		p
		0-50 (4 farms)	51-100 (5 farms)	
NEFA mmol/l	Early	0.69±0.15	0.42±0.17	<0.05
	Mid	0.35±0.09	0.3±0.13	NS
BHB mmol/l	Early	0.92±0.14	0.75±0.11	<0.05
	Mid	0.66±0.11	0.54±0.12	<0.1
Glucose mmol/l	Early	2.11±0.4	2.77±0.44	<0.05
	Mid	3.22±0.33	3.56±0.39	<0.1
Urea mmol/l	Early	3.22±0.44	3.36±0.57	NS
	Mid	4.9±0.6	5.7±0.59	<0.1
Ca mmol/l	Early	2.11±0.34	2.21±0.25	NS
	Mid	2.5±0.22	2.7±0.4	NS
Bilirubin µmol/l	Early	9.97±1.61	8.51±1.32	<0.05
	Mid	5.66±1.1	5.8±0.9	NS
Cortisol ng/ml	Early	17.9±1.2	16.3±1.6	<0.1
	Mid	12.5±2.1	11.1±1.9	NS

Table3: Concentration of metabolic parameters between cows originating from farm with low and high welfare scores according to principle of good health

Metabolites and hormone	Period of lactation	Welfare score-good health		p
		0-50 (6 farms)	51-100 (3 farms)	
NEFA mmol/l	Early	0.68±0.13	0.49±0.16	<0.05
	Mid	0.46±0.09	0.31±0.11	<0.1
BHB mmol/l	Early	0.89±0.14	0.75±0.12	<0.1
	Mid	0.61±0.15	0.42±0.17	<0.05
Glucose mmol/l	Early	2.26±0.51	2.89±0.46	<0.05
	Mid	3.45±0.28	3.49±0.32	NS
Urea mmol/l	Early	3.2±0.4	3.5±0.4	NS
	Mid	5.1±0.8	5.6±0.32	NS
Ca mmol/l	Early	2.12±0.3	2.19±0.25	NS
	Mid	2.4±0.35	2.62±0.3	NS
Bilirubin µmol/l	Early	8.39±1.2	8.56±1.4	NS
	Mid	5.3±1.13	5.2±0.09	NS
Cortisol ng/ml	Early	18.9±2.3	16.5±1.9	<0.05
	Mid	12.3±2.2	10.3±2.1	<0.05

CONCLUSION

The metabolic characteristics of cows depend on providing the principle of welfare on farms. Cows from farm with lower welfare scores showed higher cortisol concentration and signs of metabolic stress, such as higher NEFA, BHB and bilirubin concentration and lower concentration of glucose and urea. The most important principles, which significantly affect these characteristics, are the principles of good health and good feeding. These results show that it is possible to estimate the benefit of cows based on metabolic status, which requires further investigation.

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METABOLIČKE KARAKTERISTIKE KRAVA NA FARMAMA SA RAZLIČITIM NIVOOM DOBROBITI

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Izvod

Dobrobit životinja je značajan faktor koji utiče na produktivnost i zdravlje. Savremena istraživanja u oblasti dobrobiti životinja podrazumevaju analizu parametara dobijenih od samih životinja na farmi. Cilj ovog rada je da se ispituju metaboličke karakteristike kod krava na farmama sa različitim nivoom dobrobiti. Merenje nivoa dobrobiti vršeno je upotrebom protokola *Welfare Quality® scoring system*. U ogled je uključeno 9 farmi. Ispitan je svaki princip dobrobiti posebno. Na osnovu skora dobrobiti farme su podeljene u dve kategorije: farme sa niskom ocenom dobrobiti (0-50) i farme sa visokom ocenom dobrobiti (51-100). Razlika u srednjoj vrednosti metabolita između krava koje potiču sa navedene dve kategorije farmi ispitana je t-testom. Kod krava na farmama sa nižim skorom dobrobiti postoji viša koncentracija kortizola i znaci metaboličkog stresa krava koji se ogledaju u povećanoj koncentraciji NEFA, BHB i bilirubina i sniženoj koncentraciji glukoze i uree. Najvažniji principi dobrobiti koji utiču na metabolički status krava su princip dobrog zdravlja i princip dobre ishrane. Dobijeni rezultati pokazuju da je moguće koristiti metaboličke parametre krava u proceni dobrobiti, što zahteva dodatna istraživanja.

Ključne reči: krave, dobrobit, metabolički stres, kortizol.

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CONCENTRATION OF MERCURY IN MEAT AND SLOVAK TRADITIONAL MEAT PRODUCTS*

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SUMMARY: Mercury contents have been determined by atomic absorption spectrometry in the Selected ham, Frankfurters, “Lovecka” salami, “Malokarpatska” salami. The highest concentration of mercury in raw materials (beef, pork, pork bacon) was beef. Increasing concentrations of mercury was found after the addition of additives, spices and curing compounds causing a threefold increase in the concentration of mercury in finished products. We found statistically significant differences ($P < 0.001$) in the concentration of mercury between the starting materials and the finished product.

Key words: mercury, meat, meat products, AAS.

INTRODUCTION

Meat and meat products are important for human diet in many parts of the world because they contribute to solve the global food problem and provide the well-known proteins, minerals, vitamins and trace element contents (Alturiqui et al., 2012). Contamination with heavy metals is a serious threat because of their toxicity, bioaccumulation and bio magnifications in the food chain (Demirezen et al., 2006). In recent years, much attention has been focused on the concentration of heavy metals in fish, and other foods in order to check for those hazardous to human health (Alcaide-Castineira et al., 1995). Metals such as iron, copper, zinc and manganese are essential metals since they play an important role in biological system, whereas mercury, lead and cadmium are toxic, even in trace amounts. Lead, cadmium, mercury are among the main toxic metals which have a cumulative effect. The essential metals can also produce toxic effects at high concentrations (EC, 2001). Pastirma and sausage were analysed for heavy metals, their levels depend on factors such as environmental conditions, type of pasture and genetic characteristics of organisms (Demirezen and Aksoy, 2004). Foods may be contaminated by

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chemicals from many different sources (soil, sediment, water, air). Using different kinds of food (meat, dairy products, fish, vegetables and fruits), animals and human may be also contaminated with chemicals (Abou-Arab, 2001).

Mercury occurs as elemental mercury and as inorganic and organic compounds, all having different toxicological properties. Total mercury can be analysed in water, air and biological material (Massányi et al. 2003). The toxic properties of mercury vapour are due to mercury accumulation in the brain causing neurological signs. At high exposure levels, mercury tremor is seen accompanied by severe behavioural and personality changes, increased excitability, loss of memory and insomnia (Nordberg et al., 2007).

The aim of the present study was to evaluate the concentration of mercury in the traditional and popular meat products in Slovakia (selected ham and frankfurters, Lovecka and Malokarpatska salami) during the technological processing.

MATERIAL AND METHODS

Sampling was done so that the samples were representative that to have the average composition and characteristics of the goods from which they were collected. The collection sample during the manufacturing process was carried out under the following scheme.

“Selected ham” was sampled starting raw material (pork thigh), then samples homogenized meat with additives (salt and Sodium nitrite, Sodium pyrophosphate, sodium tripolyphosphate, Sodium Ascorbate). Finally, the actual sample of the finished product after heat treatment, cooling to 25°C and after drying in climates with $a_w = 0.95$. *“Frankfurters”* were sampled raw materials (beef, pork, pork bacon and emulsion of skin), then followed by homogenized samples with the addition of substances (salt, Sodium ascorbate, polyphosphates, ground pepper). *“Lovecka salami”* – were collected basic raw (beef, pork and pork bacon); then samples mixed meat with additives (salt, Sodium Ascorbate, Erythorbic acid, ground black pepper, sugar, garlic, starter culture) and finally the actual sample of the finished product after heat treatment, cooling to 25°C and drying in climates with $a_w = 0.95$. *“Malokarpatska salami”* – were collected basic raw (beef, pork and pork bacon); then samples mixed meat with additives (salt, spice extracts, Sodium Nitrite, highlighter flavor, Lactobacillus) and finally the actual sample of the finished product after heat treatment. The concentration of mercury was determined in a total of 45 samples of raw materials respectively finished product. Samples after collected were packed to plastic bags, and frozen (-18°C). Concentrations of total mercury in samples were measured with cold vapour atomic absorption spectrometer (Nippon Instrument Corporation MA-2). 30-50 mg of meat or homogenized meat samples and final products were used in the protocol. The material was not mineralized before the measurement and the analyses were performed with the wet weight (w/w) of the material. The samples were supplemented with two additives: additive M (Wako Pure Chemicals Industries Ltd. for NIC 286-61845) and additive B (Wako Pure Chemicals Industries Ltd. for 282- 98 62665) to minimize potential interferences. Limit of detection established for the whole procedure was 0.170 ng of total Hg. The accuracy of the method was checked against the certified reference material (BCR-463). Final results were given in ppb ($\mu\text{g.g}^{-1}$) for meat and other samples

We set the basic variation statistical values (arithmetic mean, standard deviation, coefficient of variation, maximum and minimum value). The significant differences

between means were calculated by a one way analysis of variance using Duncan's multiple range test at $P<0.001$.

RESULTS AND DISCUSSION

The concentrations of mercury in the selected ham are presented in Table 1. Mercury in the selected ham was present in the range of 5.231–1.159 ppb. The highest concentration was detected in finish product selected ham 6.236 ± 0.724 ppb. The lowest concentration of mercury was determined in samples of pork 1.159 ± 0.242 ppb. Results obtained in this study were lower than those obtained by Noël et al.(2011). They were quantified in 97% samples. The mean Hg concentrations in white meat ranged between 76 ppb for king crabs and 151 ppb for swimming crabs.

Table 1. Basic variation statistical characteristics of mercury concentration in the raw materials and final product Selected ham

	Selected ham		
	pork thigh/Hg	homogenized samples/Hg	finish product/Hg
\bar{X}	2.198 ^{***A, B}	6.108 ^{***A}	6.236 ^{***B}
SD	0.38	0.623	0.724
CV	17.34	10.20	11.61
SEM	0.14	0.220	0.256
MIN	1.65	4.89	4.98
Median	2.215	6.108	6.13
MAX	2.58	6.87	7.45

Legend: \bar{X} - mean SD – standard deviation, CV(%) – coefficient of variation, MIN – minimum, MAX – maximum value, ^A pork thigh versus homogenized samples; ^B pork thigh versus finish product ^{***} $P<0.001$

The concentrations of mercury were observed in the samples frankfurters starting from raw materials (beef, pork, pork bacon, emulsion skin), homogenized samples and finish product are as presented in Table 2. The highest concentration of Hg was found in the finish product (5.231 ± 0.720 ppb) and the lowest level was observed in the pork samples (1.159 ± 0.720 ppb). From raw materials was found the highest concentration of mercury in the beef (2.048ppb). Ingestion of contaminants with various environmental pollutants, especially heavy metals by animals causes deposition of residues in meat. Due to the grazing of cattle on contaminated soil, higher levels of metals have been found in beef (Sabir et al., 2003). In Nigeria, the main source of metals in chicken meat and meat of goat, sheep and beef arises from contamination of feed and drinking water (Akan et al., 2010). In animal food products one of the most important factors that influence the content of toxic metals in animal products is the life span of the animals. Animals with long life span such as horse, older cattle and game, can accumulate some inorganic contaminants (Lukáč and Massányi, 2011).

Table 2 Basic variation statistical characteristics of mercury concentration in the raw materials and final product Frankfurters

	Frankfurters					
	beef/Hg	pork/Hg	pork bacon/Hg	emulsion of skin/Hg	homogenized samples/Hg	finish product/Hg
\bar{X}	2.048 ^{***ABC}	1.159	1.459	4.61 ^{***A}	4.762 ^{***B}	5.231 ^{***C}
SD	0.835	0.242	0.289	0.836	0.954	0.720
CV	40.76	20.86	19.84	18.13	20.04	13.76
SEM	0.264	0.007	0.009	0.264	0.302	0.223
MIN	1.25	0.95	0.95	3.54	3.57	4.2
Median	1.55	1.0	1.5	4.68	4.715	5.231
MAX	3.27	1.54	1.89	6.15	6.54	6.51

Legend: \bar{X} - mean, SD – standard deviation, CV(%) – coefficient of variation, MIN – minimum, MAX – maximum value, ^A beef versus emulsion of skin; ^B beef versus homogenized samples; ^C beef versus finish product ***P<0.001

The concentrations of mercury in the “Lovecka” salami are presented in Table 3. Hg levels ranged between 1.239±0.2251 ppb in the pork bacon, 1.787±0.2163 ppb in the pork, 2.334±0.2897 ppb in the beef, 6.986±1.342 in the homogenized samples and 7.463±1.438 ppb in the finish product. We found statistically significant differences (P<0.001) in the concentration of mercury between beef versus finish product; pork versus finish product; pork bacon versus finish product. Result of concentration of mercury in samples Malokarpatska salami are shown Table 4. From raw materials the highest concentrations of mercury had beef 2.751±1.095 ppb. The average concentration of mercury was higher in homogenized samples with addition additives and spices 6.159±1.530 ppb and finish product Malokarpatska salami 9.295±2.367 ppb. Significant differences at the significance level P<0.001 in the concentration of mercury between the raw materials and finished product. Endo et al. 2003 surveyed the total mercury levels in red meat. All concentrations in all red meats were present in the range 0.52– 0.54 ppb. Hg concentrations in the “Lovecka” salami exceeded level of the total mercury levels in red meat, the most popular cetacean products in Japan.

Table 3. Basic variation statistical characteristics of mercury concentration in the raw materials and final product “Lovecka” salami

	Lovecka salami				
	beef/Hg	pork/Hg	pork bacon/Hg	homogenized samples/Hg	finish product/Hg
\bar{X}	2.334 ^{***A}	1.787 ^{***B}	1.239 ^{***C}	6.986	7.463 ^{***ABC}
SD	0.2897	0.2163	0.2251	1.342	1.438
CV	12.41	12.10	18.17	19.21	19.27
SEM	0.1183	0.0883	0.0919	0.548	0.5872
MIN	1.858	1.458	0.851	5.489	5.231
Median	2.335	1.866	1.253	6.935	7.756
MAX	2.687	2.045	1.458	8.678	9.248

Legend: \bar{X} - mean SD – standard deviation, CV(%) – coefficient of variation, MIN – minimum, MAX – maximum value, ^Abeef versus finish product, ^B pork versus finish product; ^Cpork bacon versus finish product ***P<0.001

Table 4. Basic variation statistical characteristics of mercury concentration in the raw materials and final product “Malokarpatska” salami

	Malokarpatska salami				
	Beef/Hg	Pork/Hg	Pork bacon/Hg	Mixed samples/Hg	Finish product/Hg
\bar{X}	2.751 ^{***A}	1.494 ^{***B}	1.364 ^{***C}	6.159	9.295 ^{***ABC}
SD	1.095	0.511	0.262	1.530	2.367
CV	39.82	34.17	19.21	24.85	25.47
SEM	0.346	0.1614	0.0828	0.484	0.749
MIN	1.25	1.05	0.98	2.58	7.15
Median	2.69	1.23	1.35	6.54	8.76
MAX	4.27	2.41	2.01	7.84	15.4

Legend: \bar{X} - mean SD – standard deviation, CV(%) – coefficient of variation, MIN – minimum value, MAX – maximum value, ^A beef versus finish product; ^B pork versus finish product; ^C pork bacon versus finish product ***P<0.001

According to the Codes of Alimentarium in the Slovak republic, the maximal admitted or contaminating mercury in meat and meat product is 0.05mg.kg⁻¹. In our analysis concentration of mercury in raw materials and finished products were below the permitted level of Hg. According Alturiqi and Albedair mercury contents of samples chicken's meat ranged between 0.009-0.015ppb. Hg concentrations obtained from this study were higher than the permitted mercury limit of study Alturiqi and Albedair. The levels estimated in our meat products are lower to those observed by Barreto et al. (2009) (160- 610 ppb in white meat products).

CONCLUSIONS

Results from this study show that mercury concentrations in Selected ham, Frankfurters, “Lovecka” salami, “Malokarpatska” salami were systematically below the maximum level of 0.05 mg.kg⁻¹. Their levels depend on factors such as environmental conditions. From the results of this study, finished products were found the highest significant levels of mercury and raw materials the lowest levels. Technological treatments are important for levels of mercury in meat products. Heavy metals transfer to animals and humans through the food chain JECFA, 2004. Technological process of processing meat can create a potential source of heavy metals in finish products.

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KONCENTRACIJA MERCURY-a U MESU I TRADICIONALNIM SLOVAČKIM PROIZVODIMA OD MESA

ANETTA LUKÁČOVÁ, ĽUKASZ BINKOWSKI, JOZEF GOLIAN

Izvod

Sadržaj merkurijske je određena automatskom apsorpcionom spektrometrijom, u selektovanim šunkama, Frankfurterima, "Lovecka" salami i "Malokarpatska" salami. Najviša koncentracija merkurijske, u svežem materijalu, (junetina, svinjetina i svinjski bekon), ustanovljena je u junetini. Povećanje koncentracije merkurijske je ustanovljena posle dodavanja aditiva, što povećava koncentraciju merkurijske u gbotovim proizvodima od mesa. Ovo povećanje koncentracije merkurijske, u gotovim proizvodima, u odnosu na sirovo meso, bila je statistički značajna ($P < 0.001$).

Ključne reči: mercury, meat, meat products, AAS.

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APPLICATION OF AN INTEGRAL METHODOLOGY IN DETERMINING TMDL FOR DISSOLVED OXYGEN IN THE BEČEJ-BOGOJEVO CANAL

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SALVAI¹

SUMMARY: Water quality (WQ) in the canal network in Vojvodina is not satisfactory. The most important WQ parameter is the concentration of dissolved oxygen, which indicates suitability for life of aquatic organisms. The reach of Bečej-Bogojevo canal has been chosen since it receives seriously polluted water from the Vrbas-Bezdan canal. In the research an integral methodology has been applied consisting of WQ modeling using QUAL2K, Geographic Information Systems (GIS) mapping of spreading of dissolved oxygen concentration and calculating Total Maximum Daily Load (TMDL) values of polluting substances. The calibration of the model has been conducted using the data of WQ parameters obtained from field work, previously imported into GIS database. Besides, using data on concentrations of polluting substances from the cadastre of polluters a few simulations has been conducted for various scenarios and as a result GIS maps have been obtained showing changes in concentrations of dissolved oxygen along the reach. Finally, the TMDL values of major polluting substances affecting the concentration of dissolved oxygen have been determined.

Key words: integral methodology, dissolved oxygen, canal network, modeling, QUAL2K.

INTRODUCTION

The concentration of dissolved oxygen (DO) represents one of the most important WQ parameters for sustaining aquatic life, especially for non photosynthetic organisms. The basic canal network of the Hydrosystem Danube-Tisa-Danube (HS DTD) consists of huge canals which are formed as slow flowing streams with poor aeration, due to small slope and relatively large amount of water that depends on seasonal variations. This explains variable concentration of DO

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ranging from super saturation to low concentrations, and deficient. Because of the mentioned processes macrophytic vegetation grows. Džigurski et al. (2009) have investigated canal vegetation diversity which is also conditioned by controlled water regime and different depth of some sections. Besides, canal network has significant potential for receiving and diluting waste water, but in some cases, because of excessive pollution, it represents a problem and often causes algal blooms or vegetation overgrowth. This is predominantly influenced by effluents from food processing industries, farms, pollutants coming from municipal waste waters, as well as urban and agricultural runoff and other sources (Piperski and Salvai, 2008; Piperski et al., 2010; Savić et al., 2010). The problem of WQ in the canal network is complex, and in order to overcome the problem, modern integral approach needs to be considered. The integral approach is based mostly on the requirements of Water Framework Directive (WFD) of the European Union, as well as on the concept of Total Maximum Daily Load (TMDL) developed by the United States Environmental Protection Agency (US EPA). The research was focused upon the application of an integral methodology for determining acceptable oxygen concentration in correlation to pollution loads along a reach of the canal Bečej-Bogojevo.

MATERIAL AND METHOD

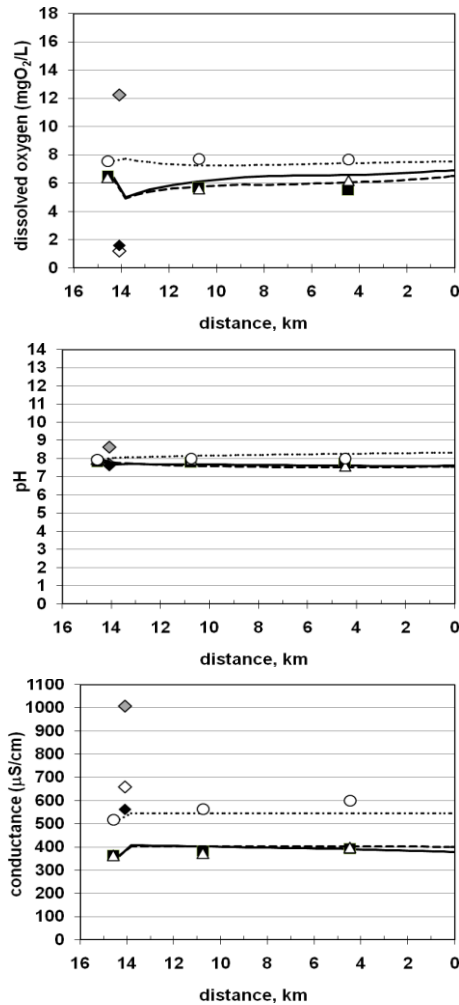
The canal Bečej-Bogojevo receives highly polluted water from the canal Vrbas-Bezdan after the point of their confluence – Triangle. In the downstream Triangle the processes of dilution and self-purification occur. The research was conducted on a 14.58 km long reach where the most intense processes of self purification take place.

The data for major pollutants (total P, inorganic P and ammonium) have been obtained from the cadastre of polluters of the PPMC “Vojvodina Vode”. Data on canal flow rates measured on the locks Vrbas for the canal Vrbas-Bezdan and Kucura for the canal Bečej-Bogojevo were obtained from the same institution. Since flow rates within the whole HS DTD have been controlled and conditioned by the users’ needs, hydrometeorological conditions have only partial significance. In the past decade 2000-2010 canal flow rates were analyzed during four years as representative: 2000, 2003, 2007, and 2009 partly because these give good reflection on variable meteorological conditions and because the systematized data on flow rates exist only for these years. Generally, according to the average daily precipitation measured by hydrometeorological station Bečej, the years 2000 and 2003 could be characterized as dry ones, 2007 as extremely wet, while 2009 was with average precipitation according to the database of the Republic Hydrometeorological Service of Serbia. The average daily flow rate for both locks for mentioned years is 5.21 m³/s, while the maximal one is 17.24 m³/s.

Three data sets of hydrometric and WQ measurements were used for calibration and validation: for calibration from May 28th, 2009, and for validation measurements conducted on September 23rd, 2009, and August 13th, 2010 (Figure 1). The Hydrometeorological data on temperature and cloudiness were obtained by direct field measurements simultaneously with WQ measurements, while the data on wind speed and humidity were taken from the database of the Republic Hydrometeorological Service of Serbia for the meteorological station Bečej for the same hours. Figure 1 presents results of calibration and validation for DO,

temperature, conductivity and pH. The complete modeling procedure using QUAL2K was explained in details in the previous research (Grabić, et al., 2011).

The simulations applying QUAL2K have been performed using different combinations of input data for canal flow rates and for pollution loads (Table 1). The QUAL2E has been well known and widely used for modeling DO concentrations (Cox, 2003), but for this research its improved version QUAL2K has been chosen since it is easier for application and enables more modeling options.



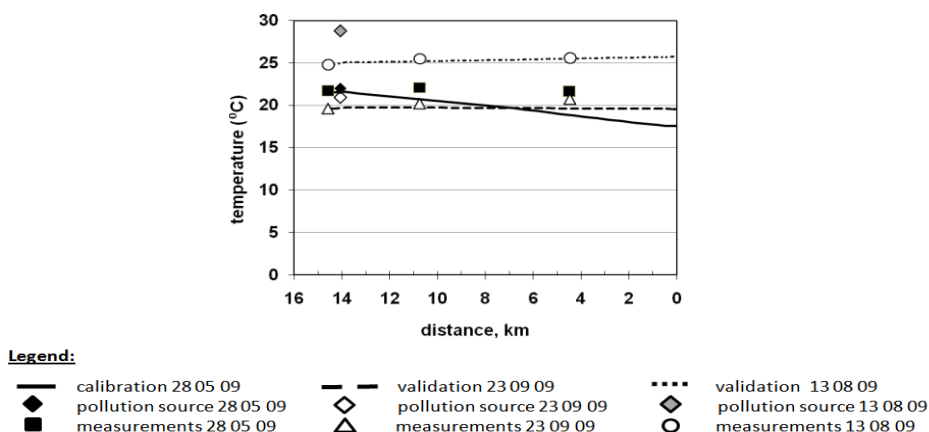


Figure 1. Calibration and validation of the WQ model for DO, pH, conductivity and temperature

Table 1. Input data for simulations

Simulations:	Canal flow rates		Pollution loads			
	descriptive	(m ³ /s)	descriptive	Total P (kgP/dan)	Inorg. P (kgPO ₄ ⁻ /dan)	NH ₄ -N (kgN/dan)
1	average	5.21	minimum	73.59	0.12	55.04
2	average	5.21	average	110.47	7.07	602.38
3	average	5.21	maximum	1346.04	55.58	6558.42
4	maximum	17.24	minimum	73.59	0.12	55.04
5	maximum	17.24	average	110.47	7.07	602.38
6	maximum	17.24	maximum	1346.04	55.58	6558.42

RESULTS AND DISCUSSION

The Fish Directive (Directive 2006/44/EC) defines the concentration of dissolved oxygen in Cyprinid waters as guide and mandatory. A guide concentration in 50 % has to be ≥ 8 and 100 % ≥ 5 , while mandatory, 50 % ≥ 7 mgO₂/l. When the oxygen concentration falls below 4 mgO₂/l, the competent authority must prove that this situation will have no harmful consequences for the balanced development of the fish population.

According to the simulations outputs it can be concluded that the most favourable conditions for DO concentrations are in cases of minimal pollution loads and maximal and average canal flow rates, when DO concentration drops only along 2km below 7 mgO₂/l, as well as for average pollution loads and maximal canal flow rate (Figure 2a, 2b). In the simulation where average pollution loads and average canal flow rate is employed for the majority of the reach, DO concentration is between 6-7, while only at the end of the reach it raises below 7 mgO₂/l (Figure 2b). The worst scenario is for the maximal pollution loads where, in case of the average canal flow rate, DO concentration is below 2 for majority of the reach, while a bit

better situation is for the maximal canal flow rate and maximal pollution loads, when DO concentration is above 4 mgO₂/l (Figure 2c).

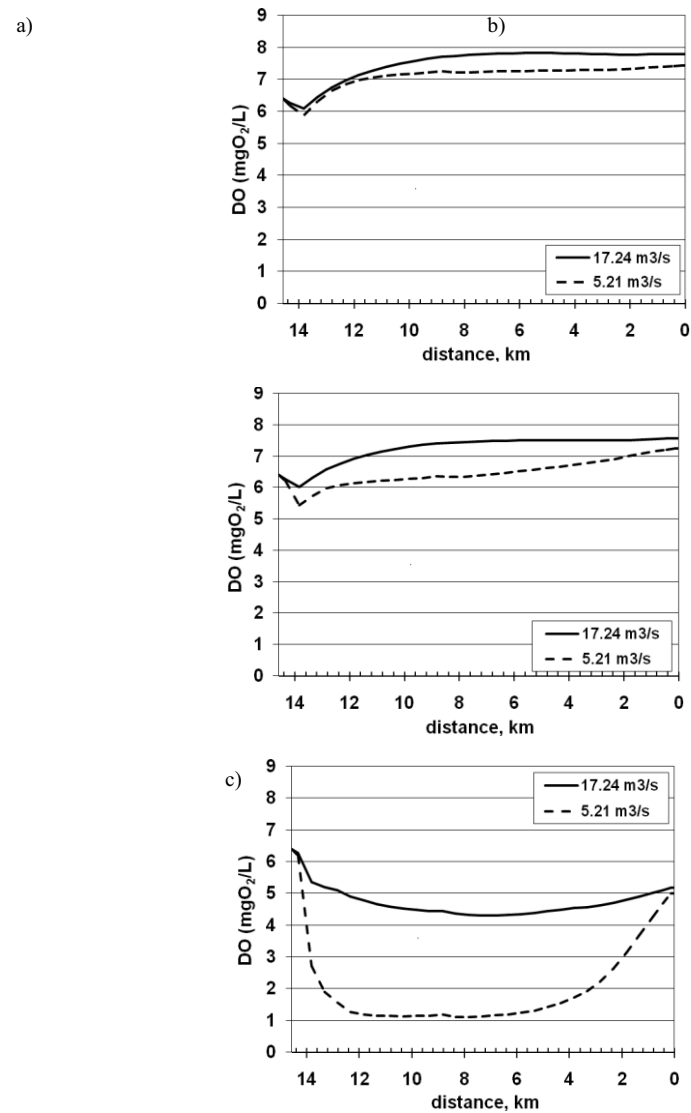


Figure 2. Changes in concentration of dissolved oxygen along the reach after receiving a) minimal, b) average and c) maximal pollution loads for maximal and average canal flow rates

TMDL values have to be calculated in a way following changes in instream canal conditions. In this paper six possible scenarios are presented and the TMDL has been calculated for the most common case – Simulation 2, for which the allowable loads of pollutants are: 58.52kg/day of total P, 21.57kg/day of inorganic P and 351.11kg/day of ammonium.

Linking GIS with WQ models brings a new opportunity in a form of maps where DO concentration changes could be easily observed along the canal reach. Differently colored ranges of DO concentrations belonging to corresponding WQ classes could help concluding whether the applied scenarios are favorable or not. Figure 3 presents changes of DO concentration along the reach after the results of Simulation 3. Results are colored in accordance with WFD coloring scheme and classification for artificial water bodies according to contemporary Serbian regulation adjusted to WFD requirements (Sl. Glasnik RS, br.74/2011).

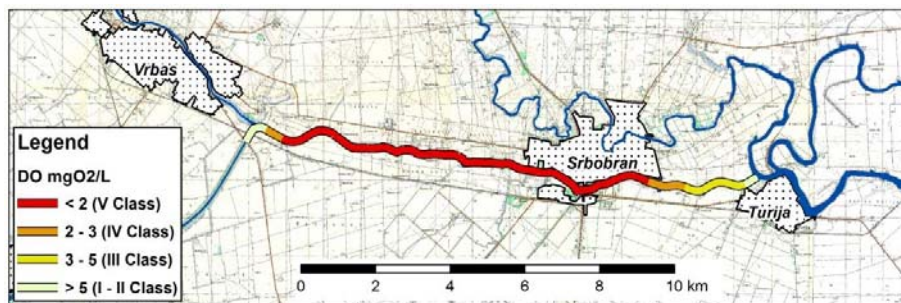


Figure 3. GIS presentation of Simulation 3 results for DO concentration changes along the canal reach

CONCLUSION

DO concentration changes along the reach of the Bečej-Bogojevo canal obtained in the research by application of the integral methodology showed that it is highly dependent on pollution loads of nitrogen and phosphorus compounds. Therefore, determination of TMDL values for polluting substances is of great importance for canal WQ management in order to maintain good WQ. Besides, linking GIS with WQ model brings a new quality in the form of presenting modeling outputs on GIS maps, which eases further data analysis. The methodology could be successfully applied on the whole canal network in Vojvodina and on the modeling various WQ parameters.

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PRIMENA INTEGRALNE METODOLOGIJE PRI ODREĐIVANJU TMDL VREDNOSTI ZA RASTVORENI KISEONIK U KANALU BEČEJ-BOGOJEVO

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SALVAI

Izvod

Kvalitet vode u kanalskoj mreži u Vojvodini nije zadovoljavajući. Najznačajniji parametar kvaliteta vode je koncentracija rastvorenog kiseonika, koja ukazuje na pogodnost za život vodenih organizama. Deonica kanala Bečej-Bogojevo je odabrana pošto prima vodu veoma opterećenu zagađenjem iz kanala Vrbas-Bezdan. Primenjena je integralna metodologija koja se sastoji iz modeliranja modelom kvaliteta vode QUAL2K, kartiranja rasprostiranja koncentracije rastvorenog kiseonika u GIS-u i određivanja TMDL vrednosti za zagađujuće materije. Kalibracija modela je sprovedena korišćenjem podataka preaćenja kvaliteta vode koji su prethodno uneti u GIS bazu podataka. Pored toga, korišćenjem podataka o zagađujućim materijama iz katastra zahgađivaća urađeno je nekoliko simulacija za različite scnearije, a kao rezultat su dobijene GIS karte koje pokazuju promenu koncentracije rastvorenog kiseonika duž deonice kanala. Na kraju su određene vrednosti za TMDL za najznačajnije zagađujuće materije koje utiču na koncentraciju rastvorenog kiseonika.

Ključne reči: integralna metodologija, rastvoreni kiseonik, kanalska mreža, modeliranje, QUAL2K.

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INTERRELATIONSHIP AND PATH COEFFICIENT ANALYSIS OF MORPHO-PHYSIOLOGICAL TRAITS AMONG MAIZE (*Zea mays* L.) DIALLELIC CROSSES IN THE SOUTHERN GUINEA SAVANNA OF NIGERIA

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SUMMARY: This study was explored to determine the effect of the grain yield and its related characteristics on 10 open-pollinated maize varieties and their 45 F₁ hybrids using correlation and path coefficient analysis. A two-year study was conducted on maize genotypes at the University of Ilorin Teaching and Research Farm Ilorin, Nigeria, during 2005 and 2006 growing seasons. Positive and significant phenotypic and genotypic correlations were found for days to 50% tasselling with plant and ear height, and grain yield with plant height, number of grains ear⁻¹ and ear weight. Positive and significant environmental correlation was also recorded for grain yield with plant and ear height, and ear weight. The path analysis revealed that, days to 50% silking, ear weight and number of grains ear⁻¹ had the highest direct effect on grain yield, while number of grains ear⁻¹ had the highest moderate indirect negative effects on grain yield. Days to flowering, plant and ear height, number of grains ear⁻¹ and ear weight could be the important selection criteria in improving open pollinated maize varieties and hybrids for high grain yield.

Key words: Phenotypic, genotypic correlation, open pollinated varieties, yield characters.

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INTRODUCTION

Maize (*Zea mays* L.) is an important staple food crops and provide bulk of raw materials for the livestock and many agro-allied industries in the world. Plant breeders are interested in developing cultivars with improved yield and other desirable agronomic and phenological characters. In order to achieve this goal, the breeders had the option of selecting desirable genotypes in early generations or delaying intense selection until advanced generations (Puri et al., 1982). The selection criteria may be yield, or one or more of the yield component characters. However, breeding for high yield crops require information on the nature and magnitude of variation in the available materials, relationship of yield with other agronomic characters and the degree of environmental influence on the expression of these component characters. Grain yield in maize is quantitative in nature and polygenically controlled therefore effective yield improvement and simultaneous improvement in yield characters are very important (Bello and Olaoye, 2009). Selection on the basis of grain yield character alone is usually not very effective and efficient. However, selection based on its component characters could be more efficient and reliable (Muhammad et al., 2003). Knowledge of association between yield and its component traits and among the component parameters themselves can improve the efficiency of selection in plant breeding. Correlation coefficient measures the mutual association between a pair of variables independent of other variables to be considered. Where more than two variables are involved, correlation coefficient alone does not give complete picture of the interrelationship (Fakorede and Opeke 1985). To determine relationships, correlation analyses are used such that the values of two characters are analyzed on a paired basis, results of which may be either positive or negative. The result of correlation is of great value in the evaluation of the most effective procedures for selection of superior genotypes. When there is positive association of major yield characters, component breeding would be very effective but when these characters are negatively associated, it would be difficult to exercise simultaneous selection for them in developing a variety (Nemati et al., 2009).

Path coefficient analysis is a standardized partial regression coefficient that allows partitioning of correlation coefficient into direct and indirect effects of various traits towards dependent variable, and also helps in assessing the cause-effect relationship as well as effective selection. Path analysis plays an important role in determining the degree of relationship between yield and its components. Studies of correlation and path analysis have recently been conducted in groundnut by Izge et al., (2004); in sorghum by Ezeaku and Mohammed, (2006); in pear millet by Izge et al., (2006) and in pea by Togay et al., (2008). Researchers have been attempted also in determine association between the characters for selection of high maize grain yield. Findings from two independent studies, (You et al., 1998; Annapurna et al., 1998) revealed positive and significant correlations between grain yield and number of rows ear⁻¹, number of grains row⁻¹. Khatun et al. (1999) and Batool et al. (2012) reported that grain yield plant⁻¹ was positively and significantly correlated with number of grains ear⁻¹, ear weight, 1000-grain weight and ear height. Orlyan et al. (1999) and Gautam et al. (1999) suggested that most important characters in improving maize grain yield are number of grains row⁻¹, number of grain ear⁻¹ and plant height. However, Mohammad et al. (2003) determined the interrelationship between grain yield and its components from eighteen maize

lines/hybrids, using genotypic correlation and path coefficient analysis. Grain yield was positively and significantly associated with plant and ear height, ear diameter, number of grains ear⁻¹, number of grains row⁻¹ and 1000-grain weight. Geetha and Jayaraman (2000) studied direct and indirect effects of different quantitative traits on grain yield in 90 hybrids and reported that number of grains ear⁻¹ exerted a maximum direct effect on grain yield. They therefore suggested that selection of number of grains ear⁻¹ could be highly effective for improvement of grain yield. Kumar and Kumar (2000) also stressed that plant height with higher number of grains row⁻¹, ear weight, and number of grains ear⁻¹ could be selected for high yielding.

Review of literatures shows that determining relationships between yield and its components is essential for high maize yield. Although all the experimental results were not in agreement with one another, but in most experiments, some yield parameters such as number of grains ear⁻¹ and ear weight feature prominently in improving grain yield. Thus, by determining association between maize grain yield and yield components and between yield components themselves as well as recognition of the parameters that have significant effect on yield, is a prerequisite plan for a meaningful breeding programme. The objectives of the present research therefore was to determine the association between maize grain yield and some other agronomic characters with the view to identifying characters whose selection could be used in improving grain yield.

MATERIALS AND METHODS

Ten open pollinated maize varieties (OPVs) developed for grain yield and adaptation to abiotic (drought) and biotic (Stalk rot, Striga and Downy mildew) stresses were obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. They are early to medium maturing white cultivars with maturity period of 90 to 100 days. The origin, genetic background, breeding emphasis and ecological adaptation of the maize parents are shown in Table 1. The ten varieties were crossed in a partial diallel to generate 45 F₁ hybrids during 2004 and 2005 growing seasons at the Teaching and Research (T and R) farm of University of Ilorin (Latitude 8° 29'N, Longitude 4° 35'E and annual average rainfall of 945 mm). The resultant hybrids were harvested, processed and stored in the cold room prior to field evaluation. It is worthwhile to note that the ultimate goal is not diallel analysis in which using pure lines as parents is necessary. Soil samples were collected from the trial site before cropping and were analyzed in the laboratory for selected physical and chemical properties and presented in Table 2. The soil texture is loamy sand. At 0-15cm depth, the amounts of silt, sand and clay were 8, 84 and 8 respectively, with soil pH = 7.30 and CEC = 2.83 (Cmol kg⁻¹).

Table 1. Origin, characteristic features and ecological adaptation of 10 open pollinated maize varieties

	Genotypes	Origin and characteristic features	Ecological adaptation
1.	Acr 90 Pool 16-Dt	Early white dent International Maize and Wheat Improvement Centre CIMMYT cultivar with stalk rot, <i>striga</i> and drought tolerance.	Forest and savannah.
2.	Tze Comp 4-Dmr Srbc2	Early maturing white and semi dent cultivar with higher yield and <i>striga</i> tolerance.	Forest and savannah.
3.	Tze Comp4 C2	Early maturing white and semi dent cultivar with higher yield and <i>striga</i> tolerance.	Forest and savannah.
4.	Acr 97 Tze Comp3 C4	Early white flint dent cultivar with higher yield, downy mildew and <i>striga</i> tolerance	Forest and savannah.
5.	Hei 97 Tze Comp3 C4	Early white flint dent cultivar with higher yield, downy mildew and <i>striga</i> tolerance.	Forest and savannah.
6.	Acr 94 Tze Comp5	Early white flint dent cultivar with <i>striga</i> tolerance.	Savannah.
7.	Tze Comp3 Dt	Early white flint dent cultivar with drought tolerance.	Forest and savannah
8.	Tze Comp3 C2	Early white and flint dent cultivar with downy mildew and <i>striga</i> tolerance.	Forest and savannah.
9.	Ak 95 Dmr-Esrw	Early maturing and flint dent cultivar with downy mildew and <i>striga</i> tolerance.	Forest.
10.	Tze Msr-W	Early white semi dent cultivar with higher yield and <i>striga</i> tolerance	Forest and savannah.

Source: IITA Archival Report 1988-1992.

Table 2. Selected physical and chemical characteristics of the soil before cropping of maize

Soil properties	Value of 0-15cm depth	Value of 15-30cm depth
Texture	Loamy sand	Loamy sand
Soil PH (water)	7.30	6.30
Sand %	84.00	88.00
Clay %	8.00	8.00
Silt %	8.00	4.00
Exchangeable Ca ²⁺ (Cmol kg ⁻¹)	1.10	2.10
Exchangeable Mg ²⁺ (Cmol kg ⁻¹)	1.60	2.10
Exchangeable Na ⁺ (Cmol kg ⁻¹)	0.18	0.19
Exchangeable K ⁺ (Cmol kg ⁻¹)	0.01	0.01
Total acidity H ⁺ (Cmol kg ⁻¹)	0.04	0.04
Cation exchange capacity (Cmol kg ⁻¹)	2.83	4.44
% Organic Carbon	0.26	0.33
% Total Nitrogen	1.30	0.90
Available P (Mg kg ⁻¹)	4.10	3.80

The parents and hybrids were evaluated using a randomized complete block design (RCBD) with 4 replicates in 2005 and 2006. Entries were made in 4-row plots of 5 x 1.5 m each and planted at inter-row spacing of 75 cm and within row spacing of 50 cm to enhance a population of about 53,333 plants ha⁻¹. Three seeds were initially planted per hill and were thinned to two plants per hill. Fertilizer was applied in split-dosage at three and seven weeks after planting (WAP) at the rate of 80 kg N, 60kg P and 60kg/ha K respectively from compound NPK fertilizer (20-10-10). Agronomic parameters measured in each year were seedling emergence, days to 50% tasselling, pollen shed and silking; anthesis-silking interval, plant and ear height (cm), number of grains ear⁻¹ and ear weight. Plant height was measured from soil level to the node of the flag leaf and to the highest ear-bearing node respectively at harvest stage. Days to 50% tasselling, pollen shed and silking were calculated as the number of days from planting to when 50% of the population have tasseled, shed pollen and silked respectively. Anthesis-silking interval was estimated as the difference between days to pollen and silking, and grain yield (t/ha) measured after adjusting to 12% moisture content. Combined analysis of variance and means over years were computed using SAS PROC, (1999) for the parents OPVs and hybrids with respect to grain yield and other agronomic parameters respectively. The phenotypic (rph), genotypic (rg) and environmental (re) correlation coefficients were estimated from the mean squares and mean across products as suggested by Mode and Robinson (1959). The correlation coefficients were partitioned into direct and indirect effects using the path coefficient analysis according to Dewey and Lu (1959).

RESULTS AND DISCUSSION

In the study, phenotypic and genotypic correlation coefficients calculated among examined characteristics in maize genotypes are presented in Table 3 and 4. The correlation coefficients of the pairs of characters revealed the presence of significant and positive ($p < 0.05$) phenotypic correlation of grain yield ha⁻¹ with days to 50% tasselling (rph= 0.34*), plant height (rph=0.56*) and ear height (rph=0.45*), number of grains ear⁻¹ (rph= 0.59*), and ear weight (rph= 0.46*) (Table 3). This indicated that by increasing these attributes, could invariably increase grain yield. Khatun et al. (1999) and Babaji et al. (2010) found that grain yield plant⁻¹ was positive and significantly correlated with number of kernels ear⁻¹, ear weight and ear insertion height. High correlation of grain yield with plant height is also reported by other researchers (Annapurna et al., 1998; Gautam et al., 1999; Batool et al. 2012; Sali et al. 2012). The relationship between seedling emergence and number of grains ear⁻¹ was also positive and significant, indicating that early emergence genotypes could result in increased number of grains ear⁻¹ and consequently increase grain yield. Days to anthesis was positive and significantly ($P < 0.05$) associated with plant and ear height and number of grains ear⁻¹. Troyer and Larkins (1985) observed that plant height was positively correlated with days to flowering morphologically, as internodes' formation stops at floral initiation, and that early flowering maize varieties are usually shorter in height. Genotypic correlation coefficients (Table 4) followed a similar trend in magnitude and significance with that of phenotypic coefficients, except plant and ear height with negative and non-significant correlation with number of grains ear⁻¹. There were positive significant phenotypic and genotypic correlations of days to flowering with plant and ear height; and grain

yield with plant height, number of grain ear⁻¹ and ear weight. This suggests that genetic factors are responsible for these associations. Therefore, plant height, number of grain ear⁻¹ and ear weight could also be considered for selection and improvement for high yielding varieties. Even though, both phenotypic and genotypic correlations are comparable in magnitude, the genotypic correlations are of higher magnitude than their corresponding phenotypic correlations, indicating a strong inherent relationship among the characters studied. This corroborates with the findings of Sali et al. (2012) and Saleem et al. (2012). Environmental correlation coefficients between studied traits illustrated in Table 5 showed that plant and ear height, and ear weight have highly positive and significant correlation ($r_e=0.87^*$, 0.75^* and 0.61^*) with grain yield. Days to plant emergence was also positive and significantly correlated with ear height. Westermann and Crothers (1977) reported that changes in yield and yield components had been attributed to plant's response to its environment which may or may not permit full genetic expression of each character. Thus, plant and ear height, and ear weight could be considered in improving maize yield in the breeding programmes. Significant positive correlations between yield and other agronomic characters that can improve yield are quite desirable in plant breeding, because it facilitates selection process and gains from selection.

Table 3. Analysis of phenotypic correlation for maize grain yield and other agronomic characters combined across year

	Seedling emergence	Days to 50% tasselling	Days to 50% pollen shed	Days to 50% silking	Anthesis-silking interval	Plant height (cm)	Ear height (cm)	Number of grain ear ⁻¹	Ear weight (t/ha)	Maize grain yield (t/ha)
Seedling emergence	1.00									
Days to 50% tasselling	0.02	1.00								
Days to 50% pollen shed	0.01	0.07	1.00							
Days to 50% silking	0.03	0.06	0.05	1.00						
Anthesis-silking interval	0.05	0.02	0.02	0.05	1.00					
Plant height (cm)	0.04	0.57*	0.04	0.04	0.04	1.00				
Ear height (cm)	0.02	0.72*	0.12	0.07	0.08	0.11	1.00			
Number of grain ear ⁻¹	0.07	0.45*	0.45	0.08	0.05	0.47*	0.53*	1.00		
Ear weight (t/ha)	0.05	0.05	0.08	0.67*	0.05	0.71*	0.02	0.03	1.00	
Maize grain yield (t/ha)	0.04	0.34*	0.04	0.05	0.06	0.56*	0.45*	0.59*	0.46*	1.00

*Significant at < 0.05 level of probability.

Table 4. Analysis of genotypic correlation for maize grain yield and other agronomic characters combined across years

	Seedling emergence	Days to 50% tasselling	Days to 50% pollen shed	Days to 50% silking	Anthesis-silking interval	Plant height (cm)	Ear height (cm)	Number of grain ear ⁻¹	Ear weight (t/ha)	Maize grain yield (t/ha)
Seedling emergence	1.00									
Days to 50% tasselling	0.02	1.00								
Days to 50% pollen shed	0.12	0.02	1.00							
Days to 50% silking	0.02	0.05	0.45*	1.00						
Anthesis-silking interval	0.01	0.07	0.02	0.03	1.00					
Plant height (cm)	0.05	0.74*	0.05	0.45*	0.42*	1.00				
Ear height (cm)	0.02	0.54*	0.07	0.02	0.06	0.09	1.00			
Number of grain ear ⁻¹	0.35*	0.02	0.02	0.05	0.02	-0.07	-0.02	1.00		
Ear weight (t/ha)	0.02	0.05	0.04	0.43*	0.35*	0.34*	0.37*	0.02	1.00	
Maize grain yield (t/ha)	0.02	0.08	0.02	0.03	0.08	0.44*	0.05	0.34*	0.43*	1.00

*Significant at < 0.05 levels of probability.

Table 5. Analysis of environmental correlation for maize grain yield and other agronomic characters combined across years

	Seedling emergence	Days to 50% tasselling	Days to 50% pollen shed	Days to 50% silking	Anthesis-silking interval	Plant height (cm)	Ear height (cm)	Number of grain ear ⁻¹	Ear weight (t/ha)	Maize grain yield (t/ha)
Seedling emergence	1.00									
Days to 50% tasselling	0.04	1.00								
Days to 50% pollen shed	0.05	0.07	1.00							
Days to 50% silking	0.12	0.05	0.03	1.00						
Anthesis-silking interval	0.15	0.11	0.03	0.02	1.00					
Plant height (cm)	0.12	0.13	0.04	0.12	0.03	1.00				
Ear height (cm)	0.57*	0.11	0.02	0.02	0.03	0.02	1.00			
Number of grain ear ⁻¹	0.15	0.03	0.06	0.13	0.11	0.03	0.02	1.00		
Ear weight (t/ha)	0.04	0.05	0.04	0.15	0.02	0.01	0.03	0.02	1.00	
Maize grain yield (t/ha)	0.04	0.12	0.03	0.11	0.03	0.87*	0.75*	0.34	0.61*	1.00

*Significant at < 0.05 levels of probability.

Path coefficient analyses was also used to obtain further information on the interrelationships among traits and their effects on grain yield and are presented in Table 6. Ear weight showed the greatest direct effect on grain yield ($p = 0.582$), followed by number of grain ear⁻¹ ($p = 0.525$) and days to 50% silking ($p = 0.443$). Many researchers (Alvi et al. 2003; Nemati et al. 2009; Sreckov et al. 2010; Batool et al. 2012) found that ear weight has direct effect on grain yield. They opined that by increasing ear weight due to more absorption of photo assimilates, the most portion of assimilates remobilizes to grains, and invariably increase grain weight. Days to 50% silking showed high negative direct effect on grain yield ($p = -0.443$). The number of grains ear⁻¹ had the highest moderate indirect positive effects on grain yield by ear weight ($p = 0.426$), while ear weight had the highest moderate indirect negative effects on grain yield by days to seedling emergence ($p = -0.013$). This is in agreement with findings of previous researchers (Bocanski et al. 2009; Khazaei et al. 2010). It is also shown that, plant height had the highest moderate indirect positive effects on grain yield by ear weight ($p = 0.256$), while number of grain ear⁻¹ had the highest moderate indirect negative effects on grain yield by ear height ($p = -0.007$). This is in line with results of earlier workers (Saidaiyah et al. 2008; Bocanski et al. 2009). However, It is obvious that other variables could have effects on grain yield. In this study, days to flowering, plant and ear height, number of grains ear⁻¹ and ear

weight appeared to be the prominent characters that could be used in selecting for high yield, because of their highly significant genotypic and phenotypic correlations with grain yield. Similar results are reported by Saidaiah et al. (2008), Sreckov et al. (2010) and Batool et al. (2012). These characters also had the highest direct and indirect effects through most of the other characters. It is therefore, concluded that these agronomic parameters could be considered as important selection criteria in improving open pollinated maize varieties and hybrids for high grain yield.

Table 6. Path coefficient analysis of grain yield of maize genotypes combined across years

Character	Means	Direct effects	Total effects									Total effect
			1	2	3	4	5	6	7	8	9	
1. Seedling emergence	38	0.191		0.035	-0.04	0.023	-0.03	0.045	0.011	0.034	0.056	-0.257
2. Days to 50% tasselling	52	0.034	0.044		0.04	0.032	0.056	0.033	0.054	0.045	-0.03	0.854
3. Days to 50% pollen shed	53	0.182	-0.06	0.047		0.034	-0.04	0.067	-0.07	0.067	0.065	0.452
4. Days to 50% silking	55	-0.443	0.073	0.085	0.012		0.044	0.056	0.034	-0.06	0.034	0.245
5. Anthesis-silking interval	3	0.147	0.046	0.047	0.041	0.045		0.072	0.005	0.127	0.033	0.342*
6. Plant height (cm)	121	0.314	-0.06	0.057	0.034	0.057	0.046		0.006	0.072	0.256	-0.234
7. Ear height (cm)	35	0.176	0.001	0.067	-0.05	-0.08	0.089	0.018		0.194	0.023	0.045
8. Number of grain ear ⁻¹	54	0.525	0.003	0.043	0.06	0.053	-0.08	-0.083	-0.007		-0.14	0.786*
9. Ear weight (t/ha)	6.4	0.582	-0.01	0.082	0.045	0.052	0.098	0.045	0.005	0.426		0.843**

***Significant at < 0.05 < 0.01 level of probability, respectively.

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**POVEZANOST I ANALIZA MORFO-FIZIOLOŠKIH OSOBINA IZMEĐU
DIALELNIH HIBRIDA KUKURUZA (*Zea mays* L.) U JUŽNOJ GUINEA
SAVANI NIGERIJE**

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AFOLABI, M. SEGUN

Izvod

Ispitivan je prinos i njegov uticaj na karakteristike 10 varijeteta i njihovih 45 F1 generacije hibrida, u južnom delu savane Guinea u Nigeriji. Ogled je izveden tokom 2 godine (2005. i 2006.), na eksperimentalnoj farmi Univerziteta Ilorin u Nigeriji. Značajane pozitivne fenotipske i genotipska korelacije, ustanovljene su kod 50 % testiranih biljaka, u pogledu prinosa zrna i visine biljke, kao broja zrna i težine klipa. Pozitivan i značajan ekološki korelacija zabeležen je i za prinos zrna po biljci, visine biljke i težine klipa. Analiza je pokazala da težina klipa i broj zrna u klipu, ima najveći direktni efekat na prinos zrna. Broj dana do cvetanja, položaj i visina klipa na stabljici, broj zrna u klipu i težina klipa, mogu biti važni kriterijumi za izbor u poboljšanju otvorenog oprašivanja sorti i hibrida kukuruza za visok prinos zrna.

Ključne reči: *Fenotipske, genotipske korelacije, otvorene polinatni varieteti, karakteristike prinosa.*

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POSSIBILITIES FOR THE IMPROVEMENT OF THE SOYBEAN PRODUCTION IN SERBIA*

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SUMMARY: Current trends in human nutrition and growing needs of livestock production, especially pig and poultry production, drive a demand for the soybean both on a global scale and in Serbia too. It is, therefore, necessary to examine the possibilities of improving this crop production at the national level. This paper analyses the yield of soybeans in Serbia in the period 1949-2012, as the main indicator of the production results. The main aim of the paper is to examine the level and stability of the soybean yields in the observed period, in order to show some possibilities for further improvement of the production of this crop. The conducted analysis has showed that an average soybean yield achieved in Serbia in the period 1949-2012 was 1,730 kg/ha; an average annual growth rate was 1.82 %, while the yield varied from 366 kg/ha (yield achieved in 1950) to 3,177 kg/ha (achieved in 2010). On the short term basis, the situation was somewhat better, i.e., in the last decade (2003-2012) the average yield was 2,448 kg/ha. This can be assessed as relatively satisfactory, when the national average yield is compared with the world, European and neighbouring countries averages, respectively. However, the demonstrated variability in the achieved yields and the fact that there are countries with higher average yields indicate that there is room for the further growth of this production. To exploit these possibilities, it is crucial that decision makers (managers) know well the specific conditions (both natural and socio-economic) in which the production takes place. Only if they constantly monitor the production process (on site), the limiting factors of yield growth can be recognized on time and the optimal management decisions for specific production conditions can be made. Thus, it can be concluded that high yields of the soybean require an optimal combination of agro-ecological conditions and adequate management decisions (for the specific conditions of production).

Key words: soybean, yield, management decisions.

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INTRODUCTION

Current trends in human nutrition and growing needs of livestock production, especially pig and poultry production, as well as the expansion of the bio-fuel industry, drive a demand for oilseeds on a global scale and in Serbia (Keyzer et al., 2002; Gelder et al., 2008; Knežević and Popović, 2012). In this respect, the soybean is particularly important, given that it is the most important grain oilseed in terms of production and trade (Masuda and Goldsmith, 2009a; U.S. Soybean Export Council, 2011; Čurović, 2012). The continuously growing demand for soybeans creates the need to examine the possibilities of improving the production of this crop in Serbia.

According to Reljin et al. (1997), there are two ways to increase soybean production - an extensive one (by the extension of cultivated area) and an intensive one (by increasing the yield per unit area). The fact that the amount of agricultural land in our country (Tomić, 1993; Bošnjak and Rodić, 2002, 2010, 2011), as well as worldwide (Rodić et al., 2008), decreases slowly but steadily stresses the necessity for more efficient use of available land resources.

Škorić (2009), Rodić et al. (2008) and Popović (2007) emphasize that the absolutely necessary growth of production per unit area and the more intensive use of resources (especially land) should not be achieved at the expense of natural resources and the environment, i.e., the intensification of production must respect environmental limitations. These requirements, on the one hand, and the fact that the obtained yield is a significant economic factor of production efficiency (Bošnjak and Rodić, 2006; 2010b, Munćan et al., 2010) on the other hand, emphasize the importance of the need for continuous monitoring and analysis of the production process and assessment of the possibilities for its improvement. In this respect, the analysis of obtained yields is always relevant and important, especially if one bears in mind that the yield per unit area is a result of natural conditions, but also of the efforts made by producers (Marko et al., 1986; Nenadić et al., 2007). Therefore, the authors of this paper tried to review trends and assess the yield stability over a longer period of time and, based on that, to indicate possibilities for the improvement of the soybean production in the Republic of Serbia.

RESEARCH METHOD AND DATA SOURCES

The research method has been chosen in accordance with the defined research objectives. The analytical-comparative method has been applied, using the main descriptive statistics indicators (mean, extreme values, interval and coefficient of variation, average annual growth rate). The time series analysis of the obtained average yield of soybean covers the period 1949-2012. The analysis has also been done for certain ten-year sub-periods, with a special emphasis on the last decade of the study period. The spatial analysis covers primarily the region of the Republic of Serbia, but certain characteristics of the soybean production in the Autonomous Province of Vojvodina, the world, Europe and the EU have also been analysed. To accomplish the research objectives, the main sources of data databases of the NBS - National Bureau of Statistics (www.stat.gov.rs) and FAO (www.fao.org) were used. The research results obtained are presented in tables and figures.

RESULTS AND DISCUSSION

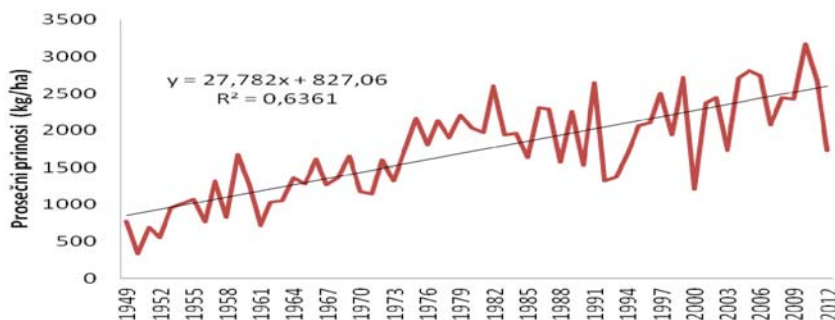
Average yield of soybean. The results achieved in agricultural production can be presented in a different way. Appropriate indicators should be chosen depending on the level and purpose of measurement. When the results of certain production (in this case soybean) are to be presented, commonly used indicators of production results are the total yield and the yield per unit of land capacity. Given the fact that the obtained yield in crop production always depends on a complex of different factors, the average yield per hectare is often considered the most appropriate quantitative indicator of success in production and as such is used most often.

The conducted analysis shows that during the observed period (1949-2012) the average soybean yield, as well as the yields of other main field crops, demonstrates an upward trend (Table 1). The pace of the increase is not particularly emphasized because the phenomenon which Marko et al. (2011) called 'yield fatigue' occurred in certain periods. An average soybean yield in the period 1949-2012 was 1,730 kg/ha. It showed a growing tendency (annual growth rate 1.82%) and fluctuated within the range of 366 kg/ha (the average yield in 1950) to 3,177 kg/ha (in 2010). In comparison with other main field crops, the soybean yields have had the most intensive development in the observed period (Table 1).

Table 1. The main characteristics of average annual yields of the major field crops in Serbia (1949-2012)

Crop	The mean value (kg/ha)	Coefficient of variation (%)	Variation interval (kg/ha)		Average annual growth rate (%)	R ²
			Min	Max		
Wheat	2,973	34.82	759	4,593	1.10	0.60
Corn	3,687	36.33	646	5,954	1.41	0.48
Sugar beet	34,872	29.54	7,005	50,729	1.02	0.42
Sunflower	1,778	25.32	583	2,558	0.90	0.44
Soybean	1,730	37.20	336	3,177	1.82	0.61

Source: (www.stat.gov.rs and authors' own calculation)



Graph 1. The average annual yield of the soybean in Serbia (1949-2012)

The extreme values of the yields (minimums and maximums) are farthest from each other in the production of soybeans and corn (1:9), followed by sugar beet (1:7) and wheat (1:6). The difference is much lower (1:4) in the production of sunflower. As can be seen from Table 1 and in Graph 1 (which shows the average annual yield of soybean in Serbia for the entire observed period of 64 years), the soybean yields showed the greatest variation of all major field crops (this is confirmed by the coefficient of variation $CV = 37.2\%$).

The presented variability of the soybean yield and its trend of growth indicate that the desired and possible yields have still not been reached. This conclusion is confirmed by the distribution of the obtained soybean yields in certain periods (Table 2). Namely, in the production of soybeans in the observed period, during more than a half of that time, the achieved yields were within the range of 1.01 to 1.50 t/ha (in 16 years), and 1.51 to 2.00 t/ha (in additional 17 years).

Table 2. The distribution of the average soybean yields in Serbia by the amount and periods of achievement (1949-2012)

Yield t/ha	1949- 1952	1953- 1962	1963- 1972	1973- 1982	1983- 1992	1993- 2002	2003- 2012	Frequ ncy	Structur e %
								64	100
<0.50	x							1	1.56
0.51- 1.00	xxx	xxxx						7	10.94
1.01- 1.50		xxxx x	xxxx xxx	x	x	xx		16	25.00
1.51- 2.00		x	xxx	xxxx	xxxx x	xx	xx	17	26.56
2.01- 2.50				xxxx	xxx	xxxx	xxx	14	21.88
2.51- 3.00				x	x	xx	xxxx	8	12.50
> 3.01							x	1	1.56

Source: (www.stat.gov.rs and authors' own calculation).

The average yield of 1.5 t/ha was exceeded in 2003 and such a low level of the average yield has not repeated ever again. In the last decade of the observed period (2003-2012), the soybean yield even exceeded 3 t/ha, which had not been recorded in the past. The fact that in the same decade the average yield was only two times between 1.51 and 2.0 t/ha is an additional argument for the claim that the average yield of the soybean in Serbia is moving to higher levels. This is particularly seen in Vojvodina, which should not be surprising since 94% of the total production of the soybean in Serbia is produced in this region. These yield changes could be evaluated as positive. They occur as a result of continuous improvements in the soybean production. This is especially visible on family farms, which have become increasingly important factors in the total soybean production in recent years (Bošnjak and Rodić, 2011).

According to the FAO data, Serbia was the 4th biggest European and the 16th biggest world soybean producer in the last decade. In other words, this means that

more than 10% of the soybean produced in Europe was produced in Serbia, while Serbian contribution to the global soybean production was 0.16% (Table 3)

Table 3. Regional characteristics of harvested area, yield and production of soybean (2003-2012)

Region	Harvested area			Yield			Production		
	Harvested area (ha)	Growth rate (%)	Coeff. of variation (%)	Yield (kg/ha)	Growth rate (%)	Coeff. of variation (%)	Production (t)	Growth rate (%)	Coeff. of variation (%)
World	96,155,663	2.22	6.92	2,375	0.90	4.57	228,714,011	3.25	10.14
Europe	2,132,237	10.41	31.99	1,666	0.80	9.36	3,578,425	11.41	35.68
EU	373,471	-1.98	17.50	2,607	0.70	10.94	968,978	-0.99	18.72
Serbia	146,957	3.25	11.01	2,448	0.20	18.56	360,789	3.46	23.77
Vojvodina	136,566	3.05	10.55	2,466	0.20	18.74	337,792	3.25	23.90

Source:FAO (www.fao.org) and NBS (www.stat.gov.rs) databases and authors' own calculations.

The average yields of the soybean in Serbia can be assessed as relatively satisfactory. This claim is based on earlier studies of Bošnjak and Rodić (2010b), and the fact that the average yields of the soybean in Serbia, especially in Vojvodina (2003-2012), were higher than the world and European averages (Table 3). The fact that Serbia had the highest average soybean yields among neighbouring countries supports the previous statement².

However, as could be seen from the data given in Table 3, the average yields of the soybean in Serbia and in Vojvodina lag behind the average yields of this crop in the EU. The determined difference in the yields of the soybean in Serbia and the EU cannot be considered as significant since according to the FAO data, the Republic of Serbia holds the 15th place among the world producers with the highest level of yield per hectare. On the other hand, there is still room for further improvement, especially given the fact that Serbia has very favourable agro-ecological conditions for the soybean production.

Characteristics of soybean yield by sub-periods. According to Masuda and Goldsmith (2009b), the increase of the world average yield of the soybean from 1961 to 2007 was not very considerable. The average annual growth rate during that period was rather low, i.e. 1.5%. These authors emphasize the different pace of change in this yield in certain sub-periods. For example, from 1990 to 1995, the average growth rate of yields on a global level was 1.4%, while in later years the rate decreased; from 1995 to 2000 it was 1.3; from 2000 to 2005 there were no changes and the rate was 0.0; while from 2005 to 2007 the growth rate was negative -0.9%.

²The average yields of soybeans in the 2003-2012 period in the neighbouring countries were: Croatia 2,362 kg/ha, Hungary 2,149 kg/ha; Romania 1,948 kg/ha, Bosnia and Herzegovina 1,865 kg/ha, Macedonia 1,833 kg/ha, Albania 1,681 kg/ha, Bulgaria 1,389 kg/ha

The analysis conducted in this paper shows that the increase in the average yield of the soybean achieved in Serbia from 1949 to 2012 was also characterized by different dynamics, if the yield is analysed by shorter time intervals, in this case the decades of study period ³ (Table 4).

Table 4. The main characteristics of the average yield of the soybean in Serbia by subperiods

Indicators	Period					
	1953-1962	1963-1972	1973-1982	1983-1992	1993-2002	2003-2012
Variation interval (kg/ha)	720-1,676	1,053-1,647	1,321-2,607	1,322-2,647	1,205-2,717	1,720-3,177
Year of minimum yield	1961	1963	1973	1992	2000	2003
Year of maximum yield	1959	1969	1982	1991	1999	2010
Average yield kg/ha)	1067	1352	1986	1944	2040	2448
Coefficient of variation (%)	25.78	14.9	16.19	20.85	23.13	18.56
Growth rate (%)	0.60	1.31	4.39	-1.0	3.25	0.20

Source: (www.stat.gov.rs and authors' own calculation)

The first decade of the study period (1953-1962) was marked by the greatest variation ($C_v=25.78\%$) and a slight increase in the soybean yield. According to Marko (1987), that increase, as well as the increase in the yield of other field crops at the time, could be explained both by different measures undertaken in order to increase the yield (intensification) and by the very low starting point. In the following two decades, the variation of yields was significantly reduced and the growth in average yields accelerated (especially in the third decade, when the annual growth rate was 4.39%). This progress can be attributed to the fact that after 1975 the expansion of the soybean areas was conducted mainly in Vojvodina (Reljin et al., 1997) where agro-ecological conditions for this crop are very favourable, but also to the fact that a number of the soybean varieties were introduced from the United States (Nenadić et al., 2007).

The dynamics of the yield growth, unfortunately, was not continued. In the fourth decade (1983-1992) the soybean yield even decreased slightly (annual growth rate was -1.0%). The decreasing tendency was coupled with a high coefficient of variation, indicating that the decade was not favourable for the soybean production is in question. It is undisputed that natural factors are very important in the yield formation. However, in this sub-period the main causes of the soybean yield reduction were not natural conditions, but the reduced use or even the omission of basic inputs. Earlier researches also indicate that the level of applied agricultural technology was one of the limiting factors of the soybean yields during that period (Reljin et al., 1997; Hrustić et al., 2002; Bosnjak and Rodic, 2006). The following

³The first four years of the study period (1949-2012) are not included in this analysis. In this period the achieved average soybean yield is very low (588 kg/ha) as a result of both adverse climatic conditions and the low level of applied agricultural technology.

decade (1993-2002) was characterised by 'revitalisation' of the soybean production (expressed through both the average achieved yield (2,040 kg/ha) and the annual growth rate (3.25%)). Yet, the extreme values of the yield in that period and the determined coefficient of variation indicate that the desired stability in soybean production was not achieved. During the last decade of the observed period (2003-2012) the positive trend continued. The identified growth rate (0.20%) indicates certain yield 'fatigue', while the decreased coefficient of variation indicates certain stabilization in the soybean production. An annual average soybean yield reached 2,448 kg/ha, which is 20% more than the average obtained in the previous decade, or 2.5 times more than in the first decade of the observed period (1953-1962). Once during this period, the average soybean yield per hectare was even over 3 tons (3.18 t/ha in 2010), which is rather a proof that such a yield is possible and an indication of what should be striven for.

The analysis of the average soybean yields in this 64-year-long period has shown that two characteristic sub-periods could be identified. The first one was until 1975. It was characterized by the gradual introduction of soybeans into planting structure, a lack of knowledge about the technology of production, and consequently relatively low and unstable yields. After that, in the last 35 years, the soybean production increased significantly, due to the technological progress. Owing to that, the average soybean yield unimaginable in the past was achieved. Analysing the soybean yields at individual production units, Nenadić and Zeković (2009) point to a significant increase in yield and emphasize that many producers in Serbia could obtain a yield as high as 4.0 to 5.91 t/ha, which supports earlier findings of Nenadić et al., 2007 that farmers in Serbia should strive to yields as high as 5-6 t/ha. Hrustić et al., 2009 have also pointed to the significant growth of the soybean yield in the past, noting that as a good basis for further growth.

Possibility for further growth of soybean yield. The expressed variability (CV = 37%) and registered growth of the soybean yields over the long term (average annual growth rate 1.82%) are clear evidence that the constant striving of farmers to increase the efficiency of this production with the help of technical progress has brought some respectable results. However, having in mind favourable natural conditions for the soybean production and yields achieved in most developed countries, it can be concluded that there is still room for further improvement. Ever growing demand for the soybean and the fact that it could be multipurposely used are strong motivating factors for further improvement in this production. However, considering numerous factors that could influence yields, there are no quick and predefined solutions to increase production. When considering options for further growth, all the factors of influence should be identified, their effects examined and systematic activities should be taken in order to adapt production to specific circumstances. Therefore, it is clear that the role of good management decisions is substantial. That is why, as far back as in 1985, Borojević (quoted by Marko et al., 1986) emphasized the importance of knowledge and personal presence on site for the achievement of high yields.

Good management practices and right agronomic decisions in the future should primarily aim at the reduction of the number of environmental stresses. Only then it could be expected that favourable natural conditions, created soybean varieties (with high yield potential) and modern technical-technological solutions will come to the fore, which will result in higher average yields. In recent years, there have been

more and more opinions that sufficient rainfall in August is not a guarantee of a high yield by itself, as used to be stated before. Much greater impact on yields is exerted by proper agronomic decisions, such as selection of varieties, the optimum time of sowing and harvesting, the analysis of soil fertility, proper crop rotation, weed and pest control, etc.⁴ Making right management decisions is certainly easier if the decision maker is personally present on site. Knowing and constantly monitoring production could provide insight into factors limiting yields and thus allow efficient management decisions in specific production conditions. Therefore, it can be concluded that the high soybean yields require both an optimal combination of agro-ecological conditions and adequate (for the specific conditions of production) management decisions. Of course, in order to improve the soybean production, it is necessary to invest in irrigation systems, as well as to attract this sector's major international companies with market access, modern technology and management know-how⁵.

CONCLUSION

The yield of soybeans in Serbia in the long term (1949-2012) varied from 366 kg/ha (in 1950) to 3,177 kg/ha (in 2010). The average annual yield was 1,730 kg/ha, with an increasing tendency (average annual growth rate 1.82%). The pace of increase in soybean yields is uneven during the observed period, i.e., there are significant differences in average annual growth rates observed in individual decades.

The analysis conducted has shown that the yield level of 1.5 t/ha was exceeded in 2003 and has not been reappeared afterwards. An average soybean yield in Serbia in the last ten years (2003-2012) was 2,448 kg/ha, which could be assessed as relatively satisfactory, if compared with the world, European and the average yield in neighbouring countries. However, the expressed variability of the soybean yield and significantly higher yields achieved by some developed countries indicate that the desired level of production has not been reached yet, i.e., that there is room for further improvement.

Knowledge and constant monitoring of production by decision makers on site provide insight into factors limiting yields and contribute to making effective management decisions in specific production conditions. Therefore, it can be concluded that high yields of the soybean require an optimal combination of agro-ecological conditions and adequate management decisions (for the specific conditions of production).

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⁴<http://extension.agron.iastate.edu/soybean/documents/HighYield.pdf>

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MOGUĆNOSTI UNAPREĐENJA PROIZVODNJE SOJE U SRBIJI

DANICA BOŠNJAK., VESNA RODIĆ, JELENA KARAPANDŽIN

Izvod

Savremeni trendovi u ljudskoj ishrani i rastuće potrebe stočarske proizvodnje, posebno proizvodnje svinja i živine, utiču na rast tražnje za sojom, kako u globalnim razmerama, tako i u Srbiji. Zbog toga se nameće potreba sagledavanja mogućnosti unapređenja proizvodnje ovog useva na nacionalnom nivou. U radu se analiziraju prinosi soje postizani u period 1949-2012, kao osnovni pokazatelj proizvodnih rezultata. Osnovni cilj rada je da se oceni nivo i stabilnost ostvarenih prinosa soje u posmatranom periodu, kako bi se ukazalo na neke mogućnosti daljeg unapređenja u proizvodnji ovog useva. Izvršena analiza pokazuje da je prosečan prinos soje ostvarivan u Srbiji u periodu 1949-2012 bio 1,730 kg/ha; prosečna godišnja stopa rasta prinosa 1.82 %, uz variranje prinosa od 366 kg/ha (koliko je ostvareno 1950. godine) do 3,177 kg/ha (2010. godine). Posmatrano na kraći rok situacija je bila nešto bolja, odnosno u poslednjoj deceniji (2003-2012) prosečan prinos je bio 2,448 kg/ha. Ovaj nivo prinosa može se oceniti kao relativno zadovoljavajući, ukoliko se nacionalni prosečan prinos poredi sa svetskim, evropskim i prosekom zemalja iz okruženja. Međutim, prisutna varijabilnost u ostvarenim prinosisima i činjenica da se u nekim zemljama, sa sličnim ili čak lošijim agroekološkim uslovima za proizvodnju soje, postižu i veći prosečni prinosi ukazuju na prostor za dalji rast. Da bi se te mogućnosti iskoristile od presudnog je značaja da donosioci odluka, odnosno menadžeri dobro poznaju specifične uslove (i prirodne i društveno-ekonomske) u kojima se proizvodnja odvija. Ograničavajući faktori rasta prinosa mogu se uočiti na vreme i optimalne upravljačke odluke doneti samo ukoliko se process proizvodnje dobro poznaje i neprekidno nadgleda i to na samoj parceli. Stoga se može konstatovati da je za rast i visoke prinose soje neophodna kombinacija optimalnih agroekoloških uslova i adekvatnih upravljačkih odluka, odnosno onih koje će u maksimalnoj meri uvažiti konkretne uslove proizvodnje.

Ključne reči: soja, prinosi, menadžerske odluke.

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PREVALENCE OF *MALASSEZIA SPP.* IN SMALL ANIMAL PRACTICE*

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IŠTVAN PALINKAŠ¹

SUMMARY: *The aim of this study was to evaluate the prevalence of Malassezia spp. in dogs and cats, and to assess the relationship between the Malassezia infection and skin diseases and/or external ear canal diseases. A total of 100 animals were examined (67 dogs and 33 cats). The cytological examination of the external ear canal and skin specimens was performed in order to diagnose Malassezia spp. The prevalence of Malassezia spp. in the external ear canal of all examined animals was 49% (49/100), with the significant difference between the occurrence of Malassezia spp. in healthy and diseased animals ($p < 0.05$). The prevalence for animals with skin diseases was 54.5% (18/33), while the prevalence for animals with diseases of external ear canal was 90.9% (10/11). Statistically significant relationship was shown between the findings of Malassezia spp. on cytological examination and the appearance of erythema in dogs ($p < 0.05$) and of excoriation in cats ($p < 0.05$).*

Key words: *Malassezia*, skin diseases, diagnosis, cytology, dog, cat.

INTRODUCTION

Malassezia yeasts are commensal organisms on the skin of humans and animals, but if cutaneous microclimate changes, their number will rise and they will become pathogens (Eidi et al., 2011). The knowledge of them being present in most cases of generalized dermatitis led to the use of antimycotic drugs in therapy, and to the higher number of cured animals (Gaitanis et al., 2013). In some cases, the presence of these yeasts may suggest that animal have some systematic disease (Nuttall, 2003; Prado et al., 2008). In addition to the clinical importance of this yeast in small animal practice, the interest for *Mallassezia spp.* is increased due to their zoonotic potential.

Original scientific paper / *Originalni naučni rad*

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According to our knowledge, the study of the presence of *Malassezia spp.* in our country have only been studied in dogs with atopic dermatitis (Milčić-Matić et al., 2010). The aim of this study was to determine the prevalence of *Malassezia spp.* on skin and in the external ear canal in group of animals (healthy and animals with the signs of dermatitis/otitis externa) using simple methods of diagnosis that are available in clinical practice on every day basis.

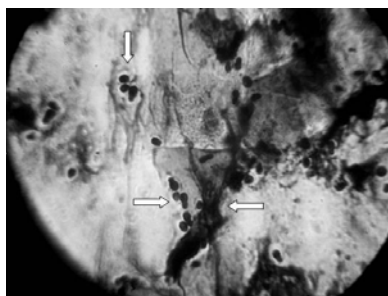
MATERIAL AND METHODS

In this study, a total of 100 animals were examined (67 dogs and 33 cats). There were 60 clinically healthy animals and 40 animals (32 dogs and 8 cats) with the signs of dermatitis/otitis. For all examined animals, clinical dermatological exam records were made. Dermatological diagnostic methods, such as skin scraping, acetate tape impression smears, cytological examination of skin specimens and native/cytological microscopic examination of specimens collected from external ear canal, were performed. From all examined animals, specimens from the external ear canal were taken, and from animals that showed signs of dermatitis, skin specimens were collected as well. In order to diagnose *Malassezia spp.* cytological examinations of the skin and external ear canal were performed. A sterile cotton swabs were used to collect the material from chosen skin area and the external ear canal. For the preparation of slides for cytology examination, after specimens were air dried, the method of heat fixation was used. May - Grünwald Giemsa method was used for staining the slides, with phosphate buffer pH 7.2. Then, slides were examined by using a microscope immersion oil lens (1000x). Slides were examined for the presence of the *Malassezia spp.* as well as for the presence of inflammatory cells. The result of ≥ 4 *Malassezia spp.* per high power field was used as a benchmark for clinically significant result. In the diagnostic process, results of microscopic examination of slides were considered together with information collected from anamnestic data and clinical exams.

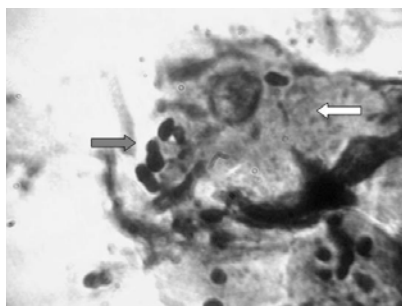
The results were analyzed by statistical software SPSS 17. Student's t-test was performed for numerical data, and Chi-square test was performed to assess the strength of the relationship between certain parameters. Differences of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

There is no established diagnostic standard for the diagnosis of *Malassezia spp.* (Bensignor et al., 2002). Previous research (Cafarchia et al., 2005) has shown that cytological diagnostic method is highly specific, fast to perform, non invasive, cheap and easily available. Due to all of these, cytological diagnosis of *Malassezia spp.* has an important role in everyday clinical practice.



Picture 1. Cytological exam of external ear canal (upper and left arrow: *Malassezia spp.*; right arrow: corneocyte), orig.



Picture 2. Cytological exam of skin samples (left arrow: *Malassezia spp.*; right arrow: achantolytic cell), orig.

Due to all of these, cytological diagnosis of *Malassezia spp.* has an important role in everyday clinical practice. The false positive results connected with cytological examination are insignificant. On the other hand, false negative results can be obtained if there are errors in the way of collecting samples and making slides for microscopic exam (Cafarchia et al., 2005).

The prevalence of *Malassezia spp.* in external ear canal of all-examined animals was 49% (49/100). The literature data show that the prevalence of *Malassezia spp.* is in the range from 10% (Bond et al., 1997) to 49% (Crespo et al., 2000) in healthy animals, and from 19% (Greene, 1998) to 83% (Crespo et al., 2000) in animals with otitis externa. In our study, the prevalence of *Malassezia spp.* in external ear canal (picture 1) of diseased animals was 57.5% (23/40), and 43.3% (26/60) for healthy ones. There was a statistically significant difference between the occurrence of *Malassezia spp.* in the external ear canal of healthy and diseased animals ($p < 0.05$). This result is in agreement with results of previous studies (Cafarchia et al., 2005; Nutall, 2003). The prevalence of *Malassezia spp.* in external ear canal of healthy dogs was 37.1% (13/35), and 52% (13/25) in ear canal of healthy cats. The earlier data have shown that the prevalence of this yeast in the external ear canal of cats range from 10 – 40% (Crosaz et al., 2013).

Table 1: Etiological factors of dermatitis in dogs

Etiological factors	Number of diseased dogs	Malassezia spp. primary factor	Malassezia spp. secondary factor
Malassezia spp.	5	5	0
Demodex canis	12	0	5
Sarcoptes scabiei	2	0	1
Intertrigo	2	0	1
Pyoderma	2	0	1
Atopic dermatitis	4	0	2
Total	27	5	10

On the skin of animals (dogs and cats) with dermatitis (picture 2) *Malassezia* spp. occurred in 54.5% (18/33) cases. For dogs with dermatitis, the prevalence was 55.5% (15/27) of which in 33.3% cases *Malassezia* spp. occurred as primary, and in 66.6% cases as secondary pathogen. For cats, the prevalence was 50% (3/6), with 25% of cases having *Malassezia* spp. as primary and 75% of cases as a secondary factor.

Table 2: Etiological factors of otitis externa in dogs

Etiological factors	Number of diseased dogs	Malassezia spp. primary factor	Malassezia spp. secondary factor
Malassezia spp.	6	6	0
Otodectes cynotis	1	0	1
Demodex canis	1	0	1
Total	8	6	2

In animals with otitis, the prevalence of *Malassezia* spp. was 90.9% (10/11). In dogs with otitis, *Malassezia* spp. was present in all eight cases (100%). Etiological factors of otitis are shown in table 2. In all cases of otitis, the cerumen was dark brown, accompanied by an unpleasant smell. In cats with otitis, *Malassezia* spp. was present in 66.6% (2/3) cases.

There was no statistically significant difference between the presence of *Malassezia* spp. and the way in which animals were kept (indoor or outdoor), as well as the presence of previous diseases.

Location of skin lesion due to *Malassezia* infection was mainly in the area of ear lobes and the head in diseased dogs, and ear lobes, head, neck and tail in diseased cats (chart 1 and 2). In generalized form, *Malassezia* spp. has only been presented in diseased dogs. In diseased dogs, alopecia was the most dominant primary skin lesions (87.5%), and from secondary skin lesions the most common were erythema (66.6%) and excoriations (58.3%). There was a statistically significant relationship between the occurrence of *Malassezia* spp. and appearance of erythema in dogs ($p < 0.05$). In diseased cats, among primary skin lesions, the most common was alopecia (62.5%), while secondary skin lesions such as excoriations and crusts were present in 87.5% cases, followed by erythema (75%). Statistically significant relationship was shown between the findings of *Malassezia* spp. on cytological

examination and excoriation in cats ($p<0.05$). Similar results were obtained in previous researches (Nuttall, 2003; Čonková et al., 2011). These studies have shown the occurrence of skin lesions in the area of ear lobes is 44.8%, and in head area this value is 25%.

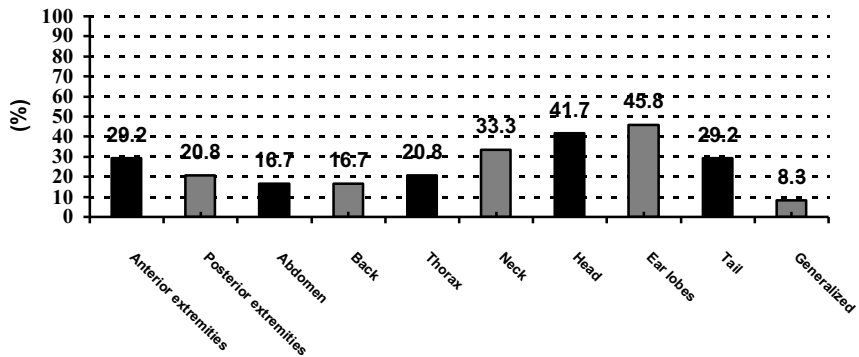


Chart 1. Localization of skin lesions in diseased dogs

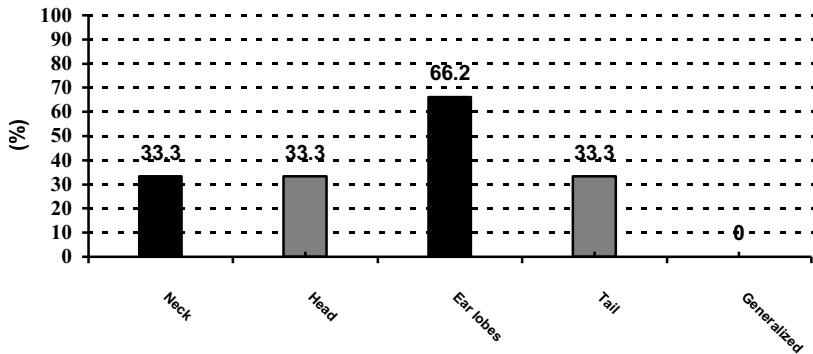


Chart 2. Localization of skin lesion in diseased cats

Cytological exam of skin samples taken from dogs with dermatitis/otitis showed that cellular infiltrate consisted of mastocytes was presented in 37.5% of cases, while neutrophils and eosinophils were presented in 33.3% of dogs, and basophils in 29.2% of dogs. Achantolytic cells were present in 16.6% of dogs. In 62.5% of cases coccoid microorganisms were revealed along with *Malassezia spp.* There was no statistically significant relationship between the presence of *Malassezia spp.* and occurrence of these cells.

Cytological exam of samples taken from the external ear canal of diseased dogs showed the incidence of mastocytes as dominant cellular infiltrate (50% of cases), while basophils, eosinophils, neutrophils and macrophages as dominant cellular infiltrate were presented in 12.5% of cases. In three cases, coccoid microorganisms were found along with *Malassezia spp.* (37.5%). There was no statistically significant relationship between the presence of *Malassezia spp.* and occurrence of these cells.

Cytological exam of skin samples taken from cats with dermatitis/otitis showed that cellular infiltrate of neutrophils and basophils was dominant in 25% of cases, while mastocytes and eosinophils were found in 12.5% cases. Achantolytic cells appeared in 25% cases, while in 37.5% of cases coccoid microorganisms were found along with *Malassezia spp.* There was no statistically significant relationship between the presence of *Malassezia spp.* and occurrence of these cells.

Cytological exam of samples taken from the external ear canal of diseased cats showed that in 66.7% cases cellular infiltrate was consisted mostly of neutrophils. Macrophages, eosinophils and basophils were present in 33.3% cases. In all cases, the coccoid microorganisms were presented. There was no statistically significant relationship between the presence of *Malassezia spp.* and occurrence of these cells.

Previous study have shown that *Staphylococcus spp.* often accompanied *Malassezia spp.* dermatitis/otitis (Petrov and Mihaylov, 2007).

Some limitation of this study should also be addressed. The main limitation is relatively small number of diseased animals, which limit the conclusion on the subject of clinical importance of *Mallassezia spp.* presence in the animals. Besides, the cytological examination was used as the sole method for *Malassezia* diagnosis. It could be presumed that the prevalence of *Malassezia spp.* would be higher with usage of both cytological examination and mycological culture methods. Further studies are needed in order to define possible connection between the occurrence of *Malassezia spp.* and the chemicals used for the maintenance of hygiene in animals.

CONCLUSION

The prevalence of *Malassezia spp.* in external ear canal of all examined animals was 49%, with statistically significant difference between the occurrence of *Malassezia spp.* in external ear canal of healthy and diseased animals. In diseased animals with dermatitis the prevalence of *Malassezia spp.* was high (54.5%), with the higher prevalence in diseased dogs (55.5%) then in diseased cats (50%). The prevalence of *Malassezia spp.* in animals with otitis was 90.9%, with the prevalence of 100% and 66.6%, in dogs and cats respectively. Skin lesions due to *Malassezia* infection are mainly localised in the area of ear lobes and head. The most common primary lesion was alopecia, while erythema, excoriations and crusts were the most common secondary skin lesions, with statistically significant relationship between the occurrence of *Malassezia spp.* and appearance of erythema in dogs and between the occurrence of *Malassezia spp.* and appearance of excoriations in cats.

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PREVALENCA *MALASSEZIA SPP.* KOD ŽIVOTINJA U MALOJ PRAKSI

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IŠTVAN PALINKAŠ

Izvod

Ovo istraživanje je sprovedeno u cilju ispitivanja prevalencije *Malassezia spp.* kod pasa i mačaka, kao i utvrđivanja postojanja veze između prisustva *Malassezia spp.* i pojave dermatitisa/otitisa. Ukupno 100 životinja je uključeno u istraživanje (67 pasa, 33 mačke). Radi dijagnostikovanja *Malassezia spp.* vršen je citološki pregled uzoraka kože i spoljašnjeg ušnog kanala. Prevalenca *Malassezia spp.* u spoljašnjem ušnom kanalu svih pregledanih životinja je iznosila 49% (49/100), sa tim da je postojala statistički značajna razlika između prisustva *Malassezia spp.* u ušnom kanalu bolesnih i zdravih životinja ($p < 0,05$). Kod životinja obolelih od dermatitisa prevalenca *Malassezia spp.* je iznosila 54.5% (18/33), dok je kod životinja obolelih od otitisa iznosila 90.9% (10/11). Postojala je statistički značajna razlika između prisustva *Malassezia spp.* i pojave eritematoznih promena kod pasa ($p < 0,05$), kao i prisustva *Malassezia spp.* i pojave ekskrijacija kod mačaka ($p < 0,05$).

Ključne reči: *Malassezia*, bolest kože, dijagnoza, citologija, pas, mačka.

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PEROXIDASE ISOENZYME POLYMORPHISM IN THE GENUS *PRUNUS*, SUBGENUS *CERASUS**

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SUMMARY: *The polymorphism of peroxidase was studied in 31 cherry accessions, representing six following species Prunus cerasus, Prunus avium, Prunus fruticosa, Prunus mahaleb, Prunus serrulata, Prunus gondouinii and two widely-used standard cherry rootstocks 'Gisela 5' and 'Colt'. Six 'Oblačinska'sour cherries, four wild sweet cherries, five ground and one mahaleb genotypes were selected from the natural populations of Serbia. Inner barks from one-year-old shoots and young actively growing leaves were used for protein extraction. The polymorphism of peroxidase was obtained both for leaf and inner bark tissues. The analysis of the leaf material showed the unique zymograms for all six species and two interspecies hybrids. Higher numbers of polymorphic loci and banding patterns were detected when protein was extracted from the leaves, than from inner bark. Obtained results indicate that the polymorphism determination of genus Prunus, subgenus Cerasus can be done on the basis of peroxidase, but it would not be useful for discrimination of different genotypes and clones.*

Key words: peroxidase, electrophoresis, polymorphism, cherry, *Prunus spp.*

INTRODUCTION

Conservation of genetic resources, essential for future breeding programs, requires a good characterization of the genetic diversity of germplasm and a proper assignment of individual genotypes to species (Tavaud et al., 2004). A description of morphological

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characteristics is the usual method for preliminary evaluations of genetic diversity because it is fast and simple, but it can be used only among morphologically distinguishable accessions. The morphological variation, a product of the genotype-environment interaction, is an important parameter, but much diversity that remains unexpressed morphologically can be revealed by biochemical and molecular markers. Isoenzymes were among the first markers applied in horticultural science. They allow the identification of plants in early stages of development and are not affected by environmental conditions. They can be separated and analyzed due to their differences in electrophoretic mobility. Isoenzymes are used as genetic markers of the genus *Prunus* because of their stability, co-dominant expression and reproducibility (Martinez-Gomez et al., 2003). For these reasons, isoenzymes are useful for the identification of genetic polymorphism (Daeil, 2004). Over the past decade, different types of molecular markers, such as AFLPs (Tavaud et al., 2004), RFLPs (Bouhadida et al., 2007), SSRs (Ercisli et al., 2011) and RAPDs (Zamani et al., 2012), have been used for the genetic characterization of the *Prunus* germplasm and the establishment of genetic relationships between cultivars and species, but the data obtaining through isozymes is relatively inexpensive compared to DNA which analysis require sophisticated instruments.

The isoenzyme analysis is used in genetics and breeding of the genus *Prunus* for identification of cultivars (Milatović et al., 2009, Nikolić et al., 2010), phylogenetic relationships among species (Daeil, 2004) and for analyzing the genetic variability of native populations (Čolić et al., 2010, 2012). Beaver et al. (1995) conducted a research on characterizations of the genus *Prunus* subgenus *Cerasus*, based on isoenzymes. They studied seven isoenzyme systems in sweet, sour and ground cherries, verifying that this technique was efficient in detecting polymorphism among them. Moreover, Corts *et al.* (2008) pointed out that problems with synonymies and homonymies frequently present in the characterization of cultivars can be solved on the basis of isoenzyme genotypes. Čolić et al. (2012) reported that ADH, IDH and SDH were the most polymorphic and most useful to identify genetic variability in the genus *Prunus* subgenus *Cerasus*.

Peroxidases are enzymes with numerous biochemical and physiological roles in higher plants. They participate in plant growth as well as in differentiation and development processes, including auxin catabolism, ethylene biosynthesis, plasma membrane redox system and generation of H₂O₂, cell wall edification, lignifications and suberization, and response to pathogen (Has-Schön et al., 2005). Peroxidase isoenzymes are tissue-specific (Manganaris and Alston, 1991, Zapata et al., 1995) and developmentally regulated (Smila et al., 2007). A study of peroxidase isoenzyme profiles in some sweet cherry rootstocks and cherry varieties conducted by Güçlü and Koyuncu (2012) showed that peroxidase profiles were similar in scions and rootstocks.

Based on the above background, this study was undertaken to compare the peroxidase variation revealed from two tissues (leaf and inner bark tissue), and to establish the usability of peroxidase for evaluating genetic diversity of the genus *Prunus* subgenus *Cerasus*, in order to make germplasm evaluation more efficient.

MATERIAL AND METHODS

The plant material (Table 1) consists of 31 cherry genotypes representing six following species, *Prunus cerasus* (12), *Prunus avium* (11), *Prunus fruticosa* (5), *Prunus mahaleb* (1), *Prunus serrulata* (1), *P. gondouinii*(1) and two interspecies hybrids (Gisela 5 and Colt). Six ‘Oblačinska’ (autochthonous and heterogeneous cultivar), four wild sweet cherry, five ground cherry genotypes and one mahaleb genotype were selected from natural populations in different parts of Serbia. The selection of genotypes was done according to the observed diversity of phenological and morphological traits of trees and fruits.

Two types of the plant material, inner barks from one-year-old shoots in dormant stage and young actively growing leaves, were used for the extraction and evaluation of peroxidase (PRX) activity. Vertical PAGE was used for the isoenzyme analysis. Polyacrylamide gel containing 8% acrylamide was used for separation. Sample preparation and staining procedures were done in accordance with the protocols given by Bošković et al. (1994) for stone fruit species. The loci of the same enzyme system were numbered progressively, beginning with locus 1 at the most anodal position. Gels were visually observed and bands that represent isoenzyme patterns were analyzed.

RESULTS AND DISCUSSION

The polymorphism of peroxidase (PRX) was established both for the leaf and inner bark tissues. As expected, the two types of tissues revealed different patterns of variation. Our results are agreeable with the findings of Smila et al. (2007) who observed a number of tissue specific isoforms (present in the root and leaf tissues of various pearl millet varieties) for both esterases and peroxidases at each stage of development, where different isozyme banding patterns were obtained. That implies differential activation of genes involved in synthesis of these enzymes at diverse development stages. Greater polymorphism was obtained for the leaf tissues having 12 types of zymograms, while the inner bark tissues showed eight banding patterns. Also, the number of polymorphic loci was higher for the leaf (Figure 1) than for the inner bark (Figure 2) tissues.

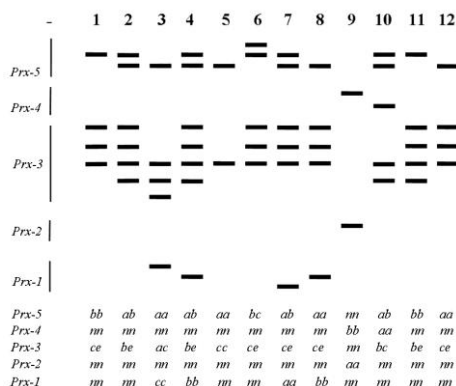


Figure 1. Types of peroxidase (PRX) zymograms obtained from leaves

The results of the electrophoresis were presented in Table 1. The analysis of the leaf material showed the unique zymograms of all six species and two interspecies hybrids. Further, unique activity in locus *Prx-2* was detected only in Colt. Obtained polymorphism for PRX is higher than Čolić et al. (2012) established for dehydrogenase. That makes leaves PRX very useful as potential marker for discrimination of species in subgenus *Cerasus*.

All the *P. avium* (Figure 1, zymogrames 1, 11) and *P. fruticosa* genotypes (Figure 1, zymogrames 8, 12) had zymograms that lacked in "a" or "b" bands at the locus *Prx-5*, respectively, while both bands were found in the sour cherry genotypes (Figure 1, zymogrames 2, 4, 10,). Moreover, the existence of the homozygous locus *Prx-1* that had a "b" band both in sour and ground cherries supports the fact that sour cherries arose from hybridization between ground and sweet cherries.

P. avium showed two types of zymograms and among eleven genotypes only KK 6/10 showed pattern 11 (Table 1), with an extra "b" band at the locus *Prx-3* (Figure 1). The position of the locus *Prx-3* and three alleles of the cultivated genotypes correspond to the results of Granger et al. (1993), but our results indicate that the number of alleles of wild forms is higher.

Three banding patterns (Table 1) were observed in the sour cherry genotypes. On the basis of activity for the locus *Prx-1*, the commercial *P. cerasus* cultivars can be distinguish from the 'Oblačinska', an autochthonous and heterogeneous cultivar. The ornamental genotype BNS lacked "d" and "e" bands at the locus *Prx-3*, and it showed activity only in the locus *Prx-4*.

The *Prunus fruticosa* genotypes showed two types of zymograms (8 and 12). Activities were recorded at *Prx-3* and *Prx-5* – the loci with the same alleles. The two genotypes had an additional zone of activity - *Prx-1*.

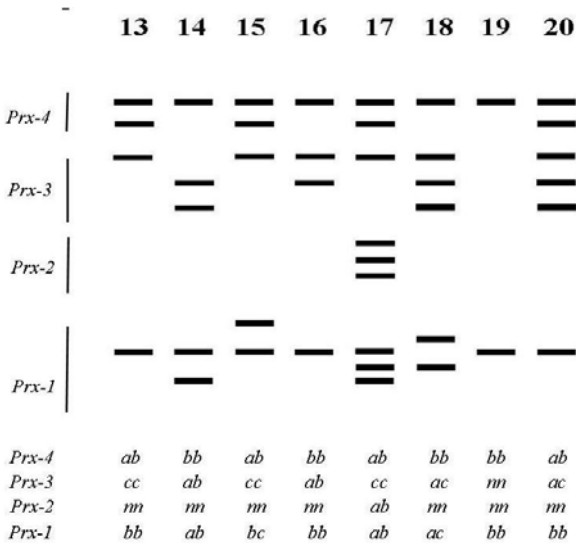


Figure 2. Types of peroxidase (PRX) zymogrames obtained from inner bark

The analysis of inner bark tissues resulted in three zones of activity, except for *P. serrulata* that showed additional activity only at the locus *Prx-2*. As with leaves, the following species could be determined according to their characteristic PRX phenotype: *P. avium*, *P. serrulata*, Colt, NS KK 6/10. Also, Maynard (*P. cerasus*) can be distinguishing from Oblačinska and commercial sour cherry cultivars on the basis of additional bands in loci *Prx-1* and *Prx-3*.

Greatest polymorphism and three banding patterns were found in *P. avium* (Figure 2, zymogrames 13, 15 and 19). This is not in accordance with the results of Güçlü and Koyuncu (2012), who evaluated a local Turkish rootstock based on *P. avium* and *P. mahaleb*, MaxMa 14, MaxMa 60, CAB 6P and Gisela 5 rootstocks, as well as a cherry variety "0900 Ziraat" and reported that peroxidase system was monomorphic. Also, for *P. avium* and *P. mahaleb*, the obtained polymorphism of PRX was higher than reported by Şeker (2008), who detected a high activity only in one zone.

The number of polymorphic loci varied in depends of a species and tissue used for the analysis, as presented in Table 2. Ten out of the eleven *P. avium* genotypes were monomorphic at the locus *Prx-3*, while an additional allele "b" was found for NS KK 6/10 in the leaves samples. When barks were used, variability was detected for the presence or absence of the allele "b" at the locus *Prx-1*, activity at the locus *Prx-3* and an allele "a" at the locus *Prx-4*.

Table 1. Zymogram patterns of peroxidase (PRX) of the investigated cultivars and genotypes

	Cultivar/genotype	Species/Interspecies hybrid	Type of zymogram	
			Leaves	Inner bark
1	Drogan's yellow	<i>P. avium</i>	1	13
2	Celeste	<i>P. avium</i>	1	13
3	Victoria	<i>P. avium</i>	1	13
4	Early Star	<i>P. avium</i>	1	15
5	Vera	<i>P. avium</i>	1	13
6	Sara	<i>P. avium</i>	1	13
7	DT X9-wild cherry	<i>P. avium</i>	1	13
8	DT X3-wild cherry	<i>P. avium</i>	1	13
9	DT X7-wild cherry	<i>P. avium</i>	1	13
10	DT K9-wild cherry	<i>P. avium</i>	1	13
11	NS KK 6/10-dwarf	<i>P. avium</i>	11	19
12	Lara	<i>P. cerasus</i>	2	16
13	Montmorency	<i>P. cerasus</i>	2	16
14	Rexelle	<i>P. cerasus</i>	2	16
15	Keleris 16	<i>P. cerasus</i>	2	16
16	Oblačinska UD 6	<i>P. cerasus</i>	4	16
17	Oblačinska UD 8	<i>P. cerasus</i>	4	16
18	Oblačinska D1 R	<i>P. cerasus</i>	4	16
19	Oblačinska D4 R	<i>P. cerasus</i>	4	16
20	Oblačinska II/10 R	<i>P. cerasus</i>	4	16
21	Oblačinska XI/3 R	<i>P. cerasus</i>	4	16
22	Maynard-dwarf	<i>P. cerasus</i>	4	18
23	BNS-ornamental	<i>P. cerasus</i>	10	16
24	SV 1	<i>P. fruticosa</i>	8	16
25	SV 2	<i>P. fruticosa</i>	12	16
26	SV 3	<i>P. fruticosa</i>	12	20
27	SV 5	<i>P. fruticosa</i>	8	20
28	SV 7	<i>P. fruticosa</i>	12	20
29	Radmilovac	<i>P. gondouinii</i>	5	16
30	Amonagawa	<i>P. serrulata</i>	6	17
31	TT	<i>P. mahaleb</i>	3	14
32	Colt	<i>P. avium</i> x <i>P. pseudocerasus</i>	9	15
33	Gisela 5	<i>P. cerasus</i> x <i>P. canescens</i>	7	16

The *P. cerasus* genotypes were monomorphic at the locus *Prx-5*, polymorphic at *Prx-3* and they varied for presence or absence of activity at *Prx-1* and *Prx-4* when leaves were used. Bark samples showed polymorphism for *Prx-1* and *Prx-3*. Our results indicate that a considerable variability of pomological and technological properties established by Nikolić et al. (2005) and Rakonjac et al. (2010) for the 'Oblačinska' sour cherry are not followed by PRX polymorphism.

The *P. fruticosa* genotypes were separated into two groups according to their *Prx-1* activity in leaves. The bark samples were polymorphic at *Prx-3* and *Prx-4*.

Table 2. Polymorphic loci of peroxidase (PRX) in genus *Prunus*, subgenus *Cerasus*

Leaves		Inner bark of one-year-old shoots	
Species	Polymorphic loci	Species	Polymorphic loci
<i>P. avium</i>	<i>Prx-3</i>	<i>P. avium</i>	<i>Prx-1, Prx-3, Prx-4</i>
<i>P. cerasus</i>	<i>Prx-1, Prx-3, Prx-4</i>	<i>P. cerasus</i>	<i>Prx-1, Prx-3</i>
<i>P. fruticosa</i>	<i>Prx-1</i>	<i>P. fruticosa</i>	<i>Prx-3, Prx-4</i>

CONCLUSION

The results of our study confirmed the organ specificity of PRX isoenzyme pattern *P. mahaleb* and *P. serrulata* were distinguished from other species for their PRX polymorphism. A unique PRX profile was also determined for NS KK 6/10. Morphological differences among *P. avium*, *P. fruticosa* and *P. cerasus* were followed by PRX polymorphism. We found the polymorphism of PRX, as biochemical markers, sufficient for identifying the genetic diversity in the genus *Prunus* subgenus *Cerasus*, but not useful for further discrimination of different genotypes and clones within the same species.

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POLIMORFIZAM PEROKSIDAZA RODA *PRUNUS*, PODROD *CERASUS*

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Izvod

Polimorfizam peroksidaza proučavan je kod 31 genotipa roda *Prunus* u okviru šest vrsta podroda *Cerasus*: *Prunus cerasus*, *Prunus avium*, *Prunus fruticosa*, *Prunus mahaleb*, *Prunus serrulata* i *Prunus gondouinii*, kao i kod dva interspecies hibrida koji se koriste kao standardne podloge za trešnju: Gisela 5 i Colt. Šest genotipova Oblačinske višnje, četiri divlje trešnje, pet genotipova stepske višnje i jedan genotip magrive su selekcionisani iz prirodnih populacija u Srbiji. Za ekstrakciju proteina korišćena je unutrašnja kora jednogodišnjih grančica i mlado lišće. Polimorfizam peroksidaza je uočen u oba tkiva. Analizom lista dobijeni su jedinstveni peroksidazni zimogrami za svaku od ispitivanih vrsta i oba interspecies hibrida. Veći broj polimorfnih lokusa i tipova zimograma utvrđen je kada je za proteinski ekstrakt korišćeno lišće, u poređenju sa unutrašnjom korom jednogodišnjih grančica. Dobijeni rezultati ukazuju da je na osnovu polimorfizma peroksidaza moguće determinisati vrste roda *Prunus*, podrod *Cerasus*, ali ne i utvrditi razlike među genotipovima i klonovima.

Ključne reči: peroksidaze, elektroforeza, polimorfizam, trešnja, *Prunus* spp.

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COLLECTION and CONSERVATION of MAMMALIAN OOCYTES and EMBRYOS for ANIMAL GENETICS RESOURCE PRESERVATION *EX SITU* (a review)

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SUMMARY: In the last few decades, farm animal genetic diversity has rapidly declined. Therefore, it is in the interest of the international community to conserve the livestock genetics. In situ (live animals herds) model of genome conservation is expensive and limited for practical usage. Because, ex situ (ex vivo) conservation model are developed to cryopreserve animal genetic resources in genome (gene banks) to regenerate a particular population in future. Although significant progress has been made in oocyte and embryo cryopreservation of several domestic species, to date a standardized procedure has not been established. Successful long-term cryopreservation of oocytes and embryos would preserve the genetic material from unexpectedly dead animals and facilitate many assisted reproductive technologies. There are the biological, economical and moral imperative and interest of the international community to conserve the livestock genetics.

Key words: oocyte, embryo, genetic resources, preservation, ex situ.

INTRODUCTION

Almost all farm animal breeds are experiencing a significant decrease of genetic diversity in the last few decades (Prentice and Anzar, 2011). This is a result of intensive genetic selection for small number of productive and reproductive traits (Buerkle, 2007), application of modern biotechnologies in reproduction, that allowed the production a large number of progeny from a single individual, as well as use the effective methods of transport and long-term storage of sperm cells, oocytes and early embryos (Patterson and Silversides, 2003). Because, biodiversity preservation in domestic animal breeds and gene banks formation is in the interest of the international community (Prentice and Anzar, 2011).

Scientific review paper / *Pregledni naučni rad*

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Gene banks are defined as systematic and organized collection, preservation and exploitation of genetic material, by *in situ (in vivo)* or *ex situ (ex vivo)* methods. The *in situ (in vivo)* method involves preservation and reproduction the small herds of various animal species, breeds, and lines (Bulla, 1996; Wildt, 1999; Stančić, 1999). Method *ex situ (ex vivo)* involves long-term storage of gametes (sperm cells and oocytes) (Johnston, and Lacy, 1995; Stančić, 2000, Stančić et al., 2001; Stančić et al., 2002; Stančić et al., 2005; Stančić and Dragin, 2011; Stanković, 2012) or early embryos by cryopreservation technology (Stančić, 2004; Boettcher, et al, 2005; Pereira and Marques, 2008; Prentice and Anzar, 2011) as well as by cryopreservation of testicular or ovarian tissue somatic cells (Andrabi and Maxwell, 2007; Pereira and Marques, 2008). Longt-term preservation of oocytes and embryos is primarily use for biodiversity preservation and for using genetic material from animals after their biological death, in various selection programmes.

The aim of this paper is to review the modren biotechnologies for collection and long-term cryopreservation of domestic animlas oocytes and early embryos *ex situ*.

OOCITES COLLECTION

There are two basic methods for collecting oocytes from domestic animal females: (a) oocytes collection after superovulation induction by treatment with exogenous gonadotrophins (eCG i hCG) and (b) oocyte extractin from the antral ovarian follicles (Stančić et al., 1992; Laurinčik et al., 1992; Stančić et al., 2007).

By the method of superovulation limited number of oocytes can be obtained. Furthermore, there are significant variation in superovulation rate, depending on the animal species and breed, body condition, health status as well as gonadotropin preparations dosage. Additionaly, hormonal treatment can result with various ovarian function disorders. In the cow, it can be obtained average 8.7 ovulation per treatment, with variations from 2 to 50 ovulations. In the sheep, superovulation rate varing from 2 to 15 ovulations, and in the sow superovulation rate varing from 25 to 46 ovulations (Stančić et al., 1992; Šahinović, 1995; Stančić et al., 1998; Stančić and Veselinović, 2002).

Significantly higher number of oocytes per one female can be obtained by using methods for oocyte extraction from antral ovarian follicles (so-called „follicular oocytes“). This method is often used in pigs, because hormonal induced superovulation gets only a slightly more oocytes than after spontaneous ovulation (10 to 51 oocytes in gilts and 18 to 24 in sows). On the contrast, in the cow, it can be obtained 8 to 14 follicular oocytes (Wiebke, 1993).

It has been obtained average 7.9 oocytes per ovary by aspiration of antral follicles, and 45 oocytes per ovary by total ovary resection, in the sexually mature gilts, post mortem (Stančić et al., 1993). The advantage of this method is the possibility of getting a large number of oocytes from sacrificed animals and avoiding the harmful effects of exogenous hormone treatment. Namely, it has been demonstrated that superovulation induction with higher doses of gonadotropins, frequently result with increasing number of degenerated oocytes, as well as increasing number of unovulated and/or cystic follicles (Moor et al., 1985; Stančić et al., 1991). However, the crucial disadvantage of this method is the fact that follicular oocytes are not possible for fertilization immediately afeter extraction from the ovarian follicles. Namely, the nucleus of more than 95% ovarian oocytes is in the germinal vesicle stadium (GV), i.e. diploten of the first meiotic division (Crozet,

1991). Therefore, it is necessary to perform *in vitro* maturation (IVM) of obtained follicular oocytes, to reach the metaphase of second meiotic division (so-called MfII-oocytes). These oocytes are capable for fertilization, i.e. for activation by sperm penetration (McDonald, 1989). Before *in vitro* cultivation, follicular oocytes must be denuded (i.e. cumulus cells complex must be removed from the zona pellucida surface). The quality of cumulus complex directly influence the *in vitro* maturation rate of follicular oocytes. Only oocytes with compact (i.e. GV-oocytes) or expanded cumulus (i.e. GVBD-oocytes, germinal vesicle broke down) are capable for successful *in vitro* maturation (Laurinčik et al., 1992). After 24h to 48h of cultivation, about 80 to 90% of oocytes mature, i.e. reach the MfII stage of nuclear division (Fukui, 1989). The number of matured oocytes can be improved by cultivation medium supplementation with p-FSH (Laurinčik et al., 1993), FSH and LH (Šahinović et al., 1994), follicular fluid (Nagai, 1994), epidermal growth factor or other bioactive substance (Singh et al., 1993),

EMBRYOS COLLECTION

Early embryos, 5 to 8 days of age (morula or early blastocyst stage), are used for long-term cryopreservation in the liquid nitrogen, at temperature -196°C (Paynter et al., 1999). Such embryos can be obtained by: (a) flushing from oviducts or uterus of superovulated and inseminated donor female or (b) after *in vitro* fertilization of follicular oocytes or oocytes obtained by flushing donor oviducts. Embryo flushing can be done by laparotomic or laparoscopic approach to reproductive organs (i.e. surgical method), as well as by transcervical flushing of uterus (nonsurgical method) (Besenfelder et al., 1998). Nonsurgical method is usually performed in large animals (cow, mare), and surgical in small animal (sheep, goat, pig) (Stančić and Veselinović, 2002).

In vitro fertilization (IVF) of *in vitro* matured (IVM) oocytes, or oocytes obtained after hormonal superovulation of donor females, mainly depend of matured oocyte (MfII-oocytes) quality, proper sperm *in vitro* capacitation, composition of maturation and fertilization medium and conditions of cultivation microclimate (temperature, composition and percentage proportion of gases, such as O_2 , CO_2 and N_2) (Thibault et al., 1988; Courot and Vallard-Nail, 1991). Success of *in vitro* fertilization is calculated by the percentage of monospermic penetrated oocytes, as well as by the number of oocytes reached 2-blastomere or 4-blastomere stadium of embryo division. In the cows, more than 70% monospermic and about 4% polyspermic penetrated oocytes have been obtained after 48h of *in vitro* follicular oocytes cultivation (Šahinović, 1995). However, in the pig, about 85% oocytes were polyspermic penetrated, after *in vitro* fertilization of follicular oocytes. Polyspermic oocytes is not possible to undergo normal embryo development (Šahinović, 1995; Šahinović et al., 1997). This phenomenon is not yet clearly elucidated, but methods of sperm *in vitro* precultivation and capacitation, as well as sperm and oocyte coincubation procedure, seems to be of the major influence (Besenfelder et al., 1998; Cshum et al., 1990). Additionally, success of IVF is influenced by cultivation medium composition, period of cultivation and microambient cultivation conditions (Edwards, 1989).

OOCYTE and EMBRYOS CRYOPRESERVATION

Cryopreservation involves preservation of oocytes, early embryos or whole tissues at very low temperatures, usually in liquid nitrogen (- 196°C) (Woods et al., 2004). At such a low temperature, biological activity is effectively stopped, and the cells functional status may be preserved for centuries. However, to avoid the intracellular ice formation, that influence cell death, freezing technology must include crioprotect substances addition in semen extender, dehydration, freezing point depression, supercooling, and intracellular vitrification (Wolfe and Bryant, 2001). Other factors, such as osmolarity changes, toxicity of cryoprotectants, increasing intracellular electrolites and other sterссора can also result in cells damage or death (Vajta, 2000). It has been demonstrated that antifreezing-proteins, sugars or antioxidants act to stabilize the cell membrane (Ledda, 2001).

During cryopreservation, a significant number of oocytes or embryos being damaged and, after thawing, is incapable of further development. The degree of damage depends on the shape and size of the cell, oocytes or embryos quality, permeability of cell membranes, and these factors vary depending on the type of animal (Vajta and Kuwayama, 2006). Previous studies have shown that early embryos are more tolerant to cryopreservation than oocytes. The exact cause is not established, but one reason could be the difference the structure and/or plasmal membrane osmotic potencial, between oocytes and embryos (Chen et al., 2003).

The maintenance potential for normal oocytes development after thawing varies between the animal species, developmental satages and origin (Critser et al., 1999, Pereira and Marques, 2008). It would ppear that the stage of embryo development and the number of cells exposed to the stersss of cryopreservation could play an important limiting role for normal embryo development after thawing (Paynter et al., 1999).

Table 1: Differences of animal oocyte and embryo cryopreservation resistance among species, developmental stages, and origin.

	More resistance	Less resistance
Species	Bovine, ovine	Porcine, equine
Developmental stages	Morula, YBL, and BL	Hatched BL and oocytes
Origin	<i>In vivo</i> derived embryos	<i>In vitro</i> produced embryos, micromanipulated embryos

YBL: young blastocyst; BL: blastocyst; *Pereira and Marques (2008)*.

CONCLUSION

According to FAO reports, in all species of domestic animals is observed an increasing decline in biodiversity. Therefore, there is an increasing demand for efficient biotechnological research methods of long-term conservation of the genomes of existing species, breeds and lines of farm animals.

Preservation of genetic resources is carried out using method *in situ* (*in vivo*), forming small herds of certain species of animals, or *ex situ* (*in vitro*), using long-term cryopreservation of sperm, oocytes, embryos or reproductive tissue somatic

cells (testis and ovary). Thus it is possible to perform multiplication of desirable genotypes, when the need arises, although the donor animals were dead for a long time. Although cryopreservation technology has progressed in recent decades, the success of survival of frozen oocytes and embryos, are still not satisfactory. Previous studies have shown that early embryos are more tolerant to cryopreservation than oocytes. In addition, cryopreservation technology is complex and expensive, and not available for widespread use. It is therefore necessary to combine the use of methods of *in situ* and *ex situ*, with the aim of successful conservation of biodiversity of domestic animal breeds.

Conservation of genetic biodiversity of domestic animals is a global imperative in the biological, economic and moral sense. Biologically, because biodiversity is a key condition for survival of life on our planet. Economically, because a human population uses a huge number of animal species for food, medicines, chemicals, technological materials and energy. Moral, because man, as dominant species, is responsible for the maintenance and protection of all other species of living organisms, with which it must live on this planet.

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PRIKUPLJANJE I KONZERVACIJA OOCITA I EMBRIONA SISARA ZA ČUVANJE ŽIVOTINJSKIH GENETSKIH RESURSA *EX SITU* (pregled)

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IZVOD

U poslednjih nekoliko dekada, genetski diverzitet domaćih životinja rapidno opada. S tim u vezi, postoji interes međunarodne zajednice za očuvanje genetike domaćih životinja. Model *in situ* čuvanja genoma je skup i značajno limitiran za praktičnu primenu. Zbog toga se razvija model *ex situ* (*ex vivo*) krioprezervacije animalnih genetskih resursa (banke gena) za regeneraciju pojedinih populacija u budućnosti. Iako postoji značajan napredak u krioprezervaciji oocita i embriona pojedinih vrsta domaćih životinja, do danas nije ustanovljena standardna procedura ove tehnologije. Uspešna dugotrajna krioprezervacija oocita i embriona će omogućiti očuvanje genetskog diverziteta i primenu brojnih tehnologija asistiranе reprodukcije domaćih životinja. Postoji biološki, ekonomski i moralni imperativ i interes međunarodne zajednice za očuvanje genetike domaćih životinja.

Ključne reči: oociti, embrioni, genetski resursi, čuvanje, *ex situ*.

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RESISTANCE TO BETA LACTAM ANTIBIOTICS IN GRAM-NEGATIVE MICROORGANISMS, A BRIEF REVIEW*

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*SUMMARY: Resistance mechanisms to β -lactam antibiotics are briefly presented, emphasizing the importance of extended spectrum β -lactamases. In two independent research works from our laboratories, ESBLs were not detected among *Salmonella enterica* serovar Enteritidis and resistance to ampicillin was quite low (out of 878 isolates included in the study, 2.2% were resistant to AMP). As food producing animals are reservoirs of ESBL producing bacteria, antibiotic treatment in veterinary practice has to be selective and the prudent use of antimicrobials is strongly recommended.*

Key words: extended spectrum β -lactamase, bacteria, antibiotics, humans, animals.

HISTORICAL PERSPECTIVE

The Penicillin's are the oldest antibiotics introduced in medical practice. A broad range of antibiotics, including penicillin derivatives cephalosporins (I, II, III and IV generation), monobactams, carbapenemes and β -lactam/ β -lactamase inhibitor combinations are available for therapy. All classes of β -lactam antibiotics contain a β -lactam ring in their molecular structures. β -lactam antibiotics act by inhibiting the cell wall synthesis in bacteria. The resistance develops after bacteria produces enzymes termed β -lactamases, which cleave β -lactam ring and prevent antibiotics to bind to penicillin binding proteins (Frye and Jackson, 2013). β -lactamase enzymes are chromosomally mediated or encoded from transferable plasmids and they belong to diverse or genetically similar molecules that are subsequently classified in different groups and derivatives. Widespread application of antibiotics has worsened therapeutic options since bacteria emerged in a whole variety of resistant clones.

Review paper / Pregledni rad

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This work is a short review of enzymes produced by Gram-negative bacteria as a response to β -lactam antibiotics.

Extended-Spectrum β -lactamase (ESBLs) enzymes are an important part of the defense mechanism in Gram-negative rods. They hydrolyze penicillins, oxyimino-cephalosporins and oxyimino-monobactam (aztreonam), are often plasmid-mediated and transferable between bacterial species. Classification of major groups of β -lactamases is described in a research work of Bush (2001). According to Ambler molecular classification, ESBL enzymes belong to the group A having serine at the active site. Since there is a growing evidence of new β -lactamases the classification suggested by Bush, Jacoby and Medeiros was adopted. Their classification is based on biochemical properties, molecular structures and sequence of the target genes. In that respect, the ESBL enzymes that hydrolyze oxyimino-cephalosporins, monobactams and are inhibited by clavulanic acid are classified as a group 2be. The "oldest" ESBL enzyme was found in *E. coli* in 1960. This enzyme was termed TEM-1 because it was discovered in a patient from Greece named Temoniera (Bradford, 2001). It is a plasmid encoded and incorporated into transposon, enabling its successful spread in the environment. The next enzyme, which is frequently found in gram-negative rods, is SHV-1. In *Klebsiella pneumoniae*, it is found on chromosome, while in *E. coli* it is mostly mediated by the plasmid. Most of the ESBLs evolved as derivatives from TEM and SHV enzymes. In SHV variants, amino acid exchanges are most frequently found at position 238 where glycine is replaced by serine. In the variant SHV-5, substitution is attributed to exchange of glutamate for lysine at 240. Both amino acid exchanges are also found in TEM derived ESBLs. Amino acid serine at 238 is important for successful hydrolysis of ceftazidime, while lysine at 240 is critical for hydrolysis of cefotaxime (Bradford, 2001). Phenotype characterization of ESBLs producers is very important and provides the first evidence of the capacity of bacterial enzymes to develop resistance mechanisms.

β -lactamase inhibitors were introduced in therapy owing to their synergistic effect and to enhance therapeutic options. However, inhibitor-resistant β -lactamase has been found from TEM and SHV. The designation TEM-IRT was given to variants that are resistant to the inhibition of following combinations: amoxicillin-clavulanate, ticarcillin-clavulanate and ampicillin-sulbactam. They have emerged in France and some other European countries since 1990. The SHV-10 variant with the inhibitor resistant phenotype was discovered in *E. coli* from urine of a hospitalized diabetic patient, which received amoxicillin-clavulanate therapy (Bradford et al., 2001).

Nowadays, the most frequent mechanism of resistance to β -lactam antibiotics is attributed to new groups of ESBLs - the CTX-M enzymes. They differ genetically from TEM and SHV (40 % of identity) and hydrolyze better cefotaxime than ceftazidime (Bradford, 2001; Chong et al., 2011). These enzymes are widespread in members of *Enterobacteriaceae* and are found in *Salmonella enterica* serotype Typhimurium. The CTX-M family of enzymes is divided into several major groups: CTX-M-1 type, CTX-M-2, CTX-M-8 and Toho 1 and Toho2 enzymes. The most prevalent type is CTX-M-15. It was originally found in *E. coli* and its clonal spread is attributed to the *E. coli* ST131, harboring IncFII plasmid, determining resistance to several classes of antibiotics (Chong et al., 2011). The OXA enzymes belong to the molecular class D and to a functional group 2d (Bush, 2001). These enzymes confer resistance to ampicillin, cephalothin (first generation of cephalosporins) and

they hydrolyze oxacillin and cloxacillin. They are modestly inhibited by clavulanic acid. Variants in OXA genetic group differ from the main ancestor the OXA-10 type, by one, two or more amino acid exchanges. Comprehensive overview of OXA group of enzymes and brief information about other new families of ESBLs could be found in a review paper from Bradford et al. (2001).

Next important group of bacterial enzymes, the AmpC type, is structurally different from ESBLs and according to Ambler classification they belong to the group C. These enzymes confer resistance to the third generation of cephalosporins and to β -lactam/ β -lactamase inhibitor combinations. In fact, they emerged as a consequence of therapy with antibiotic/inhibitor combinations. The resistance is due to the mutational insertions in the promoter region of ampC, leading to constitutive expression of the gene and subsequent extended-spectrum cephalosporin-resistant (ESCs) phenotype (Seiffert et al., 2013).

Carbapenemases are very important enzymes produced by bacteria. They are classified into molecular group A and a subgroup 2f. These enzymes are inhibited with EDTA and contain at least one Zinc atom at the active site. Such molecular construction enhances hydrolysis of a bicyclic β -lactam ring and their catalytic function are different from enzymes that have serin at the active site. Sometimes they are prescribed for therapy in patients infected with highly resistant Gram-negative microorganisms (Livermore 2008). For detailed information about this family of enzymes, a comprehensive review of Queenan and Bush (2007) is recommended for readers.

Resistance of *Salmonella* Enteritidis to beta lactam antibiotics in Southern Bačka and Srem County

In two independent research works, quinolone resistant *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) was selected to obtain resistotyping. First, a collection of randomly selected *S. Enteritidis* was prepared from stool, food and poultry isolates to perform molecular typing. In this collection, resistance to ampicillin was found in two poultry and three stool isolates. One poultry isolate was resistant only to ampicillin while the other was multiple resistant to several classes of antimicrobials, i.e. resistance was found to ampicillin, cephalothin (a first generation of cephalosporin antibiotics), nalidixic acid (NAL) and tetracycline. Three isolates from human stool were either resistant to ampicillin only or multiple resistant to ampicillin, tetracycline and trimethoprim-sulphamethoxazole (one isolate) and ampicillin, trimethoprim-sulphamethoxazole, NAL and tetracycline (one isolate). Resistance to quinolones was established in 15% of isolates from the collection (Kozoderović et al., 2011). In another work, 878 isolates of *S. Enteritidis* were tested for quinolone resistance. Only 2.2% *S. enteritidis* were found to be resistant to NAL and resistance to β -lactam antibiotics was found in two strains only. One isolate was resistant to ampicillin, trimethoprim-sulphamethoxazole and NAL and one isolate was resistant to ampicillin, cephalothin and trimethoprim-sulphamethoxazole. Because resistance to penicillins and cephalosporins was not significant in NAL resistant strains, we did not determine genes responsible for development of resistance to β -lactams (Kozoderović et al 2012). More comprehensive research regarding β -lactam resistance needs to be done for the members of *Enterobacteriaceae* in the future. Clinical and veterinary isolates of *Escherichia coli* and *Klebsiella* spp are of particular interest since those bacteria

emerge as multiple resistant in nosocomial infections as well as in animals treated with β -lactam antibiotics.

Resistance to beta lactam antibiotics in food animals-recent findings

Most antimicrobials used for the treatment of animals belong to classes that are also used in human medicine (cloxacillin, gentamicin, ampicillin, amoxicillin etc). The usage of antimicrobial drugs in food producing animals could results in antimicrobial resistance among pathogenic and commensal bacteria in these animals, and the resistant bacteria may then be transmitted to humans through the food chain and increase risk of treatment failures (Petrović et al., 2008; Petrović et al., 2011). Resistance to β -lactam antibiotics, new generation of cephalosporins and also to carbapenems, may cause significant problems in the treatment of nosocomial infections and community or environmentally acquired infections all around the globe (Pitout and Laupland 2008). Person to person transmission in hospital settings and food-borne transmission in the environment and communities is the primary cause of spreading ESBLs (Liebana et al., 2013). Therefore, the emergency of new clones with mobile genetic elements in food animals as well as among humans is extremely important and careful monitoring of resistance patterns through national monitoring programs is warranted (Velhner et al., 2010, Velhner et al., 2012, Liebana et al., 2013). A comprehensive review about dissemination of ESBLs as well as the occurrence of resistant Gram-negative bacteria in livestock industry and its role in food and environmental contamination is published in a work of Seiffert et al. (2013). We briefly present here the research from Germany, Canada, USA and Korea, dealing with ESBLs profiles in food animals. A collection of 22.679 *Salmonella enterica* isolates from Germany, for a period 2003 to 2007, was tested for ESBLs and AmpC production by Rodríguez et al., (2009). These isolates were found in foods, food producing animals, feed, environment, humans or were from unknown sources. All isolates that obtain MIC to ceftiofur of $>4\text{mg/L}$ were further analyzed. Among 16 isolates found to be ESBL positive the *bla*_{CTX-M-1} gene was found in 15 isolates: 9 *Salmonella* Typhimurium (ST), 2 *Salmonella* Anatum, 2 *Salmonella* Paratyphi BdT+, 1 *Salmonella* Infantis and 1 *Salmonella* London. One ST isolate posses *bla*_{CTX-M-15} gene. Five *Salmonella* Agona and 1 *Salmonella* Kentucky were found to harbor *bla*_{CMY-2} gene. In 2 *Salmonella* Paratyphi BdT+ isolates the *bla*_{TEM-20} gene was found while *bla*_{TEM-52} gene was found in 1 *Salmonella* Paratyphi BdT+ and 1 *Salmonella* Virchow. In this research, the genetic position of the aforementioned genes in mobile genetic elements was studied as well. *Salmonella enterica* and *E. coli* isolates from several European laboratories were selected for molecular typing on their respective plasmids belonging to the incompatibility group IncI1. These plasmids are responsible for dissemination of ESBL genes and posses also genes relevant to the virulent potential of Shiga-toxin producing *E. coli*. The following ESBL genes were found in those isolates: *bla*_{CMY-2}, *bla*_{CTX-M-15}, *bla*_{CTX-M-1}, *bla*_{CTX-M-14}, *bla*_{TEM-52}, *bla*_{SHV-12}, *bla*_{TEM-1}. Plasmids were further classified by restriction fragment length polymorphism (RFLP method) and sorted accordingly, whereas for multilocus sequence typing several alleles were selected to further organize plasmid in different genetic groups. It was found that the source for IncI1 plasmids and its reservoirs are associated with poultry pathogens such as *Salmonella* spp and *E. coli* (Garcia-Fernandez et al., 2008). Frey and Jackson described resistance patterns in *Salmonella* spp, *E. coli* and *Enterococcus*

spp in the USA. The clonal spread of multiple resistant *Salmonella* carrying IntI1 integrons or IncA plasmids are the most widespread among multiple drug resistant strains (MDR) in the USA. This includes *Salmonella* Typhimurium Definitive Phage Type 104 and *Salmonella enterica* serovar Newport (*S.* Newport). The spread of MDR *S.* Newport is probably associated with antibiotic treatment of cattle with ceftiofur. This clone had a MDR-AmpC phenotype encoded by *bla*_{CMY-2} gene from IncA/C plasmid. Poultry and turkeys may be a source of *Salmonella enterica* serovar Heidelberg. This serovar was found to have IncI1 plasmids with the *bla*_{CMY-2} gene, but MDR phenotype is less frequently expressed. Therapy of neonatal calves and pigs with β -lactams and cepheims is of great concern since these antibiotics are also used in human medicine. In recent years in Canada, plasmid mediated AmpC beta lactamase resistance is more frequent than ESBLs. In that respect, Mataseje et al. (2010) made comparison between different plasmids from *Salmonella* and *E. coli* found in humans, feedlots and water. The isolates included were from different provinces in Canada. The multi locus sequencing strategy combined with the RFLP profiles have shown that genetically similar plasmids are found in *E. coli* and *Salmonella* across Canada. The most frequent clusters were as following: I1, A/C, K/B and the unknown type. Resistance patterns were almost identical in *Salmonella* and environmental *E. coli* isolates and were attributed to the following antimicrobials: ampicillin, amoxicillin/clavulanic acid, cefoxitin and ceftiofur, while in clinical *E. coli* the resistance patterns were related to: ampicillin, cefazolin, cefoxitin and ceftriaxone. In Japan MDR serovars of *Salmonella* spp were also found. The most prevalent among broiler chickens was *S. Infantis* exhibiting resistance to 3 or more different classes of antibiotics. For the first time cephalosporin resistance in Japan from food producing animals was reported. The *S.* Senftenberg isolate was isolated from feces of a broiler chicken and particulate isolate carried CTX-M-2 gene (Ishihara et al., 2009). The goal of the research conducted in Korea was to monitor MDR phenotype in nontyphoid *Salmonella* from poultry in 2 provinces and from humans in one province. MDR phenotype was attributed to the following classes of antibiotics: ampicillin, cephalothin, ceftiofur, aztreonam, genatamycin, streptomycin, tetracycline and sulfametoazole. In most of the isolates the CTX-M-15 β -lactamase gene prevailed and was associated with MDR and self transferable plasmid (Tamang et al., 2011).

Wild animals have been recognized as a possible source of multiple resistant *E. coli* and resistance to β -lactam antibiotics was found. We have described previously the possible spread of resistant *E. coli* from wild birds and small mammals in nature (Velhner et al., 2012). Several factors play important role in dissemination of resistant bacteria in wildlife. Wild birds that reside close to the urban area and are fed with the waste and sewage are more frequently reservoirs of resistant microorganisms. There have been some seasonal variations for possible spreaders and those parts of Europe with higher population density present significant risk for contaminating the environment comparing to less urban zones. It is very important to prevent our planet from further pollution and to preserve wildlife in the best possible way taking care of their natural habitat and survival.

CONCLUSION

Numbers of reports are dealing with resistance to β -lactam antibiotics and extended-spectrum cephalosporins. Food producing animals are important reservoirs

of infection with ESBL resistant clones. In human medicine, the prudent use of antibiotics is critical, while empirical therapy and easy access to antibiotics present substantial risk in spreading resistant microorganisms in hospitals and community. Therapy with new generations of antibiotics needs to be very restricted and in animal husbandry forbidden or prescribed carefully in order to obtain lower risk of resistance development in bacteria.

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OTPORNOST GRAM-NEGATIVNIH MIKROORGANIZAMA NA BETA LAKTAM ANTIBIOTIKE (Pregled)

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Izvod

U radu je prikazan mehanizam otpornosti gram.negativnih mikroorganizama na β -laktam antibiotike, sa isticanjem značaja širokog spektra β -laktamasa. U dva odvojena ogleda u našoj laboratoriji, ESBLs nije detektovana između *Salmonella enterica* serovar Enteritidis, a otpornost na ampicilin je bila vrlo niska (od 878 izolata, uključenih u istraživanja, svega 2.2% je bilo otporno na AMP). Kako su životinje, od kojih se proizvodi hrana za ljude, rezervoar ESBL, antibiotski tretman u veterinarskoj praksi mora biti selektivan, pa se predlaže vrlo oprezana primena antibiotika.

Ključne reči: širok spetar β -laktamase, bakterija, antibiotici, ljudi, životinje.

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CUSTOMER RELATIONSHIP MANAGEMENT (CRM): POTENTIAL APPLICATION IN VETERINARY SERVICE SECTOR*

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SUMMARY: The CRM concept has initially been applied in service sector emerging in time into the business philosophy that is widely practiced through a range of activities and business branches. Establishing of a successful long-term relationship with customers is highly important aspect in modern business administration and can be successfully used in all business spheres. In modern market economy, along with the advancement of information technologies, Internet and database software programs a two-way communication between the product/service supplier and customer has been identified as the priority in business activity. Although the CRM conception still has different definitions, all the approaches are focused on client/customer satisfaction rather than to the product itself, as it was in the past. Implementation of CRM in the sphere of veterinary service would offer benefits not only to veterinary institutes, administrative and professional service departments but also to agricultural and veterinary stations and cooperatives, farmers and animal owners in a view of improved and easier communication and cooperation with the professional staff employed in the field of agriculture, veterinary medicine or food industry. CRM system database would provide a baseline for further research and analysis as well as the support to the decision-making process.

Key words: customer relationship management, CRM concept, process.

INTRODUCTION

The CRM (*Customer Relationship Management*) concept has been widely practiced around the globe since 20 years through all business spheres. In modern market economy, along with the advancement of information technologies, Internet and database software programs a two-way communication between the

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product/service supplier and customer has been identified as the priority in business activity. CRM conception can be defined in many ways; however, it is essentially a comprehensive approach for attracting, satisfying and maintaining the clients/customers. CRM concept relies on two basic elements – documenting and management of client/customer relationship and maintaining the database on each particular client/customer. To that aim, a wide range of data storage and management software programs has been developed. Appropriate database and well-defined procedures create a structure that enables standardization of working process, and the comprehensiveness is related not only to collecting information on the clients but to involving the entire organization into the process in a view of changing the business philosophy in the entire company and is not singled out to individual department (e.g. sales and marketing). In Serbia, this concept was first recognized by emerging small and medium enterprises and has been substantially growing since the last few years (Vidić, 2011). Thus, one can notice specialized employment advertisements looking for professionals to run the CRM in the company. Regrettably, many companies first realize the need and importance of CRM technology after they have already lost their customers. Even though CRM concept has initially been applied only in service sector, it has emerged into the business approach that is practiced through the wide range of business activities and branches.

Implementation of CRM in the sphere of veterinary service would offer benefits not only to veterinary institutes, administrative and professional service departments but also to agricultural and veterinary stations and cooperatives, farmers and animal owners in a view of improved and easier communication and cooperation with the professional staff employed in the field of agriculture, veterinary medicine or food industry. The key benefit of CRM is very simple yet highly personalized communication with clients. It enables providing animal owners with prompt and most current information on modern accomplishments in relevant field. In this way, the client obtains not only timely and high-quality service, but also the possibility to improve his production to the highest level in line with the good production practice.

The objective of this paper is to describe and understand the concept of client/customer/buyer relationship management and its potential application in veterinary practice.

DEFINITION

CRM (*Customer Relationship Management*) is a concept that primarily originates from the private sector and is related to the management of client/buyer/customer relationship (Lovreta et al., 2010). The term of CRM was first defined by mid 1990s, though the technique itself has been well known for decades and centuries back (Bergeron, 2002). Namely, manufacturers and service suppliers in the past (as well as the majority of present ones) were well aware that building of strong relationship with their clients/buyers/customers and identifying and fulfilling their wishes, habits and needs is the prerequisite and a “safe road” to business success (Anderson and Kerr, 2002). The difference is reflected by the fact that nowadays the number of clients and buyers dramatically increased and the contact and communication ways are different (mostly internet, mail or phone instead of “face-to-face” contact) thus it is impossible to know individual customers in person.

CRM concept was developed to respond to this emerging trend, and new technical and technological developments gave rise to its wide application.

Greenberg (2004) defined the CRM as „philosophy and business strategy“ that, supported by technology, strives to improve the relationship between both sides in business environment. Reynolds (2002) considers CRM not only business strategy, but also process, culture and technology“ that enables an organization to optimize the income and increase the value through „understanding and satisfying individual needs of the customer“. Anderson and Kerr (2002) define CRM as „comprehensive approach to creating, maintaining and expanding relationship with clients and customers“. In spite of a lacking unique definition of CRM, all the approaches are focused on client/customer satisfaction rather than on the product itself, as it was in the past (Kirchler et al., 2009). In that respect, nurturing of the relationship with clients based on mutual trust results in his need to share his demands, problems and ideas with the manufacturer, i.e. service supplier (Reynolds, 2002). Such an approach enables substantial savings through increased efficacy, as well as further positive effects on financial outcome (e.g. expansion of company and market, faster investment payback etc.) and increased effectiveness in achieving company's goals (Payne and Frow 2005; Kirchler et al., 2009)

Another definition considers CRM a strategic approach aimed at creating profit for stakeholders through development of strong bonds with key customers. CRM encompasses information technology and marketing strategies to create profitable long-term relationships. CRM enables use of the data and information with an aim of understanding the clients/customers requirements and developing of marketing relations. It requires an integrated process-oriented approach of staff members, operations, processes and marketing options, which is enabled through use of novel technologies and application improvement (Payne, 2005).

ELEMENTS AND FEATURES OF CRM

Disregarding that CRM is applied in a wide range of different business branches such as banking (Ćirić, 2009), telecommunication (Kujović and Dulović, 2011) or hotel industry (Andrews, 2007), the concept itself relies on two basic elements (Kirchler et al., 2009; Anderson and Kerr, 2002).

- *documenting and managing the relationships with the client/customer* – documenting is accomplished through creation and maintaining the database on each individual client/customer. The database encompasses information on both each person and every communication with him/her. To the purpose of data storage and database management numerous software programs were developed, and collected data are appropriately stored to be readily available in relevant form and structure. In this way, the communication process is supported with actual and reliable facts and figures and still focused on single client. Mutual conversation should point out potential weaknesses that are related either directly or indirectly to the service/product in question and issues to be addressed in order to satisfy client's needs.
- *structured and comprehensive approach*: the database and precisely defined procedures build the structure that enables the standardization of working process, which results in the improvement in efficacy and effectiveness of business activities. At the same time, the comprehensiveness does not pertain only to collecting the information on

clients but also to involvement of the entire company into the process, changing the overall company's philosophy beyond the sales and marketing department only

The main issues addressed by CRM include collecting data on customers/clients from all available and appropriate sources, storing into the data storehouse in a way that enables safe and easy access and management of the data, protection from unauthorized use and misuse, extraction of hidden information and its application in marketing activities and customer/client support (Alić, 2003). These tasks are fulfilled by the three CRM forms (Payne, 2005):

- *Operational CRM* is intended for improvement in efficacy through automating of marketing, service support and sales activities. It enters data on interactions with the customer via different databases and applications for monitoring customers' activities, yet without data processing and analysis.
- *Analytical CRM* - encompasses acceptance, storage, organizing, analysis, interpretation and usage of the data collected within the operative CRM. Highest level of integration between operative and analytical CRM is of particular importance for proper functionality of entire system (ibid). Performing of aforementioned operations within analytical CRM creates a picture of each particular client, his wishes and needs, with an aim of establishing closest possible connection and relationship with him.
- *Collaborative CRM* - deals with collaborative services and infrastructure to enable synchronization and integration of company/customer and company/its employee's interactions. Communication with clients involves a range of available communication channels (mail, phone, fax, e-mail, SMS, Web) (Dragović and Ivković, 2008). Thus, for example, the system delivers notices, offers etc. to the clients / customers and feedback is shared through the operative CRM into the system

If compared with other traditional techniques, application of CRM stands out through some unique features (Payne, 2005):

- *contact intensity*: contact with the client is at much higher level, which is mainly due to application of novel technologies and high level of automation (Payne, 2005). Novel software systems enable creation of huge number of personalized letters (the letter is highly personalized, the headline contains the name of the recipient , relevant personal data pertaining to particular recipient are automatically inserted from the database at predefined custom fields, etc.) in very short time, thus saving required manpower for this task and enabling the moving of the employees to other tasks (e.g. increasing the number of delivered letters at annual basis or performing completely different tasks). Besides the direct letters, new communication channels such as internet, e-mail, call-centers or various social networks enable frequent contacts with clients and information exchange, thus resulting in better understanding of their wishes, needs and problems. In this way, the contact is not only intense (Manić and Riznić, 2011) but also continuous and without communication gaps (see below).
- *contact continuity*: the purpose of CRM is establishing of long-term relationships with clients, which implicates high level of continuity in

interaction with customers (Parvatiyar and Jagdish 2003). CRM encompasses accomplishing and continual update of the knowledge related to client's needs, motivation and behavior as well as application of this knowledge in continuous performance improvement (service, product) by „learning from mistakes“ method (Milošević, 2006). Every communication is recorded in „contact history“ thus providing continuity in cases of personal changes in the organization-supplier, organizational reforms, etc (Parvatiyar and Jagdish 2003).

- *contact quality*: Application of CRM implies highly standardized and well-established procedures and processes that minimize potential errors and maximize client's satisfaction (Grbac and Meler, 2007). Moreover, proper implementation and application of CRM results in well trained and highly motivated employees and use of novel technologies, and hence increased quality of the contact and service support (Payne, 2005).
- *resource consumption* (time, manpower): implementing of CRM significantly reduces time required for particular activity, since: firstly, important information are stored in one base, i.e. in a „CRM data warehouse“ (Payne, 2005) thus enabling fast, easy and accurate search. Secondly, human resource savings are significantly increased due to highly automated and standardized communication with clients as well as the data processing (ibid). Thirdly, in spite of virtually high investment in software solutions (ibid), there is numerous disproving evidence to this attitude (ibid), that is, application of quite simple software solutions results in more than satisfactory outcome.
- *control and monitoring of contact intensity and quality*: highest level of data documenting as well as standardization of procedures and processes within the CRM system enables their continuous monitoring and analysis and identifying of failures, errors and potential improvements, which significantly influences the quality of every subsequent contact and service (Parvatiyar and Jagdish 2003).
- *lapse in data*: maintaining and updating of the data in a database through an intensive and continuous contact with clients is an essential aspect of CRM, which prevents potential lapse (Payne, 2005). Moreover, CRM system identifies and eliminates irrelevant and unnecessary information, thus preventing storage of lapsed or irrelevant data in the database (Domazet, 2007)
- *application of new technologies*: new developments in information technology, internet and software programs for database creation directed business activities towards the two-way communication between the organization and the client (Domazet, 2007). In that respect, the assigned username and password enable the client to enter the desired data by himself, to perform the search and communicate with the service supplier in the most appropriate way for him. Software programs that enable collecting, sending, integrating and using of processed data are the prerequisite for such communication (Grbac and Meler, 2007). Moreover, all kinds of digital content (e.g. digital cards, etc.) related to individual clients can be stored in the database and are accessible any time. For example, typing of client's name opens the whole range of information that is accessible depending on the purpose and desired details.

POTENTIAL APPLICATION OF CRM IN AGRICULTURAL SECTOR

In Serbia, this concept was first recognized by emerging small and medium enterprises and has been substantially growing since the last few years. Thus, one can notice specialized employment advertisements looking for professionals to run the CRM in the company. Regrettably, many companies first realize the need and importance of CRM technology after they have already lost their customers. However, such approach is mainly characteristic for the representative and branch offices of foreign companies in Serbia, which first establish the call-centers and then the appropriate CRM systems. Client's benefit from the implementation of CRM technology is better attention he receives from the seller / supplier. Great majority of companies in our country use tabular programs, into which they enter data on their clients. However, such data are mostly not integrated, that is, the company management has no overall insight in the system. CRM enables the overall insight in business activity through: (1) marketing communication, (2) sales communication and (3) follow-up communication within customer support (clients one has already won).

In spite that CRM concept has initially been applied in service sector, it has emerged into the business philosophy that is widely practiced through a range of activities and business branches. Implementation of CRM offers benefits not only to institutes, administrative and professional service departments but also to agricultural and veterinary stations and cooperatives, farmers and animal owners in a view of improved and easier communication and cooperation with the professional staff employed in the field of agriculture, veterinary medicine or food industry. Implementing of CRM in the aforementioned sectors would enable better monitoring and control of field conditions, which is of highly valuable for both everyday practice and science (Savić-Jevđenić et al., 2007; Vidić et al., 2008b; Boboš et al., 2013; Vidić et al., 2013). CRM system database would provide a baseline for further research and analysis as well as support to the decision-making process. This concept ensures making proper decisions, which are highly dependent on appropriate analysis of most current data on the situation in the field and actual problem of all interested parties.

APPLICATION OF CRM IN VETERINARY SERVICE SECTOR

If looking the literature sources and the spectrum of topics and branches they cover, it is apparent that CRM is mainly practiced in so called „business“ sector, particularly in the field of services such as banking, mobile telecommunications, etc. This, however, does not mean that CRM covers only a narrow spectrum of applications. On the contrary – CRM is considered a concept of unlimited application range. Previous chapter mainly addressed the CRM concept itself, and in this one, we shall consider its potential application in the veterinary service sector.

The development of marketing in veterinary practice was significantly supported by the articles published in the journal *Veterinary Economics* and numerous books published mostly in the U.S. (Ackermann, 2007). These articles and books address all issues relevant for veterinary practice, from the moment of taking decision to establish a practice and the best way to do it, everyday activities in the practice and

adjusting to variable working environment to making decision to sell the practice. In Serbia, marketing in veterinary practice has been rarely discussed issue among professionals (Vidić, 2012a). Inadequate application of marketing principles in veterinary practice is likely due to lack of knowledge as well as the necessary financial assets. In practice, the majority of veterinary organizations are reluctant to such investment (Vidić, 2012b). Since recently, veterinary practice has been considered a business requiring high quality of service since strong competition imposes the necessity of using marketing tools in order to achieve competitive advantage, survive and grow in the market (Vidić et al., 2008a).

Regardless of the reasons for practicing veterinary medicine (e.g. love for animals or other) it should be considered a business. Clients are the most important aspect of every business and it is necessary to focus on client relationships and to meet their needs (Međugorac, 2009).

Implementation of CRM in the sphere of veterinary service would offer benefits to veterinary institutes, clinics, veterinary stations and cooperatives, farmers and animal owners in a view of improved and easier communication as goods and service suppliers to animal owners. CRM would enable creation of databases that would provide a baseline for more effective veterinary services offered to end users (Vidić et al., 2008c; Savić et al., 2009). The system would contain all relevant data on the clients, particularly the following ones:

1. Data on animal owner
2. Data on organizing system
3. Number, animal species, breed/race, category
4. Purpose and extent of production
5. Number of visits to animal owner
6. Epizootiological history / anamnesis
7. Diagnoses, laboratory findings, therapy and control
8. Duration of cooperation with individual client
9. Average number of hours dedicated to the client
10. Time intervals of client's contacts / visits
11. Total turnover related to particular client
12. Fulfilling of financial obligations by the client
13. Other notices and comments of the service supplier (veterinarian)

The aforementioned data enable profile creation for each individual client and allow fully personalized subsequent access to this client (Vidić, 2012b). In practice, it means that a vet, who has hundreds of clients, can access all relevant information about individual owner, i.e. animal by one single mouse click. The vet gets an overview of all relevant data that could help him in making prompt and accurate decisions, which further results in good treatment result and satisfied client. In this way, satisfied clients get tightly bound to this veterinary organization, i.e. service supplier, which is further reflected in good financial results of the company.

The key benefit of CRM is very simple yet highly personalized communication with clients. Animal owners are provided with prompt and most current information on modern accomplishments in relevant field, seminars, education programs, possibilities of applying for various program and projects for production improvement, etc. In this way, the client obtains not only timely and high-quality service, but also the possibility to improve his production to the highest level in line

with the good production practice (Lazić et al., 2009). Successful implementation of CRM poses specific technical requirements such as integration and interconnection, safety insurance, report on system functionality, availability as well as support and upgrade.

CONCLUSION

CRM is not a novelty. Successful professionals have been applying this technique since long time ago, being aware of the fact that keeping the customer, maintaining the close relationship and personal contact with client is the key of success. Nowadays, when number of customers constantly increases, this goal is difficult to achieve; however, modern technological developments enable efficient management of customer contacts since the purpose of business is creating customers. CRM is an integrated approach for creating, maintaining an expanding customer relationships. Clients are today well educated, have access to extremely wide range of products and services offered by competitors, huge variety of information and communication possibilities thus CRM could be the solution for satisfying such clients. CRM is a philosophy and business strategy supported by technology, and is designed to improve the relationships between people in business environment. Implementation of CRM is a long-term process that is never completed, as customers' needs and requirement increase with the advancement of technology.

Establishing the relationship with clients is an important aspect of modern veterinary business, providing competitive advantage at service market. Implementation of CRM would increase and improve the relationship with clients, i.e. users of veterinary service. This would result in increased the range of services offered, improved marketing, increase in sales volume, better monitoring of sales of goods and services, faster delivery of goods and services, better quality of service aimed at customer's satisfaction.

Information on application and importance of CRM in modern business presented in this paper strongly suggest indisputable advantages of this approach that results in improvement and strengthening of links with animal owners. In spite that CRM concept has initially been applied in service sector, it has emerged into the business philosophy that is widely practiced through a range of activities and business branches. Implementation of CRM offers benefits not only to animal owners in a view of improved and easier communication and cooperation with the professional staff employed in this sector, but also to entire veterinary service sector, offering possibilities for better monitoring and control of field conditions, which is of highly valuable for both everyday practice and science. CRM system database would provide a baseline for further research and analysis as well as support to the decision-making process. This concept ensures making proper decisions, which are highly dependent on appropriate analysis of most current data on the situation in the field and actual problem of all interested parties.

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UPRAVLJANJE ODNOSIMA SA KLIJENTIMA (CRM): POTENCIJALNA PRIMENA U SEKTORU VETERINARSKIH USLUGA

VASO VIDIĆ, SARA.SAVIĆ, BRANKA VIDIĆ, ŽIVOSLAV GRGIĆ

Izvod

CRM koncept je u početku bio rezervisan za upotrebu u uslužnom sektoru, ali je vremenom prerastao u poslovnu filozofiju koja je našla svoju primenu u širokom spektru aktivnosti i delatnosti. Uspostavljanje odnosa sa korisnicima usluga je veoma važan i značajan aspekt savremenog poslovanja i sa uspehom se može primenjivati u svim sferama poslovanja. U savremenoj tržišnoj privredi, paralelno sa razvojem računarske tehnologije, Interneta i softvera za kreiranje baza podataka, prioritet poslovne aktivnosti postaje dvosmerna komunikacija davaoca usluga i proizvođača i korisnika usluga i kupaca. I pored toga što jedinstvene definicije za CRM nema, zajedničko za sve njih jeste to da u fokusu imaju zadovoljnog klijenata/kupca a ne sam proizvod, kao što je to ranije bio slučaj.

Koristi od implementacije CRM-a u veterinarskoj službi imali bi kako veterinarski instituti, zavodi, stručne službe, poljoprivredne i veterinarske stanice, zadruge, poljoprivredni proizvođači, vlasnici i držaoci životinja, koji bi lakše ostvarivali komunikaciju i saradnju sa stručnim licima iz svake oblasti, poljoprivredne, veterinarske, prehrambene delatnosti. Baza podataka koja nastaje stvaranjem CRM sistema postala bi polazna osnova za sve vrste ispitivanja i analiza i pružala podršku u procesu donošenja odluka. Prednost upotrebe CRM je i u veoma jednostavnoj, a visoko personalizovanoj komunikaciji sa korisnicima usluga. Na ovaj način omogućeno je da vlasnici životinja u najkraćem vremenskom periodu dobiju najaktuelnije informacije o najnovijim saznanjima iz oblasti kojom se bave. Zadovoljni klijenti postaju dugoročno vezani za veterinarsku organizaciju, odnosno davaoca usluga, što za rezultat daje i uspešno finansijsko poslovanje organizacije.

Ključne reči: upravljanje odnosima sa korisnicima usluga, CRM koncept, procesi.

HORMONAL METHODS FOR ESTROUS CYCLE MANIPULATION IN DAIRY COWS*

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STANČIĆ, ALEKSANDAR BOŽIĆ, IVAN JOVANOVIĆ, BRANKO
ATANASOV, ADAM ŠULUBURIĆ¹

SUMMARY: Reproduction efficiency in dairy cows is declining all over the world. Epidemiologic studies suggest that diseases have greater effect on herd fertility compared to the parameters like milk production. Immediate solution to the problem of infertility may be the application of some method of hormonal manipulation of estrous cycle in dairy cows. Reproductive system ultrasound examination enables today's veterinarians to visualize ovarian changes during the estrous cycle, giving them a chance to intervene with exogenous hormonal inhibition and/or stimulation of temporary ovarian structures at the appropriate time. Summarizing possible hormonal methods for estrous cycle manipulation we have analyzed following protocols: prostaglandin based protocols, prostaglandin + progestagens in combination, regimes using prostaglandin + gonadotropin-releasing hormone (GnRH) and prostaglandin + GnRH+ estradiol protocols. In dairy herds where estrus detection does not represent a significant problem, prostaglandin based or Select Synch protocols are methods of choice for the hormonal manipulation of estrous cycle. However, if there are problems regarding estrus detection Ovsynch or Presynch + Ovsynch could be an effective alternative for the hormonal manipulation of estrous cycle. Furthermore, fixed time artificial insemination (TAI) could be modified to coincide with the second GnRH injection, thus reducing the time and number of visits.

Key words: dairy cows, estrous cycle, hormonal manipulation.

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Decline in reproduction efficiency

Genetic selection in dairy cows has apparently created a conflict situation where reproduction performance is reduced while milk production is increasing (Lucy 2001; Royal et al., 2002). Dairy cows are usually inseminated during the maximum of lactation and pregnancy is mandatory in order to achieve optimal production. The two parameters of reproduction efficiency that could illustrate the decline in reproduction performance are first-service conception rate and number of services per conception. Both of them are worsening worldwide during the last several decades. The first service conception rate in New York State decreased from 65% in 1951 to 40% in 1996, according to data presented by Butler (1998). This process is also evident in other parts of the world like Ireland (Roche, 2000), United Kingdom (Royal et al., 2000) and Australia (Macmillan et al., 1996). Summarizing records from dairy herds in Kentucky (USA) Silvia (1998) has reported an increase in number of services per conception (SPC) from 1.62 in 1972 to 2.91 in 1996. Another basic parameter of reproduction efficiency is calving interval (CI), and maximal milk production should aim at CI of 12-13 months. Data from USA are clearly indicating prolonged CI as well as increased number of inseminations needed for successful pregnancy (figure 1).

Similar negative trends of reproduction efficiency parameters have also been detected in Serbia. Data from large dairy farms agglomeration near Belgrade indicated that days open period (DO) and number of services per conception (SPC) are increased (176 and 2.7; DO and SPC, respectively; Stojić et al., 2011), and DO between 137-146 days, and SPC value of 2.8 have been recorded by Gvozdić et al., (2011). However, epidemiologic studies suggest that diseases have greater effect on herd fertility compared to nondisease parameters like milk production (Gröhn et al., 2000), and our results corroborated that opinion (Gvozdić et al., 2011; Gvozdić et al., 2012).

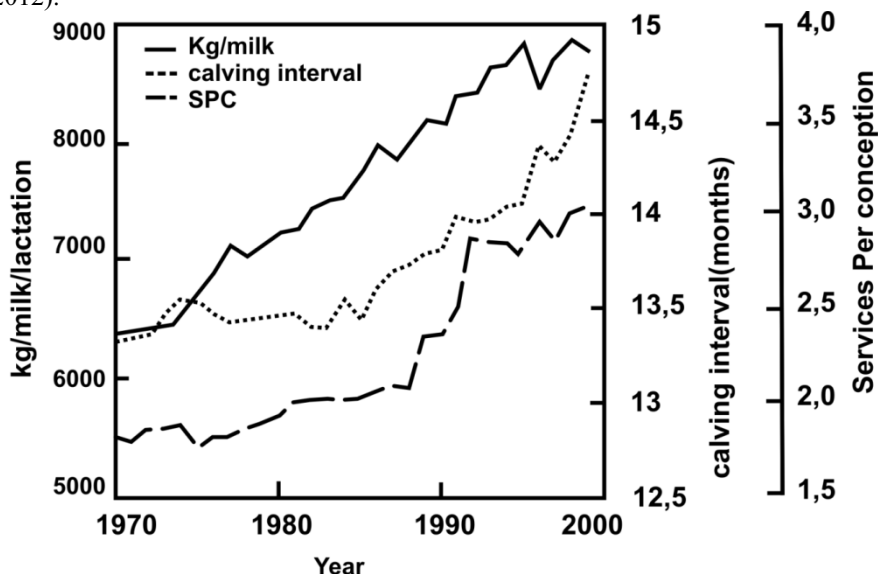


Figure 1. Milk production, calving interval and number of services per conception in dairy cows between 1970-2000 (Modified from Lucy, 2001).

In fact, higher producing herds generally have better reproductive performance (Nebel et al., 1993; Stevenson, 1999), that can probably be attributed to better feeding and reproduction management (Lucy, 2001).

When the farmers are encountered with the decline in reproduction performance an immediate solution is to use some method of hormonal intervention in order to manipulate estrous cycle in dairy cows. An ultimate goal in every case is successful pregnancy. However, there are multiple methods for estrous cycle manipulation in dairy cows, and the aim of this paper is an attempt to summarize them.

Principles of estrous cycle manipulation

Estrous cycle in dairy cows has two distinctive phases: luteal and follicular phase, with markedly different temporary endocrine structures dominating on the ovaries. Hence, there are two general principles that can be applied to the hormonal manipulation of estrus cycle in dairy cows: 1) manipulating/imitating corpus luteum life and function, and 2) manipulating follicular development and ovulation. The earliest method for estrous cycle manipulation (developed in the 1960s) was based on the use of exogenous progestagens, which means an artificial extension of the luteal phase that blocked and postponed estrus and ovulation. However, those methods were soon abandoned since the conception rate was depressed. During the 1970s it was discovered that prostaglandin F_{2α} is locally active uterine hormone responsible for luteal regression and demise, and combined with progestagens it was used for estrous cycle manipulation. Despite considerable improvement there was still the problem with depressed conception rate in different hormonal methods for estrous cycle manipulation. Only after the technological breakthrough based on ultrasound during the 1980s it was discovered that persistent dominant follicle (DF) caused depressed fertility (Lucy, 2004).

Follicle development in dairy cows

The culprit for estrous cycle in dairy cows is the ovulation of dominant follicle (DF) with well manifested signs of estrus that enables successful insemination and pregnancy. In order to achieve better understanding of hormonal methods for estrous cycle manipulation here are several important facts about the development of follicles in dairy cows. A slow phase of follicular growth precedes their development in the estrous cycle and takes about 30 days. It is characterized by the increase in follicles diameter from 300 μm to 3-5 mm (Lussier et al., 1987). This period of follicular growth is independent on gonadotropin hormones. Further follicular growth is much faster (up to 1-2 mm/day) and it depends on follicle stimulating hormone (FSH) and luteinizing hormone (LH) for the final stages of development. Those changes can be detected using ultrasonography, and that could be the critical point for introducing hormonal treatment for estrous cycle manipulation.

Dairy cows usually have two to three, so called “follicular waves” during the estrous cycle. One follicular wave is composed of three phases (figure 2.): 1) **recruiting**, i.e. emerging of 4-8 follicles, less than 5 mm in diameter, 2) **selection**, when only one of the recruited follicles will continue to growth while others will undergo

atresia and demise, and 3) **dominance**, with inhibition of follicles recruitment and development, accompanied with one DF development. Only the DF from the last follicular wave during the estrous cycle will reach the final stage of development, with the possibility to ovulate and shed the oocyte. Dominant follicles from the first (and second, if there are three waves) follicular wave will undergo atresia before they reach final stage of development. Atresia of DF will initiate an increase in FSH secretion, since growing follicle secretes protein hormone inhibin that suppresses FSH secretion. The trigger of DF final development and maturation is luteal regression and a sudden decrease of progesterone blood level. The enlargement and maturation of DF with increase estradiol production will signal the preovulatory LH surge, inducing manifest estrus and ovulation.

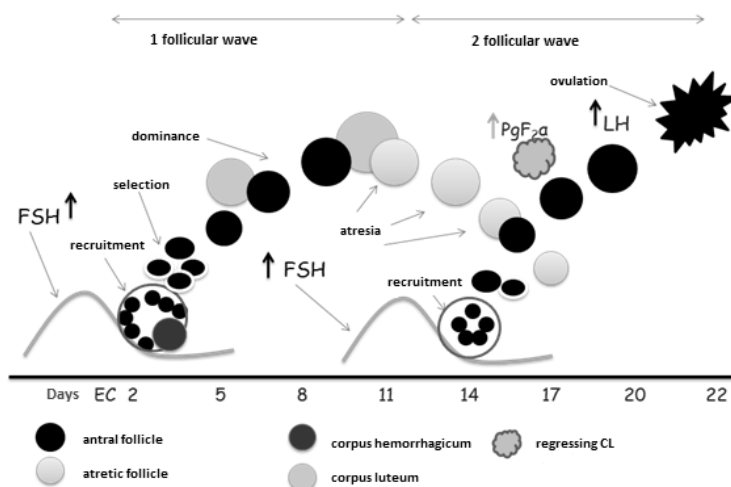


Figure 2. Follicular waves, CL development and regression (EC, estrous cycle)
Modified from *Ptaszynska, 2009*.

Corpus luteum

Corpus luteum (CL) is formed after ovulation at the place of DF. The CL is possible to distinguish from the ovarian tissue as soon as two days after ovulation as an echogenic spherical or ellipsoid structure. In this phase of development CL is filled with blood coagulum (*corpus hemorrhagicum*). Function of the CL is manifested as an increase in blood progesterone level above 1 ng/mL and starts about 5 days after ovulation. Corpus luteum is increasing in size and function, to more than 25 mm CL diameter, and reaches maximal level of progesterone production at day 12 after ovulation. Luteolysis is a process of functional CL (*corpus luteum periodicum*) decomposition that occurs between days 17-19 of estrous cycle in dairy cows, if there was no previous successful insemination. Prostaglandin F_{2α} (PGF_{2α}) secreted from the endometrium of ipsilateral uterine horn is local causative luteolytic agent. It is also the most extensively used hormone in different estrous cycle manipulation protocols.

The decrease of plasma progesterone concentration after initiation of luteolysis in nonpregnant dairy cows is faster than CL morphological changes, causing less experienced veterinarians to make mistakes regarding its function. Using measurements of blood progesterone as the indicator of CL function it was found that 25-39% of dairy cows classified as having functional CL (assessed by rectal palpation) were not producing high amounts of progesterone. Furthermore, between 15-21% of dairy cows classified as having nonfunctional CL had high blood progesterone (Senger, 2003). During ultrasound examination it is not unusual to find CL with fluid filled central cavity that is mistakenly named cystic corpus luteum. It has been proven that this finding is not pathological structure, and it could be detected as well in pregnant animals (Grygar et al., 1997; Dovenski, 1997).

PGF_{2α} in hormonal manipulation of estrous cycle

Natural prostaglandin (PGF_{2α}) is effective luteolytic agent that can be used for hormonal control of estrous cycle in dairy cows. The prerequisite for successful treatment is that cow is cycling and has functional CL. It is also important to know that cow is nonpregnant, otherwise the treatment will cause embryonic mortality or abortion, since functional CL is necessary for normal pregnancy. Another disadvantage of prostaglandin based protocol is that it has no direct effect on follicular waves. Parenteral application of PGF_{2α} causes luteolysis, shortening luteal phase of the estrous cycle and indirectly creating favorable conditions for subsequent follicular development. Furthermore, prostaglandin treatment is effective only during specific period in the estrous cycle. This “window” in normal healthy cycling cows is opened from days 5-7 until days 17-18 after estrus. That 11-12 days period represents the time during which CL is responsive to prostaglandin treatment since appropriate receptors are present. If prostaglandin treatment is issued during the first 5 days after estrus or after days 17-18 of the estrous cycle there will be no answer: at the first 5 days of estrous cycle CL is not matured enough, and after days 17-18 CL in nonpregnant dairy cows has already gone through physiologic luteolysis with no need for exogenous luteolytic agent. Hence, PGF_{2α} treatment is usually consisted of two injections with 11 days interval in between (figure 3). If there are two follicular waves during the estrous cycle and PGF_{2α} is given within the appropriate time frame, there could be three possible outcomes: either prostaglandin injection is given early (days 7-9), at the middle (days 10-13) or late (days 14-16) in the estrous cycle. It is favorable if PGF_{2α} is given at the early or late days, because it is the time when DF on the ovaries can be expected, with high chance for maturation and ovulation, and 2-5 days are usually needed until the manifestation of estrus. If prostaglandin treatment is issued in the middle of the estrous cycle (days 10-13) it will take 3-7 days until estrus, since first follicular wave DF has undergone regression and atresia, while the second wave DF is underdeveloped and needs more time for maturation (Lucy, 2004).

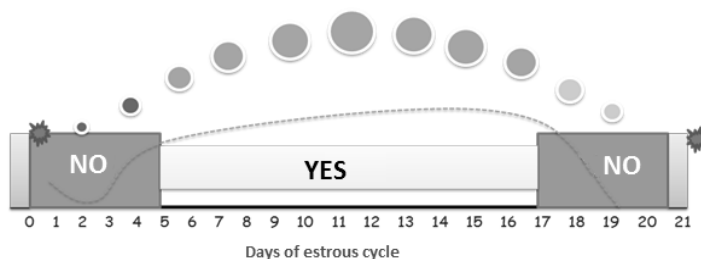


Figure 3. Prostaglandin treatment in the cycling nonpregnant dairy cows is effective during “window” between days 5-17 of the estrous cycle (marked with YES); during the first 5 days and after day 17, prostaglandin treatment is ineffective or useless (marked with NO). *Modified from Senger, 1998.*

Prostaglandin treatment alone in the cycling dairy cows requires estrus detection. Estrus signs are less pronounced in the postpartum dairy cows in negative energy balance (Westwood et al., 2002). There is also the problem with variation of luteal phase duration in high producing dairy cows (Lucy, 2003), contributing to the limited use of prostaglandin treatment alone for hormonal manipulation of estrous cycle.

Relatively simple protocol for hormonal manipulation of the estrous cycle utilizing prostaglandin is known as “monday morning” regime (figure 4). That means prostaglandin treatment is issued for noninseminated cycling dairy cows (after voluntary waiting period, VWP) at monday morning, and estrus signs are monitored during the next 3-5 days, responders are inseminated and hopefully impregnated. If there is no estrus (no response) after the first prostaglandin treatment it is repeated 14 days later (second monday after first treatment), with estrus detection and successful insemination. The response to the first breeding treatment could be improved if one prostaglandin injection is given 14 days prior to the first insemination treatment.

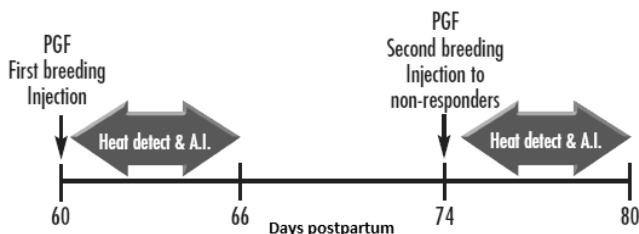


Figure 4. Monday morning protocol for hormonal manipulation of estrous cycle using PGF_{2a} alone

Modified from DeJarnette, 2013.

Progestagens and PGF_{2a} in the hormonal manipulation of estrous cycle

Hormonal manipulation of estrous cycle using progestagens is mimicking luteal phase progesterone secretion. Progesterone is important in the hormonal regulation of estrous cycle since it has a strong negative feedback on the hypothalamus, reducing the frequency of the basal episodic secretion of gonadotropin-releasing

hormone (GnRH). However, the amplitude of LH pulses (together with FSH secretion), induced by the tonic GnRH secretion, is high enough to allow the development of follicles during the luteal phase. These follicles do not reach the preovulatory status until the progesterone block is removed (luteolysis). Blood progesterone concentration higher than 1 ng/mL is needed to suppress the preovulatory LH surge and estrus (Lucy, 2004). Treatment with progesterone for 14-21 days resulted in high estrus response within 3 days of progesterone removal (Macmillan et al., 1993). The negative aspect of progesterone treatment of such duration is lower conception rates, which can be overcome with shortening the time of exposure to increased progesterone level to 7 days (Lucy, 2004). Reduced fertility after long progesterone treatment originates from the fact that DF are exposed to elevated gonadotropin stimulation beyond the time when either ovulation or atresia would have occurred (Lucy, 2004, Bridges et al., 2003). Those follicles have been termed “persistent DF” and there is also negative effect on the oocyte in such follicles that could cause unsuccessful outcome of insemination (Mihm et al., 1999). Besides the reduction in duration progesterone (progestagens) treatment is usually combined with prostaglandin that removes endogenous source of progesterone and synchronize follicular waves.

There are several routes for progestagens administration: peroral treatment (Melengeterol Acetate, MGA), intravaginal administration (Controlled Internal Drug Release, CIDR; (Progesterone-Releasing Intravaginal Device, PRID), or subcutaneous application (subcutaneous ear implants, Synchro-Mate B, Crestar method). In spite of the success in development of the MGA protocols, the use of MGA as part of any estrus synchronization protocol in beef cows represents an extralabel use of medicated feed that is prohibited in the USA by the Animal Medicinal Drug Use and Clarification Act and regulation 21 CFR 530.11(b). The feeding of MGA is specifically approved by the Food and Drug Administration (FDA, USA) regulations for estrus suppression in heifers only (0.5 mg/daily for up to 24 days).

The CIDR[®] (Eazi-Breed[™] CIDR[®], Zoetis, USA) is a T-shaped vaginal insert impregnated with natural progesterone (1.38g/insert). It is inserted intravaginally for seven days period, imitating luteal phase progesterone secretion. One day before the removal of CIDR cows are treated with the prostaglandin for the elimination of potential endogenous source of progesterone. The removal of progesterone (exogenous as well as endogenous) should create favorable conditions for the final stages of dominant follicle development and maturation. It is expected that during the next 3-5 days most of the animals exhibit estrus and can be inseminated. Furthermore, fixed time artificial insemination (TAI) at 48-64 hours after CIDR removal could be utilized. Progesterone treatment using CIDR can be used for hormonal manipulation of estrous cycle and first postpartum insemination as well as for resynchronization of nonresponsive animals (figure 5).

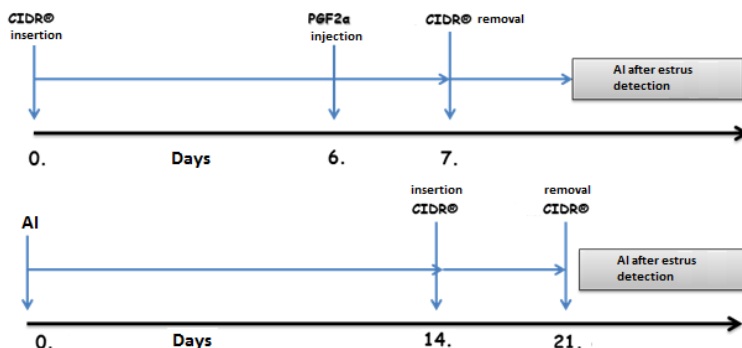


Figure 5. Progesterone treatment using CIDR for estrus synchronization and resynchronization in dairy cows. *Modified from Ptaszynska, 2006.*

The CIDR inserts have also been recommended in dairy farms where fixed time insemination protocols (TAI) were used, especially for resynchronization programs. However, the results of progesterone treatments are not unanimous: in 3 experiments inclusion of CIDR inserts to TAI protocols improved number of pregnancies/artificial insemination (P/AI) (El-Zarkouny et al., 2004, Melendez et al., 2006; Stevenson et al., 2008), whereas in 3 experiments CIDR inserts had no effect (El-Zarkouny et al., 2004, Exp. 2; Galvao et al., 2004; Stevenson et al., 2006). Furthermore, response to CIDR inserts seems to be farm dependent (Stevenson et al., 2006). Although CIDR-treated cows had greater progesterone concentrations after AI in one study (Melendez et al., 2006), the reasons why the treatment with CIDR inserts during the TAI protocol improves pregnancies per AI (P/AI) are still not clear enough. The fact that anovular cows treated with CIDR inserts were more likely to resume cyclicity in comparison with untreated cows (Gumen et al., 2005; Chebel et al., 2006; Cerri et al., 2009), could indicate possible explanation, especially if we consider the fact that CIDR treatment provided priming with progesterone that reduced the incidence of short luteal phases after AI (Rhodes et al., 2003; Cerri et al., 2009).

GnRH and PGF_{2α}

Hormonal manipulation of the estrous cycle using GnRH and PGF_{2α} in dairy cows affects both temporary ovarian endocrine structures – follicles and CL. During the follicular phase of regular estrous cycle ovulation is induced by the massive GnRH secretion from hypothalamic surge center. This mode of GnRH secretion terminates current follicular development and allows emerging of the new follicular wave. The same situation is created after exogenous GnRH analogue application, when new follicular wave starts one or two days later. When PGF_{2α} is applied seven days after GnRH injection all treated cows should be in the same phase of follicular development. The end point of treatment is the regression of CL induced by PGF_{2α} and presence of DF that finalizes its development, hopefully ovulating during the next 48-72 hours. Besides that, the GnRH induces luteinization of DF that could stimulate cyclicity in many anestrus cows (Stevenson et al., 2000). There are several variations of GnRH-PGF_{2α} based breeding programs commonly used in

dairy herds. Each system operates from the same basic framework of GnRH and PGF_{2α} administered at seven-day intervals, but vary in how animals are handled for estrus detection and A.I. One of the most extensively applied combinations of hormones is known as Ovsynch, that has been developed by Pursley et al., (1995) and tested in many experimental conditions (Pursley et al., 1997; 1998; Stevenson et al., 1999). This method is also known as GPG protocol (Gonadotropin-Prostaglandin-Gonadotropin). It is based on the GnRH-PGF_{2α} regime, with the additional (second) GnRH injection 48 hours after the prostaglandin injection. The second GnRH injection induces ovulation of the DF recruited after the first GnRH injection. No estrus detection is needed and animals are inseminated at 8-18h after the second GnRH (Figure 6). Cows expressing estrus before the scheduled TAI should be inseminated like any cow in heat and do not need the second dose of GnRH. Despite a relatively modest results in pregnancy rate (30-40%) Ovsynch protocol has been widely used mainly because it does not require estrus detection. Furthermore, data from USA suggest that average dairy producer detects only 40% of eligible cows in estrus, with conception rate of only about 40%. Based on those data it can be calculated that in 21-day period the effective pregnancy rate is about 16% (DeJarnette, 2013). Comparing these results with 30-40% pregnancy rate from GPG protocol, with no need for estrus detection and single TAI, it is a very good effect.

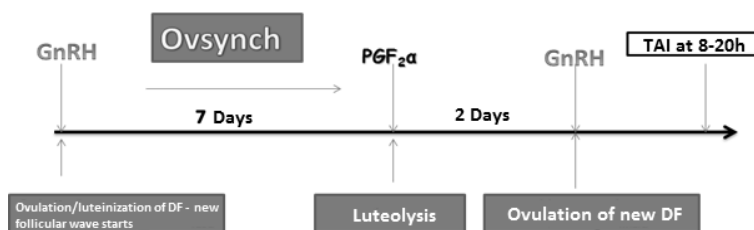


Figure 6. Ovsynch protocol (GPG method) for hormonal manipulation of estrous cycle in dairy cows (TAI – fixed time artificial insemination). *Modified from Ptaszynska, 2006.*

There are several alternative modifications to GPG protocol named: Presynch, CO-Synch, Select-Synch and Heatsynch (figure 7), and each of them should be utilized in specific situation.

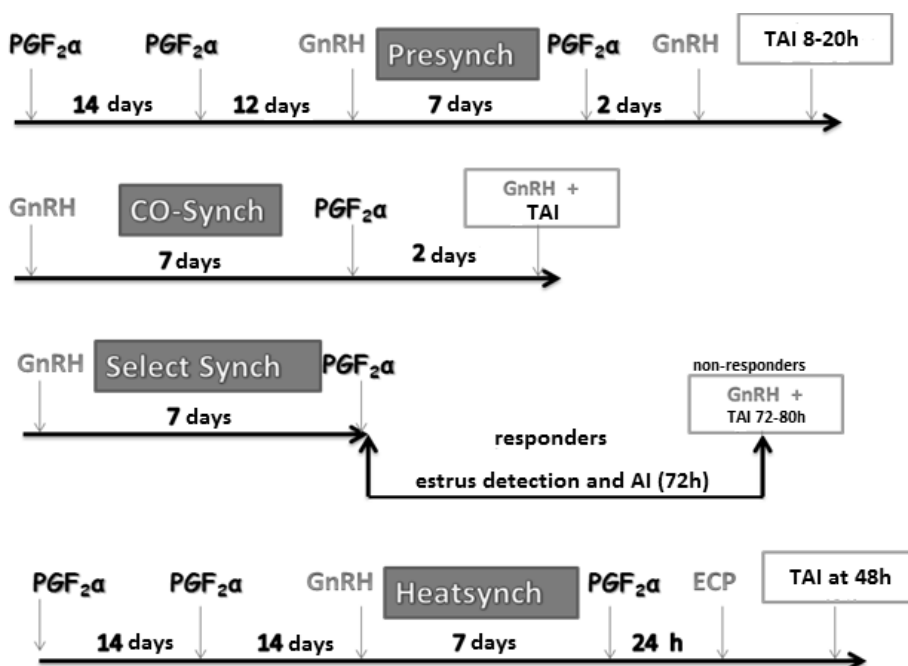


Figure 7. Alternative modifications to GPG protocol: Presynch, CO-Synch, Select-Synch and Heatsynch (ECP – estradiol cypionate). *Modified from Ptaszynska, 2006.*

Presynch is modification of the GPG protocol that includes two prostaglandin injections with 12-14 days interval between them before the start of Ovsynch. The reasoning for such modification is based on the fact that only about 50% of cows between days 13-17 of estrous cycle have follicles able to respond to the first GnRH injection (Geary et al., 2000; Vasconcelos et al., 1999). It means that prostaglandin pretreatments actually “pre-synchronizes” cows so they will be all in the early stage of estrous cycle at the time of first GnRH injection of GPG protocol. The problem with Presynch is that it should start before the end of VWP, when it could induce luteolysis in cows having functional CL during the time of uterine involution. Then they can respond with estrus and if we inseminate them it will likely result in reduced conception rate. Therefore, introducing Presynch might actually worsen reproduction efficiency in the herd. The obvious solution to this problem is to postpone the start of Presynch until the end of VWP, which puts us in dilemma (DeJarnette, 2013): are we introducing Presynch or we implement prostaglandin based method for hormonal manipulation of estrous cycle with Ovsynch for all cows not detected in estrus? The solution is not a simple one, since Presynch can improve pregnancy rate after Ovsynch (Moreira et al., 2000; El-Zakouny et al., 2004). If we accept possible drawbacks of Presynch early start we can count on improved pregnancy rate after Ovsynch that should be satisfactory. Possible answer to this dilemma might be the CO-Synch protocol, where the cows are originally inseminated at the time of second GnRH injection (figure 7). Modifying CO-Synch protocol with Presynch treatment starting at 32-37 days postpartum, and timing the AI and second GnRH injection at 72 hours after the last prostaglandin treatment Portaluppi et al., (2005) achieved pregnancy and calving rate of 31.4% and 29.3%, respectively.

At dairy farms where estrus detection is not a problem and AI is conducted in standing estrus Select-Synch is a method of choice for hormonal manipulation of estrous cycle. Cows responding to the treatment are detected in estrus during 3 days after prostaglandin injection and inseminated. Nonresponding cows are inseminated at fixed time between 72-80 hours after prostaglandin treatment with concurrent GnRH injection (DeJarnet et al., 2001). Using this protocol for hormonal manipulation of estrous cycle it is possible to inseminate most of the cows in standing estrus (50-70%), while the remaining animals are given the possibility to achieve pregnancy after fixed time artificial insemination. It is obvious that this protocol can save money because the second GnRH injection is used only for nonresponding dairy cows.

Major difference between Heatsynch and other protocols is in the use of estradiol cypionate at 24h after prostaglandin injection. Estradiol and its derivatives like estradiol-cypionate in this context is used instead of GnRH since it can cause increase of endogenous GnRH secretion that can induce LH surge and DF ovulation. In 75% of estradiol-treated cows submitted to the Heatsynch protocol ovulation occurred between 48-72h after ECP injection. This protocol is an alternative to GPG since combined with Presynch is comparable to Ovsynch regarding pregnancy rate (Pancarci et al., 2002).

An extensive meta-analysis of many different estrous cycle manipulation protocols (71 treatments and control comparisons) conducted by Rabiee et al., (2005) led to the conclusion that conception and pregnancy rates obtained by prostaglandin based protocols, Select-Synch and different modifications of Ovsynch protocol were comparable to the results of Ovsynch. It was also indicated that in dairy herds with suboptimal detection of estrus a modification of Ovsynch protocol using presynchronization (Presynch) and TAI at the time of second GnRH injection could be an effective alternative for the hormonal manipulation of estrous cycle.

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HORMONALNE METODE ZA MANIPULACIJU ESTRUSNIM CIKLUSOM KOD MLEČNIH KRAVA

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BLAGOJE STANČIĆ,
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Izvod

Efikasnost reprodukcije kod mlečnih krava ima tendenciju pogoršanja u svim delovima sveta pa i u Republici Srbiji. Epidemiološka ispitivanja ukazuju da se razlozi za smanjenje reproduktivne efikasnosti pre moraju tražiti u parametrima vezanim za pojavu različitih oboljenja nego u porastu proizvodnje mleka. Međutim, trenutno rešenje za problem smanjene plodnosti mlečnih krava najčešće se traži u primeni različitih hormonalnih metoda za manipulaciju estrusnim ciklusom kod mlečnih krava. Ultrazvučni pregled reproduktivnog trakta kod mlečnih krava je omogućio veterinarima da direktno prate promene na janjicima i uterusu tokom estrusnog ciklusa, otvarajući prostor za pravovremenu primenu hormona radi inhibicije/stimulacije privremenih endokrinih struktura na jajnicima. U našem pokušaju da sumiramo najznačajnije hormonalne metode za manipulaciju estrusnog ciklusa kod mlečnih krava analizirali smo protokole bazirane isključivo na aplikaciji prostaglandina, metode zasnovane na kombinovanju prostaglandina i progestagena, režime manipulacije bazirane na primeni kombinacije prostaglandina i gonadotropnog oslobađajućeg hormona (GnRH), kao i primenu prostaglandina, GnRH i estradiola. U stadima mlečnih krava gde je optimalna detekcija estrusa metoda izbora za hormonalnu manipulaciju estrusnog ciklusa je tzv. Select Synch protokol. Ukoliko je na farmi ustanovljen prisutan problem detekcija estrusa može se primeniti Ovsynch protokol ili kombinacija Presynch i Ovsynch protokola za hormonalnu manipulaciju estrusnog ciklusa. Fiksno vreme veštačkog osemenjavanja koje se sprovodi u toku Ovsynch protokola može se pomeriti tako da se vrši u vreme druge aplikacije GnRH, čime se smanjuje broj fizičkih intervencija i skraćuje trajanje protokola.

Ključne reči: mlečne krave, estrusni ciklus, hormonalna manipulacija.

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PORK AS A SOURCE OF HUMAN INFECTION WITH *TOXOPLASMA GONDII**

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SUMMARY: *Toxoplasma gondii* is parasitic coccidia capable of invading almost every species, including humans. In Serbia, undercooked pork is considered to be the main source of human infection. Serological screening of pigs for *T. gondii* antibodies is one of the measures, proposed by European Food Safety Authority, that should contribute to better control of the parasite. Currently, most promising tests in this field are enzyme-linked immunosorbent assay and indirect immunofluorescence antibody test.

Key words: *Toxoplasma gondii*, pork, enzyme-linked immunosorbent assay, indirect immunofluorescence antibody test.

INTRODUCTION

Toxoplasma gondii is intracellular, parasitic coccidia from the family *Sarcocystidae*, phylum *Apicomplexa*. Despite its worldwide distribution and wide range of hosts, it represents the only species within the genus *Toxoplasma*. The discovery of the parasite occurred at the first decade of the 20-th century, as a result of independent work of two groups of scientists: Charles Jules Henry Nicolle (1866-1936) and Louis Herbert Manceaux (1865-1934), from the Pasteur Institute in Tunis and Alfonso Splendore (1871-1953), from Brasil (Morissette i Ajioka, 2009). Initially, both groups identified the protozoan as *Leishmania*. However, Nicolle soon realised they discovered a new organism and named it *T. gondii*, based on its morphological characteristics (mod. L. *tox*o = arc or bow, *plasma* = life) and the host from which it was isolated (African rodent *Ctenodactylus gundi*). Soon, other scientists started to report *Toxoplasma*-like parasites in various species, including humans (Ferguson, 2009), but it was Abner Wolf and his colleagues which, in 1939., first isolated *T. gondii* from an infant girl deceased from encephalomyelitis and

Review paper/Pregledni rad

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conclusively identified it as a human pathogen (Weis and Dubey, 2009). Further research confirmed their findings and pointed many scientists' interests towards better understanding of morphology, biology, genetics, virulence and pathogenicity of *T. gondii*, as well as development of new diagnostic techniques, therapeutics and preventive measures (Morissette i Ajioka, 2009).

TOXOPLASMA GONDII LIFE CYCLE AND HOSTS

To date, infection with *T. gondii* has been confirmed worldwide, in numerous mammalian and avian species, including arctic foxes from Svalbard (Prestrud et al., 2008). Most of these species, together with man and meat producing animals, act as intermediate hosts, in which parasite forms tissue cysts that contain slowly dividing forms – bradyzoites. Sexual phase of the life cycle may only be completed in the small intestine of *T. gondii*'s definite host, felids. Sexual phase results in the formation of oocysts, environmentally resistant and orally infective forms. After ingestion of oocysts, gastric juices release sporozoites, which transform into tachyzoites (quickly dividing form, present in the acute phase of the disease and its remission) and disseminate through organism infecting all tissues, including placenta of the gravid uterus and, consequently, the foetus. In tissues, tachyzoites transform into bradyzoites and form tissue cysts which are capable of surviving in the affected host for years or even through its lifetime (Dubey and Hill, 2002). Although cysts can develop in virtually all tissues of the infected host, there seems to be greater predilection for muscle and neural tissue.

There are three main routes of transmission of *T. gondii* between the hosts: feco-oral (ingestion of food and water contaminated with oocysts from cat feces), congenital (transplacental passage of tachyzoites, from mother to foetus) and carnivorism (ingestion of meat which contains viable tissue cysts). In humans, congenital infection was first described, followed by foodborne cases, while the existence and role of oocysts were last elucidated (Ferguson, 2009). Cases of acquiring infection through blood transfusion, transplanted organs and consumption of unpasteurized goat milk have also been described in the literature, while other routes, such as sexual transmission, are considered unlikely (Hill and Dubey, 2002).

IMPACT OF TOXOPLASMA GONDII ON HUMANS AND ANIMALS

Clinical toxoplasmosis may be considered relatively rare in humans, bearing in mind that almost one third of the overall human population is estimated to be infected. When it does occur, disease usually affects immunocompromised or congenitally infected individuals. Infected immunocompetent humans express clinical signs in only 10% to 20% of cases (Weiss et al., 2009) and in most of them infection is mild and self-limiting (Đurković-Đaković et al., 2013). However, despite the low incidence, toxoplasmosis is currently considered to be the most significant parasitic foodborne pathogen and its consequences, estimated on approximately 620 DALY-s (disability-adjusted life-years per year) parallel those of salmonellosis (approximately 670 DALY-s) (Đurković-Đaković et al., 2013). Reasons for such high DALY scoring of toxoplasmosis lie in the severe consequences of congenital infection, such as neonatal death, hydrocephalus, psychomotor or mental retardation, chorioretinitis, myocarditis, hepatitis, polymyositis and possibility of the occurrence of disseminated, potentially lethal, form of disease in immunocompromised

individuals (Weiss et al., 2009). According to recent studies, similar symptoms are increasingly becoming evident in immunocompetent humans as well, while many authors find *Toxoplasma* to be responsible for various psychiatric and neurological disorders, especially schizophrenia (Mc Allister, 2005; Flegr, 2013).

Majority of animals infected with *T. gondii* also remain asymptomatic. Still, some individual animals and species (Australian marsupials, arctic foxes, European brown hare etc.) may develop acute, potentially lethal form of the disease or suffer from reproductive disorders, such as abortions or congenital malformation, as is frequently the case with sheep and goats. From the aspect of human health, however, more important is the risk of acquiring *Toxoplasma* infection after consumption of raw or undercooked meat of affected animals, which, according to the results of some European studies, occurs in 30% to 63% of the cases (OIE, 2008).

PIGS AS A SOURCE OF HUMAN INFECTION WITH *TOXOPLASMA GONDII*

Like the majority of other hosts, infected pigs usually act as asymptomatic carriers of *T. gondii*. If clinical signs do appear, they tend to be mild, with increased body temperature, loss of appetite and tachypnea. Other signs, like reproductive disorders, pneumonia, myocarditis, encephalitis, weakness of the hind limbs and mortalities are rare (EFSA 2007, Basso et al., 2013).

Together with sheep and goats, pigs are considered to be the meat-producing animals that most frequently harbour infective cysts of *T. gondii* in their tissues. This is one of the reasons why consumption of raw or undercooked pork is recognised as an important risk factor for human infection (Tenter et al., 2000; Bobić et al., 2003; Klun et al., 2011).

In Serbia, pork is most frequently consumed meat (50% of the total meat consumption) and therefore believed to impose a greater risk to human health than mutton and beef, although *T. gondii*-specific antibodies were more frequently found in sheep (84.5%) and cattle (76.3%), than in pigs (28.9%) (Klun et al., 2006). Lack of programs for the slaughter inspection of pigs for *T. gondii* (routine methods of meat inspection are unable to detect microscopic cysts of the parasite) and Serbian tradition of home-curing of the meat, where no standardised concentrations of salt and spices are used and people often tend to check the taste of the raw product during its preparation, additionally increases the existing risk of acquiring *Toxoplasma* infection through pork meal.

Studies conducted in Europe during the last 30 years estimate that prevalence of *T. gondii* specific antibodies in pigs varies between the countries, from 0% to 64% (EFSA, 2007). The same studies report that, during the last ten years, some countries, such as Netherlands, Austria and Germany, significantly decreased the number of seropositive pigs or even completely eliminated infection from their farms, by implementing following biosecurity measures: (a) keeping producing animals indoors through their life-time, (b) keeping the sheds free of rodents, birds and insects, (c) feeding meat-producing animals on sterilised food, and (d) no pet animals in sheds and feed stores (Tenter et al., 2000). On the other hand, consumers' demands for organically produced meat are constantly increasing. Consequently, it is to be expected that the number of pigs reared in extensive systems will also increase in the future, exposing more pigs to the environment and *T. gondii* oocysts (EFSA, 2007). Reports from Switzerland support these expectations, since they experienced

an increase of 13% in prevalence of infected pigs after introduction of animal welfare regulations (Berger-Schoch et al., 2011).

CONTROL OF *TOXOPLASMA GONDII*

Currently, there is no commercially available vaccine against *T. gondii* (except the one registered for sheep) and the efficiency of traditional therapy is limited to treating the acute form of the disease i.e. proliferative form of the parasite, without possibility of eliminating tissue cysts (Đurković-Đaković et al., 2013). Therefore, preventive measures, which hinder intake of the infective forms of *T. gondii*, still remain the best protection from toxoplasmosis. In the case of meatborne infections, European Food Safety Authority (EFSA 2007, EFSA 2011) suggested the following: improvement of biosecurity measures at the farm level, monitoring of human infections, monitoring of meat producing animals at the farm and abattoir level, implementation of prescribed hygienic practices and following recommended temperature regimes during cooking (67°C) and freezing (-12 °C) of meat destined for human consumption. In their recommendations for animal health monitoring techniques, EFSA (2007) gives priority to indirect (serological) techniques, over the methods intended for direct detection of the parasite in tissues (bioassay, PCR, histology and immunohistochemical staining). Although direct methods proved to be highly specific, small size of the sample significantly reduces their sensitivity due to random distribution and often low density of parasite in tissues. Moreover, direct methods are generally time-consuming and require special equipment for their performance, which, together with earlier mentioned reduction of sensitivity, makes them less suitable for screening a large number of samples in contrast to indirect methods. On the other hand, serological methods (indirect immunofluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), modified agglutination test (MAT), Western blott (WB), latex agglutination (LA), indirect haemagglutination (IHA)) which are based on the detection of specific antibodies, are mainly highly sensitive, easy to perform, cheap, and therefore more suitable for the monitoring purposes. However, it should be emphasized that, currently, none of this serological techniques is sufficiently sensitive or specific to confirm or exclude infection on the individual level with 100% security.

SEROLOGICAL TESTS

Serological methods are the key in the routine diagnosis of toxoplasmosis in pigs (Pardini et al., 2012). They have been traditionally used to screen pig sera, however, meat juices are slowly emerging as an equally interesting alternative matrix, from numerous reasons: (a) procedure of meat juice extraction from muscle is more simple than that of blood sampling and sera preparation; at the same time it is possible to automate the procedure and meet the requirements of large-scale analyses; (b) there will be situations when meat will be the only available sample; (c) after extraction of juices, meat could be reused for parasitological examinations. Among all available sampling sites, diaphragm stands out as well vascularized muscle, easy to collect, with no exchange value, available from all fresh carcasses, including the ones from abroad (Villena et al., 2012).

IFAT, MAT and ELISA are the main tests used for anti-*T. gondii* antibody detection in pig sera and meat juices. However, when compared with each other,

IFAT and ELISA stand out from this trio due to their superior specificity and sensitivity (Garcia et al., 2008). While some authors (Basso et al., 2013) found ELISAs to be more specific and sensitive than IFAT, others (Veronesi et al., 2012) report almost perfect agreement between these two tests.

Many authors (Garcia et al., 2006; Veronesi et al., 2012) use IFAT as a reference test i.e. a „gold standard“ for detection of anti-*T. gondii* antibodies in pigs. IFAT produces titers comparable with the Sabin–Feldman dye test which is the longest established serological method, considered as a „gold standard“ in humans, but is, at the same time, much safer than the dye test as it uses formalin-fixed (killed) tachyzoites (Hamzavi et al., 2007). Utilization of the whole tachyzoites also increases the specificity of IFAT, since the surface antigens of the apicomplexan species are considered more specific than the intracellular ones (Björkman i Uggla, 1999). For this very reason IFAT has no affinity towards cross-reactions with other coccidia as it is the case with indirect ELISA tests which use soluble antigens of *T. gondii*. Finally, IFAT has the ability of early detection of antibodies. In studies conducted on experimentally infected pigs IFAT detected specific antibodies in all examined animals as soon as 7th day after experimental infection, while ELISA and MAT managed to do so at 14th and 21st day, respectively (Garcia et al., 2006). Attempts have been made (Pardini et al., 2012) to achieve earlier detection of antibodies with ELISA by using of native affinity purified TgSAG1 surface antigen, that also has potential to reduce number of cross-reactions; however, once more IFAT performed better on this field.

On the other hand, IFAT is considered subjective since the interpretation of results directly depends on the amount of experience and personal judgement of the researcher due to the visual reading of the results (EFSA, 2007). Furthermore, lack of the standardised cut-off values (values above which test is considered positive) which range from 1:16 to 1:200 (Dubey, 2008) additionally complicates interpretation and comparison of results between different studies.

By contrast, reading of the results of ELISA is objective and does not require skilled personnel. Unlike IFAT, it can be semi automated and therefore more suitable for examination of a large number of sera.

According to Italian authors (Ranucci et al., 2012; Veronesi et al., 2012) both IFAT and ELISA showed good performance in detecting *Toxoplasma*-specific antibodies in meat juices of naturally infected pigs. However, they emphasize that results obtained on meat juices could not be used as an alternative method to serum to guarantee the absence of the parasite from the pork, but could be applied for monitoring purposes (Ranucci et al., 2012). Finally, monitoring programs based on serological investigation of muscle fluids have already been successfully implemented for other foodborne pathogens, such as *Salmonella*, *Trichinella*, Aujeszky virus etc. (Villena i sar., 2012).

CONCLUSION

Undercooked pork meat represents significant risk for acquiring human infection with *T. gondii*. However, this risk could be significantly reduced by applying adequate control measures. One of these measures is monitoring of the health status of herds and using obtained results for advising farmers with regard to improve the existing biosecurity measures on their farms and/or expose the meat from seropositive farms to the stricter processing conditions. Both IFAT and ELISA

have good potential as serological tests that could be used for monitoring the presence of anti-*T.gondii* antibodies in pigs, however, in order to implement any of them in the monitoring program, they first must be subjected to adequate standardization and harmonization.

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SVINJSKO MESO KAO IZVOR INFEKCIJE LJUDI SA *TOXOPLASMA GONDII*

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Izvod

Toxoplasma gondii je parazitska kokcidija koja je u stanju da inficira gotovo svaku vrstu, uključujući i čoveka. U Srbiji, termički nedovoljno obrađeno svinjsko meso smatra se glavnim izvorom infekcije za ljude. Serološko testiranje svinja na prisustvo antitela protiv *T. gondii* je jedna od mera, predloženih od strane Evropske Agencije za Bezbednost Hrane (EFSA), koja treba da doprinese poboljšanju kontrole ovog parazita. Trenutno, testovi koji najviše obećavaju na tom polju jesu imunoenzimski test i test indirektne imunofluorescencije.

Ključne reči: *Toxoplasma gondii*, svinjsko meso, imunoenzimski test, test indirektne imunofluorescencije.

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THERAPEUTIC USE OF GARLIC (*Allium sativum* L.): NEW POSSIBILITIES*

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SUMMARY: *Garlic (*Allium sativum* L.) has been seriously investigated in purpose to find an alternative measure for control of various diseases in humans and animals. Use in cardiovascular therapeutics has an even longer history over 3000 years to ancient time. The active chemical in garlic is allicin, which is produced when raw garlic is crushed, allowing the enzyme alliinase to act on the stable precursor alliin. Antidiabetic, antibiotic and perhaps anticancer effects of garlic are well-accepted over the world because there is a range of scientific literature to support this. Garlic also has hepatoprotective, antioxidant, and anthelmintic effect. The other pharmacological effect includes the anticoagulant, anti inflammatory, immunomodulatory and wound healing action of garlic.*

Keywords: *Allium sativum, garlic extract, allicin, pharmacological activities.*

INTRODUCTION

Garlic (*Allium sativum* L.) is a hardy annual monocotyledon plant, and one of the oldest cultivated vegetable crops. Over the years it has been seriously investigated in purpose to find an alternative measure of control of various human and animal diseases. It is well known fact that this plant has been used for centuries in fight against various infections (Onyeagba et al., 2006). The data about the medicinal properties of this cultivated plant has been found in the oldest records of Herodot and the founder of medical class, Hipocrates (Jones and Mann, 1963; Rabinowich, 1990). The Egyptians used it to treat diarrhoea, then ancient Greeks

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used it to treat intestinal diseases, while the ancient Japanese and Chinese used it to treat headache, flu, sore throat and fever (El-mahmond, 2009). The antimicrobial properties of garlic were first described by Pasteur in 1858, and it was used as an antiseptic to prevent gangrene during World War I and World War II. Since then, research has demonstrated its effectiveness against bacteria, protozoa, fungi and some viruses (Jaber and Al-Mossawi, 2007). Nowadays, garlic is increasingly used as an alternative medicine for treating various diseases in human medicine, to prevent and treat cardiovascular disease by lowering blood pressure and cholesterol and as a preventive agent for cancer. Garlic inhibits platelet aggregation and enhances fibrinolytic activity, reducing clots on damaged endothelium (MacDonald et al., 2004).

In phytopharmacy it is used in the form of various macerate and fermented extracts, for the suppression of certain plant pathogens.

The garlic particularly has influence on certain genera of bacteria, even in dilutions from 85,000 to 125,000 (Lazić et al., 2001).

Chemistry of garlic

Allicin (diallylthiosulfinate), the best known active compound of garlic, is the product from the secondary metabolism of amino acids containing sulfur, and it is not the the initial substance of garlic plant, but occurs after bulbs injury under the interaction of the amino acid alliin with the enzyme alliinase (Kastori 2010, Miron et al., 2002). Alliin is the primary odorless, sulfur-containing amino acid, a precursor of allicin; it is converted into thiosulfinates through enzyme reactions when raw garlic is cut or crushed.

Thus, no thiosulfinates are found in intact garlic (Amagase H., 2013). In order to activate the enzyme and start the reaction, oxygen from the air is required. For this reason the tissue must be injured so that leaking juice can come in touch with oxygen.

S-Allyl-L-cysteine (SAC) is one of the water-soluble organosulfur compounds in garlic and it has many biological effects: antidiabetic action (Saravanan et al., 2013), suppress of proliferation and metastasis of carcinoma (Ng et al., 2012), influence on placenta or retina (Yu et al., 2012; Chen et al., 2012).

Nonsulfur compounds - steroid saponins, such as eruboside-B isolated from the garlic bulb, exhibit antifungal activity against *Candida albicans* (Nakagawa et al., 1987). Studies report that the crude glycoside fraction (Matsuura H., 2001; Slowing et al., 2001) from methanolic raw-garlic extracts, which mainly contains spirostanol saponins, lowered total plasma cholesterol and LDL cholesterol without changing HDL cholesterol levels in hypercholesterolemic animal models.

Safety of garlic preparations

Although garlic has been safely used in cooking as a popular flavor and has been used traditionally for medicinal purposes, it is commonly known that excessive consumption of garlic can cause diarrhea (Adachi A., 2010) and occasional allergic reactions like dermatitis and bronchial asthma (Jappe et al., 1999; Van der Walt et al., 2013). Garlic fractions induced apoptosis of testicular germ cells and decrease of serum testosterone levels (Hammami et al., 2013). SAC, as one of the active ingredients in garlic preparations, is a safe compound and its biological effects are well researched. The U.S. National Cancer Institute tested the toxicity of SAC

compared with other typical garlic compounds and found that SAC is less toxic than allicin. The oral 50% lethal dose in mice (mg/kg body weight) is 309 in males and 363 in females for allicin and 8890 in males and 9390 in females for SAC. Thus, SAC is 24% less toxic than allicin (Amagase H., 2013).

The different constituents in various garlic preparations, have different safety characteristics and biological and pharmacological activities, which are discussed below.

Cholesterol reduction

Numerous animal studies, as well as *in vitro* ones, have demonstrated the hypolipidemic effects of garlic while clinical trials are highly inconsistent (Zeng et al., 2013). But, it has been demonstrated that 12-month treatment with garlic powder tablets results in the significant decrease of cardiovascular risk by 1.5-fold in men ($p < 0.05$), and by 1.3-fold in women (Sobenin et al., 2010). Also, some studies suggested garlic as effective in reducing total serum cholesterol and low-density lipoprotein cholesterol in individuals with elevated total cholesterol levels (>200 mg/dL). Reduction in total serum cholesterol of 8% has been observed after 2 months application of garlic. This finding is of clinical relevance as it has been associated with 38% reduction of risk of coronary diseases in fifty-year-olds (Ried et al., 2013a).

Antihypertensive potential

Hypertension (systolic blood pressure (SBP) ≥ 140 mm Hg; diastolic blood pressure (DBP) ≥ 90 mm Hg) is a main risk factor for cardiovascular diseases, it affects about 30% of adults worldwide. Blood pressure reducing properties of garlic have been linked to its hydrogen sulphide production and allicin content – liberated from alliin and the enzyme allinase, which has angiotensin II inhibiting and vasodilating effects, as shown in animal and human cell studies (Londhe et al., 2011). It may be a safe adjunct treatment to conventional antihypertensive therapy (Ried et al., 2013b). Aged garlic extract significantly reduced interstitial fibrosis in hypertensive rats (Hara et al., 2013). Allicin lowered blood pressure and triglyceride levels in spontaneously hypertensive rats, also (Elkayam et al., 2013). From that reasons garlic has played an important dietary role in human medicine.

Antidiabetic potential

Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia resulting from either a defect in the secretion or activity of insulin. Hyperglycaemia leads to an increase in the oxidative stress because of the overproduction of free radicals (Wiernsperger F., 2003). Most of the previous researches in animal models on rats have studied the physiological and biochemical effects of garlic on diabetes.

Oral administration of garlic to alloxan-induced diabetic rats for a month significantly reduced the concentrations of serum lipids and blood glucose. The supplementation of garlic oil for diabetic patients may decrease the incidence of diabetic complications, which may result from an increase of free radical activity in diabetes (Abdultawab and Ayuob, 2013). S-allyl cysteine is a potent agent against lipogenesis and glucose metabolism in alloxan diabetic rabbits (Nasri H., 2013).

From these encouraging results in animal models, garlic extract could have sufficient potential as anti diabetic agent for the patient of type 2 diabetes mellitus.

Antibacterial potential

Various preparations of garlic nowadays exhibit a wide spectrum of activity against Gram-positive and Gram-negative bacteria (Ankri and Mirelman, 1999). The most common bacterial genera that have been investigated in terms of sensitivity to garlic were *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Bacillus* and *Clostridium*. More frequent consumption of garlic prevents the development of the *Helicobacter pylori* which causes gastritis in humans and animals (Cellini et al., 1996). Increase of the number of sulphur atoms in the active substance of garlic increases the antibacterial activity against the same bacterium (O'Gara et al., 2000). It was noted that pharmacological components of garlic act synergistically with antibiotics such as streptomycin and chloramphenicol in therapy against *M. tuberculosis* (Gupta and Viswanathan, 1955). In research carried by Vlajić et al. (2013), it was noticed that raw garlic extract has proved to be more effective *in vitro* conditions against isolates of *Salmonella typhimurium* and *Salmonella enteritidis*, which are resistant to ampicillin. The data given by Ankri and Mirelman (1999) indicate that the development of resistance to betalactame antibiotics is 1000 times faster than to allicin, from the garlic. Gram-positive bacteria are less sensitive to garlic extract because of the presence of thick layer of peptidoglycan (Bakri and Douglas, 2005; Indu et al., 2006), however some isolates of Gram-positive bacteria like *S. aureus* originated from swab throat are extremely sensitive (Vlajić et al., 2013).

In the last few years it has been noticed that bacteria have become more significant pathogens of cultivated plants. The sensitivity of phytopathogenic bacteria to garlic is also very important, especially since the usage of antibiotics in therapeutic treatment of plants is prohibited by the Law of Plant Protection. The usage of specific copper compounds produces results only in prophylaxis, since they are the first of all fungicides. Highly sensitive effect to garlic extract was recorded in species *Xanthomonas euvesicatoria*, *X. a. pv. phaseoli* and *Erwinia amylovora*, a lower sensitivity was observed in *Pseudomonas syringae. pv. syringae* (Vlajić et al., 2012). The existence of hydrophilic capsules or mucoid layers in bacterial cells, in some cases prevents the penetration of allicin in the bacteria (Ankri and Mirelman, 1999).

An accurate determination of the mechanism of garlic action is very complex; the reason for that is the interaction of some various pharmacological active ingredients found in *Allium* species. As the most important organosulphur compounds (Harris et al, 2001.; Kumar et al., 1998; Kyung et al, 2001.), allicin (Ankri and Mirelman, 1999, Chung et al., 2007, Medina et al., 2006), ajoene (Nakagawa et al., 1987) and diallyl sulphide (O'Gara et al., 2000) stand out. Yin and Cheng (2003) observed that diallyl disulphide was more effective in the elimination of important pathogenic microorganisms such as *Escherichia coli* O157: H7 and *Listeria monocytogenes*. However, others have shown that the phenolic compounds also contribute to antimicrobial activity (Benkeblia, 2004). According to Feldberg et al. (1988) allicin from garlic extract, directly or indirectly blocked the synthesis of RNA, whereas had no significant effect on DNA. Therefore it is considered that the primary aim is blocking the formation of RNA.

Anticancer potential

Although the exact mechanism involved in the protective effects of garlic compounds against carcinogenesis has not been clearly understood, at present some

organosulfur compounds derived from garlic, including S-allylcysteine, have been found to retard the growth of chemically induced and transplantable tumors in several animal models (Thomson and Ali, 2003). Some results indicate that mitochondria certainly play a major role in diallyl disulfide-induced apoptosis (Nagaraj et al., 2010). Authors of population-based case-control study which was conducted in a Chinese population from 2003 to 2010 concluded that raw garlic consumption of 2 times or more per week is inversely associated with lung cancer (Jin et al., 2013).

The Iowa Women's Health Study reported that having at least one serving of garlic per week was associated with a 48% reduced risk of distal colon cancer compared to zero servings of garlic (Larsson et al., 2005).

Antiinflammatory potential

The sulfur compounds inhibited the production of nitric oxide (NO) and prostaglandin E (2) (PGE (2)) and the expression of the pro-inflammatory cytokines tumor necrosis factor- α , interleukin-1 β , and interleukin-6 in lipopolysaccharide (LPS)-activated macrophages (Lee et al., 2012). By inhibiting Th1 and inflammatory cytokines while upregulating IL-10 production, treatment with garlic extract may help to resolve inflammation associated with inflammatory bowel disease (Londhe et al., 2011).

Hepatoprotective Potential

SAC significantly induced apoptosis and necrosis of cells MHCC97L *in vitro* and *in vivo* xenograft liver tumor model demonstrated that SAC inhibited the progression and metastasis of HCC tumor (Ng et al., 2012).

CONCLUSION

The human health benefits of consuming garlic are well documented. Garlic (*Allium sativum* L.) used in cardiovascular therapeutics has an even longer history back over 3000 years. Garlic's antidiabetic, antibiotic and perhaps anticancer effects are well-accepted because of the many of scientific literature supporting these effects. Garlic also has hepatoprotective, and antioxidant effect. The other pharmacological effect includes the anticoagulant, antiinflammatory, immunomodulatory and wound healing action of garlic. Garlic has the potential of preventing or curing a man and animals from a large number of diseases.

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TERAPIJSKA UPOTREBA BELOG LUKA (*Allium sativum* L.): NOVE MOGUĆNOSTI

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Izvod

Beli luk (*Allium sativum*) je dugo istraživana kultura, upravo zbog cilja pronalaženja alternativnih mera kontrole različitih oboljenja kod ljudi i životinja. Njegova upotreba u terapiji kod kardiovaskularnih oboljenja ima dugu istorijsku tradiciju, stariju od 3000 godina. Aktivnu komponentu belog luka čini alicin, koji nastaje nakon ozlede tkiva lukovice pod dejstvom enzima alinaze na alin. Antidijabetički, antibakterijski i verovatno i antikancerogeni efekti belog luka su dokumentovani u naučnoj literaturi. Takođe, primećeno je da beli luk ispoljava hepatoprotektivno i antioksidativno dejstvo. Drugi farmakološki efekti koji su još uvek nedovoljno ispitani, uključuju antikoagulantno, antiinflamatorno i imunomodulatorno dejstvo kao i pozitivan efekat u zarastanju rana. Beli luk ima potencijal primene u lečenju ljudi i životinja od različitih bolesti.

Ključne reči: *Allium sativum*, ekstrakt belog luka, alicin, farmakološki efekti.

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SEROPREVALENCE OF MARE HERPES VIRUS IN THE BREEDING SEASON

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SUMMARY: Equine herpes virus (EHV) is a common virus in populations of equines worldwide, and the two most important viruses in this group are EHV-1 and EHV-4. It is well known that EHV-1 causes respiratory problems, abortion and neurological disease and EHV-4 is typically causing respiratory disease but may lead to abortion. The clinical picture includes: fever - which may be the only symptom, cough, increased amounts of nasal secretions, and without previous, abortions in the late stage of pregnancy and neurological problems that usually involve the last extremity, and urinary tract. These viruses can be detected in a variety of tissues in aborted foals including lung, kidney, liver, spleen and nasal swabs, placenta and blood of live horses. A variety of techniques for diagnostic have been developed which including virus isolation, serology, PCR, real-time PCR and sequencing. The aim of this work was to establish the seroprevalence of herpes virus in reproductive mares. Clinical exams and blood samples were collected from seven mares in one stable which is located in west region of Vojvodina province, the animals are currently used for mating. All the animals in the stable are with a positive finding of antibody titre. The lowest titre of antibodies have animals that have just entered the reproductive cycle. Other symptoms attributed to herpes virus infection was not present, that can be attributed to a latent infection which is present in this case in 100% of the animals in the stable. However, the differential diagnosis should be done by viral isolation from aborted material, because the reproductive problems that occur may be attributed to other viruses.

Key words: herpes virus, seroprevalence, SN test, breeding season, mare.

Case report / Prikaz slučaja

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INTRODUCTION

Equine rhinopneumonitis is the common name for the highly contagious, clinical disease occurs as result of herpes virus infection (OIE Terrestrial Animal Health Code, 2011). Equine herpes virus (EHV) is a common virus in populations of equines worldwide, and the two most important viruses in this group are EHV-1 and EHV-4 (Lohmann, 2008). It is well known that EHV-1 causes respiratory problems, abortion and neurological disease and EHV-4 is typically causing respiratory disease but may lead to abortion.

Equine herpesvirus-1 (EHV-1) was first described by Drs. W.W. Dimock and P.R. Edwards at the Kentucky Agriculture Experimental Station, Lexington in the early 1930s (Kydd et al., 2006). Equine herpesvirus-1 and 4 are DNA viruses belonging to subfamily Alphaherpesvirinae (Patel and Heldens, 2005), genus Varicellovirus, and in fact, the two viruses were considered as subtypes of one virus species until the early 1980s (Bresgen et al., 2012).

However, in the 21st century, over 70 years after its first description and despite many vaccine trials, the virus remains responsible for major economic and welfare problems, causing respiratory and neurological disease and abortion in pregnant mares (Kydd et al., 2006). Within the United Kingdom, approximately 6% of equine abortions are caused by EHV-1, with occasional further cases linked to the closely related virus EHV-4 (Smith et al., 2003).

The virus can be transmitted from an infected animal through nasopharyngeal secretions, through the fetus, fetal membranes and reproductive tract or by reactivation of the virus from the latent state (Allen, 2002). Other authors indicate that both viruses enter via the respiratory tract where a first lytic replication takes place in the respiratory epithelium (Bresgen et al., 2012). After primary replication in the nasal mucosa, EHV-1 but not EHV-4 is capable of efficient infection of leukocytes present in the tributary lymphoid tissue, which then results in a leukocyte-associated viremia. From there, EHV-1 is spread and can reach the end vessels of the pregnant uterus or the central nervous system (CNS), particularly those of the spinal cord (Edington et al., 1986; Patel and Heldens, 2005). In the case of abortion, virus can transgress layers of the maternal and fetal placenta and result in infection of the fetus and subsequent abortion, although “sterile” abortions following endothelial cell infection are also observed (Smith and Borchers, 2001; Smith et al., 1996). Viruses are infectious for all equines but donkeys are often latently infected. The virus can not be transmitted to humans. The incubation period is short from the 24 hours up to 4-6 days, but if it comes to abortion the incubation period may take several months from infection. EHV-1 and EHV-4 infections become latent involving lymphoid tissue and the sensory and trigeminal ganglions (Allen, 2006; Pusterla et al., 2010).

The most devastating manifestations of this viral infection is a mass phenomenon of abortion, and perinatal mortality with mioencefalopathy (Dixon et al. 1978; Hartley and Dixon 1979; Greenwood and Simpson 1980, Mumford et al., 1987; McCartan et al. 1995). The clinical picture includes: fever - which may be the only symptom, cough, increased amounts of nasal secretions, without previous abortions and other characters in the late stage of pregnancy, neurological problems usually involve the last extremity, and urinary tract (ataxia, urinary retention or incontinence of urine and atony of the bladder) with preserved consciousness (Lohmann, 2008).

These viruses can be detected in a variety of tissues in aborted foals including lung, kidney, liver, spleen and nasal swabs, placenta and blood of live horses. A variety of techniques have been used to detect and differentiate EHV-1 and EHV-4 in horses including virus isolation, serology, PCR, real-time PCR and sequencing (Varrasso et al., 2001; Pusterla et al., 2005; Diallo et al., 2007; Marenzoni et al., 2008; Smith et al., 2010). OIE recommend for serological diagnosis to use virus neutralizing test, ELISA or CFT (OIE Terrestrial Manual, 2008.).

Equine herpes virus can become latent in around 80% of horses (Welch et al., 1992), involving lymphocytes and the trigeminal ganglion (Borchers et al., 1999). The prevalence in aborted fetuses vary in different studies: in Turkey the prevalence was 42% (Turan et al., 2012), in France about 15% (Leon et al., 2008), in Kentucky 54% (Allen et al., 2008), in the UK 7% (Smith et al., 2003) and in the USA 4% (Giles et al., 1993).

It is suggested that transfer of maternal VN antibody could not prevent infection, at least of the upper respiratory tract, but did provide clinical protection (Kydd et al., 2006). Virus positive nasal swabs detected as early as 11 days post partum (Foote et al., 2004).

There are attenuated and inactivated vaccines, but the need for greater protection for repeated vaccinations given program depending on the manufacturer. There is quite a body of literature and as much debate as to which vaccines and vaccine regimens provide the most reliable protection against EHV-1- and EHV-4-induced disease. But only vaccine with a claim against EHV-1-induced abortion is an inactivated EHV-1/EHV-4 combination vaccine (Heldens et al., 2001)

MATERIALS AND METHODS

Clinical exams and blood samples were collected from seven mares in one stable which is located in west region of Vojvodina province. All mares are kept loose in a common outlet during the day and at night in individual pens. The animals are currently used for mating, but in the past they were used for shows and racing. There were no action in any form of competition for more than two years for each of the mares. On all animals were conducted a thural clinical exam with emphasis on respiratory and neurological disorders. Along with taking the blood samples and clinical exams there was conducted anamnestic questioning of the owner and trainers of these animals and with an emphasis on reproductive, respiratory and neurological disorders.

Blood samples were taken from v. jugular with all the principles of asepsis and antisepsis and serological analysis was performed by serum neutralization test as directed from OIE, Chapter 2.5.9. Terrestrial Manual 2008. In this test positive are those animals who have antibody titre 1:2 or greater.

RESULTS

Table 1 shows the results of serological tests and medical history. According to the owner all mares, except the two jangest, had trouble with the conception. All animals has no other clinical findins then reproductive.

Table 1. Research results shows that 5 out of 7 animals are with reproductive issues and that all animals have positive finding of antibody titre

No.	Breed	Age (year)	Origin	Vak c.	Clinical findings	No. of foals	Ab titre
1	Lipizzaner	7	Domestic	No	-	The first mating	1:16*
2	American trotter	6	Domestic	No	-	The first mating	1:32*
3	American trotter	13	Domestic	No	Death of newborn foals, poor conception	1	1:32*
4	Lipizzaner	12	Domestic	No	Death of newborn foals, poor conception, abortion (twins)	2	1:64*
5	American trotter	20	Domestic	No	Death of newborn foals	8	1:128*
6	American trotter	9	Domestic	No	Poor conception	1	1:256*
7	American trotter	14	Import	No	Death of newborn foals (2x)	3	1:256*

*All mares have titre higher than 1:2 which is considered positive.

DISCUSSION

The table shows that all the animals in the stable are with a positive finding of antibody titre which is greater than 1:2. The lowest titre of antibodies have animals that have just entered the reproductive cycle. Older animals and those that have had multiple mating shows a higher antibody titres. The animals had reproductive problems, except the youngest two mares that had just one mating. The aforementioned reproductive problems - abortion and newborn foal deaths are consistent with the findings of many authors who state that the most devastating manifestations of this viral infection is a mass phenomenon of abortion, and perinatal mortality with mioencefalopathy (Dixon et al., 1978; Hartley and Dixon, 1979; Greenwood and Simpson, 1980; Mumford et al., 1987; McCartan et al., 1995). Other symptoms attributed to herpes virus infection was not present, that can be attributed to a latent infection which is present in this case in 100% of the animals in the stable. This is consistent with findings of other authors which find thah the latent infection rate in horses is about 80% or higher (Welch et al., 1992). It is also important to mention that there was no vaccination program for this animals which may help to reduce the consequences of infection. Abortion, death of newborn foals and poor conception can be attributed to other causes, and unfortunately in this case there was no available the aborted material for definitive confirmation of diagnosis and virus isolation.

CONCLUSION

From the obtained results it can be concluded that in our herds there are a great number of latently infected animals. The predominant clinical forms of infection are reproductive problems, which include abortion, death of newborn foals and a bad conception. However, the differential diagnosis should be done by viral isolation from aborted material, because the reproductive problems that occur may be attributed to other viruses. Vaccination program is essential for managing of this infection.

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SEROPREVALENCA KONJSKOG HERPES VIRUSA KOD KOBILA U SEZONI PARENJA

MIHAJLO ERDELJAN, IVANA DAVIDOV, MIODRAG RADINOVIĆ,
MLADEN IDUŠKI, RAMIZ ČUTUK

Izvod

Konjski herpes virus (EHV) je uobičajeni virus u populacijama kopitara širom sveta, a dva najvažnija virusa u ovoj grupi su EHV-1 i EHV-4. Dobro je poznato da EHV-1 uzrokuje respiratorne probleme, abortuse i neurološke probleme a da EHV-4 obično izaziva respiratorni oblik bolesti mada može dovesti i do abortusa. Klinička slika obuhvata: povišenju temperaturu - što može biti jedini simptom, kašalj, povećanje količine nosnog sekreta, i bez prethodnih znakova može doći do abortusa u kasnoj fazi graviditeta. Neurološki problemi obično obuhvataju zadnje ekstremitete i urinarni trakt. Ovi virusi mogu biti detektovani u različitim tkivima abortiranih ždrebadi, uključujući pluća, bubrege, jetru, slezinu i briseve iz nosa, placenti i krvi živih konja. Raznovrsne tehnike za dijagnostiku su razvijene koje uključuju izolaciju virusa, serologiju, PCR, RT-PCR i sekvencioniranje. Cilj ovog rada je bio da se utvrdi seroprevalenca herpes virusa kod kobila u reprodukciji. Klinički pregledi i uzorci krvi su prikupljeni od sedam kobila u jednoj štali koja se nalazi u zapadnom delu pokrajine Vojvodine, životinje se trenutno koriste za parenje. Sve životinje u štali su sa pozitivnim nalazom antitela. Najniži titar antitela imaju životinje koje su upravo ušle u reproduktivni ciklus. Ostali simptomi pripisani herpes virusima nisu bili prisutni, što se može pripisati latentnoj infekciji koja je prisutna u ovom slučaju kod 100% životinja u štali. Međutim, diferencijalnu dijagnozu treba uraditi uz pomoć izolacije virusa iz abortiranog materijala, jer se reproduktivni problemi koji se javljaju mogu pripisati i drugim virusima.

Ključne reči: herpes virus, seroprevalenca, SN test, sezona parenja, kobila.

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