

# ЗБОРНИК

МАТИЦЕ СРПСКЕ  
ЗА ПРИРОДНЕ НАУКЕ

MATICA SRPSKA  
PROCEEDINGS FOR  
NATURAL SCIENCES

121

NOVI SAD  
2011



МАТИЦА СРПСКА  
ОДЕЉЕЊЕ ЗА ПРИРОДНЕ НАУКЕ

ЗБОРНИК  
МАТИЦЕ СРПСКЕ ЗА ПРИРОДНЕ НАУКЕ

MATICA SRPSKA  
DEPARTMENT OF NATURAL SCIENCES  
PROCEEDINGS FOR NATURAL SCIENCES

Покренут 1951 / First published in 1951.

Published as *Научни зборник*, серија природних наука until the tenth issue (1955), as the Series for Natural Science from the eleventh issue (1956) — *Зборник за природне науке*, and under its present title since the sixty-sixth issue (1984)

*Главни уредници / Editors-in-Chief*

Miloš Jovanović (1951), Branislav Bukurov (1952—1969),  
Lazar Stojković (1970—1976), Slobodan Glumac (1977—1996), Rudolf Kastori (1996—)

## 121

### Уредништво / Editorial Board

S. GAJIN  
V. JANJIĆ  
V. JOVIĆ  
L. DOVNIKOVIĆ  
D. KAPOR  
R. KASTORI  
L. LEPSANOVIĆ  
I. MAKSIMOVIĆ  
V. MARIĆ  
S. PETROVIĆ  
S. ĆURČIĆ

### Consulting Editors

A. ATANASSOV, Bulgaria  
P. HOCKING, Australia  
A. RODZKIN, Belorusija  
M. SIMMONDS, UK  
S. STOJILKOVIC, USA  
G. SCHILING, Germany  
GY. VARALLYAY, Hungary

Articles are available in full-text at the web site of Matica Srpska and in the following data bases: Serbian Citation Index, EBSCO Academic Search Complet, abstract level at Agris (FAO), CAB Abstracts, CABI Full-Text and Thomson Reuters Master Journal List.

Главни и одговорни уредник / Editor-in-Chief  
RUDOLF KASTORI

YU ISSN 0352-4906 UDK 5/6 (05)

MATICA SRPSKA  
PROCEEDINGS FOR  
NATURAL SCIENCES

121

NOVI SAD  
2011



## CONTENTS

## САДРЖАЈ

### ARTICLES AND TREATISES

### ЧЛАНЦИ И ПРИЛОЗИ

<i>Slavica S. Ćirić, Olga V. Petrović, Zoran Ž. Ilić, Božidar N. Milošević, Zvonko Lj. Spasić</i> , Examination of bacteriological status of surface freshwaters using direct and cultivation methods – Испитивање бактериолошког статуса површинских вода применом директне и култивационих метода . . . . .	7
<i>Zora Z. Jelesić, Deana D. Medić, Mira M. Mihajlović-Ukropina, Marija R. Jevtić, Vera P. Gusman, Biljana J. Radosavljević, Biljana T. Milosavljević</i> , Susceptibility to antifungal agents of <i>Candida</i> spp. from blood and feces collected in Novi Sad in 3-year period (2008–2010) – Осетљивост на антимикотике изолата <i>Candida</i> spp. из крви и фецеса прикупљених у Новом Саду у трогодишњем периоду (2008–2010) . . .	19
<i>Igor O. Gojković, Smiljana D. Paraš, Radoslav B. Gajanin, Milica D. Matavulj, Vesna K. Rajković, Goran D. Spasojević</i> , The influence of extremely low-frequency electromagnetic field on the basal ganglia structures of the rat brain – Утицај екстремно ниско-фреквентног електромагнетног поља на структуре базалних ганглија мозга пацова . . . .	27
<i>Dragica M. Stanković, Borivoj Đ. Krstić, Mirjana T. Šijačić-Nikolić, Milan N. Knežević</i> , Concentrations of manganese and iron in some woody and herbs plants – Концентрација мангана и гвожђа у неким вишегодишњим дрвенастим и зљајастим врстама . . . . .	39
<i>Aleksandra S. Bočarov-Stančić, Slavica Ž. Stanković, Jelena T. Lević, Nataša M. Salma, Vladimir R. Pantić, Saša S. Barnić</i> , <i>In vitro</i> degradation of diacetoxyscirpenol and T-2 toxin by use of <i>Mucor racemosus</i> Fresen. f. <i>racemosus</i> isolate – <i>In vitro</i> деградација диацетоксисцирпенола и Т-2 токсина посредством изолата <i>Mucor racemosus</i> Fresen. f. <i>racemosus</i> . . . . .	51
<i>Vahid Rahjoo, Maryam Parchamian, Mohammad Taghi Feizbakhsh, Majid Zamani</i> , Quantization of total fumonisins produced by <i>Fusarium verticillioides</i> strains in some maize and sorghum genotypes by ELISA – Квантизација укупних фумонизина које стварају сојеви <i>Fusarium verticillioides</i> код неких генотипова кукуруза и сирка помоћу ЕЛИСА теста . . . .	61

<i>Majid Zamani, Vahid Rahjoo, Maryam Parchamian</i> , Evaluation of the reaction of early maturing maize genotypes to common smut using artificial inoculation – Евалуација отпорности генотипова раног кукуруза на гар помоћу вештачке инокулације . . . . .	71
<i>Ferenc F. Balaž, Marija I. Bodroža Solarov, Jovana N. Vučković, Ferenc F. Bagi</i> , Effects of chemical treatments on infestation of <i>Alternaria</i> spp. and <i>Fusarium</i> spp. in corelation with technological wheat quality – Утицај хемијске заштите на интензитет заразе врстама из рода <i>Alternaria</i> spp. и <i>Fusarium</i> spp. у корелацији са технолошким квалитетом . . . . .	79
<i>Franc Čuš, Neža J. Čadež, Peter I. Raspor</i> , Fungicide residues in grapes determined the dynamics of <i>Saccharomyces cerevisiae</i> strains during spontaneous wine fermentation – Остаци фунгицида у грозђу одређују динамику сојева <i>Saccharomyces cerevisiae</i> у току спонтане ферментације вина . .	85
<i>Marija M. Škrinjar, Marijana M. Višić-Vićanović, Nevena T. Blagojev, Zorica S. Tatalović, Vesna J. Krunić</i> , Xerophilic mycopopulations of teas in bulk – Ксерофилне микопопулације чајева у ринфузи . . . . .	103
<i>Marija M. Škrinjar, Ljiljana S. Petrović, Nevena T. Blagojev, Vladislava M. Šošo</i> , Air mycopopulations in <i>Petrovská klobása</i> producing facility – Плесни у ваздуху просторија за производњу и зрење <i>Пейровачке кобасице</i> . . . . .	115
<i>Dragutin A. Đukić, Leka G. Mandić, Lidija Božarić, Bojana D. Trifunović, Marijana I. Pešaković</i> , Potentially pathogenic, pathogenic and allergenic moulds in the urban soils – Потенцијално патогене, патогене и алергене плесни урбаних земљишта . . . . .	125
Instruction to Authors – Упутство ауторима . . . . .	133

*Slavica S. Ćirić<sup>1</sup>, Olga V. Petrović<sup>2</sup>, Zoran Ž. Ilić<sup>1</sup>,  
Božidar N. Milošević<sup>1</sup>, Zvonko Lj. Spasić<sup>1</sup>*

<sup>1</sup> University of Priština, Faculty of Agriculture, Kopaonika Street bb, 38219 Lešak, Serbia,  
E-mail: slavica.ciric@open.telekom.rs

<sup>2</sup> University of Novi Sad, Faculty of Sciences, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia

## EXAMINATION OF BACTERIOLOGICAL STATUS OF SURFACE FRESH WATERS USING DIRECT AND CULTIVATION METHODS

**ABSTRACT:** The number of bacteria in surface waters reflects the current state of waters and their trophicity, and it is the first parameter that is affected by the anthropogenic contamination. Traditionally, quantification of bacteria in waters is performed using the cultivation methods. However, all these methods detect only cultivable bacteria; the results depend on the incubation conditions, and the results are obtained after several days. These deficiencies are solved by using direct methods.

This work is the first bacteriological examination of the reservoir Ćelije and its tributaries by using a direct method of quantification.

Total of 343 samples of water from the reservoir and its tributaries were processed. In each sample, the count of aerobic mesophilic bacteria was determined using cultivation method was involving inoculation of samples on high-nutrient PCA medium and on low-nutrient R2A medium, and incubation for 7 days at room temperature.

The total number of bacteria was determined by epifluorescence microscopy technique after filtration of samples previously stained with acridine orange.

Using the cultivation methods, the highest number of bacteria was recorded in the river of Blatašnica on R2A medium (56,535 CFU/ml), and lowest in the basin Vodozahvat on PCA medium (1,364 CFU/ml). According to the results from R2A, water belonged to the class of water of poorer quality compared to the results from the PCA. The results obtained by the direct method were correlated with cultivation methods.

The significantly highest number of bacteria was obtained by epifluorescence microscopy, and the lowest on PCA medium. If the application of direct method for some reason is not possible, the real results of bacteriological state of surface waters can be obtained by inoculation of samples on R2A medium with adequate incubation conditions.

**KEY WORDS:** surface waters, bacteriological status, epifluorescence microscopy, PCA medium, R2A medium

## INTRODUCTION

Bacterioplankton abundance is an indicator of trophic status of surface waters, because it is primarily responsible for turnover of nutrients and decomposition of organic matter in waters (Fisher et al., 2000). The number



of bacteria is limited by the quantity and quality of matter that enters the water, and many biotic and abiotic factors. The number of bacteria in surface waters reflects the current state of waters and their trophicity, and is also usually the first parameter that is affected by the anthropogenic contamination (K a l w a s i ń s k a and D o n d e r s k i , 2003).

Traditionally, quantification of bacteria in waters is performed by cultivation methods with different media and incubation conditions. It was shown that prolonged incubation on low-nutrient media is more suitable cultivation method for determining the bacterial count in aquatic environments as compared to high-nutrient media and shorter incubation period (Ć i r i ć , 2003; Ć i r i ć , 2009; Ć i r i ć and P e t r o v i ć , 2009). However, all cultivation methods have the same basic shortcomings: (a) only cultivable bacteria are detected; (b) results mainly depend on cultivation conditions (temperature, medium, duration of incubation); (c) results are obtained after 24 hours to over a week.

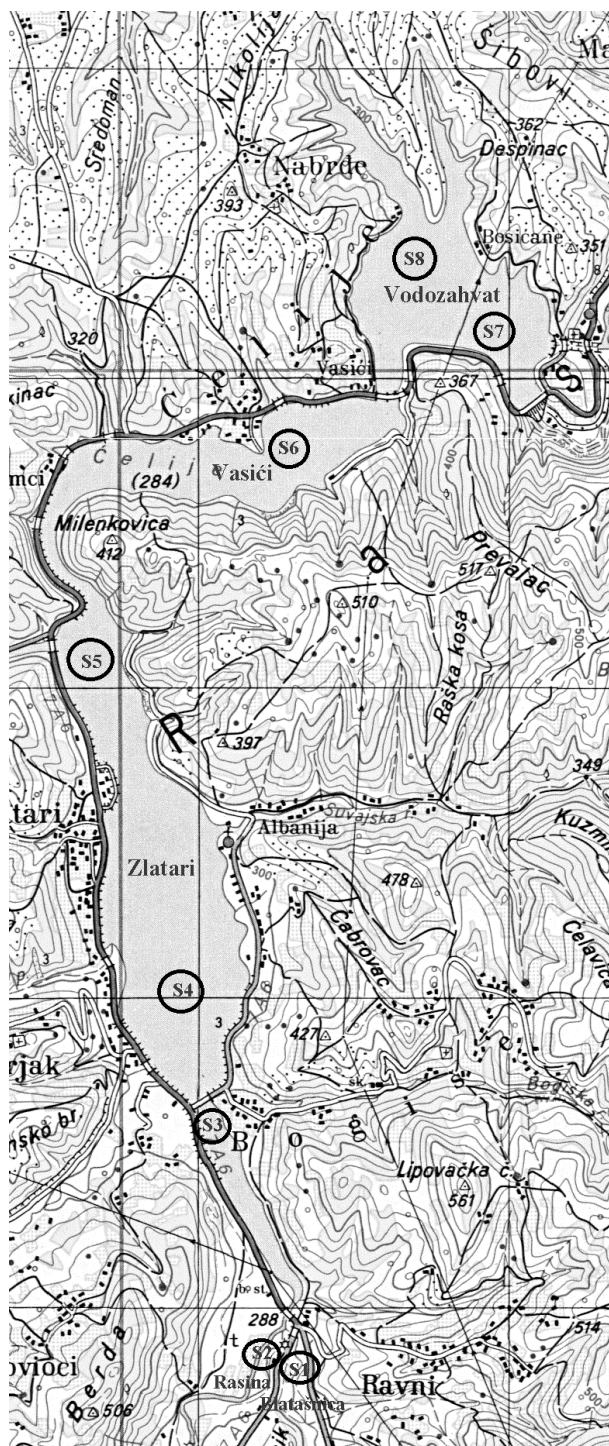
These deficiencies are overcome by direct methods of quantification of bacteria. The methods are based on the staining of water samples, their filtration through black polycarbonate filters with pore diameter of 0.2  $\mu\text{m}$ , and enumeration of bacteria on filters using epifluorescence microscope. For the staining of water samples, two dyes are commonly used: acridine orange and DAPI. The main objection to application of these dyes concerning the inability to distinguish live from dead cells on filters, so the total number including dead and inactive cells is obtained. However, it is believed that the dead cells rapidly degrade, so their presence on the filters is irrelevant (M a i e r et al., 2002). Newer dyes, designed to distinguish live from dead cells (such as LIVE/DEAD® Bac Light<sup>TM</sup>), show no difference in the total number of bacteria compared to acridine orange, but compared to DAPI they show a greater number of bacteria (B o u l o s et al., 1999).

The direct bacterial count can be determined using a scanning electron microscope after the filtration of water samples. It is a very powerful technique but quite expensive and long lasting (C a r o n , 1983); what is more important, the results obtained are not significantly different compared to epifluorescence microscopy (B o w d e n , 1977).

The aim of this study was to examine the bacteriological status of water in the reservoir of Ć elije, near the town of Kruševac, Serbia, and its tributaries, using cultivation methods and, for the first time, a direct method, and to analyze statistically the differences between these methods. In addition, the goal was to categorize the tested waters, and thus determine any differences between the applied methods.

## MATERIALS AND METHODS

During one-year study, the sampling of water in the reservoir of Ć elije and its major tributaries, the river of Blatašnica and the river of Rasina was carried out. The total of 283 samples from the reservoir and 60 samples from the rivers were taken. Samples were taken at different seasons, and from the reservoir at different depths. Sampling points are shown in Figure 1. The river



Blatašnica was sampled at one point before inflowing into the river Rasina (S1). The river Rasina was sampled before (S2) and after (S3) of the river Blatašnica inflow, just before the entrance into the reservoir Čelije. The water of reservoir Čelije was sampled in all three basins. Basin Zlatari was sampled at two points (S4 and S5); the basin Vasići was sampled at one representative point (S6), and the basin Vodozahvat (Catchment) was sampled at two points (S7 and S8).

In each water sample, the bacterial count was determined using cultivation and direct methods.

### *Cultivation method*

The samples were inoculated by pour plate technique on high-nutrient Plate Count Agar (PCA) and on low-nutrient Reasoner's 2 Agar (R2A). Each sample was inoculated in three dilutions:  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$ , and each dilution was inoculated in triplicates for both media. The amount of inoculum was 1 ml. Inoculated plates were incubated at room temperature (20°C to 22°C). Bacteria were counted after 7 days, using the colony counter BZG-30 (Windaus), through the magnifying glass with a magnification of 2 times the actual size. Counting of bacteria was performed on plates inoculated with the lowest dilution that could be read, and the obtained number was computed to 1 ml of a sample.

### *Direct method*

Direct bacterial count was determined by means of epifluorescence microscope after staining of samples with acridine orange. After sampling, the samples were diluted by adding of 1 ml of sample into a test tube containing 9 ml of solution for dilution. Solution for dilution was made as a mixture of 500 ml of distilled water and 25 ml of 50% glutaraldehyde, sterilized by membrane filtration and stored at 4°C (K e p n e r and P r a t t, 1994). Diluted samples were fixed with buffered glutaraldehyde of final concentration of 1% by adding 9 ml of diluted sample into a test tube containing 1 ml of cold fixative.

Staining of samples was performed with buffered solution of acridine orange, final concentration of 100 µm/ml. Two milliliters of diluted and fixed sample was mixed with equal volume of dye. After three minutes of dying two milliliters of stained sample was filtered through the membrane filter ISOPORE™ (Millipore Corp.), black, with diameter of 13 mm and pore diameter of 0.2 µm. As a support, the nitrocellulose filter Sartorius, white, with diameter of 13 mm and pore diameter of 0.45 µm, was used. For the filtration, Millipore steel device, with a filtration sieve 13 mm in diameter, and sterile plastic syringes, disposable, 5 ml of volume were used.

After the filtration of samples, filters were rinsed with a small amount of distilled water, previously sterilized by membrane filtration.

Counting of bacteria was performed under the epifluorescence microscope Olympus BX51, with the total magnification of 1,000x. Immersion oil

with low fluorescence, produced by ROTH (Germany), was used. Bacteria were counted from ten fields of each filter, and the average number was taken as the average number of bacteria per field. The total number of bacteria in 1 ml of water sample was calculated by the formula:

$$T = \frac{N \cdot A/a}{V},$$

where: T = total bacterial count per milliliter; N = average number of bacteria per field; A = area of filtering field (78.54 mm<sup>2</sup>); a = area of the vision field (0.031416 mm<sup>2</sup>); V = volume of filtered sample (0.09 ml).

Based on the results the percentage of number of bacteria obtained by cultivation method in the total bacterial count obtained by direct method was calculated. The TBC/AMB index was also determined. This index is the ratio of the total bacterial count (TBC) obtained by direct method, and the number of aerobic mesophilic bacteria (AMB) obtained by cultivation method (Petrović et al., 1998). Based on this index, the categorization of tested waters was performed according to the Table 1. The investigated waters were also classified based on the direct bacterial count according to Ambrazene (1976) and based on the count of aerobic mesophilic bacteria according to Kohl (1975).

Tab. 1 – Classification of waters according to the TBC/AMB index

<b>TBC/AMB index</b>	<b>Class of water</b>
> 1,000	Clean water
1,000–100	Moderately polluted water
100–10	Polluted water
10–1	Very polluted water

### *Statistical analyses*

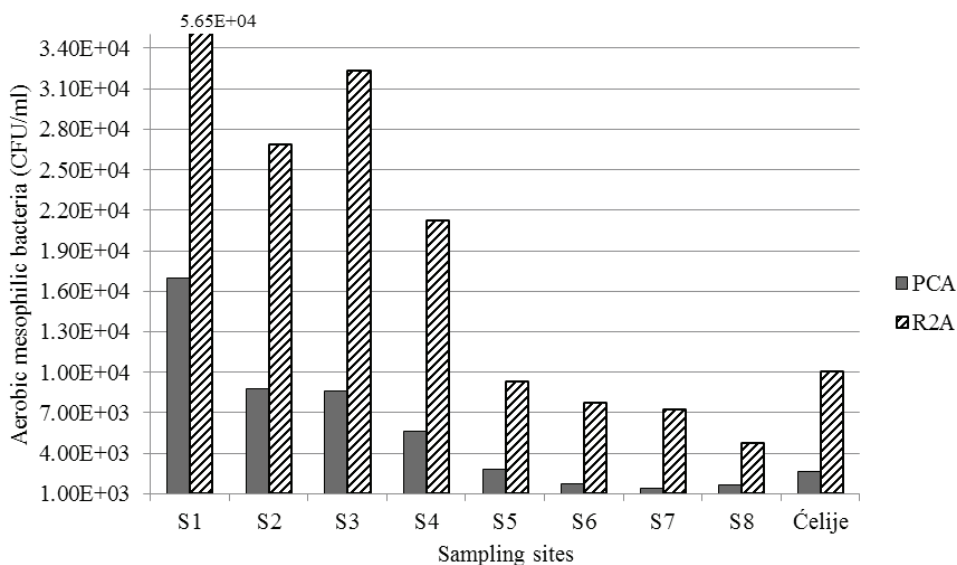
The significance of difference in the count of bacteria between different methods of quantification was tested by the statistical method of analysis of variance.

The correlation between the results obtained by the applied methods was determined by the correlation analysis.

Both statistical analyses were carried out using the software STATISTICA v. 8.0, StatSoft, Inc.

## RESULTS AND DISCUSSION

The average count of bacteria obtained by cultivation method is represented in Graph 1. The highest number of bacteria was recorded in the river Blatašnica on R2A medium (56,535 CFU/ml). In all samples, more bacteria was recorded on R2A medium compared to the PCA medium. The lowest number of bacteria was noted in the basin Vodozahvat (Catchment) on PCA medium (1,364 CFU/ml and 1,659 CFU/ml).



CFU – colony forming units

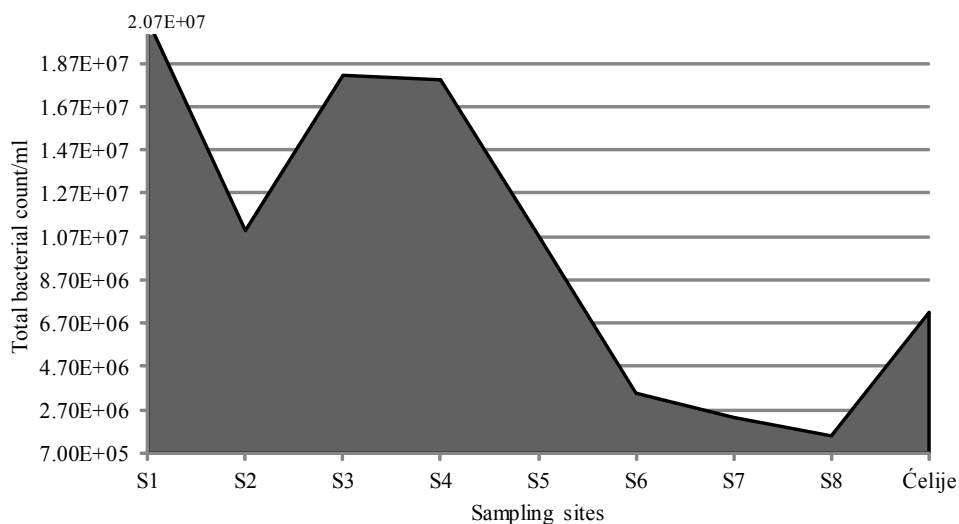
Graph 1. – Average number of aerobic mesophilic bacteria obtained using cultivation methods

The average count of bacteria obtained by direct method in investigated waters is represented in Graph 2. The highest number of bacteria was recorded in the river Blatašnica (20,694,445 bacteria/ml). The bacterial count was gradually reduced over the river Rasina and the reservoir Čelije going downstream to the basin Vodozahvat, which had the lowest value (2,340,909 bacteria/ml and 1,492,424 bacteria/ml).

Analysis of variance showed that the significantly highest number of bacteria at all sampling points was recorded using the direct method (Table 2). In the cultivation method, the significantly highest number of bacteria in all samples was noted on R2A medium compared to the PCA medium (Table 2).

The results of direct quantification of bacteria were positively correlated with those obtained by cultivation methods, with the higher coefficient of correlation recorded with R2A (Graphs 3 and 4).



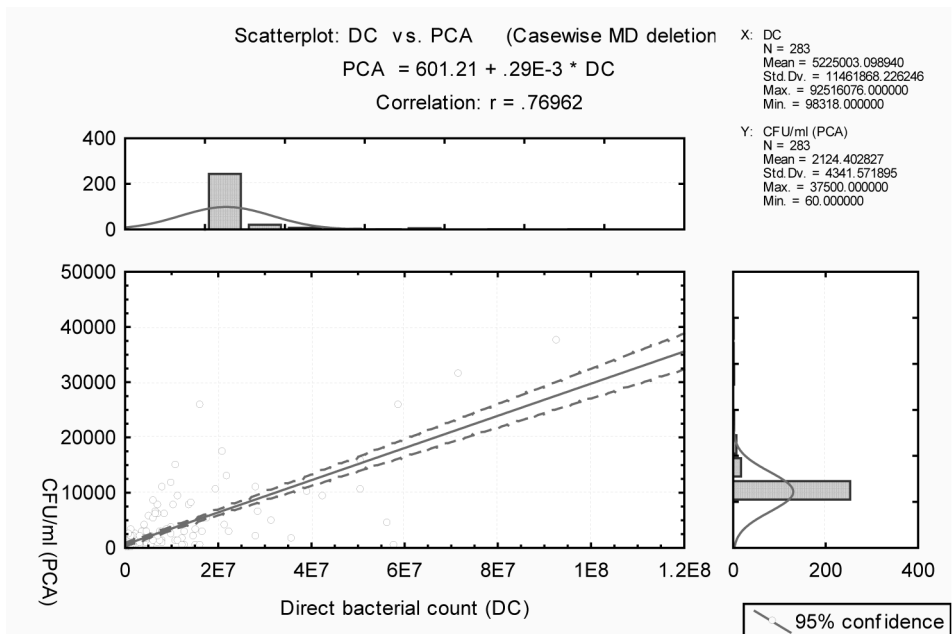


Graph 2. – Average values of total bacterial count obtained using direct method

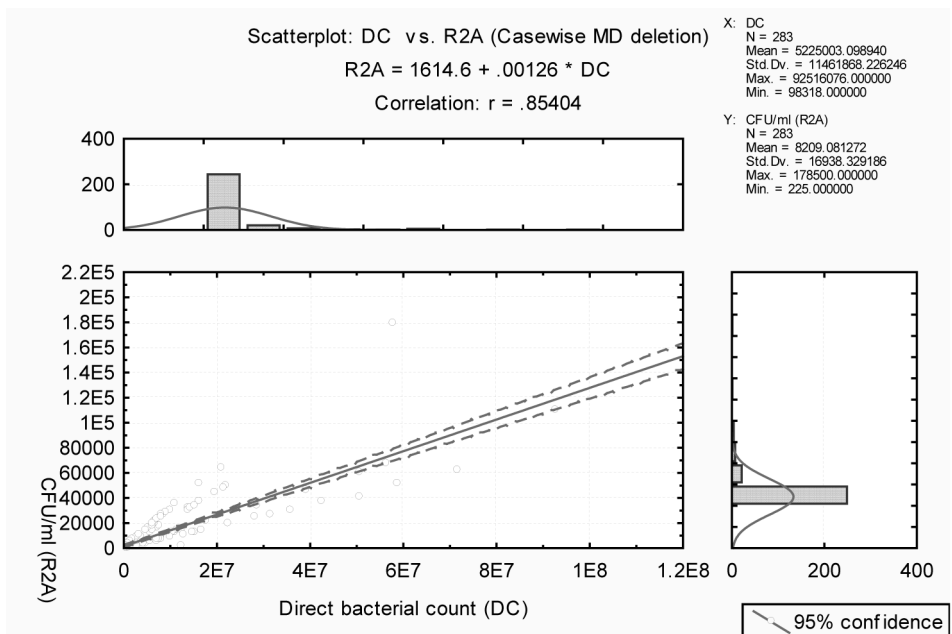
Tab. 2 – Results of analysis of variance of bacterial count obtained by different methods of quantification

Water source	Compared methods of quantification	Sources of variation	Degrees of freedom	Sum of squares	Mean square	Calculated F-value	Tabulated F-value for $\alpha=0.05$	Tabulated F-value for $\alpha=0.01$	Statistical conclusion
The river Blatnica	PCA vs. R2A	BM	1	$1.13 \times 10^{10}$	$1.13 \times 10^{10}$	12.43	4.01	7.09	GS
		WM	58	$7.83 \times 10^9$	$1.35 \times 10^8$				
		Total	59	$1.92 \times 10^{10}$					
	PCA vs. DC	BM	1	$3.38 \times 10^{14}$	$3.38 \times 10^{14}$	126.35	4.01	7.09	GS
		WM	58	$2.24 \times 10^{15}$	$3.87 \times 10^{13}$				
		Total	59	$2.58 \times 10^{15}$					
	R2A vs. DC	BM	1	$3.38 \times 10^{14}$	$3.38 \times 10^{14}$	125.87	4.01	7.09	GS
		WM	58	$2.24 \times 10^{15}$	$3.86 \times 10^{13}$				
		Total	59	$2.57 \times 10^{15}$					
The river Rasina	PCA vs. R2A	BM	1	$1.41 \times 10^{10}$	$1.41 \times 10^{10}$	11.81	4.01	7.09	GS
		WM	58	$4.38 \times 10^9$	$7.55 \times 10^7$				
		Total	59	$1.85 \times 10^{10}$					
	PCA vs. DC	BM	1	$5.37 \times 10^{14}$	$5.37 \times 10^{14}$	169.93	4.01	7.09	GS
		WM	58	$2.23 \times 10^{15}$	$3.84 \times 10^{13}$				
		Total	59	$2.76 \times 10^{15}$					
	R2A vs. DC	BM	1	$5.37 \times 10^{14}$	$5.37 \times 10^{14}$	169.43	4.01	7.09	GS
		WM	58	$2.22 \times 10^{15}$	$3.83 \times 10^{13}$				
		Total	59	$2.76 \times 10^{15}$					
The reservoir Čelije	PCA vs. R2A	BM	1	$8.62 \times 10^{10}$	$8.62 \times 10^{10}$	34.27	3.84	6.64	GS
		WM	564	$5.24 \times 10^9$	$9.29 \times 10^6$				
		Total	565	$9.15 \times 10^{10}$					
	PCA vs. DC	BM	1	$3.71 \times 10^{16}$	$3.71 \times 10^{16}$	58.76	3.84	6.64	GS
		WM	564	$3.86 \times 10^{15}$	$6.84 \times 10^{12}$				
		Total	565	$4.09 \times 10^{16}$					
	R2A vs. DC	BM	1	$3.71 \times 10^{16}$	$3.71 \times 10^{16}$	58.62	3.84	6.64	GS
		WM	564	$3.85 \times 10^{15}$	$6.83 \times 10^{12}$				
		Total	565	$4.09 \times 10^{16}$					

DC – direct count; BM – between methods; WM – within methods; GS – greatly significant ( $p < 0.01$ )



Graph 3. – Results of correlation analysis between CFU on PCA and direct bacterial count



Graph 4. – Results of correlation analysis between CFU on R2A and direct bacterial count

Tab. 3 – Categorization of investigated waters based on the results obtained using direct and cultivation methods

Sampling sites	Class of water according to Kohl		Class of water according to Ambrazene	Class of water according to TBC/AMB index	
	PCA	R2A		TBC/AMB (PCA)	TBC/AMB (R2A)
S1	II-III	III	dirty	clean	moderately polluted
S2	II	II-III	very polluted	clean	moderately polluted
S3	II	II-III	very polluted	clean	moderately polluted
S4	II	II-III	very polluted	clean	moderately polluted
S5	II	II	very polluted	clean	clean
S6	II	II	clean	clean	moderately polluted
S7	II	II	clean	clean	moderately polluted
S8	II	II	clean	moderately polluted	moderately polluted
Ćelije	II	II-III	moderately polluted	clean	moderately polluted

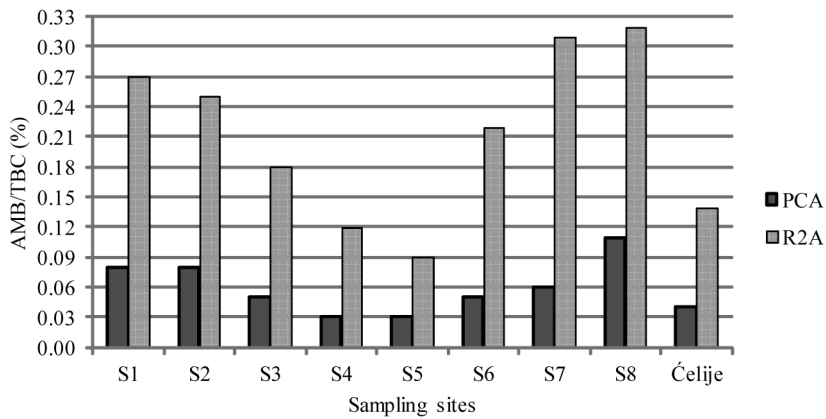
The results from two media gave the different categorization of waters according to Kohl (Table 3). Based on the results from R2A, waters belong to the class of waters of poorer quality compared to the results from the PCA, especially in the more polluted waters (rivers and the basin Zlatari).

Based on the categorization of waters according to Ambrazene, the water of the reservoir Ćelije belonged to moderately polluted waters (Table 3). Similar results were obtained by Milošević (1999) (cited by Čurčić, 2003), when based on the total bacterial count the water of Reservoir Grošnica (Serbia) was classified into the category of slightly to moderately polluted waters.

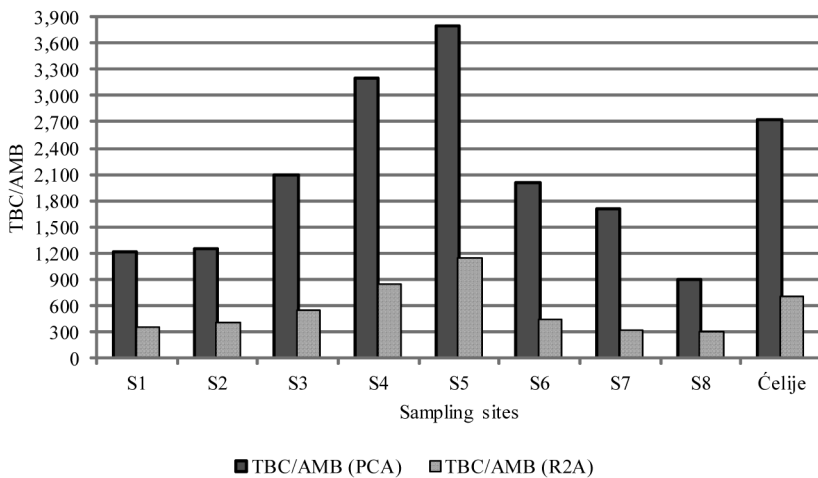
Percentage of aerobic mesophilic bacteria in the total bacterial count in the investigated waters is shown in Graph 5. The highest percentage of aerobic mesophiles in the total count was recorded applying the aerobic mesophile counts obtained on R2A agar (0.32%) in Vodozahvat basin. The lowest percentage of aerobic mesophile was noted applying the results obtained on PCA agar, in the basin Zlatari (0.03%). These results are in accordance with already published for different reservoirs. Thus, Čurčić (2003) found that the percentage of heterotrophs in the total count ranged from 0.02 to 1.99% in the reservoir of Gruža, Serbia. Čomić (1989) found that the percentage of heterotrophs ranged from 0.02 to 2.23% for the same reservoir. Gajin et al. (1991) (cited by Čurčić, 2003) noted that the percentage of heterotrophs in the total bacterioplankton was below 1% for the Reservoir of Borkovac, Serbia.

Graph 6 presents the TBC/AMB index value for the tested waters. The highest value of this index was determined in Zlatari basin when the result obtained on PCA (3,795) was taken as AMB. The lowest index value was noted in Vodozahvat basin when the result obtained on R2A (316) was taken as AMB.





Graph 5. – Percentage of aerobic mesophilic bacteria (AMB) in total bacterial count (TBC)



Graph 6. – Index TBC/AMB

It can be seen that TBC/AMB index classified the same water in different categories depending on which the cultivation method was used. Thus, the ratio of total bacterial count and the count of aerobic mesophilic bacteria obtained on PCA placed most of the tested waters into the category of pure waters, while the application of the count of aerobic mesophiles obtained on R2A categorized waters as moderately polluted (Table 3).

## CONCLUSION

The highest number of bacteria in all samples of tested waters was found using the direct quantification and the lowest by inoculation of samples on high-nutrient PCA medium.

When PCA medium was used, the categorization of waters showed unreal results with the majority of waters categorized as pure waters. The use of R2A medium produced more probable results that were more similar to the categorization obtained by direct bacterial count.

Based on the results it can be concluded that in order to determine the bacteriological state of surface waters the most appropriate method is a direct method if conditions permit. Otherwise, as in the routine monitoring, real results can be obtained using cultivation method that include inoculation of water samples on some low-nutrient medium, such as R2A.

## REFERENCES

- Амбразене, Ж. П. (1976): *Количественные взаимоотношения микроорганизмов и их использование для оценки загрязненности речных вод*, Журнал общей биологии, 37, 416-425.
- Boulos, L., Prévost, M., Barbeau, B., Coallier, J., Desjardins, R. (1999): *LIVE/DEAD® BacLight™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water*, Journal of Microbiological Methods, 37, 77-86.
- Bowden, W. B. (1977): *Comparison of two direct-count techniques for enumerating aquatic bacteria*, Applied and Environmental Microbiology, Vol. 33, No. 5, 1229-1232.
- Caron, D. A. (1983): *Technique for enumeration of heterotrophic and phototrophic nanoplankton using epifluorescence microscopy, and comparison with other procedures*, Applied and Environmental Microbiology, Vol. 46, No. 2, 491-498.
- Čomić, Lj. (1989): *Mikrobna populacija vode u slivu i akumulaciji Gruža*, doktorska disertacija, Univerzitet u Kragujevcu, Prirodno-matematički fakultet, Kragujevac.
- Ćirić, S. (2003): *Kvalitet vode za piće grada Kruševca od izvorišta do potrošača sa posebnim osvrtom na ukupan broj aerobnih mezofilnih bakterija*, magistarska teza, Univerzitet u Novom Sadu, Departman za biologiju i ekologiju, Prirodno-matematički fakultet, Novi Sad.
- Ćirić, S. (2009): *Aerobne mezofilne bakterije i niskonutritivni R2A medijum u monitoringu kvaliteta vode za piće*, doktorska disertacija, Univerzitet u Novom Sadu, Departman za biologiju i ekologiju, Prirodno-matematički fakultet, Novi Sad.
- Ćirić, S., Petrović, O. (2009): *Comparison of cultural methods on bacterial recovery in source water for water supply*, Fresenius Environmental Bulletin, Vol. 18, No. 11a, 2203-2209.
- Ćurčić, S. (2003): *Mikrobiološki indeks kvaliteta vode (mWQI) – pokazatelj stanja zajednice mikroorganizama u akumulaciji Gruža*, doktorska disertacija, Univerzitet u Kragujevcu, Institut za biologiju i ekologiju, Prirodno-matematički fakultet, Kragujevac.
- Fisher, M. M., Klug, J. L., Lanster, G., Newton, M., Triplett, E. W. (2000): *Effects of resources and trophic interactions on freshwater bakterioplankton diversity*, Microbial Ecology, 40, 125- 133.
- Kalwasinska, A., Donderski, W. (2003): *Planktonic bacteria of Lake Chelmszyńskie*, Acta Universitas Nicolai Copernici, Limnological Papers, 23, 25-39.

- Kepner, R. L. Jr., Pratt, J. R. (1994): *Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present*, Microbiological Reviews, Vol. 58, No. 4, 603-615.
- Kohl, W. (1975): *Über die Bedeutung Bakteriologischer Untersuchungen für die Beispiel der Österreich Donau*, Archiv für Hydrobiologie, 44, 392-461.
- Maier, R. M., Pepper, I. L., Gerba, Ch. P. (2002): *Environmental Microbiology*, Academic Press, An Imprint of Elsevier.
- Petrović, O., Gajin, S., Matavulj, M., Radnović, D., Svirčev, Z. (1998): *Mikrobiološko ispitivanje kvaliteta površinskih voda*, Univerzitet u Novom Sadu, Institut za biologiju, Prirodno-matematički fakultet, Novi Sad.

## ИСПИТИВАЊЕ БАКТЕРИОЛОШКОГ СТАТУСА ПОВРШИНСКИХ ВОДА ПРИМЕНОМ ДИРЕКТНЕ И КУЛТИВАЦИОНИХ МЕТОДА

Славица С. Ћирић<sup>1</sup>, Олга В. Петровић<sup>2</sup>, Зоран Ж. Илић<sup>1</sup>,  
Божидар Н. Милошевић<sup>1</sup>, Звонко Љ. Спасић<sup>1</sup>

<sup>1</sup> Универзитет у Приштини, Пољопривредни факултет, Копачичка бб,  
38219 Лешак, Србија

<sup>2</sup> Универзитет у Новом Саду, Природно-математички факултет,  
Трг Доситеја Обрадовића 3, 21000 Нови Сад, Србија

### Резиме

Број бактерија у површинским водама одражава тренутно стање вода и њихову трофичност, а такође је и први параметар који реагује на антропогену контаминацију. Традиционално, квантификација бактерија у водама се врши применом култивационих метода. Међутим, све ове методе детектују само култивабилне бактерије; резултати зависе од услова инкубације и добијају се након неколико дана. Ови недостаци се превазилазе применом директних метода.

У овом раду је први пут бактериолошко испитивање воде акумулације Ћелије и њених притока вршено применом неке од директних метода квантификације. Укупно 343 узорка воде из акумулације и њених притока је процесуирано.

У сваком узорку одређиван је број аеробних мезофилних бактерија применом одгајивачке методе која је обухватала засејавање узорака на високохранљиви *РСА* медијум и на нискохранљиви *Р2А* медијум, и њихова инкубација 7 дана на собној температури. Укупан број бактерија одређиван је техником епифлуоресцентне микроскопије након филтрације узорака претходно обојених акридин оранжом.

Применом култивационе методе, највећа бројност бактерија се бележи у реци Блаташници на *Р2А* медијуму (56.535 колонија/ml), а најмања у басену Водозахват на *РСА* медијуму (1.364 колонија/ml). На основу резултата са *Р2А* подлоге вода припада класи вода лошијег квалитета него на основу резултата са *РСА* подлоге.

Резултати добијени применом директне методе корелирају са резултатима добијеним одгајивачким методама.

Значајно највећа бројност бактерија добија се епифлуоресцентном микроскопијом, а најмања на *РСА* медијуму. Уколико примена микроскопије није могућа из неких разлога, реални резултати бактериолошког статуса површинских вода могу се добити засејавањем узорака на *Р2А* медијум уз адекватне услове инкубације.

Zora Z. Jelesić, Deana D. Medić,  
Mira M. Mihajlović-Ukropina, Marija R. Jevtić,  
Vera P. Gusman, Biljana J. Radosavljević,  
Biljana T. Milosavljević

Institute for Public Health of Vojvodina, Futoška 121, 21 000 Novi Sad, Serbia

## SUSCEPTIBILITY TO ANTIFUNGAL AGENTS OF *CANDIDA* SPP. FROM BLOOD AND FECES COLLECTED IN NOVI SAD IN 3-YEAR PERIOD (2008-2010)

**ABSTRACT:** Candidemia is an important emerging nosocomial infection in patients with risk factors. *Candida* species from nonsterile sites can give insight into the characteristics of strains that may cause invasive disease.

The aim of this study was to evaluate antifungal susceptibility of *Candida* blood and fecal isolates in Novi Sad, Vojvodina.

During a 3-year period (2008 to 2010), 424 isolates of *Candida* spp. were collected, 30 bloodstream isolates and 394 strains from fecal samples. *In vitro* susceptibility of these isolates to five antifungal agents was established using commercial ATB FUNGUS 3 (Bio-Mérieux).

Predominant species was *Candida albicans* (6 isolates from blood and 269 from feces). Resistance to one or more antifungal agents was less common in *Candida albicans* (3.63%) than in other species (24.83%). Resistance to itraconazole was the most commonly found in both groups of isolates, 9.64% strains from feces and 20% from blood samples. Twelve isolates were multiply resistant, usually to fluconazole, itraconazole, and voriconazole. Resistance to amphotericine B was extremely rare.

Although resistance to antimycotics of *Candida* spp. is rare at present, continued surveillance of antifungal susceptibility is necessary in order to monitor trends, and to choose the right empiric therapy.

**KEY WORDS:** antifungal susceptibility, candidemia, *Candida* spp.

## INTRODUCTION

Candidemia is an important emerging nosocomial infection in patients with risk factors. In the last two decades an increase of bloodstream infections caused by *Candida* species (*Candida* spp.) has been documented, with a consequent rise in related mortality and prolonged hospitalizations (Tulumoglu S. et al., 2009). Risk factors for development of candidemia are: immunocompromised host, immunosuppressive therapy and neutropenia, intensive care

units patients, central venous catheters, parenteral nutrition, and the long-term use of broad-spectrum antibacterial drugs (T a k a k u r a S. et al., 2006).

*Candida* spp. at multiple nonsterile sites often suggests colonization but these strains also may cause invasive disease. *Candida* spp. from nonsterile sites can also give insight into the characteristics of strains that cause the disease (T a s i c S. et al., 2008).

The variability in the susceptibility of clinical isolates to antifungal drugs emphasizes the importance of performing species identification and antifungal susceptibility testing (M i t r o v i c S. M. et al., 2007).

There are data which show that *Candida albicans* (*C. albicans*) maintains excellent susceptibility to all antifungal agents, but many non-albicans species have increased resistance to antifungal drugs over the past decades. (T u l u m o g l u S. et al., 2009)

There are differences in species distribution and antifungal susceptibility profiles and it is important to obtain such information for each geographic area (M u e l l e r F. M. et al., 2000)

The aim of this study was to assess the antifungal susceptibility profiles in both strains of *Candida* spp. associated with systemic disease and fecal isolates.

## MATERIAL AND METHODS

In the Center for Microbiology of Institute for Public Health of Vojvodina during a 3-year period, from January 2008 to December 2010, a total of 424 isolates of *Candida* spp. were collected, 30 bloodstream isolates and 394 strains from fecal samples.

Blood samples were collected in sterile conditions into 30 ml culture medium for BacT/Alert- BioMérieux. The bottles which were positive after incubation at 37° C for 2-5 days were transferred to blood agar and Sabouraud Dextrose Agar and incubated 24 hours at 35°C, and next 24 hours at 25°C.

Fecal samples were inoculated to Sabouraud Dextrose Agar and incubated 24 hours at 35°C, and next 24 hours at 25°C.

Colonies of *Candida* spp. were identified by examination of their microscopic and macroscopic features. *Candida albicans* was identified by the application of germination tube test in human serum. Species identification was confirmed by API 20C (BioMérieux) or Vitek2 (BioMérieux) for resistant blood isolates of *Candida* spp.

*In vitro* susceptibility of all isolates to five antifungal agents was established using commercial ATB FUNGUS 3 (BioMérieux, France) that enables the determination of the susceptibility to five antifungal agents under conditions similar to the reference method for micro-dilution according to EUCAST (European Committee on Antibiotic Susceptibility Testing) and CLSI (Clinical and Laboratory Standards Institute) recommendations. The results obtained give a MIC (Minimal inhibitory concentration) for amphotericin B (AMB), fluconazole (FCA), itraconazole (ITR), voriconazole (VRC) and/or

classify the strain as Sensitive (S), Intermediate (I) or Resistant (R) flucytosine (5FC).

The interpretative breakpoints were proposed by the manufacturer of the test. Fluconazole MICs  $\leq 8.0$  mg/L were considered susceptible and  $\geq 64$  mg/L were considered resistant, with the exception of *C. krusei*, which is considered inherently resistant to fluconazole, regardless of the MIC value. Isolates showing itraconazole MICs  $\leq 0.125$  mg/L and voriconazole  $\leq 1$  mg/L were classified as susceptible and those with MICs  $\geq 1$  mg/L for itraconazole and  $\geq 4$  mg/L for voriconazole as resistant. Although interpretative breakpoints for amphotericin B have not yet been established, isolates showing MIC of  $\geq 2$  mg/L suggest resistance.

## RESULTS

From 30 blood isolates and 394 fecal isolates predominant species was *Candida albicans*, 269 from fecal samples and 6 strains from blood (in hemocultures *Candida* spp. was more common). Resistance to one or more antifungal agents was less common in *Candida albicans* (3.63%) than in other *Candida* species (24.83%).

From total of 269 fecal isolates of *Candida albicans* 259 (96.28%) were susceptible to all antimicrobial drugs tested. Ten isolates (3.72%) had one or more resistance markers. One resistance marker had 4 strains, they were resistant to itraconazole, one was resistant to fluconazole and itraconazole, and five isolates had 3 markers and were resistant to fluconazole, itraconazole, and voriconazole. In *Candida* spp. (non-albicans) 24.80% had resistance markers, 24 (19.20%) strains with one marker, 1 (0.80%) with 2 markers, 5 (4.00%) with 3 markers and 1 (0.80%) strain with 4 resistance markers (Table 1).

Resistance to itraconazole was the most commonly found in both groups of fecal isolates, 3.72% of *Candida albicans* strains and 22.40% of *Candida* spp.

Tab. 1 – Resistance types of *Candida* isolates from feces (comparative data for *Candida albicans* and *Candida* spp.)

Number of resistance markers	Resistance types of <i>Candida albicans</i>	Number (%) of isolates	Number of resistance markers	Resistance types of <i>Candida</i> spp.	Number (%) of isolates
0		259 (96.28%)	0		94 (75.20%)
1	ITR	4 (1.48%)	1	FCA ITR VRC	2 (1.6%) 21 (16.8%) 1 (0.80%)
2	FCA, ITR	1 (0.37%)	2	FCA, ITR	1 (0.80%)
3	FCA, ITR, VRC	5(1.85%)	3	FCA, ITR, VRC AMB, FCA, ITR	4 (3.20%) 1 (0.80%)
			4	AMB, FCA, ITR, VRC	1 (0.80%)
Total number		269 (100%)	Total number		125 (100%)

Resistance to fluconazole in *Candida albicans* was established in 2.23 % and 7.20% of *Candida* spp. Resistance to voriconazole was found in 1.85% *C. albicans* and 4.80% *Candida* spp. No resistance to amphotericin B was found in *Candida albicans* and in *Candida* spp., it was rare (1.60%).

From the blood isolates 24 (80%) were sensitive to all drugs tested (all strains of *Candida albicans* were completely sensitive) and from 20% resistant isolates of *Candida* spp., 3 were identified as *C. parapsilosis*-1 isolate and *C. tropicalis* -2 isolates (10%), had 1 resistance marker and were resistant to itraconazole; 2 isolates of *C. kruzei* (6.66%) had 2 markers – fluconazole and itraconazole; and 1 *C. tropicalis* (3.33%) was resistant to 3 antimycotics – fluconazole, itraconazole and voriconazole (Table 2).

Tab. 2 – Resistance types of *Candida* isolates from blood (comparative data for *Candida albicans* and *Candida* spp.)

Number of resistance markers	Resistance types of <i>Candida albicans</i>	Number of isolates	Number of resistance markers	Resistance types of <i>Candida</i> spp.	Number of isolates
0		6	0		18
1			1	ITR	3
2			2	FCA, ITR	2
3			3	FCA, ITR, VRC	1
Total number		6	Total number		24

*Candida albicans* remains the most susceptible. All the isolates were susceptible to flucytosine and in isolates from blood no resistance to amphotericin B was found, and in fecal isolates it was rare. Cross-resistance to azoles was found in the total of 16 isolates (3.77%), and it was more common in *Candida* spp. (6.71%) than in *C. albicans* (1.81%).

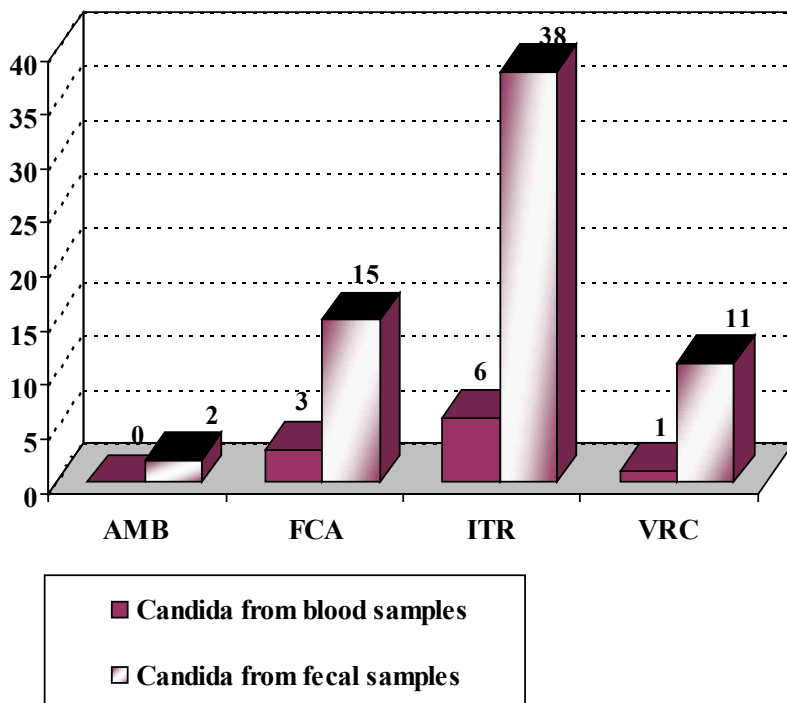
Twelve isolates were multiple resistant.

Resistance to itraconazole was dominant in both fecal and blood isolates, 38 (9.64%) strains from fecal samples and 6 (20%) strains from blood were resistant to that drug (Graph 1). Isolates resistant to itraconazole were more often found in blood than in feces, the difference is statistically significant ( $p < 0.01$ ).

Non-*albicans* *Candida* species were significantly ( $p < 0.05$ ) more frequently resistant to itraconazole and fluconazole than *Candida albicans*. To itraconazole were resistant 10 from 275 (3.63%) *C. albicans* and 33 from 149 *Candida* spp. (22.14%) ( $p < 0.05$ ). Resistance to fluconazole was less common in both groups of isolates, 6 from 275 *C. albicans* (2.18%) and 12 from 149 *Candida* spp. (8.05%) ( $p < 0.05$ ). Overall resistance to itraconazole in 424 isolates was 10.37% of isolates, to fluconazole 4.24% and to voriconazole 2.83%.



Graph 1. Total number of resistant *Candida* isolates from blood and feces



## DISCUSSION

*Candida* spp. is a member of physiological flora of the human skin and mucosal membranes. The prevalence of *Candida* colonization of the gastrointestinal tract in healthy people is very high (Tasić S. et al. 2008). There is a balance between *Candida* spp. of normal flora and immune defense mechanisms, when this balance is disturbed colonization usually results in infection (Babić M, Hukić M. 2010). Rates of candidemia have increased over the past few decades causing the morbidity and mortality for immunocompromised patients, such as those with cancer or AIDS (Safdar A. et al., 2002). This analysis included clinical isolates from bloodstream and from feces, as the most *Candida* infections arise from the hosts' endogenous microflora.

Our susceptibility results are similar to other studies: resistance is uncommon among *C. albicans*, and there is higher level of reduced susceptibility among non-*albicans* *Candida* spp.

All the isolates studied were susceptible to flucytosine, and decreased susceptibility to amphotericin B was found only in fecal isolates of non-*albicans* strains (1.60%) and resistance of all isolates tested was 0.47% that is consistent with reports from Spain, Turkey, and Mexico (Florez C. et al., 2008, Tulumoglu S. et al. 2009, Gonzalez GM. et al. 2008).



The occurrence of azole cross-resistance in clinical *C. albicans* isolates has been demonstrated by German authors (M u e l l e r F. M. et al., 2000) in children with HIV (human immunodeficiency virus). In our study, cross-resistance to azoles was found in 16 isolates (3.77%), and it was more common in *Candida* spp. (6.71%) than in *C. albicans* (1.81%). In a study from Glasgow, UK, none of the blood culture isolates was resistant to either fluconazole or itraconazole (K e n n e d y H. F. et al., 2006).

Resistance to fluconazole in this study was found in 4.24% of all isolates tested, very similar to 4.1% found in Spain (F l o r e z C. et al., 2009) and less common than 15% seen in Portugal (C o s t a - d e - O l i v i e r a S. et al., 2008).

Overall resistance to itraconazole in 424 isolates was 10.37% of isolates; it was more common in *Candida* spp. (22.14%) than in *C. albicans* (3.63%). These results are higher than in Spain and Turkey (F l o r e z C. et al., 2009, T u l u m o g l u S.) but lower than 24.7% in Lithuania (S k r o d e n i e n e E. et al., 2006), 27.6% in Venezuela (P a n i z o M. M. et al., 2009) and 43.3% in Mexico (G o n z a l e s G. M. et al., 2008).

Overall resistance to voriconazole in this investigation was low (2.83%) especially in *C. albicans* (1.81%) which is consistent to the studies in which this drug displayed potent antifungal activity (T o r t o r a n o A. M. et al., 2006 and Q u i n d o s G. et al., 2008).

In conclusion, this study shows that in Novi Sad, Vojvodina, resistant isolates were found both in *C. albicans* and in *Candida* spp. and reduced susceptibility was documented in blood as well as in fecal isolates.

Reduced susceptibility to azoles was relatively rare; most commonly found in all isolates was the resistance to itraconazole. Isolates resistant to itraconazole were more often found in blood than in feces, the difference is statistically significant. Non-albicans *Candida* species were significantly more frequently resistant to itraconazole and fluconazole than *Candida albicans*.

Voriconazole and amphotericin B were found to be very active against all species of *Candida*. Hence, these agents should be used in empirical treatment for candidemia rather than fluconazole and itraconazole.

Although resistance to antifungal agents of *Candida* spp. is rare at present, continued surveillance of antifungal susceptibility is necessary in order to monitor trends, and to choose the right empiric therapy.

## REFERENCES

- Babić, M., Hukić, M. (2010): *Candida albicans and non-albicans species as etiological agent of vaginitis in pregnant and non-pregnant women*. Bosnian Journal of Basic Medical Sciences 10 (1): 89- 97.
- Costa-de-Oliveira, S., Pina-Vaz, C., Mendoca, D., Goncalves Rodrigues, A. (2008): *A first Portuguese epidemiological survey of fungemia in a university hospital*. Eur J Clin Microbiol Infect Dis 27: 365-74.

- Florez, C., Martin-Mazuelos, E., Ruiz, M., Cisneros, J. M., Her-  
rero, M., et al. (2009): *In vitro susceptibilities of bloodstream isolates of Candida spp.: results from a multicenter active surveillance program in Andalusia*. *Enferm Infect Microbiol Clin* 27 (9): 518- 22.
- Gonzalez, G. M., Elizondo, M., Ayala, J. (2008): Trends in species distribu-  
tion and susceptibility of bloodstream isolates of *Candida* collected in Monterrey, Mex-  
ico, to seven antifungal agents: results of 3-year (2004 to 2007) surveillance study. *J Clin Microbiol* 46 (9): 2902- 5.
- Kennedy, H. F., Shankland, G. S., Bagg, J., Chalmers, E. A.,  
Gibson, B. E., et al. (2006): *Fluconazole and itraconazole susceptibilities of Can-  
dida spp. isolated from oropharyngeal specimens and blood cultures of pediatric hae-  
matology/oncology patients*. *Mycoses* 49 (6): 457-62
- Mitrović, S. M, Džamić, A. M., Arsić-Arsenijević, V. S.,  
Radonjić, I. V., Kranjčić-Zec, I. F. (2007): *Rezistencija gljiva na an-  
timikotike: mehanizmi nastanka, učestalost, prevencija i kontrola rezistencije*. *Srpski arhiv za celokupno lekarstvo* 135(7-8): 486- 94
- Mueller, F. M., Weig, M., Peter, J., Walsh, T. J. (2000): *Azole cross-re-  
sistance to ketoconazole, fluconazole, itraconazole and voriconazole in clinical Candida  
albicans isolates from HIV-infected children with oropharyngeal candidosis*. *J Antimi-  
crob Chemother* 46: 338-41
- Panizo, M. M., Reviakina, V., Dolande, M., Selgrad, S. (2009): *Can-  
dida spp. In vitro susceptibility profile to four antifungal agents. Resistance surveillance  
study in Venezuelan strains*. *Med Mycol* 47 (2): 137-43
- Peman, J., Canton, E., Gobernado, M. (2005): *Epidemiology and antifungal  
susceptibility of Candida species isolated from blood: results of 2-year multicentre study  
in Spain*. *Eur J Clin Microbiol Infect Dis* 24: 23-30
- Quindos, G., Sanches-Vargas, L. O., Villar-Vidal, M., Eraso, E.,  
Alkorta, M., et al. (2008): *Activities of fluconazole and voriconazole against blood-  
stream isolates of Candida glabrata and Candida krusei: a 14-year study in a Spanish  
tertiary medical centre*. *Int J Antimicrob Agents* 31: 266-71
- Safdar, A., Chaturvedi, V., Koll, BS., Larone, DH., Armstrong,  
D. (2002): *Prospective, multicenter surveillance study of Candida glabrata: fluconazole  
and itraconazole susceptibility profiles in bloodstream, invasive, and colonizing strains  
and differences between isolates from three urban teaching hospitals in New York City*.  
*Antimicrob. Agents Chemother* 46 (10): 3268-72
- Skrodeniene, E., Dambrauskienė, A., Vitkauskienė, A. (2006): *Sus-  
ceptibility of yeasts to antifungal agents in Kaunas University of Medicine Hospital*.  
*Medicina (Kaunas)* 42 (4): 294-9
- Takakura, S., Fujihara, N., Saito, T., Kudo, T., Iinuma, Y., et al.  
(2004) *National surveillance of species distribution in blood isolates of Candida species  
in Japan and their susceptibility to six antifungal agents including voriconazole and  
micofungin*. *Journal of Antimicrobial Chemotherapy* 53: 283-9
- Tasić, S., Miladinović-Tasić, N., Đorđević, J., Zdravković, D.,  
Paunović-Todosijević, D. (2008): *Recurrent intestinal candidosis*. *Acta Fac  
Med Naiss* 25 (4): 189-93

- Tortorano, A. M., Kibbler, C., Peman, J., Bernhardt, H., Klingspor, L., et. al. (2006): *Candidaemia in Europe: epidemiology and resistance*. Int J Antimicrob Agents 27: 359- 66
- Tulumoglu, S., Kariptas, E., Erdem, B. (2009): *Identification and antifungal susceptibility of Candida isolates from various clinical specimens in Doctor Behcet UZ Hospital*. Anatol J Clin Investig 3 (3): 170- 3

## ОСЕТЉИВОСТ НА АНТИМИКОТИКЕ ИЗОЛАТА *CANDIDA* SPP. ИЗ КРВИ И ФЕЦЕСА ПРИКЉУЧЕНИХ У НОВОМ САДУ У ТРОГОДИШЊЕМ ПЕРИОДУ (2008-2010)

Јелесић З. Зора, Медић Д. Деана, Михајловић-Укропина М. Мира, Јевтић Марија,  
Гусман П. Вера, Радосављевић Ј. Биљана, Милосављевић Т. Биљана

Институт за јавно здравље Војводине, Нови Сад

### Rezime

Кандидемија је значајна и све чешћа нозокомијална инфекција код пацијентата са факторима ризика. *Candida* врста пореклом из нестерилних подручја може да укаже на карактеристике сојева који узрокују инвазивну болест.

Циљ овог испитивања био је да се утврди осетљивост сојева *Candida* из крви и фецеса изолованих у Новом Саду, у Војводини.

У току трогодишњег периода, од 2008. до 2010. године, прикупљено је 424 изолата *Candida* spp., 30 изолата из крви и 394 сојева пореклом из узорак фецеса. Осетљивост ових изолата *in vitro* на пет антимикотика утврђено је коришћењем комерцијалног теста *ATB FUNGUS 3 (BioMérieux)*.

Најчешћа врста била је *Candida albicans* (6 изолата из крви и 269 из фецеса). Резистенција на један или више антимикотика била је ређа код *Candida albicans* (3.63%) у односу на друге non-albicans врсте (24.83%). Резистенција је најчешће била изражена на итраконазол код обе испитиване групе, утврђена је код 9.64% сојева пореклом из фецеса и 20% изолата из крви. Мултипла резистенција на три или више антимикотика доказана је код дванаест изолата, углавном на флуконазол, итраконазол и вориконазол. Резистенција на амфотерицин Б била је врло ретка.

Иако је резистенција *Candida* spp. на антимикотике код нас засад ретка појава, потребно је континуирано праћење и истраживање њихове осетљивости у циљу праћења трендова у појави резистентних изолата, али и због избора праве емпиријске терапије.

*Igor O. Gojković<sup>1</sup>, Smiljana D. Paraš<sup>2</sup>,  
Radoslav B. Gajanin<sup>3</sup>, Milica D. Matavulj<sup>4</sup>,  
Vesna K. Rajković<sup>4</sup>, Goran D. Spasojević<sup>5</sup>*

<sup>1</sup> Clinic of Orthopedic Surgery, Clinical Center Banja Luka, RS, BiH

<sup>2</sup> Department of Biology, Faculty of Science, University of Banja Luka, RS, BiH

<sup>3</sup> Department of Pathology, Clinical Center Banja Luka, RS, BiH

<sup>4</sup> Department of Biology and Ecology, Faculty of Science, University of Novi Sad, Serbia

<sup>5</sup> Department of Anatomy, Faculty of Medicine, University of Banja Luka, RS, BiH

## THE INFLUENCE OF EXTREMELY LOW-FREQUENCY ELECTROMAGNETIC FIELD ON THE BASAL GANGLIA STRUCTURES OF THE RAT BRAIN

**ABSTRACT:** We studied the influence of extremely low-frequency electromagnetic field (ELF EMF) to subcortical structures of a brain, i.e. basal ganglia, of sexually mature rats of Wistar strain. The animals were exposed to nonhomogenous ELF EMF, intensity of 50-500  $\mu$ T, 50 Hz frequency, 7 hours a day, and 5 days a week during three months. Histological and stereological analysis established a reduction in volume density of ganglia cells in the area of basal ganglia, an increase of their nucleocytoplasmatic volume ratio, and presence of an intensive edema of pericellular (perineural) type.

**KEY WORDS:** extremely low-frequency electromagnetic field (ELF EMF), basal ganglia, pericellular edema

## INTRODUCTION

Basal ganglia, located in subcortex, are consisted of three large masses of nucleuses: nucleus caudatus, putamen, and globus pallidus, as well as of their functionally connected structures such as nucleus subthalamicus, substantia nigra, and nucleus ruber that are also located immediately below cortex. There are numerous projections of motoric, premotoric cortex, and thalamus to striatum. The striatum projects to the substantia nigra and globus pallidus both directly and indirectly via the subthalamic nucleus, which also receives cortical input. The globus pallidus, from its own side through ansa lenticularis (the main efferent route from basal ganglia) projects itself to ventrolateral, to the front of the ventral nucleus of thalamus, as well as to subthalamic nucleus, nucleus ruber, and brain stem. Since the thalamus is projected to the cortex,

the functional reverberation circle with the elements of back circuit is closed in this way.

Basal ganglia are also distinctive by presence of nigrostrial dopaminergic system (the bodies of the neurons of this system are located in a dark substance while their axons are located in caudate nucleus), by high consumption of oxygen and by significant concentration of copper in their cytoplasm.

In spite of many earlier discoveries precise function of basal ganglia are still insufficient (K o r n h u b e r, 1971). It is believed that they are involved in planning and programming of movements i.e., in the processes that abstract thought, the plan of action, convert into an actual movement.

In the process of studying neurological effects of electromagnetic field (EMF) exposure different parameters of these fields as intensity, frequency, duration of EMF exposure, the co-incidence of the static magnetic field (both the natural earth's magnetic field and anthropogenic fields), the presence of the electrical field, the magnetic field, or their combination have been taken into account and, also, whether electromagnetic field is sinusoidal, pulsed or in more complex wave forms. In our daily lives we are all exposed to different sources of EMFs of extremely low frequency (ELF) (below 300 Hz) and low intensity (below 2 mT), such as residential power installations, domestic electrical appliances, etc. (G a n d h i et al., 2001, G a u g e r, 1985). Question of influence of such ELF-MEMFs on the central and peripheral nervous system remains open and there are many controversial data about this issue (C a r r u b b a et M a r i n o, 2008). The main reason for this is the obvious problem of detecting ELF-EMF effects in classical neurophysiological signals such as electroencephalograms (EEG) or evoked potentials (C o o k et al., 2004). There is evidence that neurological response to ELF EMFs influence include changes of human and monkey response time (H o m e r, 1968, G a v a l a s et al., 1970, G a v a l a s - M e d i c i et D a y - M a g d a l e n o, 1976), changes of EEG, GABA level and changes in calcium ion binding in cerebral tissue of cat and chick (B a w i n and A d e y, 1976, B a w i n et al., 1978, B a w i n et al., 1975) and also changes in expression of brain protein c-Jun in mice (S t r a š á k et al., 2009).

However, until now there are only few data on morphological changes of subcortical basal ganglia influenced by ELF EMF. In this study, we examined the effect of ELF EMF for the characteristics that are the most commonly occurring in human living and working environment, and regarding the structural characteristics of rat's basal ganglia, having in mind that many neurodegenerative diseases, primarily Parkinson disease, are related to the changes of these ganglions.

## MATERIALS AND METHODS

The experiment was performed on 26 male Wistar rats. Animals were housed in laboratory conditions with  $22\pm 2^{\circ}\text{C}$  temperature and subjected to a natural photoperiod. Access to tap water and palate food was unlimited. A

total of 13 animals were exposed to EMF from 24 h after birth, 7 hours a day (from 07:00 A.M. to 14:00 P.M.), 5 days a week for a period of three months. Thirteen animals served as controls and they were maintained in a separate room free of any appliances involved in generation of EMFs. The investigation was made with permission of the Ethical Committee on Animal Experiments of the University of Novi Sad.

The exposure system was made of a single coil of 2.5 mm thick copper wire placed on a wooden frame in 1320 turns. The coil was energized from a standard power supply of 220 V, 50 Hz, and 16 A via an autotransformer. The autotransformer provided a 60 V output and was used in order to reduce the electric field. The value of the electric field at any point in the room was less than 10 V/m. Cages with animals were placed symmetrically on both sides of the coil. The ELF EMF produced by the coil was inhomogeneous and of decaying intensity along the animal cages with a 500  $\mu$ T value on the side of the cage near the coil to 50  $\mu$ T on the opposite side.

After the decapitation, removed brain tissue was fixed in Bouin's solution and processed using a standard procedure for paraffin embedding. Samples were cut in a frontal plane on a rotary microtome (LEICA RM 2125, Leica Microsystems, Wetzlar, Germany) in 4-6  $\mu$ m thick serial sections. For the histological analysis, paraffin slices were stained with hematoxylin-eosin (HE) (both stains by Merck, Darmstadt, Germany). Histological and stereological analysis was performed on every 5<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, 20<sup>th</sup>, 25<sup>th</sup> and 30<sup>th</sup> HE stained section per animal using a multipurpose stereological grid M42 (Weibel et al., 1966) placed in the ocular of a light microscope under a total magnification of x400. The volume density of nucleus and cytoplasm of ganglion cells in subcortical nuclei was determined. The obtained numerical values were used to further calculate the total volume density of ganglion cells as well as the nucleocytoplasmic ratio. The estimations were made by the same observer. The data were statistically analyzed by Student's t-test. *p* values less than 0.05 were considered significant.

## RESULTS

Our histological studies indicated that after three months of exposure to ELF EMFs altered the structure of the rat basal ganglia cells. Damaged neurons were seen as condensed dark neurons, intermingled with normal neurons in basal ganglia (Fig. 1a-b). These dark neurons, as have been proposed by Sugimoto et al. (1990), have three main characteristics: (1) irregular cellular outlines, (2) increased density of chromatin and cytoplasm, and (3) intensely and homogeneously stained nucleus. All these properties regarding dark neuron were recorded in basal ganglia in our EMF exposed animals (Fig. 1b and 2b).



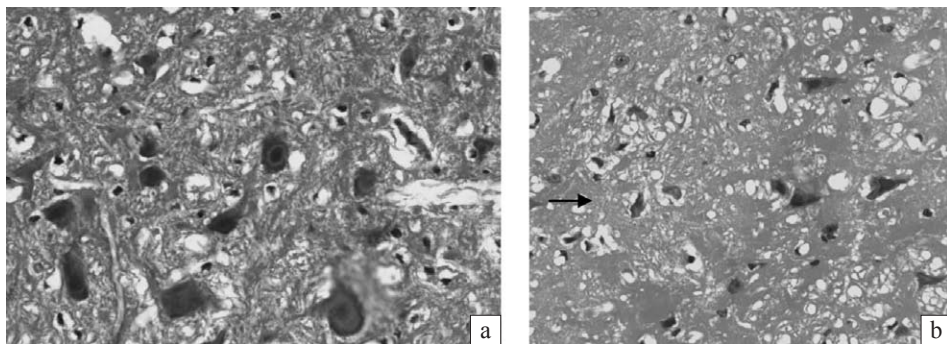


Fig. 1 – Basal ganglia cells; (a) control animal; (b) animal exposed to EMF. Among the normal big and pale blue nerve cells, there are interspersed black and shrunken nerve cells (arrow). Hematoxylin and eosin stain, x400.

Apart from the occurrence of dark neurons, in basal ganglia of the animals exposed to EMF, a great variability was also observed in the size of the cells, as well as in their shape and size of their nuclei (Fig. 2a, b).

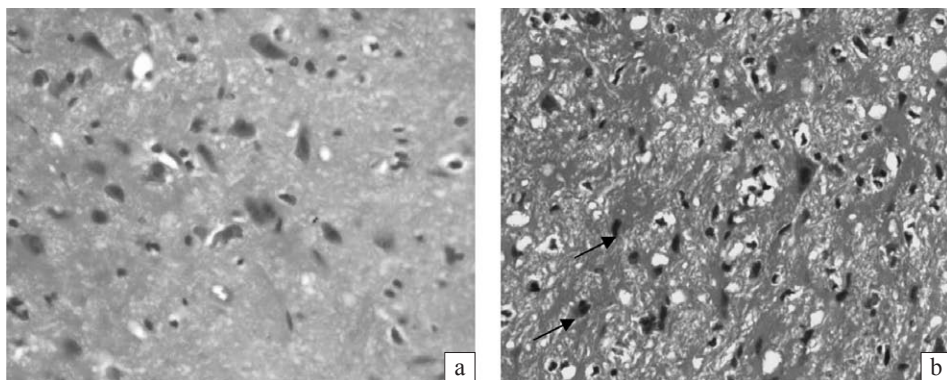


Fig. 2 – Basal ganglia cells; (a) control animal; (b) animal exposed to EMF. Note the shrunken dark blue stained (basophilic) cell bodies (arrow). Adjacent histologically intact neurons are present. Hematoxylin and eosin stain, x400.

Stereological analysis indicated statistically significant decrease of volume density of cytoplasm of EMF exposed animal basal ganglia cells compared to the control animals (Fig. 3).

However, this treatment caused statistically significant increase of volume density of basal cells nuclei compared to control animals (Fig. 4) and significant decrease of the entire volume density of these cells (Fig. 5).

The data from Fig. 3 and Fig. 4 were also used to calculate the changing mean nucleo-cytoplasmic ratio, which was found to significantly increase in animals exposed to ELF EMF (Tab. 1), due mainly to decrease in the volume density of basal cells cytoplasm in this treatment.

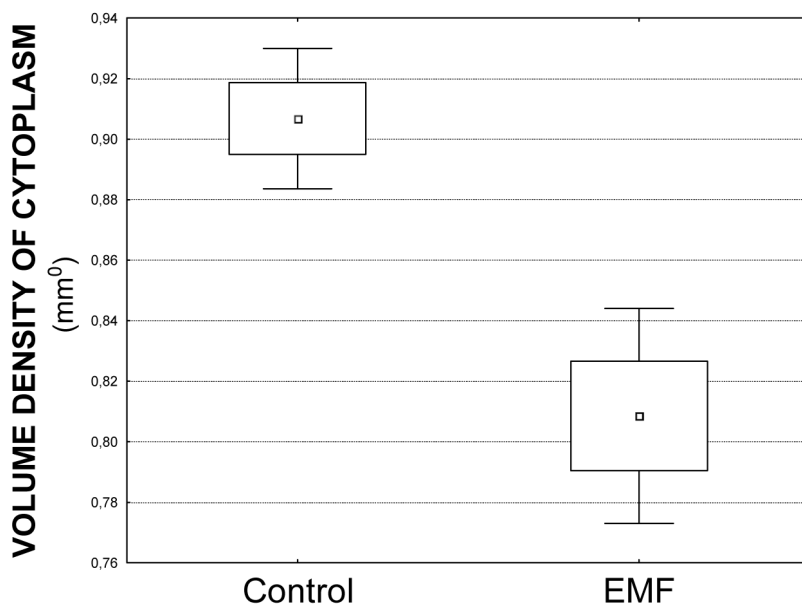


Fig. 3 – Volume density of the basal ganglia cell cytoplasm in control animals and animals exposed to EMF.

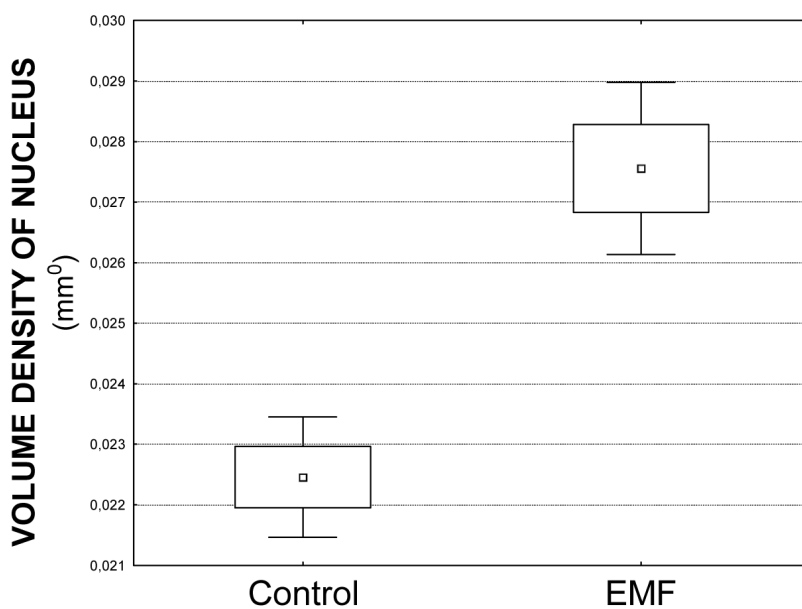


Fig. 4 – Volume density of the basal ganglia cell nuclei of control animals and animals exposed to EMF.



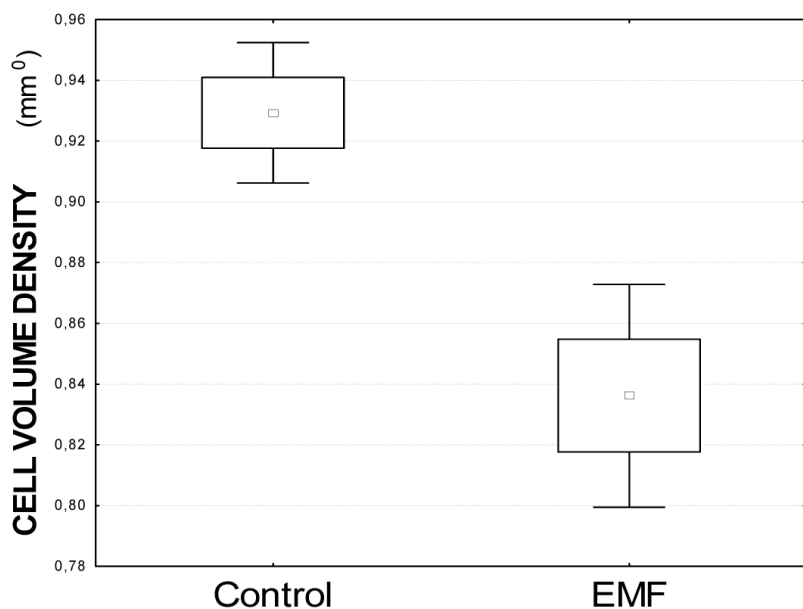


Fig. 5 – Volume density of the basal ganglia cells of control animals and animals exposed to EMF.

Tab. 1 – Mean values with the standard error of the mean (SE) of nucleo-cytoplasmic volume ratio of basal ganglion cells of control and EMF exposed animals are given.

	Control	EMF	p-level
Nucleocytoplasmic ratio	0.0248 ± 0.0007	0.0341 ± 0.0006	0.000000

Besides the change in the shape and volume of basal ganglia neurons of animals exposed to EMF, numerous small vacuole are observed in cytoplasm and, also, bubbly appearance of chromatin in their nuclei (Fig. 6).

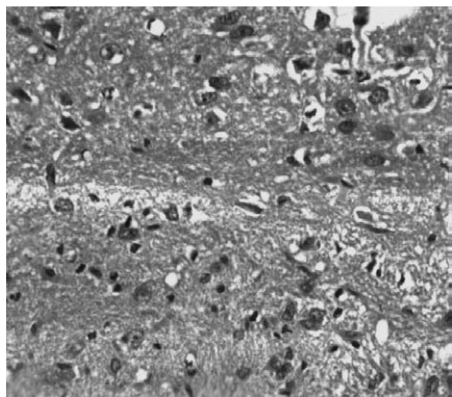


Fig. 6 – Basal ganglia cells. Animal exposed to EMF. The prominent cytoplasmic vacuolization. Hematoxylin and eosin stain, x400.

Well distinguished change in basal ganglia of the animals treated with EMF is an incidence of intensive edema of pericellular (perineural) type with clearly distinguished, blank, white, round or improperly shaped haloes, resistant to color and filled with water (Fig. 7 and 8a, b).

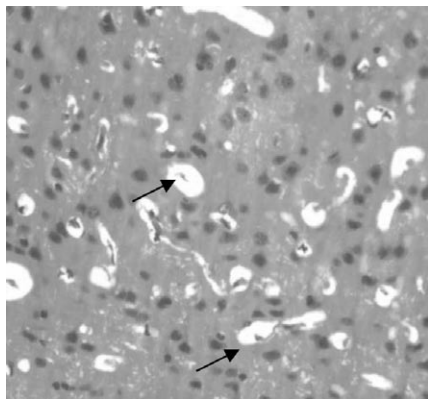


Fig. 7 – Basal ganglia cells. Animal exposed to EMF. Perineural edema (arrow) is observed. Hematoxylin and eosin stain, x400.

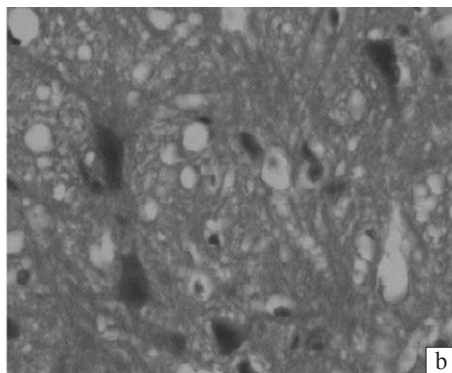
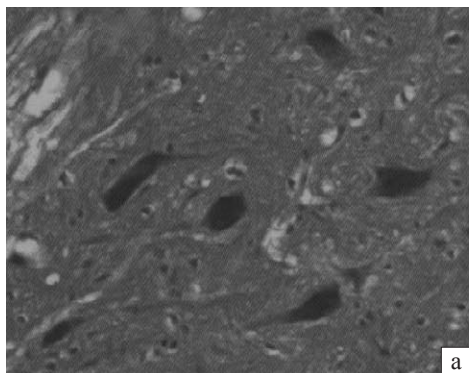


Fig. 8 – Basal ganglia cells; (a) control animal; (b) animal exposed to EMF, area with very prominent perineural edema. Hematoxylin and eosin stain, x400.

## DISCUSSION

In our study, we found evidence for neuronal damage caused by ELF EMFs. Damaged neurons and very prominent perineural edema were recorded in the basal ganglia in the brains of EMF exposed rats. Changes described here would seem to indicate a serious neuronal damage, which may be mediated through some organelle damage and blood-brain barrier (BBB) leakage.

Damaged neurons assigned as dark neurons that occurred in basal ganglia in our experimental condition also occurred after exposure to GSM (Global System for Mobile Communications) (Nittby et al., 2009, Eberhardt et al., 2008), in connection to experimental ischemia (Kövesdi et al., 2007),

hypoglycemia (Gallias et al., 2005), and epilepsy (Södenfeldt et al., 1983). Many authors suggest that the BBB leakage is the major reason for nerve cells injury and appearance of dark neurons (Fredriksson et al., 1988 Salahuddin et al., 1998, Sokrab et al., 1988, Hassel et al., 1994). Physiologically, the central nervous system (CNS) microvasculature differs from that of peripheral organs. It is characterized not only by its tight junctions, which seal cell-to-cell contacts between adjacent endothelial cells, but also by the low number of pinocytotic vesicles for nutrient transport through the endothelial cytoplasm and its lack of fenestrations; and the five-fold higher number of mitochondria in BBB endothelial cells compared to muscular endothelia in rat (Oldendorf et al., 1977). All this speaks in favor of an energy-dependent transcapillary transport. Electromagnetic fields increase permeability of BBB (Nittby et al., 2008). When this barrier is damaged in some pathological conditions, the normally excluded molecules can pass into the brain tissue. EMFs have the potency to significantly open the BBB such that the animal's own albumin passes out of the bloodstream into the brain tissue and accumulates in the neurons and in glial cells surrounding the capillaries (Salford et al., 1992).

The results obtained in this study indicate that ELF EMF affects basal ganglia, causing reduction in their volume and increasing their mean nucleocytoplasmic ratio. Observed an intensive edema, which is mainly of pericellular type, can damage the cells that at the end it results in cells death. This is a cytotoxic edema and in CNS it is always a consequence of hypoxia (Aarts and Tymiansky, 2005). As a rule, hypoxia causes cell membrane damage and also causes disturbance of cellular processes as production of high-energy phosphate and an ion balance. Hypoxia is a consequence of mitochondrion and oxidative phosphorylation cycle damage, resulting in reduced ATP production. This primary influences on active transport, which mostly depends on ATP-dependant Na/K pump. The lack of ATP causes malfunction of electrogenic pump, and that is why many Na and Cl ions enter the cell, causing an increase of intracellular osmolarity and as a consequence the water entrance to (maybe as a consequence of water APQ channels activations) cell causes its swelling. Swelling induces an increase of cell volume, damaging of cell membranes and organelles, and also induces losing of membrane phospholipids and staining ability of nucleus. Reduced ATP production is, as a rule, followed by the activation of phosphofructokinase enzyme, increased decomposition of glycogen and production of lactic acid, pH reduction, and the increase of intracellular acidosis. The acidosis caused in this way brings up the activation of non-active acid-sensitive ionic channels such as ASIC, SUR-1, NC-ca and TRP channels (Allen and Attwell, 2002). In swelling cell concentration of intracellular  $Ca^{2+}$  rises as a consequence of its increased entrance in cell and its release from depot of cell (Xiong et al, 2006). In addition, calcium enters in mitochondria and causes additional inhibition of oxidative phosphorylation (MacDonald et al., 2006).

However, despite the large number of experiments conducted, there is yet no consistent scientific evidence in support of a plausible neurocarcinogenic mechanism for ELF-EMF (50- or 60-Hz) exposure. A possible hypothesis is

that ELF-EMF affects cell membrane structure and its permeability to small molecules (Baureus Koch et al., 2003; Grassi et al., 2004; Marino et al., 2003). Also, some data from literature have described redox-related cellular changes following ELF-EMF exposure (Regoli et al., 2005; Wolf et al., 2005; Zwirska-Korczala et al., 2005). According to this theory, ELF-EMF may interfere with chemical reactions involving free radical production (Simk'o et Mattsson, 2004). These effects could be even more pronounced in neuronal cells, partly due to relatively low levels of antioxidant defenses and, but mainly, because of great amounts of polyunsaturated fatty acids in their membranes what is potential target of oxidative attack (Falone et al., 2007). It is generally recognized that neuronal cells are very susceptible to oxidative injury and, in addition, some studies have evidenced greater incidence of tumors in human nervous system after exposure to ELF-EMF (reviewed by Feychting et al., 2005). Results of Falone et al., (2007) support redox-mediated ELF-EMF biological effects. They observed a positive modulation of antioxidant defenses as well as a shift of cellular environment towards a more reduced state after exposure to these fields.

The results obtained in this study may be significant in the light of evidence that Parkinson's disease causes reduction in total number of cells in basal ganglia. Nowadays, Parkinson's disease almost becomes epidemic and cannot be interpreted only by extended life span of human race, but also by drawing attention to the environmental factors, such as electromagnetic fields present in highly urban society.

## REFERENCES

- Aarts, M. M., Tymiansky, M. (2005): *TRPM-7 and ischaemic CNS injury*. *Neuroscientist*, 11: 116-123.
- Aarts, M. M., Lihara, K., Wei, W. I., Xiong, Z. G., Arudine, M., Cerwinski, W., MacDonald, J. F., Tymianski, M. (2003): *A key role for TRPM-7 channels in anoxic neuronal death*. *Cell*, 115: 863-877.
- Allen, N. J., Attwell, D. (2002): *Modulation of ASIC channels in rat cerebellar Purkinje neurons by ischemia-related signals*. *J Physiol*, 543: 521-529.
- Baureus Koch, C. L., Sommarin, M., Persson, B. R., Salford, L.G., Eberhardt, J. L. (2003): *Interaction between weak low frequency magnetic fields and cell membranes*. *Bioelectromagnetics*, 24: 395-40
- Bawin, S. M., Adey, W. R. (1976): *Sensitivity of calcium binding in cerebral tissue to weak environmental electric fields oscillating at low frequency*. *Proc Nat Acad Sci*, 73 (6): 1999-2003.
- Bawin, S. M., Adey, W. R., Sabbot, I. M. (1978): *Ionic factors in release of  $^{45}\text{Ca}^{2+}$  from chicken cerebral tissue by electromagnetic fields*. *Proc Nat Acad Sci*, 75 (12): 6314-6318.
- Bawin, S. M., Kaczmarek, L. K., Adey, W. R. (1975): *Effects of modulated vhf fields on the central nervous system*. *Ann N Y Acad Sci*, 247: 74-81.
- Carubba, S., Marino, A. A. (2008): *The effects of low-frequency environmental-strength electromagnetic fields on brain electrical activity: a critical review of the literature*. *Electromagn Biol Med*, 27(2): 83-101.

- Cook, C. M., Thomas, A. W., Prato, F. S. (2004): *Resting EEG is affected by exposure to a pulsed ELF magnetic field*. *Bioelectromagnetics*, 25(3): 196–203.
- Eberhardt, J. L., Persson, B. R., Brun, A. E., Salford, L. G., Malmgren, L. O. (2008): *Blood–brain barrier permeability and nerve cell damage in rat brain 14 and 28 days after exposure to microwaves from GSM mobile phones*. *Electromagn Biol Med*, 27: 215–229.
- Falone, S., Grossi, M. R., Benedetta Cinque, B., D’Angelo, B., Tettamanti, E., Cimini, A., Di Ilio, C., Amicarelli, F. (2007): *Fifty hertz extremely low-frequency electromagnetic field causes changes in redox and differentiative status in neuroblastoma cells*. *Internat J Biochem Cell Biol*, 39: 2093–2106.
- Feychting, M., Ahlbom, A., Kheifets, L. (2005): *EMF and health*. *Annu Rev Public Health*, 26: 165–189.
- Fredriksson, K., Kalimo, H., Norberg, C., Johansson, B. B., Olsson, Y. (1988): *Nerve cell injury in the brain of stroke-prone spontaneously hypertensive rats*. *Acta Neuropathol. (Berl)*, 76: 227–237.
- Gallyas, F., Csordás, A., Schwarcz, A., Mázló, M. (2005): *“Dark” (compacted) neurons may not die through the necrotic pathway*. *Exp Brain Res*, 160: 473–486.
- Gavalas, R. J., Walter, D. O., Hamer, J., Adey, W. R. (1970): *Effect of low-level, low-frequency electric fields on egg and behavior in macaca nemestrina*. *Brain Res*, 18(3): 491–501.
- Gavalas-Medici, R., Day-Magdaleno, S. R. (1976): *Extremely low frequency weak electric fields affect schedule-controlled behavior of monkeys*. *Nature*, 261 (5557): 256–259.
- Gandhi, O. P., Kang, G., Wu, D., Lazzi, G. (2001): *Currents induced in anatomic models of the human for uniform and nonuniform power frequency magnetic fields*. *Bioelectromagnetics*, 22 (2): 112–121.
- Gauger, J. R. (1985): *Household appliance magnetic field survey*. *IEEE Trans Power Appar Syst (PAS)*104(9): 2436–2444.
- Grassi, C., D’Ascenzo, M., Torsello, A., Martinetti, G., Wolf, F., Cittadini, A., Azzena, G. B. (2004): *Effects of 50 Hz electromagnetic fields on voltage-gated Ca<sup>2+</sup> channels and their role in modulation of neuroendocrine cell proliferation and death*. *Cell Calcium*, 35: 307–315.
- Hamer, J. (1968): *Effects of low level, low frequency electric fields on human reaction time*. *Comm Behav Biol*, 2 (5 part A): 217–222.
- Hassel, B., Iversen, E. G., Fonnum, F. (1994): *Neurotoxicity of albumin in vivo*. *Neurosci Lett*, 167: 29–32.
- Kornhuber, H. H. (1971): *Motor functions of the cerebellum and basal ganglia. The cerebellocortical saccadic (ballistic) clock, the cerebellonuclear hold regulator and the basal ganglia ramp (voluntary speed smooth movement) generator*. *Kybernetik*, 8: 157–163.
- Kövesdi, E., Pál, J., Gallyas, F. (2007): *The fate of “dark” neurons produced by transient focal cerebral ischemia in a non-necrotic and non-excitotoxic environment: neurobiological aspects*. *Brain Res*, 1147: 272–283.
- Marino, A. A., Kolomytkin, O. V., Frilot, C. (2003): *Extracellular currents alter gap junction intercellular communication in synovial fibroblasts*. *Bioelectromagnetics*, 24: 199–205.
- MacDonald, J. F., Xiong, Z. G., Jackson, M. F. (2006): *Paradox of Ca signaling, cell death and stroke*. *Trends Neurosci*, 29: 75–81.

- Mongin, A. A., Aksentsev, S. L., Orlov, S. N., Kuacheva, Z. B., Mezen, N. I., Fedulov, A. S. (1999): *Swelling induced activation of Na, K, Cl cotransport in C6 glioma cells: kinetic properties and intracellular signalling mechanism*. *Biochim Biophys Acta*, 1285: 229–236.
- Nittby, H., Brun, A., Eberhardt, J., Malmgren, L., Persson, B. R. R., Salford, L. G. (2009): *Increase blood-brain barrier permeability in mammalian brain 7 days after exposed to radiation from GSM-900 mobile phone*. *Pathophysiology*, 16: 103–112.
- Nittby, H., Grafström, G., Eberhardt, J., Malmgren, L., Brun, A., Persson, B. R. R., Salford, L. G. (2008): *Radiofrequency and extremely low-frequency electromagnetic field effects on the blood–brain barrier*. *Electromagn Biol Med*, 27: 103–126.
- Oldendorf, W. H., Cornford, M. E., Brown, W. J. (1977): *The large apparent work capability of the blood-brain barrier: A study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat*. *Annals Neurol*, 1: 409–417.
- Pavlov, A. N., Tupitsyn, A. N., Legros, A., Beuter, A., Mosekilde, E. (2007): *Using wavelet analysis to detect the influence of low frequency magnetic fields on human physiological tremor*. *Physiol Meas*, 28: 321–333.
- Regoli, F., Gorbi, S., Machella, N., Tedesco, S., Benedetti, M., Bocchetti, R., Notti, A., Fattorini, D., Piva, F., Principato, G. (2005): *Pro-oxidant effects of extremely low frequency electromagnetic fields in the land snail *Helix aspersa**. *Free Radic Biol Med*, 39: 1620–1628.
- Salahuddin, T. S., Kalimo, H., Johansson, B. B., Olsson, Y. (1988): *Observations on exudation of fibronectin, fibrinogen and albumin in the brain after carotid infusion of hyperosmolar solutions. An immunohistochemical study in the rat indicating long-lasting changes in the brain microenvironment and multifocal nerve cell injuries*. *Acta Neuropathol (Berl)*, 76: 1–10.
- Salford, L. G., Brun, A., Eberhardt, J., Malmgren, L., Persson, B. B. (1992): *Electromagnetic field-induced permeability of the blood–brain barrier shown by immunohistochemical methods*. In: B Nordén, C Ramel, Eds., *Interaction mechanism of low-level electromagnetic fields*. Living Systems, Oxford University Press, Oxford, UK, pp. 251–258.
- Simk'o, M., Mattsson, M. O. (2004): *Extremely low frequency electromagnetic fields as effectors of cellular responses in vitro: Possible immune cell activation*. *J Cellular Biochem*, 93: 83–92.
- Strašák, L., Bártová, E., Krejčí, J., Fojt, L., Vetterl, V. (2009): *Effects of ELF-EMF on brain proteins in mice*. *Electromagn Biol Med*, 28(1): 96–104.
- Söderfeldt, B., Kalimo, H., Olsson, Y., Siesjö, B. K. (1983): *Bicuculline induced epileptic brain injury. Transient and persistent cell changes in rat cerebral cortex in the early recovery period*. *Acta Neuropathol*, 62: 87–95.
- Sokrab, T. E. O., Johansson, B. B., Kalimo, H., Olsson, Y. (1987): *A transient hypertensive opening of the blood-brain barrier can lead to brain damage*. *Acta Neuropathol*, 75 (6): 557–565.
- Sugimoto, T., Bennett, G. J., Kajander, K. C. (1990): *Transsynaptic degeneration in the superficial dorsal horn after sciatic nerve injury: effects of a chronic constriction injury, transaction and strychnine*. *Pain*, 42: 205–213.



- Ueki, Y., Mima, T., Ali, K. M., Sawada, H., Saiki, H., Ikeda, A., Begum, T., Reza, F., Nagamine, T., Fukuyama, H. (2006): *Altered plasticity of the human motor cortex in Parkinson's disease*. Ann Neurol, 59: 60–71.
- Villarini, M., Moretti, M., Scassellati-Sforzolini, G., Boccioli, B., Pasquini, R. (2006): *Effects of co-exposure to extremely low frequency (50 Hz) magnetic fields and xenobiotics determined in vitro by the alkaline comet assay*. Sci Total Environ, 361: 208–219.
- Weibel, E. R., Kistler, D. S., Shere, X. F. (1966): *Practical stereological methods for morphometric cytology*. J Cell Biol, 30: 23–29.
- Wolf, F. I., Torsello, A., Tedesco, B., Fasanella, S., Boninsegna, A., D'Ascenzo, M., Grassi, C., Azzena, G. B., Cittadini, A. (2005): *50-Hz extremely low frequency electromagnetic fields enhance cell proliferation and DNA damage: possible involvement of a redox mechanism*. Biochem Biophys Acta, 1743(1–2): 120–129.
- Zwirska-Korczala, K., Jochem, J., Adamczyk-Sowa, M., Sowa, P., Polaniak, R., Birkner, E., Latocha, M., Pilc, K., Suchanek, R. (2005): *Effect of extremely low frequency of electromagnetic fields on cell proliferation, antioxidative enzyme activities and lipid peroxidation in 3T3-L1 preadipocytes-An in vitro study*. J Physiol Pharmacol, 56 (Suppl 6): 101–118.
- Xiong, Z. G., Chu X. P., Simon, R. P. (2006): *Ca<sup>2+</sup> permeable acid-sensing ion channels and ischemic brain injury*. J. Membr Biol, 209: 59–68.

## УТИЦАЈ ЕКСТРЕМНО НИСКО-ФРЕКВЕНТНОГ ЕЛЕКТРОМАГНЕТНОГ ПОЉА НА СТРУКТУРЕ БАЗАЛНИХ ГАНГЛИЈА МОЗГА ПАЦОВА

Игор О. Гојковић<sup>1</sup>, Смиљана Д. Параш<sup>2</sup>, Радослав Б. Гајанин<sup>3</sup>, Милица Д. Матавуљ<sup>4</sup>, Весна К. Рајковић<sup>4</sup>, Горан Д. Спасојевић<sup>5</sup>

<sup>1</sup> Клиника за ортопедску хирургију, Клинички центар Бања Лука, 12 беба бб, 78000 Бања Лука, Република Српска, Босна и Херцеговина

<sup>2</sup> Одељење за биологију факултета наука, Универзитет у Бањалуци, Младена Стојановића 2, 78000 Бања Лука, Република Српска, Босна и Херцеговина

<sup>3</sup> Одељење за патологију, Клинички центар Бања Лука, 12 беба бб, 78000 Бања Лука, Република Српска, Босна и Херцеговина

<sup>4</sup> Одељење за биологију и екологију Факултета наука, Универзитет у Новом Саду, Трг Доситеја Обрадовића 2, 21000 Нови Сад, Србија

<sup>5</sup> Одељење за анатомију, Медицински факултет, Универзитет у Бањалуци, Саве Мркаља 14, 78000 Бања Лука, Република Српска, Босна и Херцеговина

### Резиме

У нашем раду смо проучавали утицај екстремно ниско-фреквентног електромагнетног поља (ENF EMP-a) на субкортикалне структуре мозга, односно базалне ганглије, полно зрелих пацова соја Вистар. Животиње су излагане дејству нехомогеног ENF EMP-a јачине 50-500  $\mu$ T, фреквенције 50 Hz, 7 сати дневно, 5 дана седмично током 3 месеца. Хистолошка и стереолошка анализа је показала смањење волуменске густине ганглијских ћелија у подручју базалних ганглија, пораст њиховог нуклео-цитоплазматског односа и присуство интензивног едема перицелуларног (перинеуралног) типа.

*Dragica M. Stanković<sup>1</sup>, Borivoj Đ. Krstić<sup>2</sup>,  
Mirjana T. Šijačić-Nikolić<sup>1</sup>, Milan N. Knežević<sup>1</sup>*

<sup>1</sup> Faculty of Forestry, University of Belgrade, Kneza Višeslava 1, 11000 Belgrade

<sup>2</sup> Faculty of Natural Sciences, University of Novi Sad, Trg D. Obradovića 2, 21000 Novi Sad.

## CONCENTRATIONS OF MANGANESE AND IRON IN SOME WOODY AND HERBS PLANTS

**ABSTRACT:** Heavy metals are the substances that indicate environmental pollution. The plants polluted with heavy metals may endanger natural environment and cause health problems in humans. In our multidisciplinary research of the concentrations of pollutants in forest ecosystems and natural environment in Belgrade, we examined the contents of heavy metals essential for plants but harmful in greater concentrations on a long-term basis. The fact that heavy metals manganese and iron are accumulated in plants to the greatest extent focused our work on determination of the level of concentrations of Mn and Fe in the vegetative parts of 8 plant types on three locations on the Avala Mountain and one location in the centre of the city of Belgrade. The analyses of heavy metals contents in plants were performed by the method of flame atomic absorption spectrophotometry. The examination of the existence of important differences between the average values was performed by implementation of Duncan's test for the level of significance of 95%. The current contents of heavy metals in plants in the area of the protected natural resource *Avala* do not represent danger that would presently cause notable damage to forests but show the tendency of the increase of concentrations. Therefore, this issue should be constantly monitored.

**KEY WORDS:** Avala mountain, protected natural resource, concentrations Fe, Mn, woody, herbs plants

## INTRODUCTION

As a two-million city, administrative, and tourist centre, Belgrade on one hand has a constant need for new areas for relaxation and recreation providing natural environment and esthetic framework. On the other hand, there is a permanent need for preservation of healthy environment in the manner of cherishing, preservation, and protection of already existent high-grade complexes.

One of such high-grade forest complexes and the only mountain near Belgrade, the favorite resort of inhabitants of Belgrade, is located as 16.5 km away from Belgrade, on the way to Kragujevac and overlooking the city and its surroundings as a guardian. Although only 511 m high (in the shape of an irregular cone), this mountain reigns the surrounding rolling terrain.



As a natural resource of common interest, it enjoys special protection. As a preserved part of nature park and landscape of outstanding features this area has permanent ecological, scientific, cultural, educational, health and recreational, as well as tourist significance.

Considering the current status of Belgrade's natural environment, the forests in the narrow as well the wider city zone must be given much greater importance.

As the causes and effects that endanger natural ecosystems of this area and their further development and natural environment are obvious to a certain extent, it was logical that our aim was to determine the pollution level of the protected area of *Avala*.

The protected natural resource *Avala* in accordance with the suggestion of the Institute for Nature Conservation of Serbia is declared the landscape of outstanding features.

According to the Special basis for management of forests (2008-2017) in the protected natural resource *Avala*, i.e., its part that is declared the landscape of outstanding features, is located on the territory Belgrade, municipality of Voždovac, and comprises certain parts of the cadastral municipalities of Beli Potok, Ripanj, Zuce, and Pinosava.

The total area of the protected natural resource is 489.13 ha; 74.35 ha (15.2%) is privately owned and 414.78 ha (84.8%) belongs to other forms of ownership.

## MATERIALS AND METHODS

Bearing in mind the importance of the quality of natural environment in the areas intended for recreation, we studied the content of heavy metals (Fe and Mn) in plant leaves on the Avala Mountain. The samples for the analysis were collected from three locations with outstanding landscape features in the area of *Avala*.

The selected locations, according to the Special basis for management of forests (2008-2017) in the protected natural resource *Avala*, belong to management unit *Avala*: location 1 – at the road entering the area of the landscape of outstanding features *Avala*; location 2 – on the top of the Avala mountain (nearby the tower); and location 3 – on the downward road from the mountain (Stari Majdan).

After review and consideration of several potential locations, we selected the location 4 as a control location in the centre of Belgrade, at the Boulevard Despota Stefana where there is high traffic frequency.

## MATERIALS AND METHODS

In the process of selecting plant species for the analysis, we performed a very detailed inventory list of all plant species present in this area. We selected 8 plant species: (1) *Tilia tomentosa* Mnch. – Silver Lime; (2) *Pinus nigra* Arn.

– European black pine; (3) *Plantago media* L. – Hoary plantain; (4) *Taraxacum officinale* Web. – common dandelion; (5) *Acer campestre* L. – Field Maple; (6) *Prunus avium* L. – wild cherry, sweet cherry, gean; (7) *Quercus petraea* (Matt.) Liebl. – sessile oak; and (8) *Pseudotsuga menziesii*; (9) (Mirb.) Franco – coast Douglas fir, common Douglas-fir, or Oregon Douglas fir.

For the purpose of control, we selected 4 plant species in the location 4 in the centre of the city as the representative sample.

Assuming again the fact that content of heavy metals in plants is directly dependant on distance from the road the plants were sampled in each location in the length of 200-300 m along the road and the depth of 15 m from the road. The samples of plant materials were collected on each location in the middle of vegetation period, at the beginning of July 2009.

For each species, only leaves were sampled and for that purpose 1-2 kg of material were collected from all locations. The samples were dried without previous washing until they became air-dry mass. The air-dry leaves were then dried further in dry kiln at 105°C, ground, and used for laboratory analyses. Mn and Fe concentrations were determined by AAS method and expressed in µg/g of dry matter.

The analyses were performed in the laboratories of the Department for Biology and Ecology, Faculty of Natural Sciences in Novi Sad and the Institute for Lowlands Forestry and Environmental Protection in Novi Sad.

The obtained data on accumulation of heavy metals were processed by implementation of the standard statistical methods, variance analysis by LSD test for the level of significance  $p=0.05$  and the testing of significance of the average values was performed by Duncan's test.

All tests were performed with the level of significance  $p \leq 0.05$ .

## RESULTS AND DISCUSSION

Environmental pollution by chemical substances is one of the most important factors of degradation of certain components of ecosystem. Therefore, it is important to be aware of accumulation and toxic effects of heavy metals in plants from ecological point of view since this is the way heavy metals enter food chain (K a s t o r i , 1997, M e m o n et al., 2001).

The level of heavy metals accumulation in plant tissue is determined by numerous biotic and abiotic factors out of which the characteristic genotype is one of the most dominant (P a j e v i ć et al., 2008; N i k o l i ć et al., 2008). For some heavy metals there apply the interspecies calibration (B e r l i z o v et al., 2007). The accumulation of heavy metals in plant tissue points to the very important role of certain plant species as (bio)indicators of environmental pollution (T e n - H o u t e n , 1983; P r a s a d and F r e i t a s , 2003).

Nowadays, more attempts are made to use woody plants for monitoring environmental pollution. Critical values for individual heavy metal, plant resistance to their presence, cumulative and perhaps synergetic activities, and many other aspects that are very little known still present a problem.

Among heavy metals that are most dangerous for human organism are arsenic, cadmium, lead, nickel, manganese, mercury, and molybdenum even when they are present in a low level. Also very toxic but with special effect on plant growth are the zinc, lead, aluminum, boron, chromium, and iron (Kastori and Petrović, 1993).

Heavy metals are the substances that signalize the issue of environmental pollution. There is still insufficient knowledge of their dispersion in nature and their role in environmental pollution.

## MANGANESE CONCENTRATIONS IN PLANTS

Heavy metals contents in herbs, moss, lichen or fungi and needles and leaves of forest trees may be used as indicators of pollution of certain components of ecosystems (Tyler, 1972). Among different plant species, there are differences in the uptake of heavy metals, which depends before all on their genetic characteristics, on the influence of the surface of root system and its capacity for absorption of ions, on the shape of root excretion and the speed of evapotranspiration (Alloway, 1995).

Manganese represents an essential element for plants, which means that in certain concentrations it is necessary for regular functioning of plants since it activates a number of enzymes. It is also very important in the transmission of electrons during photooxidation of water in photosystem II (Kastori, 2006). This element influences the level of phytohormone auxin and in that manner has an effect on elongation and plant growth. On the contrary, greater concentrations of manganese have adverse effects on plants.

The symptoms of toxicity of manganese appear on older leaves in the form of brown necrotic spots or there appear chlorotic spots near the tops of leaves. In dicotyledons, this causes the phenomenon of wavy leaves. The first signs of toxicity of manganese on plants are chlorosis and necrosis.

The role of manganese in life processes of plants is manifold, before all in activation of enzyme processes, the effect on pigment platelets (Bottrell et al., 1970), nitrogen metabolism or rather metabolism of amino acids and proteins.

There are no national regulations that refer to pollution of environment by manganese or heavy metals but there are standards and regulations in healthcare sector like The Rulebook on the quality of food and The Rulebook on the quantity of pesticides, metals, and metalloids that may be present in food products. In accordance with the regulations of the Federal Republic of Germany and the United States of America, the maximum allowed concentration of manganese in air amounts to 5 mg/m<sup>3</sup> of air (UNEP-UN/ECE 1994).

Some plant species are able to accumulate very high quantities of heavy metals in leaves before all (Baker and Brooks, 1989).

The results of laboratory analyses of manganese accumulation in the researching area (Fig 1,2,3,4) showed that concentration in the analyzed

plant species differed depending on the location where samples were collected and depending on the plant species.

We found higher manganese concentration in plant species on the locations 1, 2, and 3 (locations on the Avala) compared to the location 4 in the centre of Belgrade.

The average least concentration of manganese at the control location in the centre of Belgrade in the Boulevard Despota Stefana amounting to 48 µg/g clearly showed that the increase of concentrations of manganese in plants was not directly related to traffic as the basic source.

The manganese contents depend on plant species and have a wide range; in accordance with the data provided by K á r p á t i et al. (1967) manganese contents in the dry matter of *Lemna trisulca* amounted to 34.600 ppm and in *Plantago altissima* only 10 ppm.

The concentrations of manganese in leaves of plants by locations (Fig. 1-4) clearly showed that woody plants appeared as hyperaccumulators of manganese; oak in the locations on the Avala Mountain and lime tree (*Tilia tomentosa*) on the location 4 appeared as hyperaccumulator of manganese in comparison to other analyzed species.

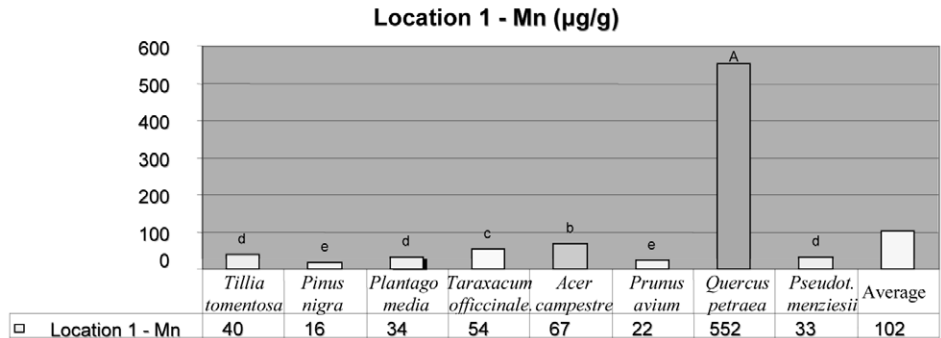


Fig. 1

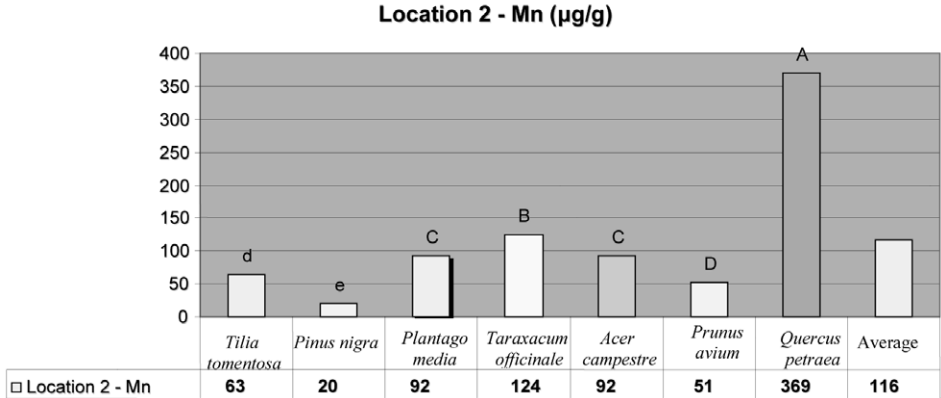


Fig. 2

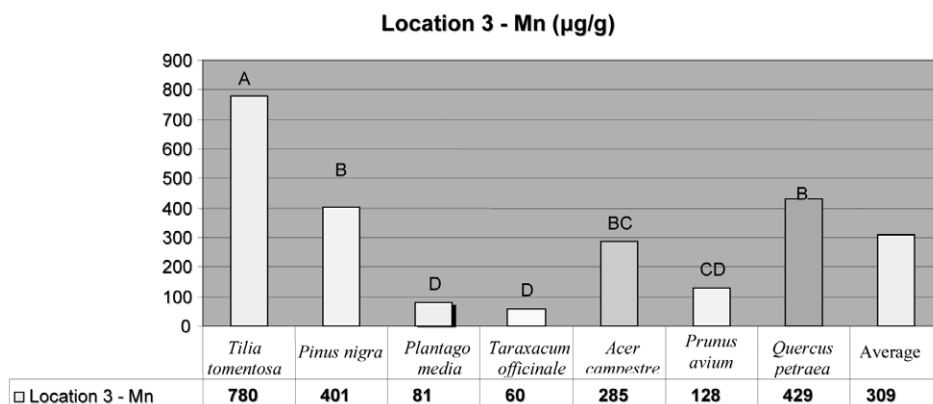


Fig. 3

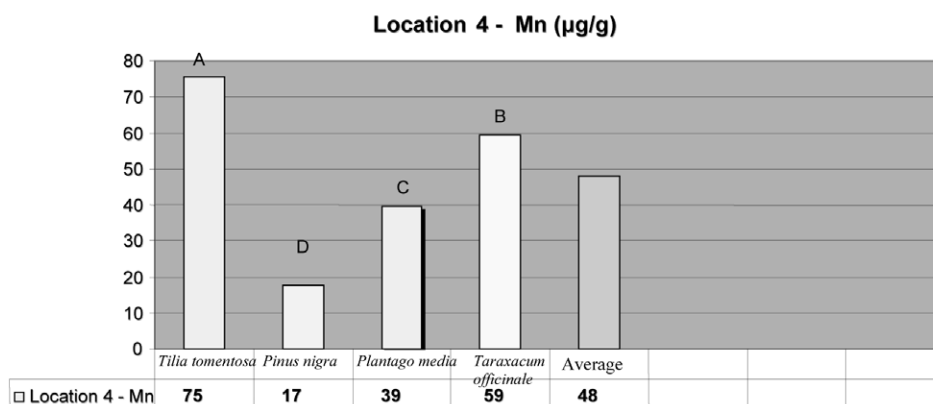


Fig. 4

The data obtained in our research on manganese concentrations by locations based of Duncan's test showed that the ranges varied on all locations from A to E.

Discussing the quantity of heavy metals in woody plants, we have to take into account that these data were obtained within a short time period of their life cycle. Manganese is transported through plants and mostly deposits in young plant organs (leaves), then in the bark, and the least deposits can be found in root or xylem juice (K a d o v i ć et al., 2002).

## IRON CONCENTRATIONS IN PLANTS

Iron is a biogenic element present in plants in low quantities. It is polyvalent and has an effect on numerous physiological and biochemical processes. It has the capacity to form chelates, which are complex compounds of metals

with certain organic compounds. Plants absorb iron from the soil in the form of ferrous ions ( $\text{Fe}^{2+}$ ), ferric ions ( $\text{Fe}^{3+}$ ), and in the form of iron chelates.  $\text{Fe}^{++}$  is physiologically active.

The high pH value, high concentration of phosphates and calcium ions influence the reduced uptake of iron. Iron plays a very important role in biosynthesis of chlorophyll, the first phases of photosynthesis, respiration, fixation of elemental nitrogen, reduction of nitrates and nitrites, metabolism of carbohydrates. It also affects cell division, elongation of plants, and root growth.

Iron contents in dry matter of plants have wide range from 50 to 1000  $\mu\text{g/g}$ . Some plants are able to accumulate iron in significantly greater quantities so that for example leaves of spinach contains up to 3000  $\mu\text{g Fe g}^{-1}$  of dry matter.

Distribution of iron within plants is very characteristic. In the aboveground plant parts, it is the most present in leaves then in stem and grain. Plants take out of the soil more Fe than any other microelement through their crops.

In case of surplus of Fe the growth of all vegetative organs is inhibited. Leaves become dark to blue-greenish, root gains umber color. The symptoms of surplus of Fe are usually followed by the signs of deficiency of phosphorous and manganese (S t a n k o v i ć, 2006).

The analysis of results (Fig. 5 to 8) showed that concentrations of iron by average values per locations ranged from 324 to 1065  $\mu\text{g/g}$ .

Iron concentrations in plant leaves by locations (Fig. 5-8) clearly showed two herbs that appeared as hyperaccumulators of iron: hoary plantain (*Plantago lanceolata*) and common dandelion (*Taraxacum officinale*) in all locations. It is also necessary to point out that on location 2 on the Avala (Fig. 6) these two species showed very high values of iron concentrations: *Taraxacum officinale* 2425  $\mu\text{g/g}$  and *Plantago lanceolata* 2765  $\mu\text{g/g}$ . Such results are in line with the results of the research of heavy metals contents on Fruška Gora Mountain where comparing plant species it can easily be noted that common dandelion shows intensive uptake of iron with the value of 1428 ppm (S t a n k o v i ć, 2008).

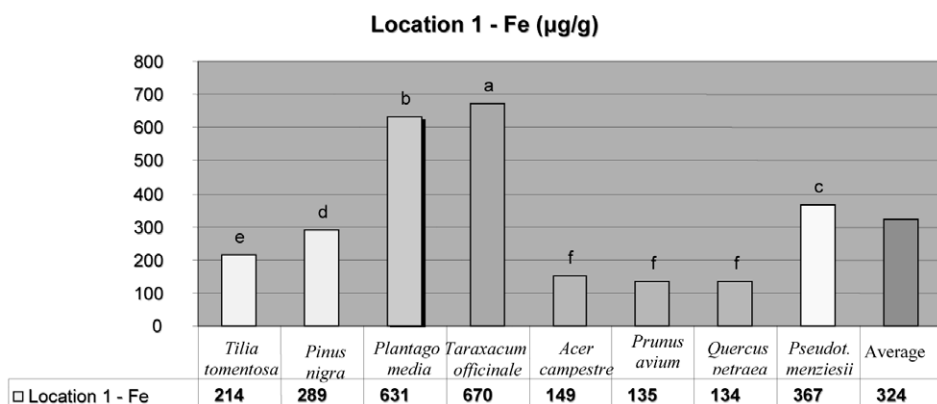


Fig. 5

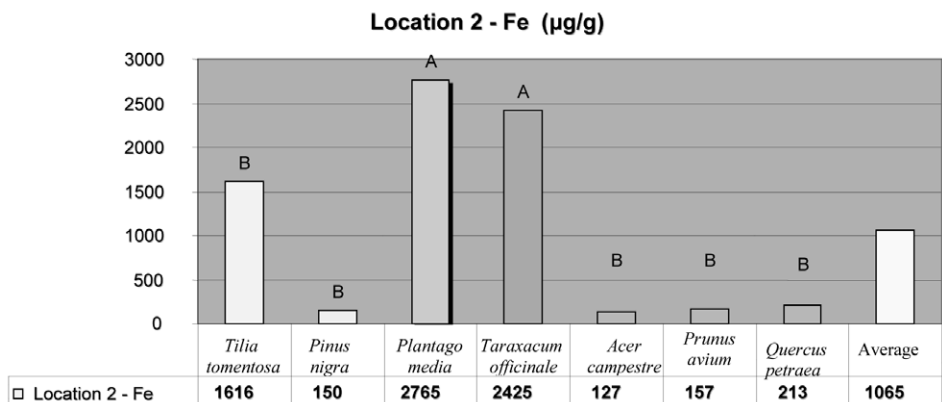


Fig. 6

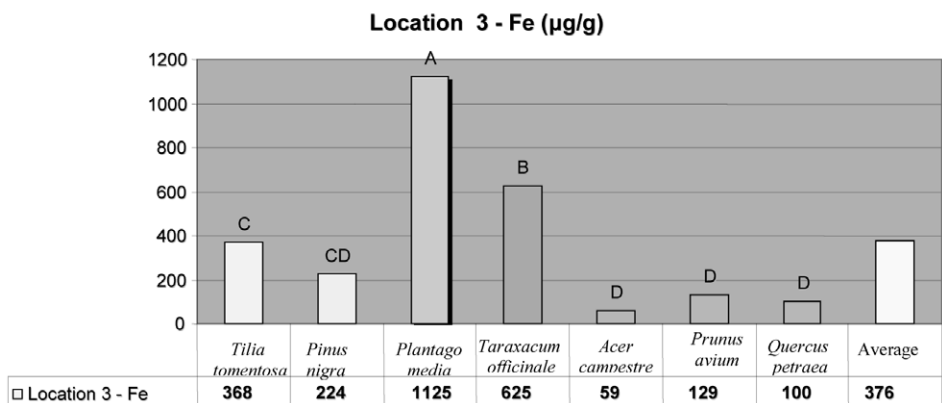


Fig. 7

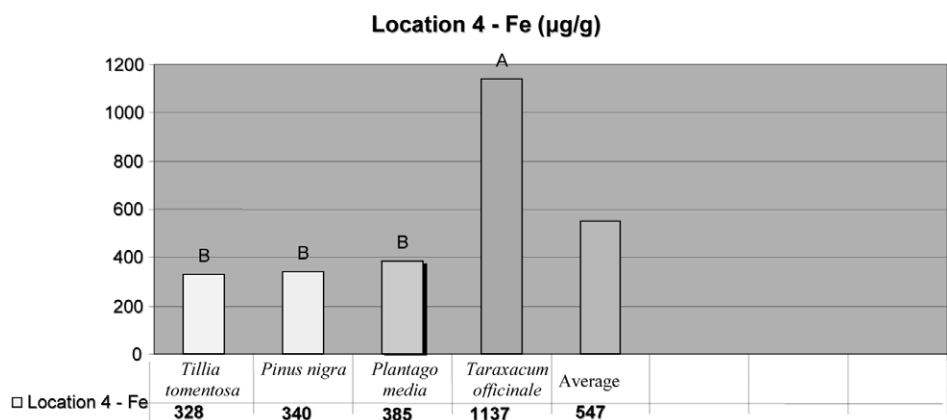


Fig. 8

It is important also to point out to the antagonism between iron and manganese i.e., the surplus of one element causes reduction of the other and vice versa.

In our study, this relation can be best seen on location 2 on the Avala (Fig. 2) where average values for manganese accumulation for all plants amounted to 116  $\mu\text{g/g}$  and the average values for Fe amounted to 1065  $\mu\text{g/g}$  (Fig. 6).

The antagonism of Fe and Mn was also clearly notable on location 4 in Belgrade where average values of Fe for all plants amounted to 547  $\mu\text{g/g}$  (Fig. 8) while the values for Mn amounted to 48  $\mu\text{g/g}$  (Fig. 4).

Regarding plant species by locations, we observed that the least range of variation in accordance with Duncan's test was present on location 4 from A-B, while on the locations on the Avala this range of variations was from A-F.

## CONCLUSION

Based on the obtained results we conclude:

- Average manganese contents in leaves on all previously mentioned locations ranged from 48  $\mu\text{g/g}$  on location 4 to 102  $\mu\text{g/g}$  on location 1. The greatest manganese concentration was found in leaves of woody plants, namely (*Tillia tomentosa*) 780  $\mu\text{g/g}$ , and then Sessile Oak (*Quercus petraea*) 552  $\mu\text{g/g}$ . The least concentrations of manganese from all locations, and especially location 1, were found in European black pine (*Pinus nigra*) 16  $\mu\text{g/g}$ .
- Average iron contents in leaves on all previously mentioned locations ranged from 324  $\mu\text{g/g}$  on location 1 to 1065  $\mu\text{g/g}$  on location 2;
- The greatest iron concentration was found in the leaves of herbs on location 2 on the Avala with especially high values amounting to 2425  $\mu\text{g/g}$  found in common dandelion (*Taraxacum officinale*) and 2765  $\mu\text{g/g}$  in hoary plantain (*Plantago media*);
- The least quantity of average accumulated iron among all locations and especially on location 3 was found in sessile oak (*Quercus petraea*) amounting to 100  $\mu\text{g/g}$  and on the same location in field maple (*Acer campestre*) we measured the least values of only 59  $\mu\text{g/g}$ ;
- On the basis of all the above mentioned and as a result of this research manganese and iron clearly appeared as antagonists;
- On the basis of the obtained results of the research for all analyzed species on all locations in general it might be concluded that traffic was not the basic polluter but that the increased concentrations of manganese and iron in plants in this complex were, besides natural contents in soil, caused by some other sources of emission.



## ACKNOWLEDGEMENTS

The researching was conducted as a part of the project *Research of the concentration of pollutants in forest ecosystems of the protected natural resource Avala in terms of environmental protection and development* funded by the Secretariat for Environmental Protection of the City of Belgrade (2009/2010).

## REFERENCES

- Alloway, B. J. (1995): *Heavy metals in soil*. Second ed., Blackie. Academic and Professional, Glasgow.
- Baker, A. J. M., Brooks, R. R. (1989): *Terrestrial higher plants wich hyperaccumulate metallic elements – A review of their distribution*, Ecology and Phytochemistry, Biorecovery, 1, 81-126.
- Berlizov, A. N., Blum, O. B., Filby, R. H., Malyuk, I. A., Tryshyn V. V. (2007): *Testing applicability of black poplar (Populus nigra L.) bark to heavy metal air pollution monitoring in urban and industrial regions*. Science of the Total Environment 372 (2-3), 693-706.
- Botrill, D. E., Possibngham, J. V., Kriedmann, P. E. (1970): *The effect of nutrient deficiencies on photosynthesis and respirations in spinach*. Plant and Soil 32, 424-438.
- Kadović, R., Knežević, M. (2002): *Teški metali u šumskim ekosistemima Srbije*. Šumarski fakultet Univerziteta u Beogradu. Ministarstvo za zaštitu prirodnih bogatstava i životne sredine Republike Srbije.
- Kárpáti, I., Kárpáti, V., Tölgyesi, Gy. (1967): *Manganese content in aquatic plants*. Acta Bot. Hung., 13, 95-112.
- Kastori, R., Petrović, N. (1993): *Uticaj teških metala na biljke*. U: Kastori R. (ured.) *Teški metali i pesticidi u zemljištu - Teški metali i pesticidi u zemljištima Vojvodine*. Poljoprivredni fakultet, Institut za ratarstvo i povrtarstvo Novi Sad, 55-73.
- Kastori, R. (ed.) (1997): *Teški metali u životnoj sredini*, Feljton, Novi Sad.
- Kastori, R. (2006): *Fiziologija biljaka*. Verzal, Novi Sad, 527.
- Nikolić, N., Kojić, D., Pilipović, A., Pajević, S., Krstić, B., Borišev, M., Orlović, S. (2008): *Responses of hybrid poplar to cadmium stress: Photosynthetic characteristics, cadmium and proline accumulation and antioxidant enzyme activity*. Acta Biologica Cracoviensia, Series Botanica 502, 95-103.
- Memon, R. A., Aktoprakgil, D., Özdemir, A., Vertii, A. (2001): *Heavy metal accumulation and detoxification mechanism in plants*. Turl. J. Bot., 25, 111-121.
- Pajević, S., Borišev, M., Rončević, S., Vukov, D., Igić, R. (2008): *Heavy metal accumulation of Danube river aquatic plants – indication of chemical contamination*. Central European Journal of Biology 3 (3), 285–294.
- Prasad, M. N. V., Freitas H. M. (2003): *Metal hyperaccumulation in plants – biodiversity prospecting for phytoremediation technology*. Electronic Journal of Biotechnology, 6 (3), 286-290, <http://www.scielo.cl/fbpe/img/ejb/v6n3/a06/bip/>

- Special basis of management of forests (2008-2017) for management unit "Avala".* Faculty of Forestry University of Belgrade. Belgrade, Serbia
- Stanković, D. (2006): *Istraživanje uticaja saobraćaja na koncentraciju polutanata u šumskim ekosistemima NP "Fruška gora", u funkciji zaštite i unapređenja životne sredine.* Doktorska teza. Prirodno matematički fakultet Univerziteta u Novom Sadu.
- Stanković, D. (2008): *Biljke i saobraćaj.* Monografija. Zadužbina Andrejević, Beograd.
- Ten-Houten, J. G. (1983): *Biological indicators of air pollution.* Environmental monitoring and assessment 3 (3-4), 257-261.
- Tyler, G. (1972): *Heavy metals pollute nature, may reduce productivity.* Ambio1, S. 52-59.
- UNEP-UN/ECE (1994): *International Co-operative Program on Assessment and Monitoring of Air Pollution Effects on Forests in the ECe-region.* GENT. Belgium.
- UNEP – <http://www.unep.org/ozone/index-en.shtml>.

## КОНЦЕНТРАЦИЈА МАНГАНА И ГВОЖЂА У НЕКИМ ВИШЕГОДИШЊИМ ДРВЕНАСТИМ И ЗЕЉАСТИМ ВРСТАМА

Драгица М. Станковић<sup>1</sup>, Боровој Ђ. Крстић<sup>2</sup>, Мирјана Т. Шијачић Николић<sup>1</sup>,  
Милан Н. Кнежевић<sup>1</sup>

<sup>1</sup> Шумарски факултет, Универзитета у Београду, Кнеза Вишеслава 1, 11000 Београд

<sup>2</sup> Природно математички факултет, Универзитета у Новом Саду,  
Трг Д. Обрадовића 2, 21000 Нови Сад

### Резиме

Авала као заштићено природно добро површине од 489 ha од изузетног је значаја различитих аспеката за становнике Београда, стога ову планину треба сачувати од контаминације полутантима пре свега антропогеног утицаја као што је на пример интензиван саобраћај. Нарочито је интересно пратити садржај тешких метала у биљкама као и неке друге полутанте (ПАН-ове).

Циљ овог рада усмерен је на утврђивање степена концентрације Mn и Fe у листовима 8 врста биљака од тога 6 дрвенастих (*Tilia tomentosa* Mnch., *Pinus nigra* Arn., *Prunus avium* L., *Quercus petraea* (Matt.) Liebl, *Pseudotsuga menziesii*) и две зељасте (*Plantago media* L., *Taraxacum officinale* Web.).

Биљке су узорковане на три локалитета на Авали и једном локалитету у центру Београда. Садржај тешких метала одређен је методом атомске апсорбционе спектрофотометрије (AAS).

Резултати истраживања за акумулацију мангана на испитиваном подручју указују да је већа концентрација мангана на локалитетима на Авали, него на локалитету у центру Београда, а то јасно показује да повећање концентрације мангана у биљкама није директно везано за саобраћај као основни извор него земљиште. Највећа акумулација мангана нађена је код биљака храста и липе а гвожђе код боквице и маслачка на свим локалитетима. У овим истраживањима јасно је уочљив и антагонизам између гвожђа и мангана. Најјасније је то видљиво на локалитету 4 у центру Београда, где су просечне вредности за све биљке код Fe износиле 547 µg/g, док су вредности за Mn на истом локалитету 48 µg/g.

Тешки метали у биљкама на подручју заштићеног природног добра „Авала” за сада не представљају опасност за настанак видљивих оштећења шуме, али показују тенденцију повећања концентрација, те их треба интезивно пратити.



Aleksandra S. Bočarov-Stančić<sup>1</sup>,  
Slavica Ž. Stanković<sup>2</sup>, Jelena T. Lević<sup>2</sup>,  
Nataša M. Salma<sup>1</sup>, Vladimir R. Pantić<sup>1</sup>,  
Saša S. Barnić<sup>1</sup>

<sup>1</sup> Centre for Bio-Ecology, Petra Drapšina 15, 23000 Zrenjanin, Serbia

<sup>2</sup> Maize Research Institute, Zemun Polje, Slobodana Bajića 1, 11185 Belgrade, Serbia

## IN VITRO DEGRADATION OF DIACETOXYSCIRPENOL AND T-2 TOXIN BY USE OF *MUCOR RACEMOSUS* FRESEN. F. *RACEMOSUS* ISOLATE\*

**ABSTRACT.** Under controlled *in vitro* conditions the capacity of the *Mucor racemosus* f. *racemosus* 1215/09 isolate to degrade type A trichothecenes (diacetoxyscirpenol – DAS and T-2 toxin) was observed in the liquid nutritive medium. According to previously performed experiments it was proved that the selected isolate, originating from sunflower meal, had the ability to degrade these fusariotoxins when growing on the modified Vogel's agar supplemented with crude extracts of DAS and T-2 toxin.

In order to determine biodegradation of fusariotoxins, the liquid nutritive medium – SPY (5% sucrose + 0.1% peptone + 0.1% yeast extract, pH 6.2) was simultaneously inoculated with the isolate *M. racemosus* f. *racemosus* 1215/09 and: a) *Fusarium semitectum* SL-B (DAS producer) or b) *F. sporotrichioides* R-2301 (T-2 toxin producer). The SPY media, inoculated with single fungal isolates, were used as a control of toxin biosynthesis. The cultures were incubated at room temperature (21-26°C) on the rotary shaker (175 rpm). After the 3-5-day incubation, the filtration of liquid cultures and the extraction of fusariotoxins from filtrates with ethyl-acetate were performed. Determinations of DAS and T-2 toxin were done by thin layer chromatography using silica gel G.

Depending on the incubation duration, *M. racemosus* f. *racemosus* in the mixed culture with *F. semitectum* degraded from 90.0 to 99.97% of DAS present in the medium (40,000-120,000 µg l<sup>-1</sup>), while in the mixed culture with *F. sporotrichioides* it degraded from 95.0 to 96.7% of T-2 toxin present in the medium (240,000 µg l<sup>-1</sup>).

Sterile filtrates of mixed cultures and single culture of *M. racemosus* f. *racemosus*, obtained by passing liquid cultures through the 0.45-µm membrane filter and added to the SPY medium, did not affect degradation of type A trichothecenes that had been biosynthesised by isolates *F. semitectum* SL-B and *F. sporotrichioides* R-2301 in the liquid medium.

**KEY WORDS:** diacetoxyscirpenol (DAS), T-2 toxin, biodegradation, *M. racemosus* f. *racemosus*, liquid medium

\* The paper was presented at the fourth international scientific meeting *Mycology, mycotoxicology, and mycoses*, which was organized in *Matica srpska, Department for natural sciences* from April 20-22, 2011.

## INTRODUCTION

Trichothecenes are a group of toxic secondary metabolites produced by several genera of *Fungi imperfecti* out of which the genus *Fusarium* is the most important. Some of trichothecenes are natural contaminants of cereal crops during their growth in fields or during grain storage under high environmental moisture. Since these compounds are toxic to both, humans and animals, it is of great interest for food and feed industry to prevent the introduction of fusariotoxins into the nutrition chain (S h i m a et al., 1997).

Despite of the application of different preventive measures that avoid the occurrence of *Fusarium* spp. and their toxins (crop rotation, ploughing, weed and insect control, appropriate sowing and harvest time, etc.), the obtained results are usually limited and unsatisfactory, hence it was necessary to develop procedures of detoxication. It is considered that biodegradation or biotransformation is the only efficient method of detoxication of mycotoxins that can only be poorly bound by various adsorbents (R o d r i g e s and B i n d e r, 2008). This group of mycotoxins also includes type A trichothecenes (diacetoxyscirpenol – DAS and T-2 toxin) in which the 12,13-epoxy ring is responsible for their toxicity.

Studies carried out by U e n o et al. (1983) and B e e t o n and B u l l (1989) point out to a possible role of soil bacteria in the transformation of T-2 toxins. Moreover, B e e t o n and B u l l (1989) reported that certain natural bacterial communities or monocultures originating from soil and freshwater represented agents that could detoxify T-2 toxin and related trichothecenes.

Swanson et al. (1988) determined that intestinal bacterial biota originating from rats completely biotransformed T-2 toxin to its de-epoxy products (de-epoxy HT-2 and de-epoxy T-2 triol). The absence of complete de-epoxidation was recorded in experiments with chicken intestinal bacteria that degraded T-2 toxin and DAS exclusively by the process of deacetylation (Y o u n g et al., 2007). In contrast to this, intestinal and fecal microorganisms of rats, cattle and pigs completely biotransformed DAS to de-epoxy monoacetoxyscirpenol. B i n d e r et al. (2000) isolated a new bacterial strain of the genus *Eubacterium* (strain BBSH 797) from a bovine rumen that had a potential to biodegrade epoxy group of trichothecenes to diene (S c a t z m a y r et al., 2006).

Beside bacteria there are other types of microorganisms that are capable of T-2 toxin biotransformation: protozoa (K i e s s l i n g et al., 1984), yeasts (R o d r i g e s and B i n d e r, 2008), and mycobiota (J e s e n s k a and S a j b i d o r o v a, 1991). J e s e n s k a and S a j b i d o r o v a (1991) identified *Alternaria* sp., *Ulocladium* sp., *Aspergillus candidus* Link, *A. flavus* Link, *Cladosporium cladosporioide* (Fr.) G. A. de Vries and *C. macrocarpum* Preuss in the group of moulds that very rapidly degraded T-2 toxin (for 48 h).

Considering the natural presence of T-2 toxin worldwide in toxicologically relevant concentrations, as well as, its powerful toxicity, the aim of the present study was to investigate the capacity of biodegradation of T-2 toxin and related metabolite DAS under laboratory conditions in the liquid medium by use of the fungal isolate *Mucor racemosus* f. *racemosus* 1215/09.

## MATERIAL AND METHODS

**Microorganisms.** The fungal isolate *Mucor racemosus* Fresen. f. *racemosus* 1215/09 obtained during the routine analysis of sunflower meal at the Department of Microbiology, Centre for Bio-Ecology, Zrenjanin, in 2009 was selected for tests of biodegradation of type A trichothecenes (DAS and T-2 toxin) having in mind our previous results (Bočarov-Stančić et al., 2010). The identification of fungal isolate was performed after Domsh et al. (1980).

The following two fungal isolates were selected for the production of type A trichothecenes in the experiments of biodegradation: a) *Fusarium semitectum* Berk. & Rev. SL-B, the Centre's isolate from the alfalfa hay, for previous studies proved that it had capability to biosynthesise DAS (Bočarov-Stančić et al., 2005) and b) *F. sporotrichioides* Sherb. R-2301, leg. Dr. D. Latus, Germany, the strain known as a good producer of T-2 toxin (Bočarov-Stančić et al., 2007). Fungal cultures were maintained on potato dextrose agar (PDA) at 4-6°C.

**Inoculation** of the liquid medium SPY (5% sucrose + 0.1% peptone + 0.1% yeast extract, pH 6.2) was done with five pieces (each 5 x 5 mm) of the fungal material taken from investigated isolates subcultured during seven days on PDA Petri plates at 27±1°C.

The first stage of the experiment encompassed studies on the ability of biotransformation of DAS and T-2 toxin by the fungal microorganism *M. racemosus* f. *racemosus* 1215/09. The variants were as follows: **I** – combination of *F. semitectum* SL-B and *M. racemosus* f. *racemosus* 1215/09, **II** – combination of *F. sporotrichioides* R-2301 and *M. racemosus* f. *racemosus* 1215/09. As a control of biosynthesis of fusariotoxins, the SPY medium was inoculated by single fungal cultures: **III** – *M. racemosus* f. *racemosus* 1215/09, **IV** – *F. semitectum* SL-B i **V** – *F. sporotrichioides* R-2301.

The second stage of the experiment included studies on capacities of extracellular enzymes of the monoculture *M. racemosus* f. *racemosus* 1215/09 (**III**), as well as mixed cultures of *F. semitectum* SL-B + *M. racemosus* f. *racemosus* 1215/09 (**I**) and *F. sporotrichioides* R-2301 + *M. racemosus* f. *racemosus* 1215/09 (**II**) to biodegrade type A trichothecenes. Sterile filtrates of cultures from the first stage of the experiment, obtained by passing through Minisart NML of 0.45 µm, in the amount of 6 ml, were added to the SPY medium immediately prior to inoculation with producers of DAS and T-2 toxin. The variants were as follows: *F. semitectum* SL-B + **I**, *F. semitectum* SL-B + **II**, *F. semitectum* SL-B + **III**, *F. sporotrichioides* R-2301 + **I**, *F. sporotrichioides* R-2301 + **II** and *F. sporotrichioides* R-2301 + **III**. As a control of biosynthesis of fusariotoxins, the SPY medium was inoculated by single fungal cultures: **IV** – *F. semitectum* SL-B and **V** – *F. sporotrichioides* R-2301.

**Incubation conditions.** A total of 100 ml of the semi-synthetic medium SPY placed in Erlenmeyer flasks (500 ml) were inoculated and incubated on a rotary shaker (175 rpm) at room temperature (21-26°C) during five and/or three days. All tests were performed in three replicates.

**Sample preparation for the analysis of fusariotoxins.** Liquid fungal mixed cultures, as well as monocultures, were filtered after incubation on the rotary shaker. Crude extracts of type A trichothecenes were obtained by use of ethyl-acetate. Further purification of DAS and T-2 toxin was carried out applying the procedure of R o m e r et al. (1978), while thin layer chromatography was performed after P e p e l j n j a k and B a b i ć (1991).

## RESULTS AND DISCUSSION

The isolate *M. racemosus* f. *racemosus* 1215/09 was selected for the experiments of biodegradation of type A trichothecenes not only because of literature data showing that various species of the genus *Mucor* could biotransform these fusriotoxins (E l - S h a r k a w y and A b b a s , 1991) but primarily due to such capacity of the selected isolate was confirmed by our previous experiments (B o ĉ a r o v - S t a n ĉ i ć et al., 2010). During the growth of *M. racemosus* f. *racemosus* 1215/09 on the modified Vogel's agar (V o g e l , 1956) to which crude extracts of DAS and T-2 toxins were added (300 µg/Petri dish) it completely biotransformed the stated fusariotoxins seven days after incubation at 27±1°C.

Tab. 1 – Biodegradation of DAS and T-2 toxin in the liquid medium by use of *Mucor racemosus* f. *racemosus* (SPY, 175 rpm, 25-26°C)

Fungus	Isolate design.	Days	Final pH	Toxin yield (µg l <sup>-1</sup> )		Yield reduction (%)
				DAS	T-2	
<i>Mucor racemosus</i> f. <i>racemosus</i>	1215/10	3	7.90	n.d. (<21)	n.d. (<21)	-
		5	8.20	n.d. (<21)	n.d. (<21)	-
<i>Fusarium semitectum</i>	SL-B	3	4.85	120,000	n.d. (<21)	-
		5	5.00	40,000	n.d. (<21)	-
<i>F. semitectum</i> + <i>M. racemosus</i> f. <i>racemosus</i>	SL-B + 1215/09	3	4.40	40	n.d. (<21)	99.97
		5	4.80	4,000	n.d. (<21)	90.0
<i>Fusarium sporotrichioides</i>	R2301	3	4.10	n.d. (<21)	240,000	-
		5	4.50	n.d. (<21)	240,000	-
<i>F. sporotrichioides</i> + <i>M. racemosus</i> f. <i>racemosus</i>	R2301 + 1215/09	3	3.90	n.d. (<21)	12,000	95.0
		5	4.45	n.d. (<21)	8,000	96.7

Significant differences in characteristics of the growth of fungal monocultures and mixed cultures were observed during their incubation in the liquid medium SPY (Table 1).

**pH value.** In case of single culture of *M. racemosus* f. *racemosus* 1215/09, the increase in pH in the SPY medium ranged from the initial value of 6.20 to the value of 7.90 and higher, while in case of single cultures of *F. semitectum* SL-B and *F. sporotrichioides* R-2301, as well as, mixed cultures of *M. race-*



*mosus* f. *racemosus* 1215/09 and these producers of fusariotoxins, pH values were reduced to 5.00 and below 5.00 during incubation in the liquid medium.

Somewhat higher pH values were observed in all combinations (mono- and mixed cultures) during prolonged incubation from 3 to 5 days.

**Biosynthesis of type A trichothecenes.** The isolate *M. racemosus* f. *racemosus* 1215/09 did not biosynthesised DAS and T-2 toxin under laboratory test conditions (Table 1).

The DAS yield in the *F. semitectum* SL-B monoculture was higher after 3-day ( $120,000 \mu\text{g l}^{-1}$ ) than after 5-day incubation ( $40,000 \mu\text{g l}^{-1}$ ) in the liquid semi-synthetic medium SPY on the rotary shaker.

On the other hand, the incubation duration under the same laboratory conditions (SPY, 175 rpm, 25-26°C), did not affect the amount of the produced T-2 toxin by use of single *F. sporotrichioides* R-2301 culture. The yield of the T-2 toxin was equal ( $240,000 \mu\text{g l}^{-1}$ ) in both cases (after three and five days of incubation).

**Biotransformation of type A trichothecenes.** Biodegradation of DAS and T-2 toxin in the liquid medium by *M. racemosus* f. *racemosus* 1215/09 was tested by mixed cultures of this fungal isolate with *Fusarium* species that were producers of the same trichothecenes (Table 1).

In case of a mixed cultures of *F. semitectum* SL-B with *M. racemosus* f. *racemosus* 1215/09, the DAS yield was significantly lower after the 3-day inoculation ( $40 \mu\text{g l}^{-1}$ ) than after 5-day incubation under the same *in vitro* conditions (Table 1). If these results are compared with the DAS yield in the single *F. semitectum* SL-B culture it is observable that *M. racemosus* f. *racemosus* 1215/09 biotransformed 90.0% of DAS present in the liquid medium after 5 days of incubation. The reduction of the yield of this type A trichothecenes was higher after 3-day incubation and amounted to 99.97%.

Similar results were also gained in case of mixed culture of *F. sporotrichioides* R-2301 with *M. racemosus* f. *racemosus* 1215/09 (Table 1). Although recorded differences in T-2 toxin yields after 3- ( $12,000 \mu\text{g l}^{-1}$ ) and 5-day inoculation ( $8,000 \mu\text{g l}^{-1}$ ) were not great, *M. racemosus* f. *racemosus* 1215/09 biodegraded over 90.0% of other type A trichothecene present in the liquid medium. The yield reduction of T-2 toxin in this mixed culture amounted to 95.0, i.e. 96.6% after 3-, i.e. 5-day-incubation, respectively.

According to El-Sarkawy and Abbas (1991) biotransformation of T-2 toxin by use of species *Aspergillus niger* van Tieg. and *Mucor mucedo* Fresen. was done by acetylation and deacetylation of this fusariotoxin, without degradation of the trichothecene skeleton, hence it is assumed that it also happened in experiments performed with our fungal isolate *M. racemosus* f. *racemosus* 1215/09.

A great capacity of the isolate *M. racemosus* f. *racemosus* 1215/09 to biodegrade DAS and T-2 toxin is the best observable if our results are compared with results gained by other authors. In the *in vitro* experiment of biotransformation of certain A- and B-trichothecenes with probiotic microorganisms (genera *Bacillus*, *Lactobacillus*, and *Saccharomyces*) Böhm et al. (2000) applied significantly lower concentrations of fusariotoxins. Those were con-



centrations that could be found under natural conditions and they amounted to 500 µg kg<sup>-1</sup> for both, DAS and T-2 toxin. However, the reduction in concentrations of tested trichothecenes was not recorded, and T-2 and DAS slowed down the growth of some strains of probiotic microorganisms.

Tab. 2 – Effects of filter sterilized liquid mono- and mixed culture *Mucor racemosus* f. *racemosus* on biosynthesis of DAS and T-2 toxin in the liquid medium (SPY, 3 days, 175 rpm, 21-23°C)

Fungus	Isolate design.	Added filtrate	Final pH	Toxin yield (µg l <sup>-1</sup> )		Yield reduction (%)
				DAS	T-2	
<i>Fusarium semitectum</i>	SL-B	-	4.76	34,000	n.d. (<21)	0
		<b>I</b>	4.55	34,000	n.d. (<21)	0
		<b>II</b>	4.75	34,000	n.d. (<21)	0
		<b>III</b>	4.70	34,000	n.d. (<21)	0
<i>Fusarium sporotrichioides</i>	R-2301	-	4.20	n.d. (<21)	184,000	0
		<b>I</b>	4.25	n.d. (<21)	184,000	0
		<b>II</b>	4.18	n.d. (<21)	184,000	0
		<b>III</b>	4.12	n.d. (<21)	184,000	0

Legend:

**I** - *F. semitectum* SL-B+*M. racemosus* f. *racemosus* 1215/09 (SPY5 days, 175 rpm);

**II** - *F. sporotrichioides* R-2301+*M. racemosus* f. *racemosus* 1215/09 (SPY, 5 days, 175 rpm);

**III** - *M. racemosus* f. *racemosus* 1215/09 (SPY, 5 days, 175 rpm).

The decline of pH values, in the medium for DAS and T-2 toxin biosynthesis, from the initial 6.20 to the values ranging from 4.12 to 4.76 at the end of incubation (Table 2), was observed in liquid cultures of *F. semitectum* SL-B and *F. sporotrichioides* R-2301 with or without addition of sterile filtrates of monoculture of *M. racemosus* f. *racemosus* 1215/09 (**III**) or mixed cultures (**I** and **II**).

Extracellular enzymes of *M. racemosus* f. *racemosus* 1215/09 present in filtrates **I**, **II** and **III** did not essentially affect the changes in the pH value, nor the yield of the same fusariotoxins in tested producers of DAS and T-2 toxin after 3-day incubation in SPY. Similar results, i.e. absence of capacities of cell free supernatants of microbial cultures to transform one type B trichothecene (deoxynivalenol) were published by Völki et al. (2004).

## CONCLUSION

The isolate *M. racemosus* f. *racemosus* 1215/09 biodegraded 99.97%, i.e. 90.0% of DAS present in the liquid medium SPY after 3-, i.e. 5-day incubation, respectively.

Under the same laboratory conditions the culture *M. racemosus* f. *racemosus* 1215/09 biotransformed 95.0%, i.e. 96.7% of T-2 toxin present in the medium after 3-, i.e. 5-day incubation, respectively.

Extracellular enzymes of the isolate *M. racemosus* f. *racemosus* 1215/09 were not capable to degrade tested type A trichothecens under laboratory conditions.

The gained results have to be considered just as a first step in the development of practically and commercially acceptable technology for decontamination of mycotoxin-containing food and feed .

## ACKNOWLEDGEMENT

This study was implemented within the project TR 31023 financed by the Ministry of Science and Technological Development of the Republic of Serbia.

## REFERENCES

- Beeton, S., Bull, A. T. (1989): *Biotransformation and detoxification of T-2 toxin by soil and freshwater bacteria*. Appl. Environm. Microbiol, 55: 190–197.
- Binder, E., M., Heidler, D., Scatzmayr, G., Thimm, N., Fuchs, E., Schuh, M., Krska, R., Binder, J. (2000): *Microbial detoxification of mycotoxins in animal feed*. Proceedings of 10<sup>th</sup> International IUPAC Symposium on Mycotoxins and Phycotoxins, 21–25 May 2000, San Paolo, Brazil.
- Bočarov-Stančić, A. S., Adamović, M. J., Đorđević, N. Ž. (2005): *Mycopopulations of alfalfa silage with particular review on toxigenic Fusarium spp.* Proc. Nat. Sci, Matica Srpska Novi Sad, 108: 59–67.
- Bočarov-Stančić, A. S., Jačević, V. M., Resanović, R. D., Bijelić, M. B. (2007): *Optimisation of laboratory conditions for biosynthesis of type A trichothecenes*. Proc. Nat. Sci., Matica Srpska Novi Sad, 113: 35–44.
- Bočarov-Stančić, A., Salma, N., Lević, J., Pantić, V., Stanković, S., Barnić, S. (2010): *Microbiological degradation of the T-2 toxin and diacetoxyscirpenol under laboratory conditions*. XIV International Symposium of Feed Technology, 19–21 October 2010, Novi Sad, Proceedings, 254–261.
- Böhm, J., Grajewski, J., Asperger, H., Cecon, B., Rabus, B., Razzazi, E. (2000): *Study on biodegradation of some A- and B-trichothecenes and ochratoxin A by use of probiotic microorganisms*. Mycotoxin Res., 16 (1): 70–74.
- Domsh, K. H., Gams, W., Anderson, T.-H. (1980): *Compendium of Soil Fungi*. Academic Press, A Subsidiary of Harcourt Brace Jovanovich Publishers, London, 76–730.
- El-Sharkawy, S., Abbas, H. K. (1991): *Metabolism of T-2 toxin by Mucor and Aspergillus sp.* Acta Pharm. Jugoslav., 41 (3): 191–201.
- Jesenska, Z., Sajbidorova, I. (1991): *T-2 toxin degradation by micromycetes*. J. Hyg. Epidemiol. Microbiol. Immunol., 35 (1): 41–49.
- Kiessling, K. H., Petterson, H., Sandholm, K., Olsen, M. (1984): *Metabolism of aflatoxin, ochratoxin, zearalenone and three trichothecenes by intact rumen fluid, rumen protozoa, and rumen bacteria*. Appl. Environ. Microbiol, 47: 120–127.

- Pepeljnjak, S., Babić, A. (1991): *Detekcija trihotecenskih mikotoksina T-2, HT-2, DON i DAS tankoslojnom hromatografijom i biološkim metodama*, Prehrambeno-tehnol. biotehnol. Rev. 29: 1070–1073.
- Rodrigues, I., Binder, E. M. (2008): *Microbiologicals for deactivating mycotoxins*. Feed Technology Update, 3 (2).
- Romer, T. R., Boling, T. M., MacDonald, J. L. (1978): *Gas-liquid chromatographic determination of T-2 toxin and diacetoxyscirpenol in corn and mixed feeds*. J. AOAC 61: 801–808.
- Scatzmayr, G., Zehner, F., Taubel, M., Scatzmayr, D., Klimitsch, A., Loibner, E. M., Binder, E. M. (2006): *Microbiologicals for deactivating mycotoxins*. Mol. Nutr. Food Res., 50: 543–551.
- Shima, J., Takase, S., Takahashi, Y., Iwai, Z., Fujimoto, H., Yamazaki, M., Ochi, K. (1997): *Novel detoxication of trichothecene mycotoxin deoxynivalenol by a soil bacterium isolated by enrichment culture*. Appl. Environ. Microbiol, 63 (10): 3825–3830.
- Swanson, S. P., Helaszek, C., Buck, W. B., Rood Jr, H., D., Haschek, W. M. (1988): *The role of intestinal microflora in the metabolism of trichothecene mycotoxins*. Food and Chem. Toxicol., 26 (10): 823–829.
- Ueno, Y., Nakayama, K., Ishii, Tashiro, F., Minoda, Y., Omori, T., Komagata, K. (1983): *Metabolism of T-2 toxin in Curtobacterium sp. Strain 114-2*. Appl. Environ. Microbiol, 46: 120–127.
- Vogel, G. H. (1956): *A convenient medium for Neurospora (medium N)*. Microb. Gen. Bull., 13: 42–43.
- Völki, A., Vogler, B., Schollenberger, M., Karlovsky, P. (2004): *Microbial detoxication of mycotoxin deoxynivalenol*. J. Basic Microbiol., 44 (2): 147–156.
- Young, C. J., Zhou, T., Yu, H., Zhu, H., Gong, J. (2007): *Degradation of trichothecene mycotoxins by chicken intestinal microbes*. Food Chem. Toxicol. 45 (1): 136–143.

## IN VITRO ДЕГРАДАЦИЈА ДИАЦЕТОКСИСЦИРПЕНОЛА И Т-2 ТОКСИНА ПОСРЕДСТВОМ ИЗОЛАТА *MUCOR RACEMOSUS* FRESN. F. *RACEMOSUS*

Александра С. Бочаров-Станчић<sup>1</sup>, Славица Ж. Станковић<sup>2</sup>, Јелена Т. Левић<sup>2</sup>,  
Наташа М. Салма<sup>1</sup>, Владимир Р. Пантић<sup>1</sup>, Саша С. Барнић<sup>1</sup>

<sup>1</sup> „Био-Еколошки Центар” ДОО, 23000 Зрењанин, Република Србија

<sup>2</sup> Институт за кукуруз „Земун Поље”, 11185 Београд-Земун, Република Србија

### Резиме

У контролисаним *in vitro* условима проучавана је способност изолата *Mucor racemosus* f. *racemosus* 1215/09 да деградује трихотецене типа А (диацетоксисцирпенол - ДАС и Т-2 токсин) у течној хранљивој подлози. Претходним експериментима је доказано да одабрани изолат, пореклом са сунцокретевог сачме, поседује способност разградње наведених фузариотоксина, који су као сирови екстракти додати у модификовану Вогелову подлогу:

У циљу утврђивања биодеградације фузариотоксина течна хранљива подлога СПК (5% сахароза + 0,1% пептон + 0,1% екстракт квасца, рН 6,2) је засејана

у исто време изолатом *M. racemosus* f. *racemosus* 1215/09 и: а) *Fusarium semitectum* SL-B (произвођач ДАС-а) или б) *F. sporotrichioides* R-2301 (произвођач Т-2 токсина). Као контрола биосинтезе токсина коришћена је СПК подлога инокулисана појединачним изолатима гљива. Културе су инкубиране на ротационој тресилици (175 о/мин) током 3-5 дана на собној температури (21-26°C). Након 3 до 5 дана инкубације вршено је филтрирање течних култура и екстракција фузариотоксина из филтрата етил-ацетатом. Детерминација ДАС-а и Т-2 токсина је рађена танкослојном хроматографијом на силика гелу Г.

Зависно од дужине инкубације, *M. racemosus* f. *racemosus* је у здруженој култури са *F. semitectum* деградовала 90,0-99,97% ДАС-а присутног у подлози (40.000-120.000  $\mu\text{g l}^{-1}$ ), док је у здруженој култури са *F. sporotrichioides* разградила 95,0-96,7% Т-2 токсина присутног у подлози (240.000  $\mu\text{g l}^{-1}$ ).

Стерилни филтрати мешаних култура и појединачне културе *M. racemosus* f. *racemosus*, добијени пропуштањем течних култура кроз 0,45  $\mu\text{m}$  мембрански филтер и додати СПК подлози, нису утицали на разградњу трихотецена типа А које су биосинтетисали изолати *F. semitectum* SL-B и *F. sporotrichioides* R-2301 у течной подлози.



*Vahid Rahjoo*<sup>1</sup>, *Maryam Parchamian*<sup>2</sup>,  
*Mohammad Taghi Feizbakhsh*<sup>3</sup>, *Majid Zamani*<sup>1</sup>

<sup>1</sup> Seed and Plant Improvement Institute (SPII), Karaj, Iran

<sup>2</sup> Plant Protection Department, Islamic Azad University, Damghan, Iran

<sup>3</sup> Agricultural Research Center of Golestan Province, Gorgan, Iran

Corresponding author, email address: vrahjoo@yahoo.com

## QUANTIZATION OF TOTAL FUMONISINS PRODUCED BY *FUSARIUM VERTICILLIOIDES* STRAINS IN SOME MAIZE AND SORGHUM GENOTYPES BY ELISA\*

**ABSTRACT:** *Fusarium verticillioides* is one of the most prevalent *Fusarium* species on maize and sorghum, causing *Fusarium* ear rot and sorghum grain mold in warm and humid regions of Iran. The pathogen produces potent mycotoxins known as fumonisins. In order to determine mycotoxin (fumonisins) production on different maize and sorghum genotypes, a field trial was carried out based on a randomized complete block design with 10 treatments and three replications for each crop at Gorgan station in 2010. The ears of corn plants were inoculated by spore suspension of the mixture of some virulent *F. verticillioides* isolates using an ear inoculation method (Nail Punch). The sorghum panicles were also inoculated by spraying of spore suspension isolates at flowering stage. All infected kernels were evaluated by ELISA kits (AgraQuant Fumonisin Kit; Romer Labs, Austria) for their total fumonisins production at the physiological maturing stage. All genotypes showed statistically significant difference in their fumonisin production in Gorgan. The results of fumonisins analysis obtained from ELISA test showed that lines 1 (Resistant) and 3 (Susceptible) with 2.4 ppm and 13.7 ppm had the least and highest amount of total fumonisins respectively among all maize genotypes. Also among all sorghum genotypes, genotypes 10 and 6 with 0.2 ppm and 4.8 ppm had the least and the highest amount of total fumonisins respectively. The results of this experiment demonstrated that fumonisin production level in maize kernels (maybe as maize kernels are the main host of this fungus) was significantly higher than sorghum kernels in Gorgan region.

**KEY WORDS:** ELISA, Corn, Mycotoxin, Sorghum

## INTRODUCTION

Maize (*Zea mays* L.) is one of the major crops in Iran with the production of approximately 1.65 million tons per year (Anonymous, 2009). Maize plants are affected by several *Fusarium* species that are responsible for diseases such

---

\* The paper was presented at the fourth international scientific meeting *Mycology, mycotoxicology, and mycoses*, which was organized in *Matica srpska, Department for natural sciences* from April 20-22, 2011.

as root rot, stalk rot, seedling blight, and ear rot. Several species of *Fusarium* belonging to section *Liseola* can cause *Fusarium* ear rot of maize, but *Fusarium verticillioides* (Sacc.) Nirenberg has been reported as the most prevalent *Fusarium* species on maize worldwide (Leslie, 1991; Chulze et al., 2000; Danielsen et al., 1998) and Iran (Zamani et al., 1998; Rahjoo et al., 2008b).

Sorghum (*Sorghum bicolor* (L.) Moench) is the world's fourth most important cereal crop after wheat, rice, and maize (Smith & Frederiksen, 2000). Diseases and insects are major factors decreasing yield of sorghum cultivars. Among the diseases, grain mold is one of the most important biotic constraints to production of grain sorghum worldwide (Thakur et al., 2006). Grain mold is caused by number of fungal species such as *Fusarium* spp. *Alternaria alternata*, *Curvularia lunata*, *Phoma* spp., and approximately 40 other fungal species. Grain mold reduces yield, seed viability, and nutritional quality. Molded grains contain mycotoxins and present health hazards to animals and human beings (Castor & Frederiksen, 1980). The use of resistant cultivars offers the most effective method for controlling the disease (Forbes et al., 1992). Grain mold has not been a great problem in Iran yet, but it can be a problem for seed production in humid regions, especially in the north of Iran.

Based on the taxonomic system of Gerlach and Nirenberg (1982) the teleomorph of *F. verticillioides* is *Gibberella moniliformis* Wineland, which corresponds to mating population A of the *Gibberella fujikuroi* species complex (Leslie, 1991; Danielsen et al., 1998). *Fusarium verticillioides* is one of the most prevalent *Fusarium* species on maize and sorghum, causing fusarium ear rot and sorghum grain mold in warm and humid regions of Iran. The pathogen produces potent mycotoxins known as fumonisins. Fumonisins are a group of mycotoxins produced by *Fusarium verticillioides* that contaminate maize and maize-based products (Nelson et al., 1993). Fumonisins were first isolated in 1988 from cultures of *F. verticillioides* strain MRC 826 by Gelderblom and colleagues (Gelderblom et al., 1988) at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) of the Medical Research Council (MRC) in South Africa (Marasas, 2001). Since the discovery of fumonisin B<sub>1</sub> in 1988, more than 10 fumonisins have been isolated and characterized. Of these, fumonisin B<sub>1</sub> (FB<sub>1</sub>), FB<sub>2</sub>, and FB<sub>3</sub> are the major fumonisins found under field conditions. Fumonisins have been linked to various animal and human mycotoxicoses, such as leukoencephalomalacia in horses, pulmonary edema in pigs, and cancer in rats and humans (Gelderblom et al., 1988; Yoshizawa et al., 1994).

Researchers use different methods for quantifying fumonisins produced in cereal kernels or foods. The production of monoclonal and polyclonal antibodies against fumonisins have permitted the development of direct and indirect competitive enzyme-linked immunosorbent assays (ELISAs) for screening foods, feeds, animal tissues and fungal cultures for fumonisins. ELISA is well suited for rapidly screening corn for fumonisins. Other advantages of the ELISA kits are their portability and ease of use (Jackson and Jablonsky, 2004).



Limited studies about fumonisins in Iran (Y a z d a n p a n a h et al., 2001; S h e p h a r d et al., 2002; G h i a s i a n et al., 2005; Y a z d a n p a n a h et al., 2006; R a h j o o et al., 2008a) indicate that fumonisins are important mycotoxins present on maize kernels before harvesting and during storage procedures.

Objectives of this study were: (i) Evaluation of relative resistance of different maize and grain sorghum genotypes to diseases and (ii) Evaluation of mycotoxin production (fumonisins B) of different maize and grain sorghum genotypes; and to estimate the correlation between disease severity and mycotoxin production level in different genotypes.

## MATERIAL AND METHODS

### *Isolation and Identification of fungal isolates*

A total of 61 *Fusarium* isolates were recovered from maize ear and sorghum panicle samples collected from different fields of the northern part of Iran. Two seeds of each sample were surface sterilized for 1 min with a 1% sodium hypochlorite solution, rinsed twice in sterile distilled water and dried. Two growth media, Potato Dextrose Agar (PDA) and Peptone PCNB Agar (PPA), were used for isolating *Fusarium* spp. The agar plates were incubated at 25°C in the dark for 5-7 days. All *Fusarium* isolates were subcultured on PDA, Spezieller Nährstoffarmer Agar (SNA), and Carnation Leaf Agar (CLA), using a single spore technique (L e s l i e and S u m m e r e l l, 2006). PDA cultures were incubated at 25°C and CLA and SNA cultures were incubated at 25°C under near UV light for four weeks. Morphological identifications of isolates were made using the criteria of G e r l a c h and N i r e n b e r g (1982) and L e s l i e and S u m m e r e l l (2006).

### *Evaluation of pathogenicity*

In order to evaluate the pathogenicity variation of *Fusarium verticillioides* strains, an experiment based on a randomized complete design with 34 treatments (20 *F. verticillioides* strains isolated from maize kernels, 12 strains isolated from sorghum kernels, one control with sterile distilled water inoculation and one control without inoculation) and three replications were conducted in greenhouse. Evaluation of pathogenicity was carried out using stem inoculation in one maize inbred line (MO17) which was susceptible to fungus. Inoculation was made using toothpick method by inserting the infected toothpicks into the first developed internode of corn stems, when the maize seedlings were two months old. The pathogenicity of each isolate was evaluated based on the necrosis length observed in stems, 10 days after inoculation (D a n i e l s e n et al., 1998).

## Evaluation of resistance to disease

In order to estimate disease severity on different maize and sorghum genotypes, a field trial was carried out based on a randomized complete block design with 10 treatments (Table 1) and three replications for each crop at Gorgan station (in the north of Iran) in 2010. The ears of corn plants were inoculated by spore suspension of the mixture of some virulent *F. verticillioides* isolates using an ear inoculation method (Nail Punch). The sorghum panicles were also inoculated by spraying of spore suspension isolates at flowering stage. All genotypes were evaluated based on the visual disease severity rating using scales 1-7 (Reid and Zhu, 2002) for fusarium ear rot and visual panicle grain mold rating (PGMR) using 1-9 scales (Sharma et al., 2010) for grain mold at physiological maturing stage.

Tab. 1 – Maize and sorghum genotypes used in this study

No.	Maize genotypes	Sorghum genotypes
1	K18	Kimia
2	K19	Sepideh
3	K19/1	KGS11
4	MO17	KGS12
5	K74/1	KGS15
6	B73rfc	KGS17
7	B73cms	KGS19
8	K166A	KGS23
9	A679	KGS27
10	K1264/1	KGS32

## Fumonisin production analysis

All infected ears and panicles were gathered from the field and mixed kernels of each genotype were randomly collected to evaluate by ELISA kits (AgraQuant Fumonisin Kit; Romer Labs, Austria) for their total fumonisins production at the physiological maturing stage.

## RESULTS AND DISCUSSION

Among *Fusarium* isolates, a total of 32 and 20 *F. verticillioides* strains were isolated from maize and sorghum kernels respectively and were identified based on the morphological criteria. After pathogenicity evaluation of isolates in greenhouse test, 8 aggressive isolates were selected for inoculating maize and sorghum genotypes.

All maize genotypes showed statistically significant difference in their susceptibility to *F. verticillioides* and fumonisin contamination in Gorgan (Figure 1).

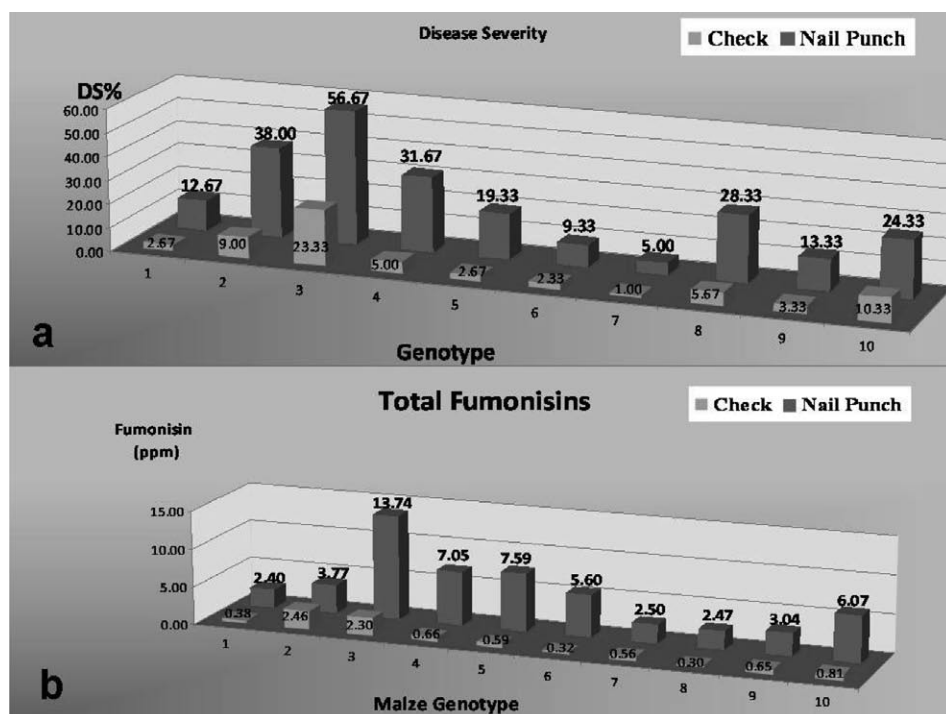


Fig. 1 – Different responses of maize genotypes to fusarium ear rot based on disease severity (%) at field experiment (a), and total fumonisin detected from maize kernels of each genotype using ELISA test (b)

The results of fumonisins analysis obtained from ELISA test showed that lines 1 (resistant) and 3 (susceptible) with 2.4 ppm and 13.7 ppm had the least and highest amount of total fumonisins respectively among all maize genotypes. On the other hand, line K19/1 (susceptible) had significantly higher levels of fumonisin in the grain and higher infection rate than the other genotypes used in this test. Fumonisin levels in maize kernels of infected ears ranged from 2.4 ppm to 13.74 ppm. No Maize line in this test was entirely free from fumonisin, so breeders should be able to find materials with better tolerance.

In addition, there were differences among sorghum genotypes in susceptibility to grain mold and also to fumonisin contamination (Figure 2). Fumonisin levels in sorghum kernels ranged from 0.2 ppm to 4.8 ppm. Among all sorghum genotypes, genotypes 10 and 6 with 0.2 ppm and 4.8 ppm had the least and the highest amount of total fumonisins respectively. This is the first report of fumonisin analysis of sorghum hybrids in Iran.

There was a relationship between fumonisin contamination and disease severity measured on different maize genotypes ( $r = +0.71$ ) and this value was estimated less ( $r = +0.59$ ) for sorghum genotypes (Figure 3). Our data are agreed with those of Bush et al., (2004) and Clements et al., (2004). Bush found that the maize hybrids reported to be more susceptible to Fusarium

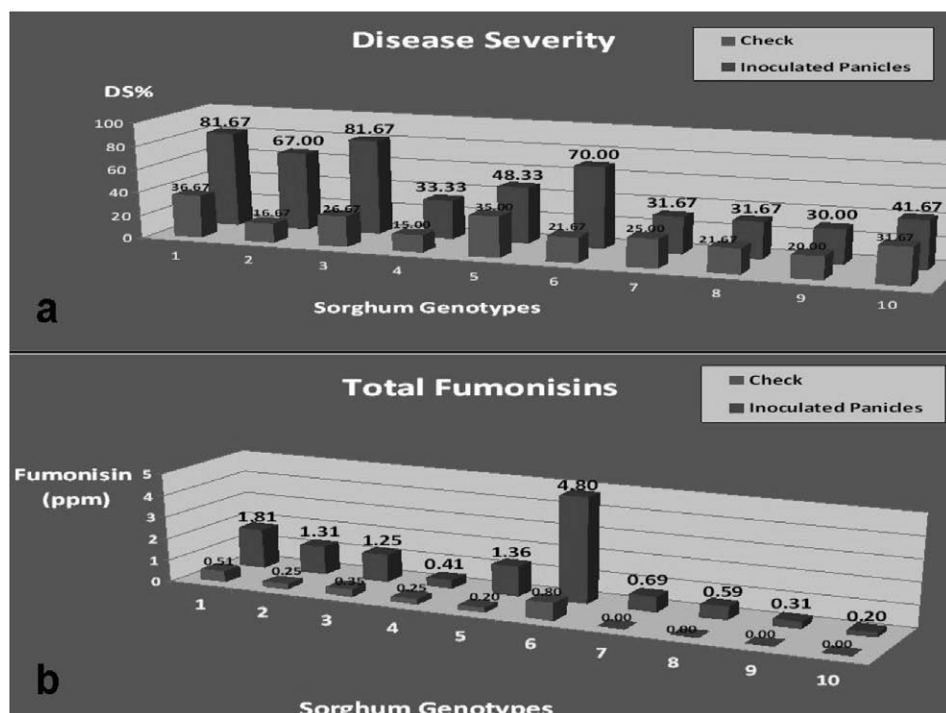


Fig. 2 – Different responses of sorghum genotypes to grain mold based on disease severity (%) at field experiment (a), and total fumonisin detected from sorghum kernels of each genotype using ELISA test (b)

ear rot had a significantly greater infection rate than either the resistant or the intermediate hybrids (Bush et al., 2004). In contrast to Keder et al., (1994) who did not find significant fumonisin production level in maize hybrids with high infection, Clements et al. (2004) found a correlation between low production levels and fusarium ear rot in some hybrids.

## CONCLUSION

As a conclusion, it seems that fumonisins are present on infected maize and sorghum kernels and even on healthy kernels in our country. Fumonisin can be considered as a great threat for consumers, especially when susceptible genotypes are used by farmers.

The results of this experiment demonstrated that fumonisin production level in maize kernels (maybe as maize kernels are the main host of this fungus) was significantly higher than in sorghum kernels in Gorgan region. There was a significant relationship between fumonisin contamination and disease severity measured on maize kernels. These results can help us to find the genotypes with low fumonisin accumulation in the future investigations. We need to continue

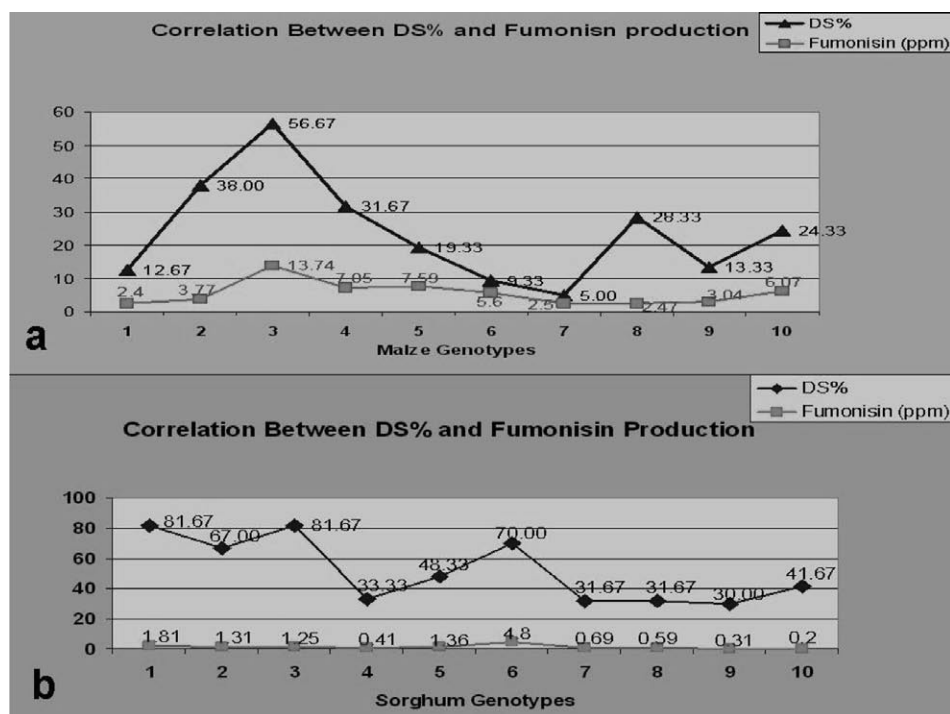


Fig. 3 – Estimated correlation between disease severity (DS %) and fumonisin production in maize genotypes (a), and sorghum genotypes (b)

our survey to quantify fumonisin levels in a broad range of genotypes at different locations and using more reliable methods like HPLC to find cultivars with low infection and fumonisin contamination.

## ACKNOWLEDGEMENTS

This work was supported by Seed and Plant Improvement Institute (SPII) and the Ministry of Jihad-e-Agriculture of the Islamic Republic of Iran, Project No. 003-03-88014.

## REFERENCES

- Anonymous (2009): *Agricultural statistical report*, Ministry of Jihad-e-Agriculture of Islamic Republic of Iran
- Bush, B. J., L. M. Carson, M. A. Cubeta, W. M. Hagler and Payne G. A. (2004): *Infection and Fumonisin production by Fusarium verticillioides in developing maize kernels*, *Phytopathology*. 94: 88-93.

- Castor, L. L. and Ferderiksen, R. A. (1980): *Fusarium and Curvularia grain molds in Texas*. Pages 93-102. in: Sorghum Diseases, a World Review. International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India.
- Chulze S. N., Ramirez M. L., Torres A., Leslie J. F. (2000): *Genetic variation in Fusarium section Liseola from no-till maize in Argentina*. Applied and Environmental Microbiology 66: 5312-5315.
- Clements, M. J., Maragos, C. M., Pataký, J. K., and White, D. B. (2004): *Sources of resistance to fumonisin accumulation in grain and Fusarium ear and kernel rot of corn*, Phytopathology. 94: 251-260.
- Danielsen S., Meyer U. M., Funck Jensen D. (1998): *Genetic characteristics of Fusarium verticillioides isolates from maize in Costa Rica*. Plant Pathology 47: 615-622.
- Gelderblom, W. C., Jaskiewicz, A., K., Marasas, W. F. O., Thiel, P. G., Horak, R. M., Vleggaar, R. and Kriek, N. P. J. (1988): *Fumonisin: novel mycotoxins with cancer-promoting activity produced by Fusarium moniliforme*, Appl. Environ. Microbiol, 54: 1806-1811.
- Gerlach W., Nirenberg H., 1982. *The genus Fusarium: a pictorial atlas*, Mitteilungen aus der Biologischen Bundesanstalt für Land-und Forstwirtschaft, Berlin-Dahlem.
- Ghiasian, S. A., Rezayat, S. M., Kord-Bacheh, P., Maghsood, A. H., Yazdanpanah H., Shephard G. S., Van der Westhuizen, L., Vismer, H. F., and Marasas W. F. O. (2005): *Fumonisin production by Fusarium species isolated from freshly harvested corn in Iran*, Mycopathologia, 159: 31-40.
- Jackson, L. and Jablonski, J. (2004). *Fumonisin*. In: Magan, M. and Olsen M. Mycotoxins in food. Woodhead publishing in food science and technology, Cambridge, England. 367-406 pp.
- Kedera, C. J., Ochor, T. E., Ochieng, J. A. W., and Kamidi, R. E. (1994b): *Incidence of maize ear rot in western Kenya*, International Journal of Pest Management, 40: 117-120.
- Leslie J. F., (1991): *Mating populations in Gibberella fujikuroi (Fusarium section Liseola)*, Phytopathology, 81: 1058-1060.
- Leslie J. F., Summerell B. A. (2006): *The Fusarium laboratory manual*, Blackwell Publishing Professional, Ames, USA.
- Marasas W. F. O. (2001): *Discovery and occurrence of the fumonisins: a historical perspective*, Environmental Health Perspective, 109 (suppl. 2): 239-243.
- Nelson, P. E., Desjardins, and R. D. Plattner R. D. (1993): *Fumonisin, mycotoxins produced by Fusarium species biology, chemistry, and significance*, Annu. Rev. Phytopathol. 31: 233-252.
- Rahjoo, V., Shephard, G. S., Klemsdal, S. S., Zad, J., Javan-Nikkhah, M., Okhovvat, S. M. and Bihamta, M. R. (2008a): *Evaluation of fumonisin production for some Fusarium isolates recovered from corn ears in Iran*, XII International Congress of Micology. 5-9 August, Istanbul, Turkey. (Abstract) p 51.
- Rahjoo, V., Zad, J., Javan-Nikkhah, M., Mirzadi Gohari, A., Okhovvat, S. M., Bihamta, M. R., Razzaghian, J. and Klemsdal, S. S. (2008b): *Morphological and Molecular identification of Fusarium isolated from maize ears in Iran*, Jorai of Plant Pathology, 90: 463-468.

- Reid, L. M., and Zhu, X. (2002): *Screening corn for resistance to common diseases in Canada*. [Online]. Available at [http://res2.agr.ca/ecorc/corn-mais/resistance/resistance\\_e.htm](http://res2.agr.ca/ecorc/corn-mais/resistance/resistance_e.htm). Agriculture and Agri-Food Canada, Central Experimental Farm.
- Sharma, R., Rao, V. P., Upadhyaya, H. D., Reddy, V. G., and Thakur, R. P. (2010): *Resistance to grain mold and downy mildew in a mini-core collection of sorghum germplasm*, Plant Dis. 94: 439-444.
- Shephard, G. S., Marasas, W. F. O., Yazdanpanah, H., Rahimian, H., Safavi, N., Zarghi, A., Shafaati, A., and Rasekh, H. R. (2002): *FBI in maize harvested in Iran during 1999*, Food Additional Contamination, 19: 676–679.
- Smith, C. W., and Frederiksen, R. A. (2000): *Sorghum: Origin, History, Technology and Production*, John Wiley & Sons, New York.
- Thakur RP, Reddy BVS, Indira S, Rao VP, Navi SS, Yang XB and Ramesh S. (2006): *Sorghum grain mold*. Information Bulletin no. 72. Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics. 32 pp.
- Yazdanpanah, H., Miraglia, M., Calfapietra, F. R., Brera, C., and Rasekh, H. R. (2001): *Natural occurrence of mycotoxins in cereals from Mazandaran and Golestan provinces*, Arch. Iranian medicine. 4: 107-114.
- Yazdanpanah, H., Shephard, G. S., Marasas, W. F. O., van der Westhuizen, L., Rahimian, H., Safavi, S. N., Eskandari, P., and Ghiasian, S. A. (2006): *Human dietary exposure to fumonisin B1 from Iranian maize harvested during 1998–2000*, Mycopathologia, 161: 395–401.
- Yoshizawa, T., Yamashita, A. and Luo, Y. (1994): *Fumonisin occurrence in corn from high-risk and low-risk areas for human esophageal cancer in China*, Appl. Environ. Microbiol. 60:1626-1629
- Zamani M., (1998): *Study on relative resistance of advanced maize lines to Fusarium ear rot*, Msc. Dissertation, University of Tarbiat Modarres, Iran.

## КВАНТИЗАЦИЈА УКУПНИХ ФУМОНИЗИНА КОЈЕ СТВАРАЈУ СОЈЕВИ *FUSARIUM VERTICILLIOIDES* КОД НЕКИХ ГЕНОТИПОВА КУКУРУЗА И СИРКА ПОМОЋУ ЕЛИСА ТЕСТА

Вахид Рахцу<sup>1</sup>, Марјам Парчамијан<sup>2</sup>, Мохамед Таги Феизбаш<sup>3</sup> и Мајид Замани<sup>1</sup>

<sup>1</sup> Завод за заштиту биља, Исламски универзитет Азад, Дамгхан, Иран

<sup>2</sup> Институт за унапређење семена и биља (SPII), Карај, Иран

<sup>3</sup> Центар за истаживање у пољопривреди провинције Горган, Иран

### Резиме

*Fusarium verticillioides* је једна од најраспрострањенијих *Fusarium* врста на кукурузу и сирку и изазива труљење клипа кукуруза и плесниност зрна сирка у топлим и влажним подручјима Ирана. Ова патогена гљива производи јаке микотоксине познате као фумонизини. У циљу одређивања стварања микотоксина (фумонизина) на различитим генотиповима кукуруза и сирка постављен је оглед по случајном *complete-block* дизајну са 10 третмана и 3 понављања за сваки род на локацији Горган 2010. године. Клипови кукуруза су инокулирани техником



бушења са суспензијом спора која представља мешавину неких вирулентних *F. verticillioides* изолата. Метлице сирка су инокулиране прскањем суспензије изолата која садржи споре у време цветања. Сва инфицирана зрна су помоћу ЕЛИСА китова (AgraQuant Fumonisin Kit; Romer Labs, Austria) оцењивана у погледу укупне производње фумонизина у физиолошкој фази сазревања. Сви генотипови су показали статистички значајну разлику у производњи фумонизина на локацији у Горгану. Резултати добијени ЕЛИСА тестом су показали да су линија 1 (отпорна) и линија 3 (осетљива) са 2,4 ppm односно 13,7 ppm линије са највећим односно најмањим садржајем укупних фумонизина код свих генотипова кукуруза. Код сирка, генотип 10 и генотип 6 са 0,2 ppm односно 4,8 ppm су имали највећи односно најмањи укупни садржај фумонизина. Резултати нашег експеримента у региону Горган су показали да је ниво стварања фумонизина у зрну кукуруза (можда због тога што је кукурузно зрно главни домаћин ове гљивице) био знатно већи него у зрну сирка.

Majid Zamani<sup>1</sup>, Vahid Rahjoo<sup>1</sup>, Maryam Parchamian<sup>2</sup>

<sup>1</sup> Maize and Forage Crops Research Department, Seed and Plant Improvement Institute, Karaj, Iran

<sup>2</sup> Plant Protection Department, Islamic Azad University, Damghan, Iran

## EVALUATION OF THE REACTION OF EARLY MATURING MAIZE GENOTYPES TO COMMON SMUT USING ARTIFICIAL INOCULATION\*

**ABSTRACT:** In order to study of the resistance of early maize genotypes, 44 lines, and hybrids in 2008 and 30 genotypes in 2009 in RCBD were conducted at Karaj and Esfahan Stations. All of the ears were inoculated by tip injection method at suitable time. Inoculation was carried out with syringe (3ml/each ear) at silking stage. Evaluation was done on ears 3-4 weeks after inoculation. The results of variance analysis and mean comparison showed that there is a different reaction among material to disease. In this study, after harvesting of ears, the resistance of each genotype was determined based on disease severity index (0-7). Among examined lines in 2008, line no. (41) (KE 77004/1-1-1) was specified as susceptible and no. (28 and 42) (KE 77003/1-8-1) and (37A) as resistant lines. Also among hybrids, K SC 400 was identified as resistant hybrid. Also, among lines in 2009, line no. (29) (KE77004/1-1-1) was specified as susceptible and line no. (27) (KE77005/4) as resistant line respectively. Among hybrids, hybrid no. (9) (KE77006/3 × K1263/1) evaluated as resistant hybrid.

**KEY WORDS:** Artificial inoculation, common smut, maize, resistance

## INTRODUCTION

Common smut is an extremely disease of different types of corn in throughout the world. Causal agent is a fungus named *Ustilago maydis*. One of the special characters of the fungus is high production of spores that the rate of it is estimated  $2.5-6 \times 10^6$  in  $1 \text{ cm}^3$  and the stability in soil has been reported between 5-7 years (Christesen, 1963). In order to pathogenecity and complementary of life cycle, mating type among compatible sporidia is necessary to change into pathogenic dikaryon mycelium, which penetrate to shoot system specially ears of susceptible genotypes to caused yield loss (Day and Anagnostakis, 1971).

\* The paper was presented at the fourth international scientific meeting *Mycology, mycotoxicology, and mycoses*, which was organized in *Matica srpska, Department for natural sciences* from April 20-22, 2011.

Losses vary with the year, geographical location, and cultivar grown. Yields of individually infected plants can be reduced 40%-100% if galls are large or formed on the ears (Pope and McCarter, 1992). Host resistance offers, the most economical means of control of common smut (Smith and White, 1988). In some cases, resistance may be a polygenic trait such as tightness and thickness of husks that involves few genes might be effective physiological and morphological characters (Kyle, 1929).

In order to determine the response of corn materials to smut in artificial infection condition, it is better to be applied sporidia suspension through tip injection so that physiological resistance of cob and kernel tissues is investigated (Pope and McCarter, 1992).

Zamani and Choukan (1990) evaluate the resistance of 60 late hybrids using cob injection method and reported K 3165/2 x MO 17 as susceptible and K 1259/3 x B 73 as resistant hybrid. The aim of this study was to evaluate early genotypes and to determine the resistance of them in pathology unit to apply in breeding programs.

## MATERIALS AND METHODS

Some infected corn ears to common smut were gathered from different locations of Iran in 2007. Teliospores from naturally infected ears were surface sterilized with CuSo<sub>4</sub> 1.5% and then were transferred on APDA and CMA media and incubated at 23-25°C. Following teliospore germination, sporidia were removed and streaked onto APDA two more times to obtain pure colonies. In order to prepare of sporidia, from galls of infected sample, 12 compatible samples were cultured and finally 6 samples were selected.

In this study, all possible pair combinations were used to give a final concentration of  $1 \times 10^6$  sporidia per mm. Then 3 ml of mixed culture was injected to each ear by injection method.

Field plots were located at Karaj and Isfahan. In this study, 44 early genotypes in 2008 and 30 genotypes in 2009 were selected and cultivated in RCBD with 3 Replications.

Routine practices were used on plots. In every year all of genotypes were planted in rows spaced 75 cm. All of the ears were inoculated by tip injections method at suitable time (Mid silking) when the silks are 5 cm. Inoculum was applied to silk channel from the tip of the each ear. The inoculum was released into ears without any wound. Inoculation was carried out with syringe (3ml each ear) at silking stage.

After incidence of symptoms (galls), evaluation of material was conducted 4 weeks, after inoculation. Disease severity determined with visual rating scale (0-7) in which 0 indicates no infection and 1-7 indicate increasing proportion of ear galled. Disease severity data **were subjected** to an angular transformation prior analysis to improve homogeneity of variance. The response of material to common smut with using disease severity is categorized six groups.

## RESULTS AND DISCUSSION

The result of variance analysis and mean comparison showed significant differences among genotypes when using disease severity index (Table 1). Also effect location x year and interaction genotypes x year, genotypes x location at 1% level was significant at 5% level. In this case is stated that common smut showed different reactions in every location due to different whether condition in disease development, whereas (D a y and A n a g n o s t a k i s , 1971) reported that the disease development at dry regions and (S m i t h and W h i t e , 1988) reported humid region for disease development, that it could be made problems for screening of maize genotypes.

Tab. 1 – Variance analysis of disease severity of common smut on late maize hybrids in 2008 and 2009 at Karaj and Isfahan stations

(S.O.V.)	(d.f)	(MS) Smut	(d.f)	(MS) Smut
	Year 2008		Year 2009	
Location (L)	1	0.371*	1	11.5*
Error (E1)	4	0.154	4	1.297
Hybrid (H)	43	4.133**	29	6.259**
L x H	43	1.469**	29	1.525**
Error (E2)	172	0.398	116	0.617
(%CV)	27.89%		29.80%	

\*, \*\*: Significant at 5% and 1% levels of probability respectively

Therefore, with using disease severity index all of genotypes were categorized in different groups (Table 2).

Disease development on lines and hybrids were satisfied in 2008 (Figure 1) where as the average of infection in K SC 301 was 3.583 as check and range of disease was 0.81 to 4.33 (Table 2).



Fig. 1 – An illustration of infected ears after artificial inoculation in field

Tab. 2 – Mean comparison and ranking of early maturity maize genotypes to common smut in 2008 based on disease severity at Karaj and Isfahan

Response	Mean of disease severity (DS)	Early genotypes	No.
MS	2.450 ABCDEF	KE 75016/2-1-1-1 x K 1264/5-1	1
MS	2.733 ABCDEF	KE 76009/1-4-1-2-3-1 x K 1264/5-1	2
S	3.050 ABCDEF	KE 76005/111 x K 1264/5-1	3
MR	1.450 CDEF	KE 76006/111 x K 1264/5-1	4
MR	1.967 BCDEF	KE 75006/212 x K 1264/5-1	5
MS	2.700 ABCDEF	KE 76009/311 x K 1264/5-1	6
MS	2.393 ABCDEF	KE 76009/321 x K 1264/5-1	7
MR	1.167 EF	KE 75006/212 x K 1263/1	8
MS	2.483 ABCDEF	KE 76009/311 x K 1263/1	9
MS	2.083 ABCDEF	KE 76009/312 x K 1263/1	10
S	3.583 ABCD	K 1271/6 x OH 43/1-42	11
MR	1.833 BCDEF	K 1263/1 x KE 75039	12
MS	2.383 ABCDEF	K 1263/1	13
MS	2.683 ABCDEF	KE 72012/12	14
MS	2.217 ABCDEF	K 1728/8	15
MR	2.000 BCDEF	K 2331	16
MR	1.500 CDEF	K 1263/2-1	17
S	3.917 AB	OH 43/1-42	18
MS	2.467 ABCDEF	R 59	19
S	3.233 ABCDE	K 615/1	20
MR	1.600 BCDEF	K 1264/5-1	21
MR	1.650 BCDEF	K 2325/1	22
MR	1.417 DEF	K 1263/17	23
MR	1.700 BCDEF	S 61	24
MR	1.650 BCDEF	R 59 x R 319	25
MR	1.967 BCDEF	37A x W 153 R	26
MR	1.083 EF	R 319	27
R	0.817 F	37A	28
S	3.850 AB	153R	29
MR	1.467 CDEF	K SC 201	30
MS	2.183 ABCDEF	DC 370	31
R	0.967 F	K SC 400	32
MS	2.150 ABCDEF	K SC 260	33
MS	2.800 ABCDEF	K SC 500	34
MS	2.033 BCDEF	K SC 302	35
S	3.767 ABC	K SC 302/1	36
MS	2.367 ABCDEF	KE 79008/5-11	37
MS	2.850 ABCDEF	KE 79006/3-2-1	38
MS	2.767 ABCDEF	KE 79017/5-2-1	39
MR	1.317 DEF	KE 79015/6-2-1	40
HS	4.333 A	KE 77004/1-1-1	41
R	0.850 F	KE 77003/1-8-1	42
MS	2.150 ABCDEF	K 722	43
S	3.583 ABCD	K SC 301	44

S: susceptible; MS: moderately susceptible; HS: highly susceptible; R: resistant; MR: moderately resistant

Among examined lines in 2008, line No. 41 was specified as highly susceptible and lines No. 28 and 42 as resistant lines. Also among hybrids, KSC400 identified as resistant hybrid. The result of Gogh (1976) also confirmed that the rate of disease is low in crosses of resistant lines (R×R); on the contrary, in susceptible lines the rate of infection is very high.

In breeding programs, resistant lines have been specified to smut, but so far mechanism and stability of resistances has not been understood. Meanwhile, morphological characters such as thick of husk cover could be affected in resistance stages (Kyle, 1929). In order to confirm resistance of some genotypes and other materials, 30 early maturity genotypes were evaluated by artificial inoculation in 2009 (Table 3).

Tab. 3 – Mean comparison and ranking of early maturity maize genotypes to common smut in 2009 based on disease severity at Karaj and Isfahan

Response	Mean of disease severity (DS)	Early genotypes	No.
MR	1/217 DE	KE 77002/1 × K1263/1	1
MR	1/700 CDE	KE77003/2 × K 1263/1	2
MS	2/333 BCDE	KE77003/3 × K 1263/1	3
MS	2/883 BCDE	KE77003/4 × K 1263/1	4
MR	1/750 CDE	KE77003/7 × K1263/1	5
MS	2/317 BCDE	KE77003/8 × K1263/1	6
MS	2/683 BCDE	KE77003/9 × K1263/1	7
S	3/450 BCD	KE77003/10 × K1263/1	8
R	0/950 E	KE77006/3 × K1263/1	9
MS	2/367 BCDE	KE770010/2 × K1263/1	10
MS	2/583 BCDE	KE77003/3 × K1264/5-1	11
MS	2/800 BCDE	KE77003/4 × K1264/5-1	12
S	3/350 BCDE	KE77003/9 × K1264/5-1	13
S	3/950 ABC	KE77003/10 × K1264/5-1	14
MS	2/667 BCDE	KE77003/12 × K1264/5-1	15
S	3/917 ABC	KE77004/1 × K1264/5-1	16
S	3/217 BCDE	KE77006/2 × K1264/5-1	17
MS	2/350 BCDE	KE77006/3 × K1264/5-1	18
S	3/200 BCDE	KE77006/4 × K1264/5-1	19
MS	2/150 CDE	KE77005/4 × K1264/5-1	20
MS	2/033 CDE	KE78002/231	21
MS	2/917 BCDE	KE8011/61231	22
HS	4/500 AB	KE78011/61232	23
MS	2/150 CDE	KE78011/611	24
MR	1/400 DE	KE77010/3	25
MS	2/567 BCDE	KE77006/4	26
MR	1/100 DE	KE77005/4	27
MS	2/050 CDE	KE77005/2	28
HS	5/767 A	KE77004/1-1-1	29
MS	2/650 BCDE	KE77003/10	30

S: susceptible; MS: moderately susceptible; HS: highly susceptible; R: resistant; MR: moderately resistant

Disease development on lines and hybrids in 2009 was better than last year, whereas range of disease in this year (2009) was 0.95 to 5.76; also the average of infection in both years was about 2.41 (Table 3). Among lines, line No. 29 was highly susceptible similar to last year and line 27 as resistant. Among hybrid, hybrid No. 9 evaluated as resistant hybrid. The greatest of DS was related to KE77004/1-1-1 and at least in KSC 400. In addition, crosses of this line (K1263/1) was resistant to smut such as KE 77006/3 x K 1263/1. In this study from two susceptible lines (K1271/6 and OH 43/1-42) was obtained one susceptible hybrid (K1271/6 x OH 43/1-42).

## CONCLUSION

It is clear that disease severity can be considered as a suitable and a stable index for evaluating of resistance of genotypes to common smut as our results demonstrated.

In general, disease development in this method (Tip injection) and its facility in applying in cornfields could be an accurate assessment for breeders to release the best and most resistant hybrids for introducing to farmers. Therefore, further investigations should be conducted to determine resistant cultivars to common smut in Iran.

## ACKNOWLEDGEMENT

This study is the part of investigation realized within the scope of project No. 0-03-03-87020 and financially supported by Seed and Plant Improvement Institute (SPII) and the Ministry of Jihad-e-Agriculture of the Islamic Republic of Iran.

## REFERENCES

- Christensen, J. J. (1963): *Corn Smut Caused by Ustilago maydis*, American Phytopathological Society, St. Paul, Minnesota, USA. Monograph 241 PP.
- Day, P. R., and Anagnostakis, S. L. (1971): *Meiotic products from natural infection of Ustilago maydis*. Phytopathology 61: 1020-1021.
- Jugenhiemer, R. W. (1976): *Corn Improvement, Seed Production and Uses*, John Wiley and Sons. New York. 670 pp.
- Kyle, C. H. (1929): *Relation of husk covering to smut of corn ears*, U. S. Department of Agriculture Technical Bulletin No.120. pp. 1-70.
- Pataky, J. K., Nankan, C., and Kerns, M. R. (1995): *Evaluation of a silk inoculation technique to differentiate reactions of sweet corn hybrids to common smut*, Phytopathology 85:1323-1328.



- Pope, D. D., and McCarter, S. M. (1992): *Evaluation of inoculation methods for inducing common smut on corn ears*, Phytopathology 82: 950-955.
- Renfro, B. L. (1983): *Genetic of Resistance to Disease in Maize*, CIMMYT (International Center for in Improvement of Maize and Wheat), Mexico.
- Shurtleff, M. C. (1980): *Compendium of Corn Diseases*, 2<sup>nd</sup> ed. American Phytopathological Society, St. Paul, Minnesota, 105 pp.
- Smith, D. R. and White, D. G. (1988): *Diseases of Corn*, Pp. Pages 687-766. in Corn and Corn Improvement. Academic Pres, New York, USA
- Thakur, R. P., Leonard, K. J., and Pataky, J. K. (1989): *Smut gall development in adult corn plant inoculated with Ustilago maydis*, Plant Disease. 73:921-925.
- Ulstrup, A. J. (1978): *Corn diseases in the United State and their control*, Agricultural Handbook, No. 199. 21 pp.
- Zimmermon, S. A., and Pataky, J. K. (1992): *Inoculation techniques to produce galls of common smut on ears of sweet corn*, Phytopathology 82:995.

## ЕВАЛУАЦИЈА ОТПОРНОСТИ ГЕНОТИПОВА РАНОГ КУКУРУЗА НА ГАР ПОМОЋУ ВЕШТАЧКЕ ИНОКУЛАЦИЈЕ

Мадид Замани<sup>1</sup>, Вахид Рахџу<sup>1</sup>, Марјам Парчамјан<sup>2</sup>

<sup>1</sup> Завод за истраживање кукуруза и сточне хране, Институт за унапређење  
семена и биља, Карај, Иран

<sup>2</sup> Завод за заштиту биља, Исламски универзитет Азад, Дамгхан, Иран

### Резиме

У циљу проучавања отпорности генотипова раног кукуруза постављен је оглед са 44 линије хибрида 2008. године и 30 генотипова 2009. године по случајном *complete-block* дизајну на локацијама у Карају и Есфahanу. Сви клипови су инокулирани инјекционом техником у одговарајуће време. Инокулација је обављена убризгавањем (3 ml по сваком клипу) у време свилања кукуруза. Евалуација клипова је урађена након 3 до 4 недеље. Резултати добијени анализом варијансе и поређење средњих вредности су показали да постоји различита отпорност испитиваног материјала на болест. Након брања кукурузних клипова одређивана је отпорност сваког генотипа на бази индекса тежине обољења (0-7). Од испитиваних линија у 2008. години као осетљива се показала линија број 41 (KE77004/1-1-1) а као отпорне линије број 28 и 42 (KE77003/1-8-1 и 37A). Од испитиваних хибрида отпорност је показао K SC 400. Од испитиваних линија у 2009. години, линија број 29 (KE77004/1-1-1) се показала као осетљива а линија број 27 (KE77005/4) као отпорна. Од испитиваних хибрида, хибрид број 9 (KE77006/3xK1263/1) је оцењен као резистентан.



*Ferenc F. Balaž*<sup>1</sup>, *Marija I. Bodroža Solarov*<sup>2</sup>,  
*Jovana N. Vučković*<sup>2</sup>, *Ferenc F. Bagi*<sup>1</sup>

<sup>1</sup>Faculty of Agriculture, Department for Environmental and Plant Protection, D. Obradovića 8 Novi Sad

<sup>2</sup>Institute of Food Technology, Bulevar Cara Lazara 1, Novi Sad

## EFFECTS OF CHEMICAL TREATMENTS ON INFESTATION OF *ALTERNARIA* SPP. AND *FUSARIUM* SPP. IN CORRELATION WITH TECHNOLOGICAL WHEAT QUALITY\*

**ABSTRACT:** In this study, the time of infestation by fungi from genus *Alternaria* spp. and *Fusarium* spp. was investigated in different stages of wheat maturity (milk, waxy, and technological maturity); the effects of different fungicides on the yield, technological properties, and content of mycotoxin DON were studied also.

The results showed that *Alternaria* spp. attacked spike and kernel in flowering and end-flowering stage, as it was already known for *Fusarium* species. Fungicide treatment increases the yield up to 20%, test weight by 3.7%, and thousand-kernel weight up to 19.1%.

High content of mycotoxin DON, above tolerable limits, was detected only in the treatment with fungicide Caramba and in untreated control.

**KEY WORD:** chemical treatment, *Fusarium*, *Alternaria*, technological quality, DON

## INTRODUCTION

In difference to the 70's and 80's of the last century, when *Fusarium* species dominated on wheat kernels, diseases caused by *Alternaria* spp. are of recent widespread. In 2004, *Alternaria* spp. and some other fungi caused extremely low technological quality of wheat kernel (Balaž, 2010).

Fungi from genus *Fusarium* have high economic significance causing decrease of yield and grain quality. In Serbia, agro-climatic conditions favor development of *Fusarium graminearum* (Schwabe) which is the main causal agent of wheat head blight (Balaž et al., 2006; 2010). According to Bagi (1999) in years with average precipitation level, disease incidence of wheat head blight is about 5%. Beside *F. graminearum*, some other *Fusarium* species as well

---

\* The paper was presented at the fourth international scientific meeting *Mycology, mycotoxicology, and mycoses*, which was organized in *Matica srpska, Department for natural sciences* from April 20-22, 2011.

as fungi from genera *Alternaria*, *Aspergillus*, *Penicillium* etc., colonize wheat kernel inducing significant losses.

As previously described, fungal contamination causes significant yield decrease, but the losses are even greater because of mycotoxins produced by these fungi. Cereals contaminated by *Fusarium* spp. and other mycotoxin producers, are not only a risk to human and animal health, but also influence reduced technological properties (Prange et al., 2005; Havlova et al., 2006; Suchowilska et al., 2007, 2010).

Haidukowski et al. (2005) consider that fungicide treatment during or immediately after wheat flowering stage can significantly reduce *Fusarium* head blight infestations and control wheat leaf diseases as well.

The aim of this research was to investigate the time of head infection by *Alternaria* spp. and *Fusarium* spp. and chemical control possibilities against pathogens, as well as their influence on technological wheat quality.

## MATERIAL AND METHODS

The experiment was conducted in 2010 on the location of Bački Petrovac using wheat variety Renesansa.

During vegetation period standard agrotechnical measures were applied. Chemical treatments were applied in flowering stage using TwenJet 11004 nozzle. In the research were used following fungicides: Osiris 2l/ha (metconazole+epoxiconazole), Prosaro 1 l/ha (tebuconazole+prothioconazole), Champion 1.2 l/ha (epoxiconazole+boskalid), Caramba 1.5 l/ha (metconazole), Bumper 1l/ha (propiconazole), Zamir 1l/ha (prochloraz+tebuconazole). Plots of each treated wheat variety were established randomly in four replications with untreated control. Each parcel was 5 m<sup>2</sup>.

Wheat kernel mycobiota was determined by phytopathological isolations in milk stage, waxy, and technological maturity. *Fusarium* head blight severity was evaluated calculating the number of infected spikes/m<sup>2</sup> during early milk stage of wheat. Immediately after harvest, in purpose of mycotoxin and quality parameters analysis and mycobiota determination, representative seed samples were taken.

Thousand-kernel weight was determined by Perten SK CS 4100 (Kernel Hardness Tester, Perten Instruments, Reno, Nevada, USA). The hectoliter weight was measured by dusting the procedure and equipment Schopper scale. Level of fungal infection was determined by Petri dish test according to Pitt and Hocking (1985). Determination of fungi to the level of genus was performed based on morphology and cultural characteristics.

Protein content was determined according to AOAC approved methods 992.23. Gluten content was determined according to ICC standard No 106/1.

Mycotoxin DON was determined from both treated and untreated wheat cultivars by ELISA (enzyme-linked immunosorbent assay) method. Screening method for analysis was done using Neogen Veratox<sup>®</sup> testing kits with limits of detection 0.25 mg/kg for DON (342 Veratox DON-a 5/5).

All data were processed using Statistica 10 software (StatSoft Inc., Tulsa, Oklahoma). Statistically significant differences between means were detected by Tukey's test after analysis of variance (ANOVA).

## RESULTS

During last two decades, in agricultural practice in Serbia, fungicide application in wheat protection has been reduced and focused on good agrotechnical measures and fertilization. Fungicides are used only in the high yield targeted production. Since *F. graminearum* is dominated pathogen, *F. culmorum* appears only in very humid years (B a g i , 1999). Beside *Fusarium* spp., *Alternaria* spp. occurs very frequently nowadays (B a l a ž et. al., 2010).

The results showed that *Alternaria* spp. infected spike and kernel in flowering and end-flowering stage such as fungi from *Fusarium* genera. That explains positive effect of chemical treatments on wheat in flowering stage. Chemical treatments were applied in flowering stage using TwenJet 11004 nozzle that enables better ear coverage with fungicides, which is in correlation with L e h o c z k i - K r s j a k et al. (2008).

Effects of fungicide treatments had high significance in all stages, i.e. less percentage of infestations by fungi in respect to the control plot. Infection was positively correlated with yield decrease.

On the control plot yield was 6.132t/ha, that is 19.7% less than yield achieved in the treatment with fungicide Osiris of 7.635 t/ha (Table 1).

Results achieved in this work are in accordance to previous researches, which showed that yield is increased by fungicides treatment quantitatively such as qualitatively (B a l a ž et. al, 2006).

Tab.1 – Infestation of *Alternaria* spp. and *Fusarium* spp. in different seed development stages of wheat with different fungicide treatments

Fungicides	SEED DEVELOPMENT STAGES						YIELD t/ha
	Milk stage		Waxy maturity		Technological maturity		
	<i>Alternaria spp.</i>	<i>Fusarium spp.</i>	<i>Alternaria spp.</i>	<i>Fusarium spp.</i>	<i>Alternaria spp.</i>	<i>Fusarium spp.</i>	
Osiris	0 a	0 a	0 a	0.5 a	0 a	1.2 a	7.635 c
Prosaro	10.1 b	0 a	5.3 b	0.8 a	0.3 a	2 a	7.582 c
Caramba	15.3 c	5.2 b	16.4 c	2.8 ab	14.9 b	4.6 b	7.254 bc
Champion	10.2 b	8.5 bc	13.4 c	6.2 b	12.7 b	7.3 b	6.972 b
Bumper	13.8 bc	10.6c	12.9 c	11.2 c	14.1 b	12.6 c	6.725 b
Zamir	22 d	15.8 d	21.7 d	17.3 d	24.1 c	16.6 c	6.725 b
CONTROL	36.4 e	26.7 e	34.8 e	25.6 e	38.4 d	28.7 d	6.132 a

Mean values in the same column followed by different letters of the same case are significantly different ( $P < 0.05$ )

In the case of 1000 kernel weight and hectoliter weight, fungicide treated cultivars had significant higher values compared to untreated control on the level of  $p < 0.05$  (Table 2).

Previously described fungi are of great importance due to toxin production, particularly DON produced by *Fusarium* spp., which was examined in many researches focused on resistance of genotypes to *Fusarium* head blight, fungicide efficacy, and mycotoxin accumulation (Simpson et al., 2001, Pirgozliev et al., 2002, Andersen and Thrane, 2006).

Deoxynivalenol is mycotoxin produced by *F. graminearum* and *F. culmorum*. Although it is considered the most frequent contaminant of wheat kernel infested by *Fusarium* head blight, it should not neglect other toxin producers harmful for animal and human health.

According to Simpson et al. (2001) and Haidukowski et al. (2005) fungicides treatments with triazoles, very effectively control *Fusarium* head blight and content of DON and combination of triazoles (tebuconazole + prothioconazole) is even more efficient stated by Hauser Hahn et al. (2008).

Tab. 2 – Effect of fungicides treatments on physical, chemical and DON mycotoxin content in wheat grain

Fungicides	Test weight (kg/hl)	1000	Protein content (%/d.m.)	Gluten content (%)	DON mg/kg
		kernel weight /sm			
Zamir	79.7 b	38.9 e	12.13 b	24.12 b	<0.25 a
Champion	79.5 b	33.4 c	12.42 bc	25.14 bc	<0.25 a
Osiris	79.7 b	34.7 d	12.23 b	24.50 b	<0.25 a
Prosaro	79.7 b	34.4 d	14.4 d	30.47 d	<0.25 a
Pumper	79.7 b	32.7 b	12.06 b	23.92 b	<0.25 a
Caramba	80.1 b	33.7 c	12.9 c	26.60 c	0.77 b
CONTROL	77.2 a	31.5 a	11.45 a	22.01 a	1.30 c

Mean values in the same column followed by different letters of the same case are significantly different ( $P < 0.05$ )

Mycotoxin DON was present above detection limit in two cases wheat sample treated with fungicide Caramba (0.77 mg/kg) and untreated control (1.3 mg/kg). This research shows that chemical treatments in flowering stage can effectively suppress the occurrence of all contaminate products of fungi (Table 2).

Chemical composition of kernel, regarding protein and gluten, also indicates the positive effect of wheat protection. Higher content of protein and gluten in wheat treated with fungicide can be explained by better conditions for the synthesis of protein compared to untreated control. The highest protein content was remarked in the treatment with Prosaro, in amount of 14.4%, which is a highly significant difference compared to untreated control 11.45% (Table 2).

## CONCLUSION

Results of this work showed that fungus from genus *Alternaria* genera attacked head in flowering and end-flowering phase respectively.

Fungicides Osiris and Prosaro applied with Twen Jet 11004 nozzle provided efficient protection against the most common wheat diseases.

Efficient protection of wheat from *Alternaria* spp. and *Fusarium graminearum* contribute to high technological kernel quality and high yield.

## ACKNOWLEDGEMENT

The authors thank the Ministry of Science and Technological Development of the Republic of Serbia for financial support (Project III 46005).

## REFERENCES

- Andersen, B., Thrane, U. (2006): *Food-Borne Fungi in Fruit and Cereals and Their Production of Mycotoxins*. In: Hocking, A.D., Pitt, J. I., Samson, R. A., Thrane, U. (eds) *Advances in food mycology*. Springer US, 137-152.
- AOAC approved methods 992.23
- Bagi, F. (1999): *Testing the population heterogeneity of fungus Fusarium graminearum Schwabe pathogen of wheat and maize*. University of Novi Sad, Master Sc. thesis.
- Balaž, F. (2010): *Alternaria spp. značajan patogen semena pšenice i mogućnosti hemijske zaštite*, X Savetovanje o zaštiti bilja, Zlatibor, Book of abstracts, 88-89.
- Balaž, F., Bagi, F., Stojšin, V., Mastilović, J., Bukvić, Ž. (2006): *Influence of chemical protection against economically important wheat diseases on yield and technological parameters of grain in 2006*. 7<sup>th</sup> Plant protection meeting, Serbian Plant Protection Society, Zlatibor, 27.11-01.12.2006., Book of abstracts, 54-55.
- Haidukowski, M., Pascale, M., Perrone, G., Pancaldi, D., Campagna, C., Visconti, A. (2005): *Effect of fungicides on the development of Fusarium head blight, yield and deoxynivalenol accumulation in wheat inoculated under field conditions with Fusarium graminearum and Fusarium culmorum*, Journal of the Science of Food and Agriculture, 85:191-198.
- Hauser Hahn, I., Dutzmann, S., Freissleben, R., Meissner, R., Goehlich, F. (2008): *Prosaro- a new fungicide for control of Fusarium and mycotoxins in cereals*, Cereal Research Communacations, 36 (Suppl. B): 711-712.
- Havlova, P., Lancova, K., Vanova, M., Havel, J., Hajslova, J. (2006): *The effect of fungicidal treatment on selected quality parameters of barley and malt*, Journal of Agricultural and food chemistry, 54: 1353-1360.
- ICC standard No 106/1
- Lehoczki-Krsjak, Sz., Toth, B., Kotai, Cs., Martonosi, I., Farady, L., Kondrak, L., Szabo-Hever, A., Mesterhazy, A. (2008): *Chemical*



- control of FHB in wheat with different nozzle types and fungicides. Cereal Research Communications, 36, (Suppl. B): 677-678.
- Pirgozliev, S. R., Edwards, S. G., Hare, M. C., Jenkinson, P. (2002): Effect of dose rate of azoxystrobin and metconazole on the development of *Fusarium head blight* and the accumulation of deoxynivalenol (DON) in wheat grain, European Journal of Plant Pathology, 108: 469-478.
- Pitt, J. I., Hocking, A. D. (1985): *Fungi and Food Spoilage*, CSIRO Division of Food Research, Sydney, Academic press, 1- 414.
- Prange, A., Birzele, B., Johannes Krämer, Meier, A., Modrow, H., Köhler, P. (2005): *Fusarium inoculated wheat: deoxynivalenol contents and baking quality in relation to infection time*, Food Control 16, 739–745.
- Simpson, D. R., Weston, G. E., Turner, J. A., Jennings, P., Nicholson, P. (2001): *Differential control of head blight pathogens of wheat by fungicides and consequences for mycotoxin contamination of grain*, European Journal of Plant Pathology 107: 421-431.
- Suchowilska, E., Kandler, W., Sulyok, M., Wiwarta, M., Krskab, R. (2010): *Mycotoxin profiles in the grain of Triticum monococcum, Triticum dicoccum and Triticum spelta after head infection with Fusarium culmorum*, J Sci Food Agric, 90, 556–565.
- Suchowilska, E., Wiwart, M., Borusiewicz, A. (2007): *The reaction of the selected Triticum aestivum, Triticum spelta and Triticum dicoccum genotypes to spike infection by Fusarium Culmorum*, Sbornik z konference „Ekologické zemědělství 2007”, 174.

## УТИЦАЈ ХЕМИЈСКЕ ЗАШТИТЕ ПШЕНИЦЕ НА ИНТЕЗИТЕТ ЗАРАЗЕ ВРСТАМА ИЗ РОДА *FUSARIUM* SPP. И *ALTERNARIA* SPP. У КОРЕЛАЦИЈИ СА ТЕХНОЛОШКИМ КВАЛИТЕТОМ

Ференц Ф. Балаж<sup>1</sup>, Марија И. Бодрожа Соларов<sup>2</sup>, Јована Н. Вучковић<sup>2</sup>,  
Ференц Ф. Баги<sup>1</sup>

<sup>1</sup> Пољопривредни факултет, Департман за заштиту биља и животне средине, Д.  
Обрадовића 8, 21000 Нови Сад

<sup>2</sup> Научни институт за прехранбене технологије, Цара Лазара 1, 21000 Нови Сад

### Резиме

Испитивано је време инфекције гљивама из рода *Alternaria* spp. и *Fusarium* spp. у различитим фазама зрелости пшенице (млечна, воштана, пуна) као и дејство примене различитих фунгицида на принос, параметре технолошког квалитета, и садржај микотоксина ДОН. Резултати овог истраживања су да и гљиве из рода *Alternaria* заражавају клас и зрно у фази формирања зрна, као што је то до сада било познато за *Fusarium* врсте. Третман фунгицидима повећава принос до 20 %, хектолитарску масу за 3,7%, масу хиљаду зрна до 19,1%. Садржај микотоксина ДОН изнад дозвољене границе утврђен је једино код примене комерцијалног препарата Caramba и код контроле без третмана.

*Franc Čuš<sup>1,2</sup>, Neža J. Čadež<sup>2</sup>, Peter I. Raspor<sup>2</sup>*

<sup>1</sup>Agriculture Institute of Slovenia, Central Laboratory, Hacquetova 17, 1000 Ljubljana, Slovenia

<sup>2</sup>University of Ljubljana, Biotechnical Faculty, Department of Food Technology, Chair of Biotechnology

## FUNGICIDE RESIDUES IN GRAPES DETERMINED THE DYNAMICS OF *SACCHAROMYCES CEREVISIAE* STRAINS DURING SPONTANEOUS WINE FERMENTATION\*

**ABSTRACT:** Impact of three fungicides against *B. cinerea* (iprodione, pyrimethanil and fludioxonil plus cyprodinil) on the population of *Saccharomyces cerevisiae* strains during the spontaneous alcoholic fermentation was studied. With regard to the use of fungicides in the vineyard at two stages of the grapevine growth we followed four different spontaneous fermentations: control, iprodione, pyrimethanil and fludioxonil plus cyprodinil. The fungicide residues in the grapes were determined by GC/MS system and the fermentations were followed by changes in yeast, sugar, and ethanol concentrations using colony counting and HPLC. The karyotype analysis of 473 isolates was done by pulsed-field gel electrophoresis. The fungicide residues in the grapes at the harvest were below the maximum residue limits. Isolates of *S. cerevisiae* were classified into 15 karyotype groups. The duration of the processes and the populations of the karyotypes differed between the fermentations. The iprodione and control fermentations lasted 36 days with the prevalence of karyotype A while the fludioxonil plus cyprodinil fermentation lasted 50 days and karyotype D led the process. In the pyrimethanil fermentation, none of the karyotypes prevailed in the must and the fermentation lasted much longer than others did (68 days). The results showed that the fungicide residues have an influence on the fermentation kinetics and selection of *S. cerevisiae* strains during the spontaneous alcoholic fermentation and therefore should be considered as an important factor that may indirectly influence the formation of fermentation aroma in the wine produced by such process.

**KEY WORDS:** *Botrytis cinerea*, Strain typing, Wine yeasts, Yeast Ecology

## INTRODUCTION

Gray mould (*Botrytis cinerea* Pers.) is an important fungal disease of the grapevine (*Vitis vinifera* L.). Its development in the grapes depends on the susceptibility of the variety grown in the vineyard and the climatic conditions during the growing season, especially at the flowering and during the grape

\* The paper was presented at the fourth international scientific meeting *Mycology, mycotoxicology, and mycoses*, which was organized in *Matica srpska, Department for natural sciences* from April 20-22, 2011.

ripening (Pearson and Goheen, 1988). Balanced growth of the vines in the vineyard and proper canopy management represent the best way to control the disease but is often not enough, particularly in the cool climate vineyards where the application of fungicides against *B. cinerea* is required. They are generally used two times during the season: at the closure of grape berries and at the beginning of grape ripening that usually represents the last application of the fungicides before the harvest (Ellison et al., 1998). Older fungicides against *B. cinerea* belong to the group of dicarboximides, while the new ones either to the group of anilinopyrimidines, phenylpyrrols or hydroxylanilides (Forster and Staub, 1996; Rosslenbroich and Stuebler, 2000). The amount of fungicide residues found in the grapes and further in the must at the beginning of the wine fermentation depends on the half period of the fungicide, climatic conditions during the grape ripening and the type of grape processing. If the safety interval and the prescribed concentration of fungicide are considered correctly, the residues normally do not reach the maximum residue limits (Cabras et al., 1997b; Cabras and Angioni, 2000).

Yeast ecology of grape berries and later in wine fermentations is influenced by several factors (Raspor et al., 2006). An impact of fungicides has been studied recently (Cadez et al., 2010) and the authors showed that they have an impact on the composition of grape berry communities after the safety interval. Further, Cus and Raspor (2008) examined the influence of fungicide pyrimethanil on the course of spontaneous wine fermentation and correlated it with an initial concentration of yeasts in must. As yeast strains notably determine the aroma of the produced wine (Lema et al. 1996; Romano et al., 2003; Howell et al., 2004) the amount of fungicides residues in fermenting must may indirectly influence the quality of wine. On the other hand the impact of the fungicide residues on the wine yeasts is difficult to measure in particular in low concentrations as they are found in the grapes and must. In most studies the inoculated fermentations contained the fungicides in higher concentrations than those normally found in the grapes (Cabras et al., 1999; Polsinelli and Vandini, 2003) or impact of fungicides on yeast growth was observed on solid media (Sapis-Domercq, 1980; Monteil et al., 1986). The latter method was improved by use of paper discs which is well established method in the inspection of growth inhibition by antibiotics (Woods and Washington, 1995; Cadez et al., 2010). In spite of all mentioned methods there is still lack of the results for indigenous yeast flora present in the spontaneous alcoholic fermentation.

Characterisation of yeast strains in the grape must during the wine fermentation is of great practical interest as yeast strains differ in their metabolic profile which significantly determines the fermentation aroma of produced wine (Egli et al., 1998; Pretorius, 2000; Romano et al., 2003). Numerous techniques of typing yeast strains on the basis of DNA polymorphism are currently available and they allow very successful discrimination (Schuller et al., 2004). One of them is karyotype analysis that is based on the chromosome separation by pulsed field electrophoresis (PFGE) and types the yeast strains with regard to the variability in their chromosomal length

(Carle and Olson, 1984). It was already successfully used for monitoring the succession of yeast strains during wine fermentations (Vezinhet et al., 1990; Schutz and Gafner, 1993; Brione et al., 1996; Raspor et al., 2002) and is considered as very useful but complex, laborious and time-consuming for the analysis of large number of isolates.

Therefore, in the present work, we examined the influence of one older (iprodione) and two newer fungicides (pyrimethanil, fludioxonil plus cyprodinil) against *B. cinerea* on the population of *S. cerevisiae* strains in the spontaneous alcoholic fermentation determined by karyotype analysis.

## MATERIAL AND METHODS

### *Grapevine variety, fungicide application and harvest*

The experiment was set in the vineyard of the white grapevine variety Rebula (*Vitis vinifera* L.) in the Goriska Brda winegrowing region. Application of different fungicides against *Botrytis cinerea* Pers. was the only factor of the experiment with four levels: control, where the grapes were not sprayed against the gray mould, iprodione, where the grapes were sprayed two times with iprodione (0.765 kg a.i./ha), pyrimethanil, in which fenhexamid (0.750 kg a.i./ha) was used at the first and pyrimethanil (0.750 kg a.i./ha) at the second spraying date and fludioxonil plus cyprodinil, where the fludioxonil (0.200 kg a.i./ha) in a combination with cyprodinil (0.300 kg a.i./ha) was applied two times. In the 2002-growing season mentioned combinations of fungicides were applied at the closure of grape berries (1<sup>st</sup> July) and at the beginning of grape ripening (13<sup>th</sup> August), taking into account the safety intervals for each fungicide. The vines were sprayed using a knapsack sprayer (SOLO Port Type 423, VA, USA). The water quantity used was 3.0 L per fungicide application. At the harvest (18<sup>th</sup> September) only healthy and undamaged grapes of each level of the experimental factor were carefully picked in new, steam-washed boxes.

## FUNGICIDES DETERMINATION

Gas chromatography combined with mass spectrometry (GC/MS) was applied to determine the fungicide residues in the grapes (Cabras, Angioni and Garau, 1997a; Fillion, Sauve and Selwyn, 2000). At the harvest approximately 2.0 kg of grapes per level was picked. The extraction of fungicide residue was done from 20.0 g of the homogenized grape sample with solvents as follows: acetone-petroleum ether-dichloromethane (1:2:2, v/v/v). Organic layer was filtered in the Soxhlet Glassware and evaporated under the nitrogen stream. The residue was dissolved in cyclohexane-ethylacetate (50+50 v/v), filtered (Minisart SRPSRP 15 – 0.2 µm, Sartorius), and cleaned with the AutoPrep<sup>®</sup> 1000 Automated GPC Cleanup System (O.I.

Corporation). The eluate was evaporated under the nitrogen stream and the residue was dissolved in acetone (Thier and Zeumer, 1992a; Thier and Zeumer, 1992b; Makovi and McMahon, 1999).

The GC/MS system was GC HP-6890 (Hewlett-Packard) combined with GCMS 5973 (Hewlett-Packard). The column used was HP-5MS (30 m x 0.25 mm id; Agilent Technologies J&W) and helium was the carrier gas at 1.2 ml/min. Calibration was done with the matrix match standards (Fillion et al., 2000).

### *Must handling and fermentation*

The grapes of each level of the experimental factor were crushed aseptically and separately. To prevent any microbial contamination by cellar equipment the mashers were not pressed but only free-run must was poured into aseptic stainless steel tank for each level and left to settle down for 12 hours at 14°C. In order to preserve the indigenous yeast population, the must were not treated with SO<sub>2</sub>. The initial sugar concentrations in the musts were 178.6 g/l (control), 178.2 g/l (iprodione), 177.4 g/l (pyrimethanil) and 186.2 g/l (fludioxonil plus cyprodinil) and pHs of the musts were 3.16 (control), 3.14 (iprodione), 3.08 (pyrimethanil) and 3.14 (fludioxonil plus cyprodinil). After the must settling, an equal volume of the sediment was drawn off from each tank and the remaining must were then stirred and aliquots of 4 L were drawn into three 5 L vessels for each level of the experiment. That time point represented the beginning of the fermentations. The karyotype analysis was performed only in the one fermentor out of three per level of the experimental factor. The control over the fermentation temperature was achieved by keeping the room temperature where the fermentations took place between 18°C and 19°C. Due to the slow processes, the fermentation was considered as completed when less than 6.0 g of sugar per L of must was detected. The produced wines were not sensory evaluated.

### *Chemical analyses of fermenting must*

The fermentation processes were characterized by our following the changes in sugar and ethanol concentrations determined by HPLC. The separation was achieved on Aminex<sup>®</sup> HPX-87H Ion Exclusion column (Bio-Rad, Hercules, CA, USA). A mobile phase used was 0.005 M H<sub>2</sub>SO<sub>4</sub>. Detection of analyzed compounds was based on the refractive index.

### *Sampling and morphotyping*

For the microbiological and chemical analyses the must samples were taken simultaneously according to the fermentation kinetics. The samples for the chem-

ical analysis were filtered through the Chromafil® filters (Macherey-Nagel, Düren, Germany) with pore diameters of 0.45 µm and 0.20 µm. The samples for the microbiological analysis were plated on the yeast extract-malt extract agar (YM; 0.3% yeast extract, 0.3% sugar extract, 0.5% peptone, 1.0% glucose, 2.0% agar). After the incubation at 28°C for three days the yeast colonies were counted and classified into the morphological groups. The isolates for karyotype analysis were sampled from one fermentor per level with regard to the fermentation kinetics. Sampling was done in three main phases of *Saccharomyces cerevisiae* appearance: when *S. cerevisiae* started to replace the non-*Saccharomyces* yeasts, at the end of the fermentation (for the pyrimethanil fermentation six days before) and in between the previously mentioned time points.

### *Karyotype analysis*

The chromosomal DNA of *S. cerevisiae* strains was prepared according to the procedure published by Carle et al. (1984) as modified by (Rasporet al., 2001). The yeast chromosomes were separated in 1% agarose gels in 0.5 x TBE buffer chilled at 12°C in CHEF-DRIII electrophoresis apparatus (Bio-Rad, Hercules, Calif., USA) at 170 V for 15 h with 60 s pulse time, 8 h with 90 s pulse time, and 1 h with 100 s pulse time. The agarose gels were stained with ethidium bromide (0.5 µg/mL) and subsequently documented by Gel Doc 2000 (BIO-RAD system, Hercules, USA). The karyotypes were normalized to the chromosome size marker *S. cerevisiae*, YPH 755 (Roche Diagnostics, Mannheim, Germany) using BioNumerics ver. 4.0-computer program (Applied Math, Kortrijk, Belgium). The processed karyotypes sharing chromosomal fragments of similar lengths were grouped manually.

## RESULTS

### *Fungicide residues*

The results of fungicide residues in the grapes are shown in the Tab. 1. Their residues were below the maximum residue limits (MRL) in the grapes and the values reached between 20-25% of the MRLs for iprodione, pyrimethanil and fludioxonil and 36.5% of MRL for cyprodinil.

### *Kinetics of the spontaneous alcoholic fermentations and the populations of wine yeast*

Spontaneous alcoholic fermentations for each level of the experimental factor were carried out in three replicates. The duration and kinetics of the fermentation did not differ between the replicates in the control (36 days),



iprodione (36 days) and fludioxonil plus cyprodinil (50 days) fermentations, while in the pyrimethanil the process finished after 68 days in two fermentors and after 137 days in the third one. *Saccharomyces cerevisiae* was earliest (day 5) detected in the must of control (4.3%) and fludioxonil plus cyprodinil (3.8%), followed by iprodione (day 6 [0.1%]) and pyrimethanil (day 12 [59.3 %]). At the first shown time for the all fermentations that mostly coincided with the shift to higher consumption rate of sugars and production of ethanol (Figs. 1-4), they particularly differed in the shares of *S. cerevisiae* compared to the non-*Saccharomyces* yeast species (the highest share of in the control [64.4 %] and the lowest share in the iprodione [13.4%] fermentation). In the next two sampling times, in the middle and at the end of the processes the fermentations mainly differed in the replacement (control, pyrimethanil and fludioxonil) or not (iprodione) of *S. cerevisiae* by *D. bruxellensis* (unpublished results).

### *Karyotype analysis*

Karyotype analysis was done for *S. cerevisiae* strains isolated from one fermentor out of three per level of the experimental factor. The total number of analyzed strains was 473, where 114 of them were isolated from the control, 116 from the iprodione, 127 from the pyrimethanil and 116 from the fludioxonil plus cyprodinil fermentations. Our goal was to determine the population of strains in three phases of the spontaneous process as described in the material and methods. At those sampling times we isolated 30-40 randomly picked colonies of *S. cerevisiae*. The only exceptions, when the lower number of isolates per sampling time was obtained, were the eighth day in the iprodione fermentation (nine isolates) (Fig. 2) and 12<sup>th</sup> day in the pyrimethanil fermentation (29 isolates) (Fig. 3).

The analyzed strains were classified into 15 karyotype groups (A, B, C, D, F, G, P, H, I, M, O, R, T, U and V) with regard to their chromosomal pattern normalized to the chromosome size marker *S. cerevisiae* (Tab. 3). The karyotypes, which were characteristic for one or two strains were unclassified and are designated by letter X. The most numerous karyotype groups were A (127 isolates), D (58 isolates), C (44 isolates), R (41 isolates) and B (34 isolates) while within the other groups 23 (M), 18 (G) and 17 (H in T) isolates were classified. Between three and 10 isolates per karyotype were isolated in the groups F, P, I, O, U and V. The 59 strains were unclassified.

### *Population dynamics of yeast strains*

Population dynamics of the *S. cerevisiae* strains in the four spontaneous alcoholic fermentations are shown in the Figs. 1-4. The results showed that the fermentations differed in the percentage and frequency of occurrence of particular karyotype and consequently also in the prevailing karyotype during



the process. The isolates for karyotype analysis were first sampled when *S. cerevisiae* started to replace the non-*Saccharomyces* yeasts in the must and the growth curves shifted from stationary to decline phase, then in the middle of the decline phase and the last sampling was done at the end of the fermentations except for the pyrimethanil where the sufficient number of strains was lastly isolated at day 62 instead at the end of the fermentation (day 68).

Karyotype A prevailed in the must of control fermentation and it was isolated in all sampling times shown in the Fig. 1. Its share was 54.1% at day 8, 34.4% at day 18 and 46.9% at day 36 of the fermentation. Other karyotypes with higher percentages in the population were C, F and R. Eight different karyotypes were detected at day 8 (A, B, C, F, G, H, M, R), nine at day 18 (A, B, C, D, F, G, H, M, R) and five at 36<sup>th</sup> day of the fermentation (A, C, D, H, R).

Karyotype A also prevailed in the must of iprodione fermentation and it was isolated at all sampling times with the exception of days 16, 18, and 28 of the process (Fig. 2). Its share was 33.3% at day 8, 60.0% at day 20, and 45.0% at day 36 of the fermentation. Other karyotypes with higher shares in the population were karyotypes C, G, M and R. Five different karyotypes were detected at day 8 (A, G, H, R, U), seven at day 20 (A, B, C, D, G, U, V), and five at day 36 of the fermentation (A, C, G, M, R).

None of the strains predominated in the must of pyrimethanil fermentation and the different prevailing strains were determined in the three main sampling times (Fig. 3). At day 12, 29 isolates were analyzed and classified in eight different groups. The highest shares had karyotypes R and A (each 20.7%) followed by karyotype C (17.2%). Ten different karyotypes were determined in the must at day 28 of the fermentation when karyotype C (37.5%) prevailed in the must, followed by karyotypes R (12.5%) and A (10.0%). The strain population was most heterogeneous at day 62 of the process when 11 different karyotypes were isolated. At the day 62 of the fermentation, the highest shares in the strain population belong to the groups M (20.0%) and P (15.0%).

Karyotype D obviously dominated in the must of fludioxonil plus cyprodinil fermentation (Fig. 4). It was isolated in all sampling times. Its share was 90.6% (day 12), 20.0% (day 22) and 27.8% (day 50). Other karyotypes with higher shares in the population were karyotypes B, T and R. Four different karyotypes were detected at day 12 (B, D, T, U), eight at day 22 (A, B, C, D, I, M, R, T), and five at day 50 of the fermentation (B, D, I, R, T).

## DISCUSSION

Many factors influence the occurrence and growth of yeasts during alcoholic fermentation and fungicide residues are also considered as one (Fleet, 2003). The results of studies for fungicides against *Botrytis cinerea* such as iprodione, pyrimethanil and cyprodinil plus fludioxonil showed that they do not inhibit the growth of wine yeasts on solid media or in inoculated alcoholic fermentations in the concentrations normally found in must (Sapich-Domercq, 1980; Cabras et al., 1999). In contrast, there are few results

of influence of some fungicides on the spontaneous alcoholic fermentation (Viviani-Nauer et al., 1997, Čuš and Raspor, 2008) or natural flora on grapes of cultivars resistant to fungi (Viviani-Nauer et al., 1995, Cadez et al., 2010). Therefore in the present work we studied the influence of three different fungicides against *B. cinerea* on the indigenous population of *Saccharomyces cerevisiae* strains during the spontaneous alcoholic fermentation.

Regarding to the use of fungicides in the vineyard we also determined their residues in the grapes at the harvest. The main reasons that the amounts of the residues were below the maximal residue limits are considering of appropriate fungicide concentrations at the application and also considering of safety intervals, which have to run out from the last application and harvest. Other authors reported too, that in such conditions there are no exceed of MRLs in the grapes/must/wine chain (Lemperle et al., 1982; Farris et al., 1992; Cabras et al., 1997b). As the concentrations of the fungicides in the must were not measured we may propose that they reached approximately 50-60 % of iprodione (Lemperle et al., 1982; Farris et al., 1992; Garcia-Cazorla and Xirau-Vayreda, 1994), 90-95 % of pyrimethanil, 45-50 % of fludioxonil and 30-40 % of cyprodinil determined in the grapes (Cabras et al., 1997b; Flori, Frabboni and Cesari, 2000).

The karyotype analysis is very useful and accurate method for typing the strains of *S. cerevisiae* (Vežinhet et al., 1990; Schuller et al., 2004). In spite of its complex and time consuming methodology we were able to follow four spontaneous fermentations of Rebula grape must (control, iprodione, pyrimethanil and fludioxonil plus cyprodinil) and determined the karyotypes of 473 strains in three different phases of each process. In the three out of four fermentations (control, iprodione and fludioxonil plus cyprodinil), at least one karyotype prevailed in the must throughout the processes regardless of their length. The most heterogeneous population was found in the must of pyrimethanil although also here few karyotype groups (A, C, R and M) were isolated in all three main sampling times, but the prevailing strain was different in each phase of the process.

Dissimilar concentrations of yeast cells, composition of yeast species and strains of *S. cerevisiae* in the musts caused different kinetics and duration of the fermentations (Figs. 1-4). Longer persistence of *Hanseniaspora uvarum* and lower concentration of yeasts cells in the must of pyrimethanil and higher shares of *Candida stellata* in the early stages of the fludioxonil plus cyprodinil fermentation together with likely weaker fermentation capacity of the strains isolated in both processes most likely caused their prolongation. To the some extent early isolation of *D. bruxellensis* in the must of pyrimethanil might also inferred with the population of *S. cerevisiae* strains. Weaker fermentation rate of pyrimethanil process resulted in the highest number of isolated strains and their most evident succession throughout the process. It is interesting that the control and iprodione fermentations lasted the same (36 days) had the identical prevailing karyotype (A) and very similar karyotypes populations (groups C and R). Probably some selection has occurred in the

population of the yeast strains in the grapes due to activity of iprodione. This had been predicted because strains of *B. cinerea* resistant to the group of dicarboximides to which iprodione belongs were already isolated (L e r o u x et al., 2002). Although this statement is has not been directly confirmed, it is also supported by the reproducibility of the fermentation kinetics in the all three replicates of the control and iprodione fermentations. Furthermore three replicates of fludioxonil plus cyprodinil and two of pyrimethanil fermentation also lasted the same and significantly longer than control and iprodione one. The similarity of the *S. cerevisiae* population dynamics in the control and iprodione fermentations may therefore be a result of very comparable starting yeasts composition that determined its further development as was already observed by others (F l e e t and H e a r d , 2002; S c h u l l e r , A l v e s , D e - q u i n and C a s a l , 2005).

## CONCLUSIONS

The results of our experiment with four spontaneous alcoholic fermentations showed that fungicide residues although they were detected below the permitted levels in the grapes, play an important role in the selection of *Saccharomyces cerevisiae* strains during the process. Consequently, the application of fungicides against *B. cinerea* in the vineyard determined the prevailing strain and the population of karyotypes in the must. Fludioxonil plus cyprodinil and especially pyrimethanil retarded the course of spontaneous fermentation in comparison to the iprodione, which are fairly longer on the market. We predicted that some selection of *S. cerevisiae* strains in the grapes or must might have occurred with regard to the use of fungicides during the season. It should also be stressed that despite the long duration of the spontaneous alcoholic fermentations it was found very suitable for the study of intrinsic factors such as fungicide residues in the must on the selection of the yeast strains. Namely, in such fermentation interactions between microorganisms are more obvious and number of yeasts cells per ml is lower and therefore the influence of different factors is easier to follow. However, some further study of investigated fungicides on the growth of isolated strains is needed, although the conditions of spontaneous fermentation are difficult to reproduce in both chemical and microbiological sense.

## Acknowledgements

The authors wish to thank to Dr. A. Gregorčič and Dr. H. Baša-Česnik and acknowledge the technical assistance of Nataša Kračun. This research was supported by the Ministry of Higher Education, Science and Technology and by the Ministry of Agriculture, Forestry and Food of Republic of Slovenia (Project no. V4-0591-01).

## REFERENCES

- Briones, A., Ubeda, J., Grando, S. (1996): *Differentiation of Saccharomyces cerevisiae strains isolated from fermenting must according to their karyotype patterns*, International Journal of Food Microbiology 28, 369-377.
- Cabras, P., Angioni, A., Garau, V. L. (1997a): *Gas chromatographic determination of cyprodinil, fludioxonil, pyrimethanil, and tebuconazole in grapes, must and wine*, Journal of AOAC International 80, 867-870.
- Cabras, P., Angioni, A., Garau, V. L., Melis, M., Pirisi, F. M., Minelli, E. V., Cabitza, F., Cubeddu, M. (1997b): *Fate of some new fungicides (cyprodinil, fludioxonil, pyrimethanil, and tebuconazole) from vine to wine*, Journal of Agricultural & Food Chemistry 45, 2708-2710.
- Cabras, P., Angioni, A., Garau, V. L., Pirisi, F. M., Farris, G. A., Madau, G., Emonti, G. (1999): *Pesticides in fermentative processes of wine*, Journal of Agricultural & Food Chemistry 47, 3854-3857.
- Cabras, P., Angioni, A. (2000): *Pesticides residues in grapes, wine, and their processing products*, Journal of Agricultural & Food Chemistry 44, 967-973.
- Cadez, N., Raspor, P., De Cock, A. W. A. M., Boekhout, T., Smith, M. T. (2002): *Molecular identification and genetic diversity within species of the genera Hanseniaspora and Kloeckera*, FEMS Yeast Research 1, 279-289.
- Cadez, N., Zupan, J. & Raspor, P. (2010): *The effect of fungicides on yeast communities associated with grape berries*, FEMS Yeast Research 10, 619-630.
- Carle, G. F., Olson, M. V. (1984): *Separation of chromosomal DNA molecules from yeast by orthogonal-field-alternation gel electrophoresis*, Nucleic Acids Research 12, 5647-5664.
- Cus, F. & Raspor, P. (2008): *The effect of pyrimethanil on the growth of wine yeasts*, Lett. Appl. Microbiol. 47, 54-59.
- Egli, C. M., Edinger, W. D., M., M. C., Henick-Kling, T. (1998): *Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines*, Journal of Applied Microbiology 85, 779-789.
- Ellison, P., Ash, G., McDonald, C. (1998): *An expert system for the management of Botrytis cinerea in Australian vineyards*, I. Development. Agricultural Systems 56, 185-207.
- Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A. (1999): *Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers*, International Journal of Systematic Bacteriology 49, 329-337.
- Farris, G. A., Cabras, P., Spanedda, L. (1992): *Pesticide residues in food processing*, Italian Journal of Food Science, 149-169.
- Fillion, J., Sauve, F., Selwyn, J. (2000): *Multiresidue method for the determination of residues of 251 pesticides in fruit and vegetables by gas chromatography/mass spectrometry and liquid chromatography with fluorescence detection*, Journal of AOAC International 83, 698-712.
- Fleet, G. H., Heard, G. M. (2002): *Yeasts – growth during fermentation*. In: Fleet, G. H. (Ed.), Wine microbiology and biotechnology, Taylor & Francis, New York, pp. 27-54.

- Fleet, G. H. (2003): *Yeast interactions and wine flavour*, Journal of Food Microbiology 86, 11-22.
- Flori, P., Frabboni, B., Cesari, A. (2000): *Pesticide decay models in the wine-making process and wine storage*, Italian Journal of Food Science 12, 279-289.
- Forster, B., Staub, T. (1996): *Basis for use strategies of anilinopyrimidine and phenylpyrrole fungicides against Botrytis cinerea*, Crop Protection 15, 529-537.
- Garcia-Cazorla, J., Xirau-Vayreda, M. (1994): *Persistence of dicarboximide fungicide residues in grapes, must, and wine*, American Journal of Enology & Viticulture 45, 338-340.
- Howell, K. S., Swiegers, J. H., Elsey, G. M., Siebert, T. E., Bartowsky, E. J., Fleet, G. H., Pretorius, I. S., de Barros Lopes, M. A. (2004): *Variation in 4-mercapto-4-methyl-pentan-2-one release by Saccharomyces cerevisiae commercial wine strains*, FEMS Microbiology Letters 240, 125-129.
- Lema, C., Garcia-Jarec, C., Orriols, I., Angulo, L. (1996): *Contribution of Saccharomyces and non-Saccharomyces populations to the production of some components of Albarino wine Aroma*, American Journal of Enology and Viticulture 47, 206-216.
- Lemperle, E., Emmanouilidis, N., Kerner, E. (1982): *Decomposition behaviour of the fungicides Ronilan, Rovral and Sumisclex on wine grapes*, II. Residues of the active ingredients in grapes, must and wine. Deutsche Lebensmittel-Rundschau 78, 51-55.
- Leroux, P., Fournier, E., Brygoo, Y., Panon, M. L. (2002): *Biodiversity and variability within Botrytis cinerea, the causal agent of grey mould*, Phytoma 554, 38-42.
- Makovi, C. M., McMahon, B. M. (1999): *Extraction with acetone, liquid-liquid partitioning with petroleum ether/methylene chloride*, In: Makovi, C. M., McMahon, B. M. (Eds.), Pesticide analytical manual: Vol. 1. R.O.W. Sciences, Inc., Rockville, pp. 302-307.
- Monteil, H., Blazy-Maugen, G., Michel, G. (1986): *Influence des pesticides sur la croissance des levures des raisins et des vins*, Sciences des Aliments 6, 349-360.
- Navarro, S., Oliva, J., Navarro, G., Barba, A. (2001): *Dissipation of Chlorpyrifos, Fenarimol, Mancozeb, Metalaxyl, Penconazole, and Vinclozolin in grapes*, American Journal of Enology & Viticulture 52, 35-40.
- Pearson, R. C., Goheen, A. C. (1988): *Compendium of grape diseases*, APS Press - The American Phytopathological Society, St. Paul, pp 87.
- Polsinelli, M., Vandini, C. (2003): *Studi sull'effetto di fungicidi su cellule di lieviti da vino*, Vignevini, 137-139.
- Pretorius, I. S. (2000): *Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking*, Yeast 16, 675-729.
- Raspor, P., Smole Mozina, S., Cadez, N. (2001): *Identification of yeast from grape/must/wine system*. In: Spencer, J. F. T., Ragout de Spencer, A. L. (Eds.), Food microbiology protocols. Humana Press Inc., Totowa, pp. 243-257.
- Raspor, P., Cus, F., Povhe Jemec, K., Zagorc, T., Cadez, N., Nemanic, J. (2002): *Yeast population dynamics in spontaneous and inoculated alcoholic fermentations of Zametovka must*, Food Technology and Biotechnology 40, 95-102.
- Raspor, P., Milek, D. M., Polanc, J., Mozina, S. S. & Cadez, N. (2006): *Yeasts isolated from three varieties of grapes cultivated in different locations of*









- the Dolenjska vine-growing region, Slovenia*, International Journal of Food Microbiology 109, 97-102.
- Romano, P., Fiore, C., Paraggio, M., Caruso, M., Capece, A. (2003): *Function of yeast species and strains in wine flavour*, International Journal of Food Microbiology 86, 169-180.
- Rosslenbroich, H. J., Stuebler, D. (2000): *Botrytis cinerea – history of chemical control and novel fungicides for its management*, Crop Protection 19, 557-561.
- Sapis-Domercq, S. (1980): *Etude de l'influence des produits de traitement de la vigne sur la microflore des raisins et des vins*, Connaissance de la Vigne et du Vin 14, 155-181.
- Schuller, D., Valero, E., Dequin, S., Casal, M. (2004): *Survey of molecular methods for the typing of wine yeast strains*, FEMS Microbiology Letters 231, 19-26.
- Schuller, D., Alves, H., Dequin, S., Casal, M. (2005): *Ecological survey of Saccharomyces cerevisiae strains from vineyards in the Vinho Verde region of Portugal*, FEMS microbiology ecology 51, 167-177.
- Schutz, M., Gafner, J. (1993): *Analysis of yeast diversity during spontaneous and induced alcoholic fermentations*, Journal of Applied Bacteriology 75, 551-558.
- Thier, H. P., Zeumer, H. (1992a): *Cleanup Method 6*. In: Thier, H. P., Zeumer, H. (Eds.), *Manual of pesticide residue analysis*, VCH, Verlagsgesellschaft, Weinheim, pp. 31-36.
- Thier, H. P., Zeumer, H. (1992b): *Organochlorine, organophosphorus, nitrogen-containing and other pesticides*. In: Thier, H. P., Zeumer, H. (Eds.), *Manual of pesticide residue analysis*. VCH, Verlagsgesellschaft, Weinheim, pp. 317-322.
- Vezinhet, F., Blondin, B., Hallet, J. N. (1990): *Chromosomal DNA patterns and mitochondrial DNA polymorphism as tools for identification of enological strains of Saccharomyces cerevisiae*, Applied Microbiology and Biotechnology 32, 568-571.
- Viviani-Nauer, A., Hoffmann-Boller, P., Basler, P., Gafner, J. (1995): *Composition and dynamics of the yeast flora on grapes from cultivars resistant to fungi*, Schweizerische Zeitschrift Fuer Obst- und Weinbau 131, 390-393.
- Viviani-Nauer, A., Hoffmann-Boller, P., Gafner, J. (1997): *In vivo detection of folpet and its metabolite phthalimide in grape must and wine*, American Journal of Enology & Viticulture 48, 67-70.
- Woods, G. L., Washington, J. A. (1995): *Antibacterial susceptibility tests: dilution and disk diffusion methods*. In: Murray, P. R., Baron, E., Pfaller, M. A., Tenover, F. C., Tenover, R. H. (Eds.), *Manual of clinical microbiology*. ASM Washington Press, Washington, pp. 1327-1341.
- Yarrow, D. (1998): *Methods for the isolation, maintenance and identification of yeasts*. In: Kurtzman, C. P., Fell, J. W. (Eds.), *The yeasts, a taxonomic study*. Elsevier Science Publishers, Amsterdam, pp. 77-102.










Tab. 1 – Maximum residue limits (MRL) and amounts of fungicide residues in the grapes of Rebula (\*ND – not detected).

Level of the experimental factor	Determined fungicide	MRL of fungicide (mg/kg)	Fungicide residue (mg/kg)
CONTROL	Iprodione	10.00	ND*
	Pyrimethanil	5.00	ND
	Fludioxonil	2.00	ND
	Cyprodinil	2.00	ND
IPRODIONE	Iprodione	10.00	2.60
	Pyrimethanil	5.00	ND
	Fludioxonil	2.00	ND
	Cyprodinil	2.00	ND
PYRIMETHANIL	Iprodione	10.00	ND
	Pyrimethanil	5.00	1.18
	Fludioxonil	2.00	ND
	Cyprodinil	2.00	ND
FLUDIOXONIL + CYPRODINIL	Iprodione	10.00	ND
	Pyrimethanil	5.00	ND
	Fludioxonil	2.00	0.43
	Cyprodinil	2.00	0.73

Tab. 2 – Karyotype groups isolated from four spontaneous alcoholic fermentations

Group	A	B	C	D	F	G	P	H
Karyotype with chromosome lengths (kbp)								
	2070	2200	2190	2200	2100	2200	2200	2070
	1480	1570				1521		1440
	1090	1230		1210	1120	1120	1230	1120
			1100					
	930	1050		1060	1020	970	1030	980
		950	960	990	990			
	800	850	810	840	880		880	830
	780		780	790	840	800	850	790
	750	800	760	770	800	790	790	740
		780				750		
		710	700	720	740		730	710
	690							
	600	630	610	630	650	690	630	610
Number of isolated strains	127	34	44	58	9	18	8	17



Group	I	M	O	R	T	U	V
Karyotype with chromosomes length							
Number of isolated strains	3	23	4	41	17	6	5

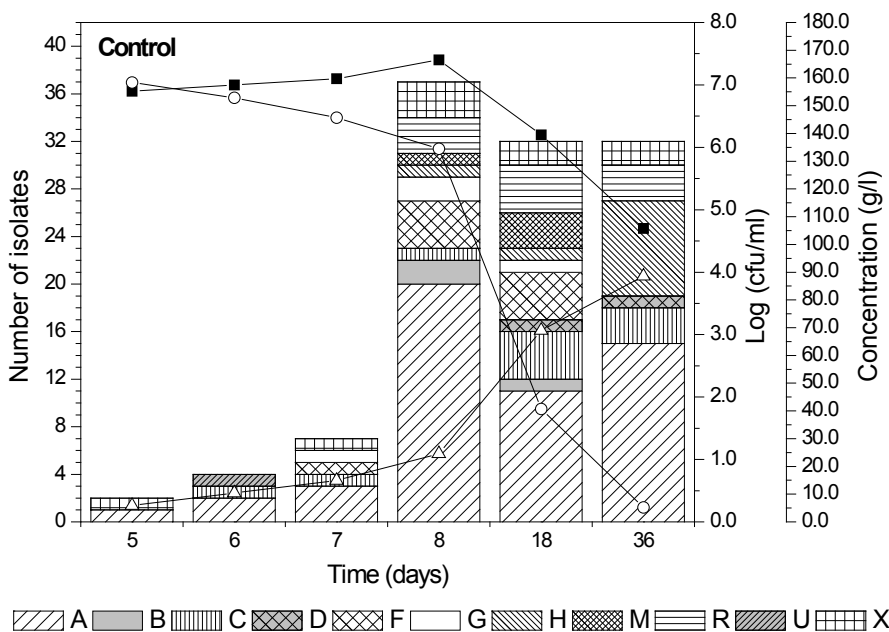


Fig. 1 – Yeasts growth (-■-), sugars utilization (-○-), ethanol production (-△-) and frequency of occurrence of different *S. cerevisiae* strains in the control fermentation

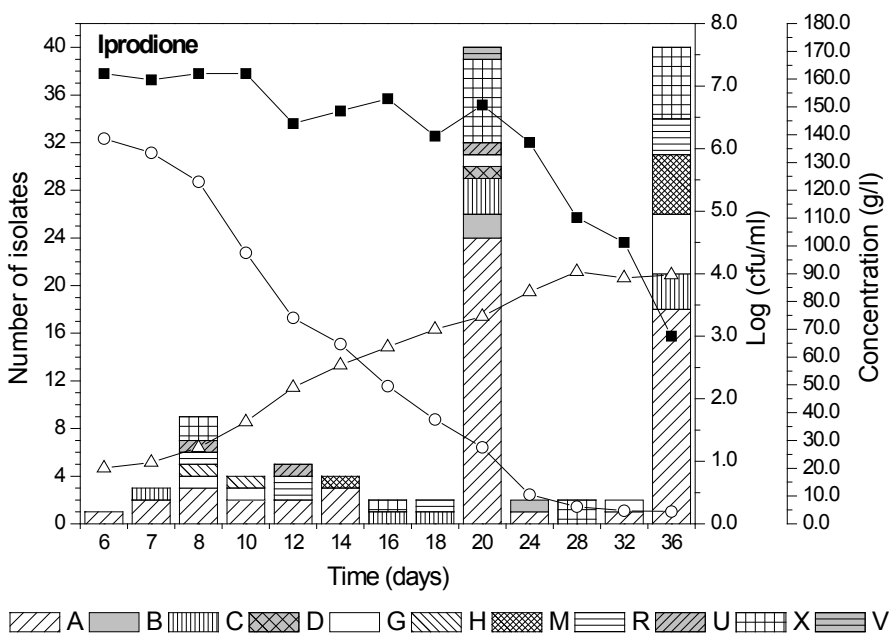


Fig. 2 – Yeasts growth (-■-), sugars utilization (-○-), ethanol production (-△-) and frequency of occurrence of different *S. cerevisiae* strains in the iprodione fermentation

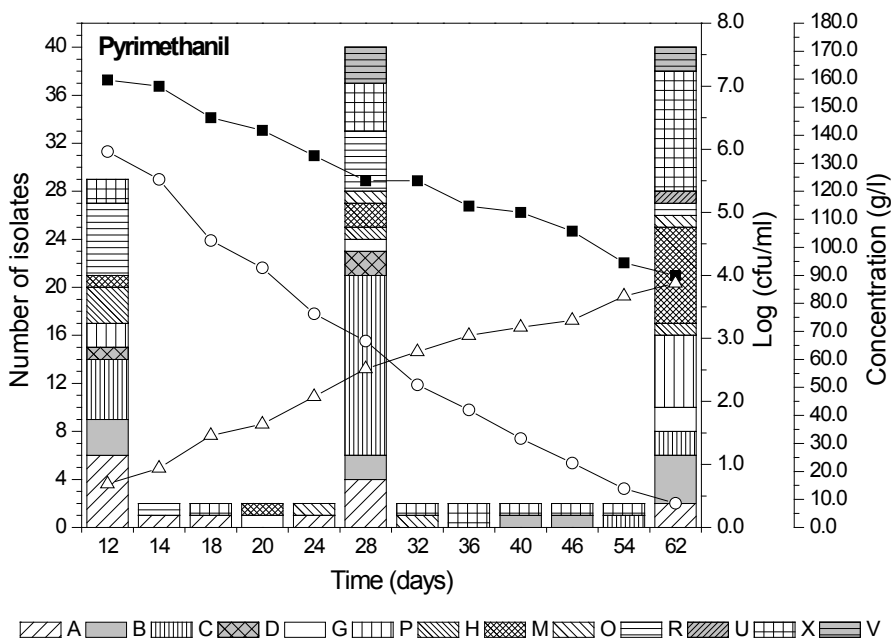


Fig. 3 – Yeasts growth (-■-), sugars utilization (-○-), ethanol production (-△-) and frequency of occurrence of different *S. cerevisiae* strains in the pyrimethanil fermentation

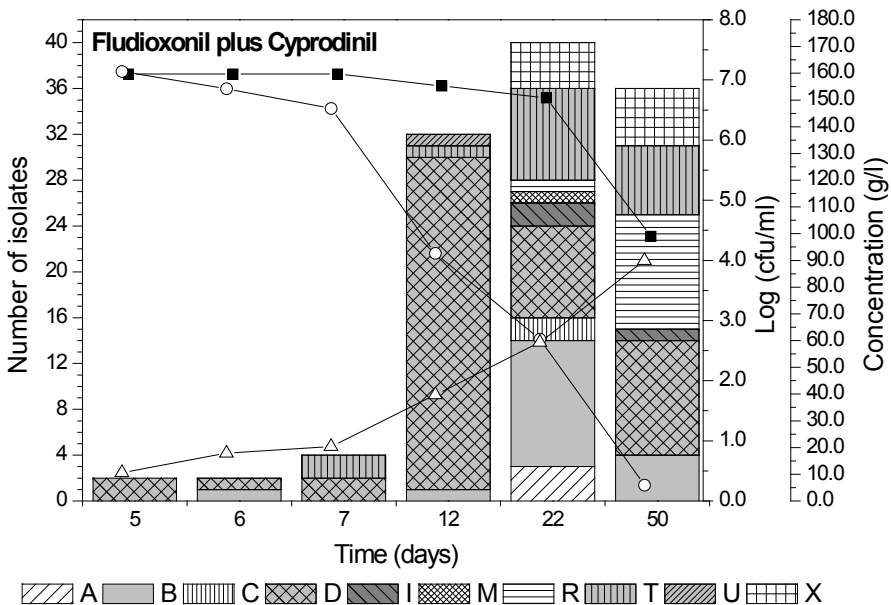


Fig. 4 – Yeasts growth (-■-), sugars utilization (-○-), ethanol production (-△-) and frequency of occurrence of different *S. cerevisiae* strains in the fludioxonil plus cyprodinil fermentation

# ОСТАЦИ ФУНГИЦИДА У ГРОЖЉУ ОДРЕЂУЈУ ДИНАМИКУ СОЈЕВА *SACCHAROMYCES CEREVISIAE* У ТОКУ СПОНТАНЕ ФЕРМЕНТАЦИЈЕ ВИНА

Франц Чуш<sup>1,2</sup>, Нежа Ј. Чадеж<sup>2</sup>, Пејтер И. Расјор<sup>2</sup>

<sup>2</sup> Универзитет у Љубљани, Биотехнички факултет,  
Департмент за технологију хране, Катедра за биотехнологију

## Резиме

Проучавали смо утицај три фунгицида на *Botrytis cinerea* (ипродион, пириметанил и флудиоксонил плус ципродинил) на популацију сојева *Saccharomyces cerevisiae* у току спонтаног алкохолног врења. С обзиром да се фунгициди у винограду користе у две фазе раста винове лозе пратили смо четири различите спонтане ферментације: контрола, ипродион, пириметанил и флудоксонил плус ципродинил. Остаци фунгицида у грозњу су одређивани GC/MS системом а ферментације су праћене према променама у квасцу, шећеру и концентрацијама етанола бројањем колона и високопритисном течном хроматографијом. За анализу кариотипова из 473 изолата користили смо гел електрофорезу у пулсном пољу. Остаци фунгицида у грозњу у време бербе били су испод границе максималне резидуалне вредности. *S. cerevisiae* изолати су класификовани у 15 кариотипних група. Трајање процеса и популације кариотипова се разликовало од ферментације до ферментације. Ипродион и контролна ферментација су трајале 36 дана уз преваленцију кариотипа А док је флудиоксонил плус ципродинил ферментација трајала 50 дана а кариотип је био водећи у процесу. Код пириметанил ферментације ни један од кариотипова није био преовлађујући у шири а сама ферментација је трајала много дуже (68 дана). Добијени резултати су показали да остаци фунгицида утичу на кинетику ферментације и избор сојева *S. cerevisiae* у току спонтаног алкохолног врења па се из тог разлога може сматрати важним фактором који може индиректно утицати на стварање ферментационе ароме вина добијеног оваквим процесом.



Marija M. Škrinjar<sup>1</sup>, Marijana M. Višić-Vićanović<sup>1</sup>,  
Nevena T. Blagojević<sup>1</sup>, Zorica S. Tatalović<sup>2</sup>,  
Vesna J. Krunić<sup>2</sup>

<sup>1</sup> Faculty of Technology, University of Novi Sad, Bulevar cara Lazara 1, Novi Sad, Serbia

<sup>2</sup> EKOLAB d.o.o., Jovana Cvijića 45, Novi Sad, Serbia

## XEROPHILIC MYCOPOPULATIONS OF TEAS IN BULK\*

**ABSTRACT:** Other the water, tea is the most popular beverage in the world today. They are used for ages, in the beginning as refreshing drinks, and later more for their healing properties. Teas have been demonstrated to show antioxidative, anti-carcinogenic, and anti-microbial properties. Considering that the teas, during the production, are not treated with any temperature, there is high risk for contamination with different type of microorganisms, especially with moulds. Moulds are ubiquitously distributed in nature and their spores can be found in the atmosphere even at high altitudes and under favorable conditions of temperature and humidity, moulds grow on many commodities including cereals, oil seeds, nuts, herbs and spices. Most of them are potential producers of mycotoxins which present a real hazard to human health.

The aim of this work was to investigate total mould count and to identify moulds isolated from teas in bulk, than from teas treated with hot, sterile, distilled water and from the tea filtrates. Tested teas were peppermint, sage, yarrow, black tea, bearberry, lemon balm, mixture of teas from Zlatibor. In teas in bulk was observed high contamination with different kinds of moulds (1.84-4.55 cfu/g), such as *Aspergillus awamori*, *A. lovaniensis*, *A. niger*, *A. phoenicus*, *A. repens*, *A. restrictus*, *A. sydowii*, *A. versicolor*, *Eurotium amstelodami*, *E. chevalieri*, *E. herbariorum*, *Penicillium chrysogenum*, and *Scopulariopsis brevicaulis*. The most frequent were species from *Aspergillus* and *Eurotium* genera. Thermal treatment with hot, sterile, distilled water reduced the number of fungal colonies. *Aspergillus awamori* was the most resistant and appeared in six samples of filtrates of tea, *Aspergillus niger* in one sample and *Penicillium chrysogenum* in one sample.

**KEY WORDS:** teas, mould contamination, thermal treatment

## INTRODUCTION

Other the water, tea is the most popular beverage in the world today. They are used for ages, in the beginning as refreshing drinks, and later more for their healing properties. Tea and tea products mainly contain tea polyphenols.

---

\* The paper was presented at the fourth international scientific meeting *Mycology, mycotoxicology, and mycoses*, which was organized in *Matica srpska, Department for natural sciences* from April 20-22, 2011.

nols, which are natural antioxidants and have been demonstrated to show anti-oxidative, anti-carcinogenic and anti-microbial properties by many researchers (McKay and Blumberg, 2002; Rietveld and Wiseman, 2003).

Considering that the teas, during the production, are not treated with any temperature, there is high risk for contamination with different sort of micro-organisms, especially with moulds, mostly xerophilic moulds.

Moulds are ubiquitously distributed in nature and their spores can be found in the atmosphere even at high altitudes, carried and disseminated by wind and air currents, or spread by insects, rodents, and other animals (Kungulovskii et al., 2011) and under favourable conditions of temperature and humidity, moulds grow on many commodities including cereals, oil seeds, nuts, herbs, and spices (Berra et al., 1998).

Most of moulds are potential producers of mycotoxins. Mycotoxins are secondary extracellular metabolites of moulds. After biosynthesis, they diffuse from mycelium into the substrate (Višić, 2007). They, as potential mutagenic, teratogenic, cytotoxic, and carcinogenic present a real hazard to human health. Mycotoxins are, also, very thermostable. Even the temperature of sterilization is too low to cause destruction of mycotoxin structures (Škrinjar, 1997).

Because of those reasons the aim of this work was to investigate total mould count and to identify moulds isolated from teas in bulk, than from teas treated with hot, sterile, distilled water, and from the tea filtrates.

## MATERIALS AND METHODS

Mycological investigations were carried out in seven samples of tea. The samples were collected from health food store (6) and from the market (1). The names and families of each sample are presented in Table 1.

Tab. 1 – Names and families of studied teas

No.	English name	Latin name	Family
1.	Peppermint	<i>Menta piperita</i>	Lamiaceae
2.	Sage	<i>Salvia officinalis</i>	Lamiaceae
3.	Yarrow	<i>Achilea millefolium</i>	Asteraceae
4.	Black tea	<i>Camellia sinensis</i>	Theaceae
5.	Bearberry	<i>Arctostaphylos uva ursi</i>	Ericaceae
6.	Lemon balm	<i>Melissa officinalis</i>	Lamiaceae
7.	Mixture of teas from Zlatibor		

In further investigation, the influence of thermal treatment on surviving of the present moulds during the preparation of beverages was investigated. Twenty grams of seven samples of tea were treated with 180 ml of hot sterile distilled water for 15 minutes. After filtration, total mould count in dry residues were determined using dilution method by Koch in duplicates, while



total mould count in 1ml of filtrate of teas were determined by membrane filter method (MFM).

Media used for total mould count determination were: **MY10-12** (maltose extract, 20 g/l; yeast extract, 5 g/l; NaCl, 100 g/l; glucose, 120 g/l; chloramphenicol, 0.05 g/l; agar, 20 g/l) and **MEA** (maltose extract, 20 g/l; pepton, 1g/l; glucose, 20 g/l; NaCl, 170 g/l; chloramphenicol, 0.05 g/l; agar, 20 g/l).

Inoculated Petri dishes were incubated for 7 (MY 10-12) and 14 days (MEA) at 25°C.

Identification of isolated fungal species was done according to Thom and Raper (1945), Klich (2002) and Samson et al. (2004).

## RESULTS AND DISCUSSION

### *Mycological investigations of tea samples in bulk*

Results for total mould count per gram of teas in bulk, expressed as log of cfu/g, are presented in Table 2 and in the Figure 1.

The most contaminated was a sample of mixture of teas from Zlatibor what is determined by applying MY10-12 medium (4.55 cfu/g), while on MEA medium, for the same tea, moulds were not isolated. Black tea was the least contaminated on both media (1.84 cfu/g on MY10-12; 1.00 cfu/g on MEA medium).

Tab. 2 – Total mould count per g of teas in bulk [ $\log_{10}$ cfu/g] determined by using MY10-12 and MEA media

Sample	MY 10-12	MEA
	Total mould count [ $\log_{10}$ cfu/g]	Total mould count [ $\log_{10}$ cfu/g]
Peppermint	2.77	2.66
Sage	2.602	2.95
Yarrow	3.56	2.93
Black tea	1.84	1.00
Bearberry	2.83	1.70
Lemon balm	3.04	2.42
Mixture of teas from Zlatibor	4.55	-

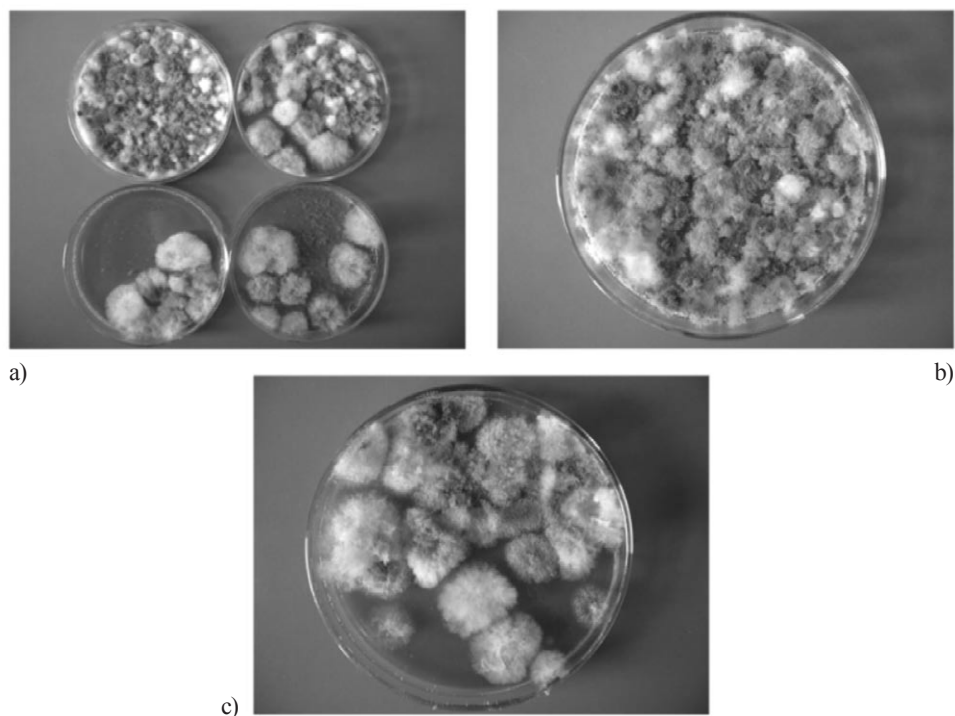


Fig. 1 – Mould contamination of teas in bulk

From studied samples a number of different moulds were isolated which are classified to 4 genera and 13 species as can be seen in Table 3.

Tab. 3 – Fungal species isolated from teas samples

Genus	Subgenus	Section	Species
<i>Aspergillus</i>	<i>Circumdati</i>	<i>Nigri</i>	<i>Aspergillus awamori</i> Nakazawa <i>Aspergillus niger</i> v an Tieghem <i>Aspergillus phoenicus</i> (Cda) Thom
<i>Aspergillus</i>	<i>Nidulantes</i>	<i>Versicolores</i>	<i>Aspergillus versicolor</i> (Vuill.) Tiraboschi <i>Aspergillus sydowii</i> (Bain and Sart.) Thom and Church
<i>Aspergillus</i>	<i>Aspergillus</i>	<i>Restricti</i>	<i>Aspergillus restrictus</i> G. Smith
<i>Aspergillus</i>			<i>Aspergillus lovaniensis</i> Biourge <i>Aspergillus repens</i> DeBary
<i>Eurotium</i>	<i>Aspergillus</i>	<i>Aspergillus</i>	<i>Eurotium amstelodami</i> (Mangin) Thom ans Church <i>Eurotium chevalieri</i> (Mangin) Thom ans Church <i>Eurotium herbariorum</i> (Wiggers) Link
<i>Penicillium</i>			<i>Penicillium chrysogenum</i> Thom
<i>Scopulariopsis</i>			<i>Scopulariopsis brevicaulis</i> (Sacc.) Bain

Fungal species isolated from teas in bulk are presented in Table 4. The most frequent were species from genera *Aspergillus* and *Eurotium*. Also, some species from genus *Penicillium* were presented.

Tab. 4 – Fungal species isolated from teas in bulk

Me- di- um	Samples						
	Peppermint	Sage	Yarrow	Black tea	Bearberry	Lemon balm	Mixture of teas from Zlatibor
MY 10-12	<i>Aspergillus awamori</i> <i>Aspergillus versicolor</i> <i>Eurotium amstelodami</i>	<i>Aspergillus awamori</i> <i>Eurotium amstelodami</i>	<i>Eurotium amstelodami</i> <i>Eurotium chevalieri</i>	<i>Aspergillus awamori</i>	<i>Aspergillus awamori</i> <i>Aspergillus niger</i>	<i>Aspergillus flavus</i> <i>Aspergillus lovaniensis</i> <i>Aspergillus niger</i> <i>Scopulariopsis brevicaulis</i>	<i>Aspergillus restrictus</i> <i>Eurotium amstelodami</i> <i>Eurotium herbariorum</i> <i>Penicillium chrysogenum</i>
MEA	<i>Penicillium chrysogenum</i>	<i>Aspergillus versicolor</i>	<i>Penicillium chrysogenum</i>	<i>Aspergillus repens</i>	<i>Aspergillus versicolor</i> <i>Eurotium herbariorum</i>	<i>Aspergillus versicolor</i> <i>Aspergillus sydowii</i> <i>Eurotium amstelodami</i> <i>Eurotium herbariorum</i>	

The most of isolated moulds are xerophilic and minimum of water activity for their growth is presented in Table 5.

Tab. 5 – Minimum temperature and water activity for growth of different fungal species

Fungal species	Temperature [°C]	a <sub>w</sub>
<i>Aspergillus flavus</i>	10	0.81
<i>Aspergillus niger</i>	12	0.77
<i>Aspergillus versicolor</i>	6	0.70
<i>Eurotium amstelodami</i>	10	0.71
<i>Eurotium chevalieri</i>	6	0.72
<i>Eurotium herbariorum</i>	4	0.78
<i>Penicillium chrysogenum</i>	4	0.84
<i>Scopulariopsis brevicaulis</i>	*	0.90

\*not determined

### *Moulds in dry residues and in tea drinks after thermal treating*

Results for total mould count per gram in dry residues after thermal treating, expressed as log of cfu/g are presented in Table 6. It is observed reduction of total mould count in all samples what can also be seen in the Figure 2. On MY10-12 medium number of colonies was ranged from 1.00 cfu/g on peppermint to 2.02 cfu/g on lemon balm, while on MEA medium growth didn't appear in all samples.

Tab. 6 – Total mould count in dry residues after thermal treating [ $\log_{10}$ CFU/g]

Sample	MY 10-12	MEA
	Total mould count [ $\log_{10}$ CFU/g]	Total mould count [ $\log_{10}$ CFU/g]
Peppermint	1.00	-
Sage	1.78	-
Yarrow	1.30	-
Black tea	1.60	-
Bearberry	1.81	-
Lemon balm	2.02	-
Mixture of teas from Zlatibor	1.30	-

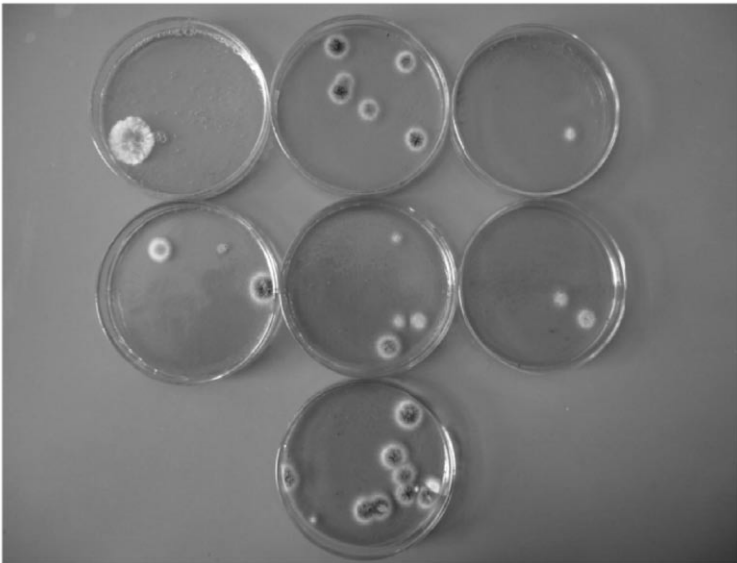


Fig. 2 – Mould contamination of dry residues after thermal treating

Isolated fungal species from dry residues after thermal treating were from genera *Aspergillus* and *Eurotium* what is presented in Table 7.

Tab. 7 – Isolated fungal species from dry residues after thermal treating

Me- di- um	Samples						
	Peppermint	Sage	Yarrow	Black tea	Bearberry	Lemon balm	Mixture of teas from Zlatibor
MY 10-12	<i>Eurotium herbariorum</i>	<i>Eurotium amstelodami</i>	<i>Aspergillus awamori</i>	<i>Aspergillus awamori</i>	<i>Aspergillus awamori</i>	<i>Aspergillus niger</i>	<i>Aspergillus niger</i> <i>Eurotium herbariorum</i>
MEA	-	-	-	-	-	-	-

Regarding filtrates of tea, growth was observed in all samples on MY10-12 medium, while on MEA medium there was no growth (Table 8, Figure 3). The least contamination was in filtrate from peppermint (0.70 cfu/g) and the highest in filtrate from mixture of teas from Zlatibor (1.84 cfu/g).

From all filtrates, except from lemon balm, was isolated *Aspergillus awamori*. In filtrate of lemon balm appeared *Aspergillus niger* and *Penicillium chrysogenum*, what is presented in Table 9.

Tab. 8 – Total mould count in filtrate of tea obtained by membrane filter method

Sample	MY 10-12	MEA
	Total mould count [ $\log_{10}$ cfu/ml]	Total mould count [ $\log_{10}$ cfu/ml]
Peppermint	0.70	-
Sage	1.33	-
Yarrow	1.69	-
Black tea	1.43	-
Bearberry	1.23	-
Lemon balm	1.56	-
Mixture of teas from Zlatibor	1.84	-

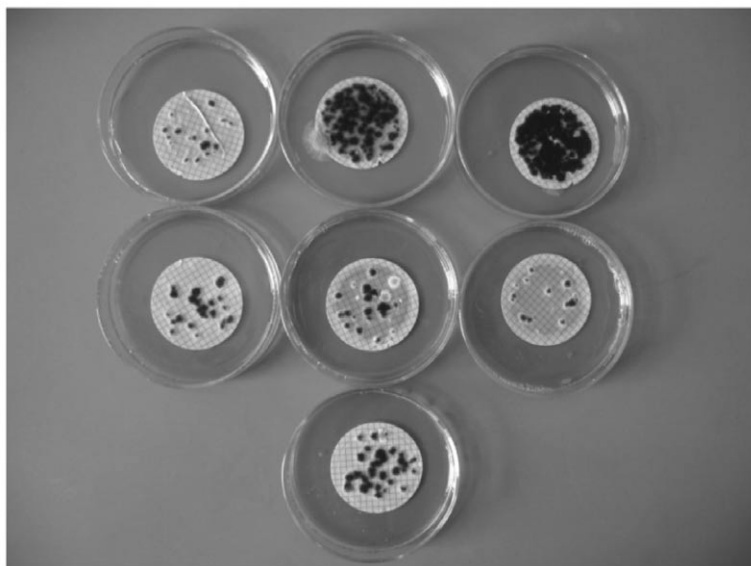


Fig. 3 – Mould contamination of filtrate of tea obtained by membrane filter method

Tab. 9 – Isolated fungal species from filtrate of tea obtained by membrane filter method

Medium	Samples						
	Peppermint	Sage	Yarrow	Black tea	Bearberry	Lemon balm	Mixture of teas from Zlatibor
<b>MY 10-12</b>	<i>Aspergillus awamori</i>	<i>Aspergillus awamori</i>	<i>Aspergillus awamori</i>	<i>Aspergillus awamori</i>	<i>Aspergillus awamori</i>	<i>Aspergillus niger</i> <i>Penicillium chrysogenum</i>	<i>Aspergillus awamori</i>
<b>MEA</b>	-	-	-	-	-	-	-

The presence of different mould genera in teas in bulk, dry residues after thermic treatment and in filtrates of teas can be seen in the Figure 4.

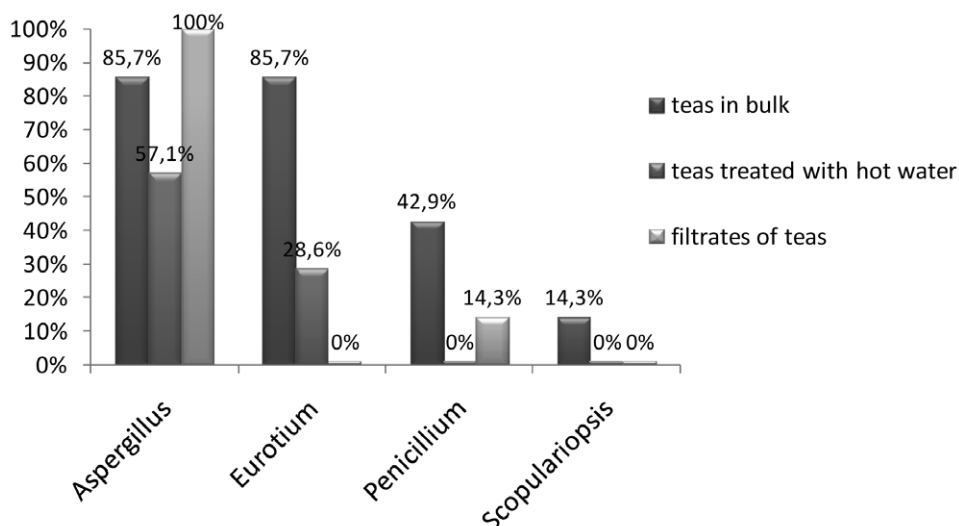


Fig. 4 – Presence of different mould genera in teas in bulk, dry residues after thermic treatment and in filtrates of teas

It is obvious that *Aspergillus* species were present in all three cases. *Eurotium* species was not present only in tea filtrates, *Penicillium* species were present in teas in bulk and in tea filtrates and *Scopulariopsis brevicaulis* was present only in teas in bulk.

All of isolated fungal species, according to different allegations, are potential producers of mycotoxins. In the Table 10 can be seen some of isolated moulds whose are potential producers of toxins.

Tab. 10 – Some of isolated moulds whose are potential producers of toxins

Moulds	Toxins
<i>Eurotium amstelodami</i>	Sterigmatocystin (traces)
<i>Eurotium herbariorum</i>	Sterigmatocystin (traces)
<i>Eurotium shevalieri</i>	Emodin, gliotoxin, physicon, xanthocillin
<i>Aspergillus flsvus</i>	Aflatoxins, aflatrem, aflavinin, aspergillic acids
<i>Aspergillus niger</i>	Maltformins, nigragilin
<i>Aspergillus sydowii</i>	Nidulotoxin, sterigmatocystin, griseofulvin
<i>Aspergillus versicolor</i>	Nidulotoxin, sterigmatocystin
<i>Penicillium chrysogenum</i>	Roquefortine C, PR-toxin, xantocillin, penicillin

All of this species are widely distributed (K l i c h , 2002).

*Eurotium* species are world-wide but predominantly in tropical and sub-tropical areas and they were reported from dried food products, spices, peas, milled rice, nuts (D o m s c h et al., 1980).

*Aspergillus flsvus* is the most widely reported food-borne fungus and from many other substrates in indoor and outdoor environments (S a m s o n et al., 2001).

*Aspergillus niger* has been reported from soils, plant litter, dried fruits, nuts and indoor environments (P i t t and H o c k i n g , 1997).

Habitat of *Aspergillus sydowii* is primary soil, but it has been reported from many other substrates in indoor and outdoor environments. *A. sydowii* is much less present in food than *Aspergillus versicolor* which occurs especially in spices, dried cereals and nuts, and is common in indoor environments (D o m s c h et al., 1980).

*Penicillium chrysogenum* has been reported from indoor environments, deserts, dried foods, cheese, salterns (S a m s o n and F r i s v a d , 2004).

## CONCLUSIONS

Different kinds of moulds were isolated from all samples of tea. Total mould count was the highest in teas in bulk (1.84-4.55 log<sub>10</sub>cfu/g). The most frequent moulds were species from *Aspergillus* and *Eurotium* genera.

After treatment with hot, sterile, distilled water total mould counts in all samples were reduced (in dry residues after thermal treating number of colonies was in range from 1.00 cfu/g to 2.02 cfu/g and in tea filtrates from 0.70 cfu/g to 1.84 cfu/g).

*Aspergillus awamori* was the most resistant and appeared in six samples of filtrates of tea, *Aspergillus niger* in one sample and *Penicillium chrysogenum* in one sample.



## REFERENCES

- Brera, C., Miraglia, M., & Colatosti, M. (1998). *Evaluation of the impact of mycotoxins on human health: sources of errors*. Microchemical Journal, 5(1), 45–49. In: B. Romagnoli, V. Menna, N. Gruppioni, C. Bergamini, Aflatoxins in spices, aromatic herbs, herb-teas and medicinal plants marketed in Italy, Food Control 18 (2007) 697-701.
- Domsch, K. H., Gams, W. and Anderson, T. H. (1980): *Compendium of Soil Fungi*. London. Academic Press.
- Klich, A. M. (2002): *Identification of common Aspergillus species*, Centraalbureau voor Schimmel cultures, Utrecht, The Netherlands.
- Kungulovski, Dz., Avramovski, O., Atanasova Pancevska, N., Kungulovski, I. (2011): *Mycotoxigenic molds in spices from Macedonian stores*. Proc. Nat. Sci, Matica Srpska Novi Sad. 120: 81-91.
- McKay, D. L., and Blumberg, J. B. (2002). *The role of tea in human health: an update*. Journal of the American College of Nutrition, 21(1), 1–13. In: Wang, R., Zhou, W., Isabelle, M., Comparison study of the effect of green tea extract (GTE) on the quality of bread by instrumental analysis and sensory evaluation, Food Research International 40 (2007) 470–479.
- Pitt, J. I. and Hocking, A. D. (1997): *Fungi and Food Spoilage 2<sup>nd</sup> ed*. London, U.K. Blackie Academic and Professional.
- Rietveld, A., and Wiseman, S. (2003). *Antioxidant effects of tea: evidence from human clinical trials*. The Journal of Nutrition, 133, 3285–3292. In: Wang, R., Zhou, W., Isabelle, M., Comparison study of the effect of green tea extract (GTE) on the quality of bread by instrumental analysis and sensory evaluation, Food Research International 40 (2007) 470–479.
- Samson, R. A. and Frisvad, J. C. (2004): *Penicillium subgenus Penicillium: new taxonomic and other extrolites*. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
- Samson, R. A., Hoekstra, E. S. and Frisvad, J. C. (2004): *Introduction to food- and airborne fungi*. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
- Samson, R. A., Houbbraken, J., Summerbell, R. C., Flannigan, B. and Miller, J. D. (2001): *Common and important species of fungi and actinomycetes in indoor environments*. In: Eds. B. Flannigan, R.A., Samson and J.D. Miller Microorganisms in Home and Indoor Work Environments, 287-292. New York: Taylor and Francis.
- Škrinjar, M. (1997): *Toksigene plesni i njihovi metaboliti u sirovinama korišćenim u industriji mesa*. Tehnologija mesa, 2-3: 59-62.
- Thom, C., Raper, K. B. (1945): *A Manual of the Aspergilli*. The Williams & Wilkins company, Baltimore.
- Višić, M. (2007): *Mogućnost stvaranja ohratoksina A i sterigmatocistina uz prisustvo kalijum sorbata*. Diplomski rad. Tehnološki fakultet, Univerzitet u Novom Sadu.

## КСЕРОФИЛНЕ МИКОПОПУЛАЦИЈЕ ЧАЈЕВА У РИНФУЗИ

Марија М. Шкрињар<sup>1</sup>, Маријана М. Вишић-Вићановић<sup>1</sup>, Невена Т. Благојевић<sup>1</sup>,  
Зорица С. Таталовић<sup>2</sup>, Весна Ј. Крунић<sup>2</sup>

<sup>1</sup> Технолошки факултет, Универзитет у Новом Саду,  
Булевар цара Лазара 1, Нови Сад, Србија

<sup>2</sup> ЕКОЛАБ д.о.о., Јована Цвијића 45, Нови Сад, Србија

### Резиме

После воде, данас је чај најпопуларније пиће на свету. Конзумира се већ дуги низ година, у почетку више као освежавајуће пиће, а у последње време више због својих лековитих својстава. Показало се да чајеви поседују антиоксидативне, антиканцерогене и антимикробне особине. Имајући у виду да чајеви током производње не пролазе кроз технолошке операције које подразумевају дејство високих температура, постоји велика могућност контаминације различитим врстама микроорганизама, посебно плеснима. Плесни су широко распрострањене у природи, а њихове споре се могу наћи у атмосфери чак и на веома великим висинама. Под повољним условима температуре и влажности, плесни расту на многим намирницама, као што су житарице, уљарице, језгровито воће, лековито биље и зачини. Многе врсте плесни су потенцијални произвођачи микотоксина, што представља велик ризик по људско здравље.

Циљ овог рада је био да се испита укупан број плесни и да се идентификују плесни изоловане из чајева у ринфузи, из чајева третираних врелом, стерилном дестилованом водом и из филтрата. Испитивани су чајеви: нана, жалфија, хајдучка трава, црни чај, увин чај, матичњак и мешавина чаја са Златибора. У чајевима у ринфузи је установљена висока контаминација различитим врстама плесни (1,84-4,55 цфу/г), као што су *Aspergillus awamori*, *A. lovaniensis*, *A. niger*, *A. phoenicus*, *A. repens*, *A. restrictus*, *A. sydowii*, *A. versicolor*, *Eurotium amstelodami*, *E. chevalieri*, *E. hermariorum*, *Penicillium chrysogenum* и *Scopulariopsis brevicaulus*. Најфреквентније су биле врсте родова *Aspergillus* и *Eurotium*. Термички третман врелом стерилном дестилованом водом је редуковао број плесни. *Aspergillus awamori* је био најотпорнија врста и појавио се у шест узорака филтрата чаја, *Aspergillus niger* у једном и *Penicillium chrysogenum* такође у једном узорку филтрата.



*Marija M. Škrinjar, Ljiljana S. Petrović,  
Nevena T. Blagojev, Vladislava M. Šošo*

Faculty of Technology, University of Novi Sad, Bulevar cara Lazara 1, Novi Sad, Serbia

## AIR MYCOPOPULATIONS IN *PETROVSKÁ KLOBÁSA* PRODUCING FACILITY\*

**ABSTRACT:** Different types of filamentous fungi periodically cause problems in small-scale facilities for traditional dry fermented sausages, such as *Petrovská klobása* from Vojvodina province (Serbia). Mould contamination can be observed during processing, ripening, and storage. Sausages may become spoiled due to visible mould colonies on the surface and off-flavours they produce. The most important – if mycotoxin production occurs it might promote a number of health disorders. Knowledge and control of filamentous fungi are, therefore, essential to produce sausages that satisfy the criteria of hygienic quality, sensorial characteristics, and food safety. The aim of this study was to survey mycoflora of a small-scale facility producing traditional dry fermented sausage – *Petrovská klobása*. The mould contamination of the air in processing unit and ripening chambers was investigated, in order to determine the important fungi in terms of spoilage of the products and ability to produce mycotoxins.

The mould contamination of air in processing unit and ripening chambers examined was in range 0.22 – 1.89 log CFU/P.d. Isolated moulds belong to 6 genera: *Aspergillus* (3 species), *Cladosporium* (1 species), *Eurotium* (2 species), *Fusarium* (1 species), *Penicillium* (12 species) and *Scopulariopsis* (1 species). The most abundant were species of *Penicillium* genus, many of which are capable for mycotoxin production.

The level and diversity of fungal contamination of air varied between samples, influenced by the general hygiene, the buildings, the airflow, the outdoor environments, and the time of year. This knowledge is crucial for the improvement of hygiene control systems in small-scale processing units.

**KEY WORDS:** mycopopulations, processing unit, air

## INTRODUCTION

Traditional dry fermented sausage production has increased overall Europe since the 1980's. The development is due mainly to the consumer's request for natural and authentic products that are made in non-industrial environ-

---

\* The paper was presented at the fourth international scientific meeting *Mycology, mycotoxicology, and mycoses*, which was organized in *Matica srpska, Department for natural sciences* from April 20-22, 2011.

ment, characterized by small-scale batch production with a limited degree of mechanization, and strongly identified with a place or region of origin (L e b - e r t et al., 2007). One of them is *Petrovská klobása*, produced in the area of Bački Petrovac (Vojvodina Province, Serbia), in the traditional way, without additives and starter cultures. Because of its specific and distinctive quality, *Petrovská klobása* has been protected with Designation of Origin (PDO) according to Serbian legislation (P e t r o v i ć et al., 2007).

The traditional procedure relies on indigenous microflora whose origins could result from the raw material used or from the environment. Each processing facility has a specific house flora composed of useful microorganisms for the fermentation and flavour of sausages, as well as spoilage and pathogenic flora. Thus, the characterization of this house-flora is crucial because safety (pathogenic flora), acceptability (spoilage flora), and sensorial quality (technological flora) of the products depend totally on it (C h e v a l - l i e r et al., 2006). This study dealt with spoilage microflora, particularly filamentous fungi. Moulds periodically cause problems in small-scale facilities for traditional dry fermented sausages production. Mould growth can be observed during processing, ripening, and storage and can be a quality problem. Sausages may become spoiled due to visible mould colonies on the surface and the off-flavors they produce. Besides, mould growth may also represent a health risk because of the possibility of mycotoxin production by several mould species (P a p a g i a n n i et al., 2007, V e s k o v i ć - M o r a č a n i n et al., 2009). Consumption of contaminated sausages might promote a number of health disorders. Knowledge and control of filamentous fungi are, therefore, essential to produce sausages that satisfy the criteria of hygienic quality, organoleptic characteristics and food safety (Š k r i n j a r et al., 2010).

Only few studies have focused on mycobiota in the processing areas of meat processing plants (I s m a i l et al., 1995; A n d e r s e n , 1995; B a t t i - l a n i et al., 2007). Therefore, the aim of this study was to survey the typical house-flora, particularly mycoflora, of a small-scale facility producing traditional dry fermented sausage – *Petrovská klobása*. The mould contamination of the air in processing unit and ripening chambers was investigated, in order to determine the important fungi in terms of spoilage of the products and ability to produce mycotoxins.

## MATERIALS AND METHODS

The processing unit investigated in this research was located in Bački Petrovac (Vojvodina Province, Serbia). Sampling of the air was carried out during the production of *Petrovská klobása* in December 2009. The first sampling was done in the unit where sausages were prepared and stuffed on the day of preparation (day 1). Produced sausages were left to ripen in chambers in Bački Petrovac (days 1-11), so the sampling of the air in ripening chamber was done on days 2, 6, and 9 of ripening. On day 11, the sausages were moved to Kucura for further ripening, so the sampling of the air in Kucura's cham-

bers was carried out on days 11, 14, 34, and 65 after sausage production. On day 65, sausages were moved to an industrial facility in Novi Sad to finish ripening process there, and the air sampling in industrial ripening chambers was done on days 65, 90, 120, and 217 of ripening (Table 1).

Tab. 1 – Air samples

Room	Day of sampling	Sign
Processing unit	1	A-1
Ripening chamber Bački Petrovac	2	A-2
	6	A-3
	9	A-4
Ripening chamber Kucura	11	A-5
	14	A-6
	34	A-7
	65	A-8
Ripening chamber An industrial facility in Novi Sad	65	A-9
	90	A-10
	120	A-11
	217	A-12

Occurrence (presence/absence) of moulds and identification of species isolated from collected samples were then carried out. Air in the sausage processing rooms was examined by gravity sedimentation onto 9 cm Petri dishes containing 15 ml of Sabouraud-maltose agar with 2% of chloramphenicol, for 20 minutes, at five different locations. After 7 days incubation at 25°C, the Petri dishes were inspected and the colonies were sub-cultured onto agar plates according to Samson et al. (2004), as follows: *Penicillium* and *Aspergillus* species were plated onto Czapek agar (CzA), *Fusarium* species onto potato dextrose agar (PDA) and others onto Sabouraud-maltose agar (SMA). Agar plates were incubated for 7 days at 25°C. The isolates were then identified by their morphological characteristics, following the methods of Ellis (1971), Nelson et al. (1983), Samson and Frisvad (2004) and Samson et al. (2004).

## RESULTS AND DISCUSSION

Results for total mould counts per Petri dish, isolated from air in *Petrovská klobása* processing unit and ripening chambers, expressed as logCFU/Petri dish, are presented in Table 2.

Tab. 2 – Total mould counts per Petri dish (TMC/P.d.), isolated from air in *Petrovská klobása* processing unit and ripening chambers, (log CFU/P.d)

Sample	Room	Day of sampling	TMC/P.d.
A-1	Processing unit	1	0.40 ± 0.14*
A-2	Ripening chamber Bački Petrovac	2	1.46 ± 0.08
A-3		6	1.31 ± 0.03
A-4		9	0.22 ± 0.2
A-5	Ripening chamber Kucura	11	1.68 ± 0.04
A-6		14	1.34 ± 0.06
A-7		34	1.53 ± 0.05
A-8	Ripening chamber An industrial facility in Novi Sad	65	0.37 ± 0.09
A-9		65	1.89 ± 0.02
A-10		90	0.94 ± 0.14
A-11		120	1.84 ± 0.04
A-12		217	0.15

\* – Mean ± standard deviation from 5 measurements

The mould contamination of air in processing unit and ripening chambers examined was in range 0.22 – 1.89 logCFU/P.d. Similar results were obtained by Sørensen et al. (2008) who investigated air contamination of some fermented sausage processing area. Their results for air contamination by filamentous fungi are in range 0.23 – 1.2 logCFU/P.d.

Table 3 presents the results obtained by identification of moulds isolated from air in processing unit and ripening chambers. Table also present the moiety of species in mycopopulations isolated from specific sample.

Isolated moulds belong to 6 genera and 20 species. Genera are: *Aspergillus*, *Cladosporium*, *Eurotium*, *Fusarium*, *Penicillium* and *Scopulariopsis*. Species are: *A. caespitosus* Raper & Thom, *A. fumigatus* Fres, *A. versicolor* (Vuill.) Tiraboschi, *C. oxysporum* Berk. & Curt, *E. chevalieri* Mangin, *E. herbariorum* (Wiggers), *Fusarium sporotrichioides* Sherb, *P. aurantiogriseum* Dierckx, *P. brevicompactum* Dierckx, *P. camemberti* Thom, *P. chrysogenum* Thom, *P. corylophilum* Dierckx, *P. glabrum* (Wehmer) Westling, *P. griseofulvum* Dierckx, *P. janthinellum* Biourge, *P. neoechinulatum*, *P. velutinum* van Beyma, *P. olsonii* Bainier & Sartory, *P. solitum* Westling and *S. brevicaulis* (Sacc.) Bain. The most abundant were species of *Penicillium* genus; all air samples tested were contaminated with at least one *Penicillium* species. It was followed by *Aspergillus*, whose species were found in 6 (50%) of tested samples, then *Eurotium*, that contaminated 5 (41.67%) samples, while species of *Scopulariopsis*, *Fusarium* and *Cladosporium* genera were isolated from one air sample each (Fig. 1).



Tab. 3 – Mycopopulation of air in *Petrovská klobása* processing unit and ripening chambers

Sample	Room	Day of sampling	Species	Isolated (%)
A-1	Processing unit	1	<i>Aspergillus fumigatus</i> <i>Penicillium chrysogenum</i> <i>Scopulariopsis brevicaulis</i> <i>Penicillium olsonii</i>	50 26 14 10
A-2	Ripening chamber Bački Petrovac	2	<i>Penicillium aurantiogriseum</i> <i>Penicillium neoechinulatum</i> <i>Penicillium glabrum</i>	69 20 11
A-3		6	<i>Penicillium aurantiogriseum</i> <i>Eurotium chevalieri</i> <i>Eurotium herbariorum</i> <i>Aspergillus caespitosus</i> <i>Fusarium sporotrichioides</i> <i>Penicillium olsonii</i>	39 26 21 10 3 1
A-4		9	<i>Penicillium janthinellum</i>	100
A-5		11	<i>Penicillium aurantiogriseum</i> <i>Penicillium chrysogenum</i>	75 25
A-6	Ripening chamber Kucura	14	<i>Penicillium aurantiogriseum</i> <i>Penicillium chrysogenum</i> <i>Penicillium velutinum</i>	65 22 13
A-7		34	<i>Eurotium herbariorum</i> <i>Aspergillus versicolor</i> <i>Penicillium griseofulvum</i> <i>Aspergillus caespitosus</i>	70 20 6 4
A-8		65	<i>Eurotium herbariorum</i> <i>Penicillium aurantiogriseum</i> <i>Aspergillus versicolor</i> <i>Penicillium glabrum</i>	37 27 20 16
A-9		65	<i>Eurotium herbariorum</i> <i>Cladosporium oxysporum</i> <i>Penicillium brevicompactum</i> <i>Penicillium solitum</i> <i>Penicillium olsonii</i> <i>Penicillium chrysogenum</i> <i>Aspergillus caespitosus</i>	23 23 18 15 13 5 3
A-10	Ripening chamber An industrial facility in Novi Sad	90	<i>Penicillium chrysogenum</i> <i>Penicillium camemberti</i> <i>Aspergillus versicolor</i> <i>Penicillium corylophilum</i>	43 39 16 2
A-11		120	<i>Penicillium aurantiogriseum</i> <i>Penicillium chrysogenum</i>	58 42
A-12		217	<i>Penicillium aurantiogriseum</i> <i>Penicillium chrysogenum</i> <i>Eurotium herbariorum</i>	42 33 25

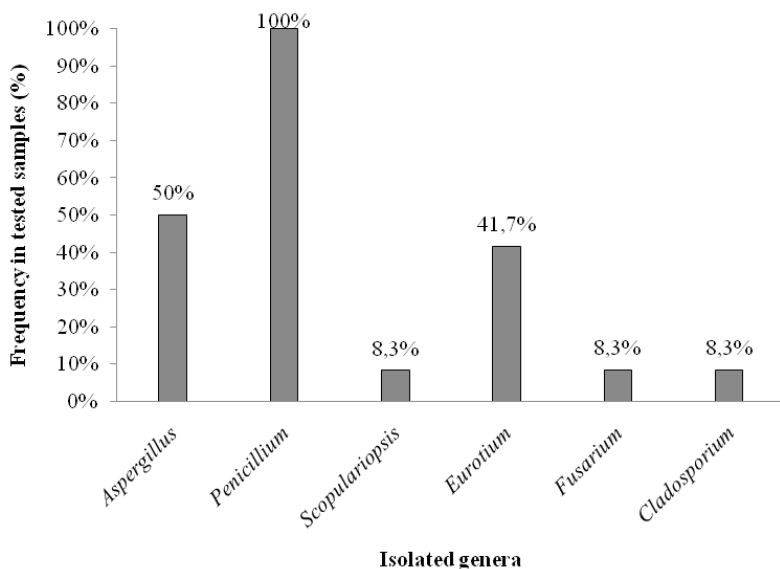


Fig. 1 – Frequency of isolated genera in samples of air in ripening chambers

The highest number of different mould species (7), which makes 35% of all identified, was isolated from ripening chamber in industrial facility in Novi Sad, on the 65<sup>th</sup> day of ripening (A-9). From sample A-3, six different species were isolated (30%), while the majority of samples (A-1, A-7, A-8 and A-10) were contaminated with four different species (20% of all identified). Samples A-2, A-6 and A-12 were contaminated with three different species (15%), samples A-5 and A-11 with two (10%), while from the sample A-4 just one species was isolated (5%), Fig. 2.

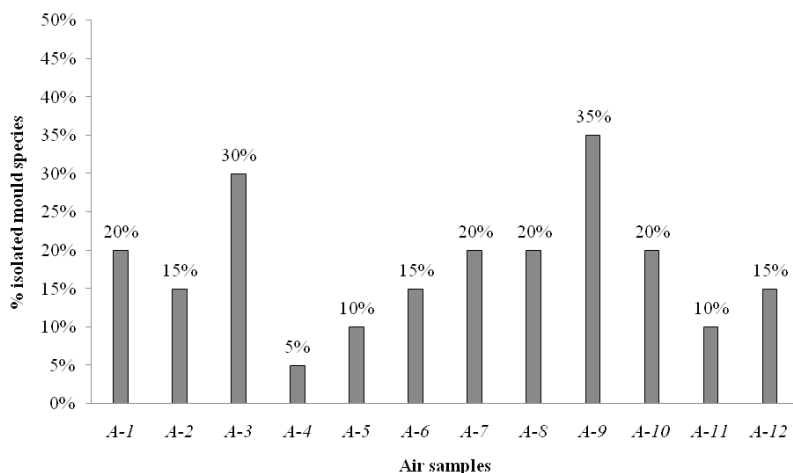


Fig. 2 – Presence of different mould species in mycopopulations isolated from air in *Petrovska klobasa* processing unit and ripening chambers

*Penicillium* species were isolated frequently from air in the processing areas and ripening chambers and it was the most frequent genus found. Among the *Penicillium* isolates, 12 species were identified. The most abundant were *P. aurantiogriseum* and *P. chrysogenum*, which were isolated from 7 samples each (58.3%). They were followed by *P. olsonii*, which was isolated from three samples (25%), and *P. glabrum*, that was isolated from two samples (16.7%). All of 8 species remain were isolated from just one sample (8.3%).

*Aspergillus* genus was found with quite low occurrence in the air. It was presented with three different species. The most abundant was *A. versicolor*, isolated from three air samples (25%), followed by *A. caespitosus* (16.67%) and *A. fumigatus* (8.33%). *Eurotium*, the perfect state of the *Aspergillus glaucus* group, was found relatively frequently. *Eurotium* generally grows well on substrates with low water activity. It was presented with two species – *E. herbariorum* was isolated from five air samples, which makes 41.67% of all tested, and *E. chevalieri* was isolated from one sample (8.33%). Genera *Scopulariopsis*, *Fusarium* and *Cladosporium* were found in one air sample each and all three genera were presented with one species – *S. brevicaulis*, *F. sporotrichioides* and *C. oxysporum*.

This work surveyed the mould contamination of air in *Petrovská klobása* processing unit and ripening chambers, and contributed to knowledge of the mycological ecosystems of food environments.

The level of fungal contamination of air in processing unit and ripening chambers varied between the individual rooms and the time of sampling, probably influenced by the general hygiene, the buildings, the airflow, the outdoor environments and the time of the year. The diversity of fungi isolated from air was relatively high. This probably reflects that fungal conidia are air-borne and are therefore easily spread. Important reservoirs can be humans, soil, dust, raw materials, drains, equipment surfaces, and ventilation ducts (Scholte et al., 2002). Many of the mould species were isolated rarely, only once or twice during the survey, which mean that they were most likely isolated by chance and were not representatives of a consistent house biota. Fungi present in more than five samples during the survey were considered as present in significant number. In that mean, the important genera were: *Penicillium*, *Aspergillus* and *Eurotium*.

Many *Penicillium* species can produce mycotoxins. At least half of the *Penicillium* species identified in this study are potentially able to produce toxic metabolites, according to Frisvad and Thrane (2002). Some species of *Aspergillus* and *Eurotium* also produce toxic metabolites. Little is known about the toxigenic potential of *Cladosporium* species. In this study, the most frequent toxigenic *Penicillium* species were *P. aurantiogriseum* and *P. chrysogenum*. Important toxic metabolites known to be produced by these species are: ochratoxin A, citrinin, penicilic acid, roquefortine C, and other (Samson and Frisvad, 2004; Marasas et al., 1984), (Table 4).

Tab. 4 – Production of mycotoxin by fungi isolated from air in *Petrovská klobása* processing unit and ripening chambers

Species	Toxins
<i>Eurotium chevalieri</i>	Emodin, gliotoxin, physicon, xantocillin X
<i>Eurotium herbariorum</i>	Sterigmatocystin (traces)
<i>Aspergillus fumigatus</i>	Fumigalclavines, fumigallin, fumigatin, fumitoxins, fumitremorgins, gliotoxin, spinulosin, verruculogen
<i>Aspergillus versicolor</i>	Nidulotoxin, sterigmatocystin
<i>Fusarium sporotrichioides</i>	Trichothecenes A, butenolide, zearalenone
<i>Penicillium aurantiogriseum</i>	Penicilic acid, xanthomegnin, viomellein, viridicatin, ochratoxin A
<i>Penicillium brevicompactum</i>	Brevianamides, mycophenolic acid, botryodiploidin
<i>Penicillium camemberti</i>	Cyclopiazonic acid
<i>Penicillium chrysogenum</i>	Roqufortine C, PR-toxin, xanthocillin X, penicillin, ochratoxin A
<i>Penicillium glabrum</i>	Citromycetin
<i>Penicillium griseofulvum</i>	Patulin, cyclopiazonic acid, roqufortine C, griseofulvin
<i>Penicillium solitum</i>	Viridicatin

## CONCLUSION

This study targeted fungal ecosystems of food processing environments, which are not usually surveyed. *Petrovská klobása* processing unit and ripening chambers showed a high variability of microbial levels in their environments, some of them with excessive levels of toxigenic species. The different cleaning, disinfecting, and manufacturing practices of the small-scale processing units could be responsible for this variability. Also, mycobiota in the processing plants showed a high diversity of fungal species, mainly belonging to the genera *Penicillium*, *Aspergillus*, *Eurotium*, etc. Many toxigenic mould species were isolated from the processing areas, which poses a high risk for human health. This knowledge is crucial for the improvement of hygiene control systems in small-scale processing units, in order to provide high level of product stability and safety.

## ACKNOWLEDGEMENTS

This study was supported by the Ministry of Science and Technological Development of Republic of Serbia, Project No. TR31032.

## REFERENCES

- Andersen, S. J. (1995): *Compositional changes in surface mycoflora during ripening of naturally fermented sausages*, J. Food Protect, 58: 426-429.
- Battilani, P., Pietri, A., Giorni, P., Formenti, S., Bertuzzi, T., Toscana, T., Virgili, R. and Kozkiewicz, Z. (2007): *Penicillium populations in dry-cured ham manufacturing plants*, J. Food Protect, 70: 975-980.

- Chevallier, I., Ammor, S., Lauget, A., Labayle, S., Castanet, V., Dufour, E. and Talon, R. (2006): *Microbial ecology of a small-scale facility producing traditional dry sausage*, Food Control, 17: 446-453.
- Ellis, M. B. (1971): *Dematiaceous Hyphomycetes*, Commonwealth Mycological Institute, Kew, Surrey, England.
- Frisvad, J. C. and Thrane, U. (2002): *Mycotoxin production by common filamentous fungi*. In: R. A. Samson, E. S. Hoekstra, J. C. Frisvad, O. Filtenborg, Eds., *Introduction to Food- and Airborne fungi*, Ed 6, Centraalbureau voor Schimmelcultures, Utrecht, pp. 321-331.
- Ismail, M. A., Abou Elala, A. H., Nassar, A. and Michail, D. G. (1995): *Fungal contamination of beef carcasses and the environment in a slaughterhouse*, Food Microbiol, 12: 441-445.
- Lebert, I., Leroy, S., Giammarinaro, P., Lebert, A., Chacornac, J. P., Bover-Cid, S., Vidal-Carou, M. C. and Talon, R. (2007): *Diversity of microorganisms in the environment and dry fermented sausages of small tradition French processing units*, Meat Sci, 76: 112-122.
- Marasas, W. F. O., Nelson, P. E. and Toussoun T. A. (1984): *Toxigenic Fusarium species – identity and mycotoxicology*, The Pennsylvania State University Press, University Park and London.
- Nelson, P. E., Toussoun, T. A. and Marasas, W. F. O. (1983): *Fusarium species*. An Illustrated Manual for Identification. The Pennsylvania State University Press, University Park and London.
- Papagianni, M., Ambrosiadis, I. and Filioysis, G. (2007): *Mould growth on traditional Greek sausages and penicillin production by Penicillium isolates*, Meat Sci., 76:653-657.
- Petrović, Lj., Džinić, N., Tomović, V., Ikonić, P. and Tasić, T. (2007): *Tehnološki elaborat o načinu proizvodnje i specifičnim karakteristikama proizvoda Petrovska klobasa (Petrovačka kobasica)*, Novi Sad.
- Samson, R. A. and Frisvad, J. C. (2004): *Penicillium subgenus Penicillium: new taxonomic schemes, mycotoxins and other extrolites*, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
- Samson, R. A., Hoekstra, E. S. and Frisvad, J. C. (2004): *Introduction to food- and airborne fungi*, Centraalbureau voor Schimmelcultures, Utrecht, pp. 1-389.
- Scholte, R. P. M., Samson, R. A. and Dijksterhuis, J. (2002): *Spoilage fungi in the industrial processing of food*. In: R. A. Samson, E. S. Hoekstra, J. C. Frisvad and O. Filteborg, Eds., *Introduction to Food- and Airborne Fungi*, Ed 6, Centaalbureau voor Schimmelcultures, Utrecht, pp. 339-356.
- Sørensen, L. M., Jacobsen, T., Nielsen, P. V., Frisvad, J. C. and Koch, A. G. (2008): *Mycobiota in the processing areas of two different meat products*, Int. J. Food Microbiol, 124: 58-64.
- Škrinjar, M., Vesković-Moračanin, S. and Nemet, N. (2010): *Mycopopulations of fermented sausages during the process of ripening and storage*, 12<sup>th</sup> International Meat Technology Symposium NODA 2010, Proceedings. 156-163.
- Vesković-Moračanin, S., Borović, B., Velebit, B., Rašeta, M. and Milićević, D. (2009): *Identification of mycobiota in Serbian slaughterhouses*. Proc. Nat. Sci. Matica Srpska Novi Sad. 117: 45-49.

## ПЛЕСНИ У ВАЗДУХУ ПРОСТОРИЈА ЗА ПРОИЗВОДЊУ И ЗРЕЊЕ ПЕТРОВАЧКЕ КОБАСИЦЕ

Марија М. Шкрињар, Љиљана С. Петровић, Невена Т. Благојев,  
Владислава М. Шошо

Технолошки факултет, Универзитет у Новом Саду,  
Булевар цара Лазара 1, 21000 Нови Сад, Србија

### Резиме

Различите врсте филаментозних гљива периодично изазивају проблеме у малим погонима за производњу традиционалних сувих ферментисаних кобасица, као што је *Petrovská klobása* из Војводине, Србија. До контаминације плесни-ма може доћи током производње, зрења и складиштења. Квар кобасица се може манифестовати у виду раста колонија на површини или промене укуса. Оно што је много важније – уколико дође до продукције микотоксина, може доћи до читавог низа здравствених поремећаја. Познавање и контрола филаментозних гљива су, стога, од великог значаја при производњи кобасица које задовољавају критеријуме хигијенског квалитета, сензорних карактеристика и безбедности хране. Циљ овог рада је био да се испита типична микофлора малог погона за производњу суве ферментисане кобасице *Petrovská klobása*. Испитана је контаминација ваздуха у производној јединици и коморама за зрење, у циљу детерминације гљива значајних са аспекта кварења производа и способности продукције микотоксина.

Контаминација ваздуха плеснима у испитаној производној јединици и коморама за зрење кретала се од 0.22 до 1.89 log CFU/P.d. Изоловане плесни сврстане су шест родова: *Aspergillus* (3 врсте), *Cladosporium* (1 врста), *Eurotium* (2 врсте), *Fusarium* (1 врста), *Penicillium* (12 врста) и *Scopulariopsis* (1 врста). Најзаступљеније биле су врсте рода *Penicillium*, од којих су многе способне да продукују микотоксине.

Ниво и разноврсност контаминације ваздуха варирали су између узорака, под утицајем опште хигијене, градње, протока ваздуха, спољашње средине и годишњег доба. Ова сазнања су од великог значаја за побољшање система контроле хигијене у малим производним погонима.

*Dragutin A. Đukić<sup>1</sup>, Leka G. Mandić<sup>1</sup>,  
Lidija Božarić<sup>2</sup>, Bojana D. Trifunović<sup>3</sup>,  
Marijana I. Pešaković<sup>4</sup>*

<sup>1</sup> Faculty of Agronomy, Cara Dušana 34, 32000 Čačak, Serbia

<sup>2</sup> Specialist Veterinary Laboratory, Podgorica, Bulevar Džordža Vašingtona bb, Montenegro

<sup>3</sup> City Administration of Urban Planning of the Čačak, Župana Stracimira 2, 32000, Serbia

<sup>4</sup> Fruit Research Institute, Kralja Petra I 9, 32000 Čačak, Serbia

## POTENTIALLY PATHOGENIC, PATHOGENIC, AND ALLERGENIC MOULDS IN THE URBAN SOILS\*

**ABSTRACT:** The dynamics of soil mould populations that can compromise the human immune system was evaluated in experimental plots located at different distances (100, 300, 500, 700 and 900 m) from the main source of pollution – the Podgorica Aluminium Plant. Soil samples were collected in July and October 2008 from three different plot zones at a depth of 0-10 cm. The count of potentially pathogenic, keratinolytic and allergenic (melaninogenic) moulds was assessed, which can significantly contribute to both diagnosis and prophylaxis.

The count of medically important moulds was higher in the urban soil than in the unpolluted (control) soil. Their count decreased with increasing distance from the main pollution source (PAP). Their abundance in the soil was considerably higher in autumn than in spring.

**KEY WORDS:** moulds, pollution, soil

## INTRODUCTION

Many microscopic moulds produce biologically active substances – enzymes, vitamins, antibiotics and toxins. Micromycetes account for 50% of the total microbial biomass in some soils (Mirčnik 1988; Zvjaginčev, 1999). They play an important role in the mineralisation of organic substances and maintenance of soil fertility. Many micromycete species are toxic (Bilai, 1989) since they produce antibiotics and phyto- and zootoxins (Bilai and Pidopličko, 1970).

Anthropogenic activities in urban areas lead to the formation of specific soils that show disturbance in the cycle of biogenic elements, depletion of the

---

\* The paper was presented at the fourth international scientific meeting *Mycology, mycotoxicology, and mycoses*, which was organized in *Matica srpska, Department for natural sciences* from April 20-22, 2011.



biodiversity of saprotrophic microorganisms (composition, structure, function) and an increase in the presence of pathogenic microorganisms, moulds in particular (Islam et al., 2004; Đukić et al., 2009 a, b). Pathogenic moulds lead a saprophytic existence in the soil, but they can cause disease in animals and humans under certain conditions.

Urban soils are inhabited by a few hundred moulds, which can be potentially pathogenic (opportunistic), pathogenic or allergenic (Litvin, 1990; Hogg et al., 2000; Đukić et al., 2011), depending on the state of the immune system of the organism (Kurup and Schmitt, 1970; Marfenina, 1996, 2000). As yet, very little is known about the distribution of medically important moulds in different soil types (Marfenina, 1996, 2000), notwithstanding the intensification of research over the past few decades, most notably due to the high importance of knowledge of the geographical distribution of diseases, which specifically contributes to their timely diagnosis and prophylaxis (Kurup and Schmitt, 1970; Hadayati et al., 2005).

Soil moulds of medical importance were evaluated in this study in order to provide disease prevention and health protection in urban populations, primarily those who are exposed to polluted soil more frequently.

## MATERIAL AND METHODS

Our aim was to study potentially pathogenic, keratinolytic and allergenic moulds in the urban soil; experimental plots were selected according to their distance (100, 300, 500, 700, 900 m) from the main source of pollution i.e. the Podgorica-based Aluminum Plant (PAP). The control soil was located outside the urban zone and was typical of the region before intensive industrialisation and urbanisation – 2100 m away from the PAP. Plot size was 25 m<sup>2</sup>. Soil samples were collected in July and October 2008 from three different plot zones at a depth of 0-10 cm. The count of potentially pathogenic and allergenic (melaninogenic) moulds was determined by inoculation of 0.5 cm<sup>3</sup> of diluted soil suspension on Sabouraud dextrose agar supplemented with dermatophytes and adequate antibiotics (cycloheximine and chlortetracycline). Keratinolytic moulds and related dermatophytes were determined using the 1952 Vanbreuseghem method (Christensen, 1989).

The evaluation of the relative abundance of potentially pathogenic, allergenic, and keratinolytic moulds was performed following the recommendations of Hogg et al. (2000) and McGinnis (2004).

## RESULTS AND DISCUSSION

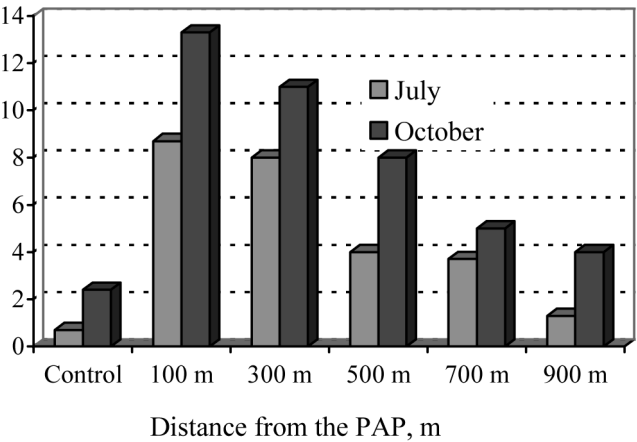
The soil in the urban city zone was found to contain a substantially higher presence of potentially pathogenic moulds of the genera *Aspergillus*, *Paecilomyces* and *Fusarium* as compared to the control soil (Tab. 1, Graph 1), which was in agreement with the research conducted by Marfenina (1996)



who reported a 25%–30% increase in their count in urban soils. The count of potentially pathogenic microfungi in the soil was higher in autumn than in summer. Moreover, it was observed to decrease with increasing distance from the main source of pollution, which was as expected, given the fact that potentially pathogenic moulds easily survive in polluted ecosystems (Ž d a n o v a et al., 1994).

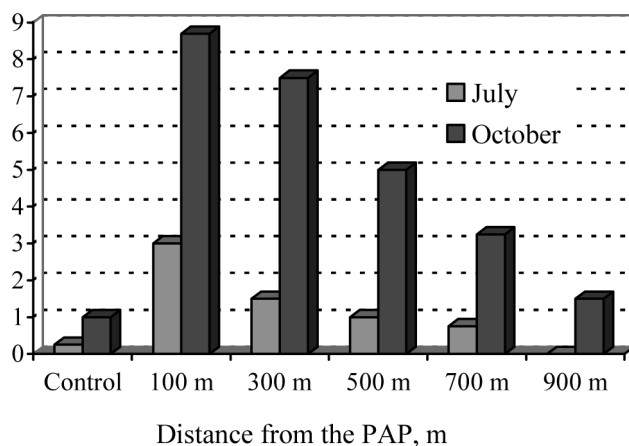
Tab. 1 – Total counts of potentially pathogenic, pathogenic, and allergenic moulds in the urban soils of Podgorica (in g absolutely dry soil)

Moulds isolated	Soil plot distance from the Podgorica Aluminum Plant, m											
	100		300		500		700		900		Control	
	July	Oct.	July	Oct.	July	Oct.	July	Oct.	July	Oct.	July	Oct.
Potentially pathogenic												
<i>Aspergillus fumigatus</i>	12	17	12	15	7	14	8	9	2	9	1	4
<i>Fusarium oxysporum</i>	5	12	3	8	–	3	–	3	2	2	1	2
<i>Paecilomyces variotii</i>	9	11	9	10	5	7	3	3	–	1	–	1
$\bar{X}$	8.7	13.3	8.0	11.0	4.0	8	3.7	5.0	1.3	4.0	0.7	2.4
Keratinolytic												
<i>Microsporum gypseum</i>	4	13	2	12	2	7	1	4	–	2	–	1
<i>Trichophyton terrestre</i>	5	15	4	13	–	11	2	6	–	2	1	2
<i>Chrysosporium keratinophylum</i>	1	4	–	3	1	2	–	2	–	1	–	–
<i>Ctenomyces serratus</i>	2	3	–	2	1	1	–	1	–	1	–	1
$\bar{X}$	3.0	8.7	1.5	7.5	1.0	5.0	0.75	3.25	–	1.5	0.25	1.0
Allergenic (melaninogenic)												
<i>Alternaria alternata</i>	17	22	17	18	13	17	6	11	–	5	–	2
<i>Cladosporium herbarum</i>	11	9	6	6	3	7	2	7	–	7	1	4
<i>Trichocladium asperum</i> spp.	6	16	6	11	2	5	2	2	2	3	–	2
$\bar{X}$	11.3	15.7	9.7	11.7	6.0	9.6	3.3	6.7	0.7	5.0	0.3	2.7



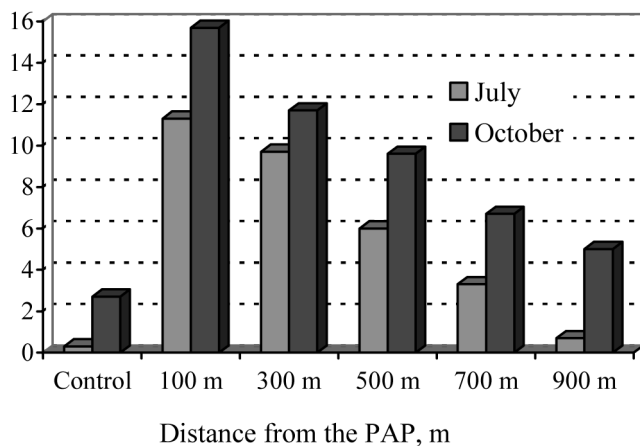
Graph 1 – The number potentially pathogenic moulds in the urban soils of Podgorica

Keratinolytic moulds are among the most important trophic groups of soil micromycetes that cause degradation of keratinous substances and skin disease. The estimated counts of medically important moulds (*Microsporium gypseum*, *Trichophyton terrestre*, *Chrysosporium keratinophilum*, *Ctenomyces serratus*) were found to be significantly higher in the polluted soil than in the control soil i.e. in October than in July, as well as to decrease with increasing distance from the pollution source (Tab. 1, Graph 2). This complied with the results of other authors (E m o n s , 1951; D e n t o n et al., 1961; K u r u p , S c h m i t t , 1970), most notably regarding the predominance of the species *Trichophyton terrestre* and *Microsporium gypseum*.



Graph 2 – The number keratinolytic moulds in the urban soils of Podgorica

The urban soil was also found to contain allergenic moulds such as melaninogenic *Alternaria alternata*, *Cladosporium herbarum*, and *Trchocladium asperum*. Their abundance was higher in the polluted soil than in the control soil i.e. in October than in July, and it decreased with increasing distance from the pollution source (Tab. 1, Graph 3). Similar results were obtained by S c h a t a et al. (1989), Ž d a n o v a et al. (1994), M a r f e n i n a (1996, 2000), K u l ' k o and M a r f e n i n a (1998) and D o m s c h et al. (1993), who suggested that the higher tolerance of melaninogenic moulds to toxic substances, radiation, drought, etc. results from the presence of melanin within their cell wall structures.



Graph 3 – The number allergenic (melaninogenic) moulds in the urban soils of Podgorica

## CONCLUSION

The results of this study suggest that the count of the test moulds was significantly higher in urban soils than in the control soil, in October than in July, as well as that it decreased with increasing distance from the pollution source.

In order to prevent undesirable effects on human health, control of all soils, urban ones in particular, should be performed on a regular basis for the presence of medically important moulds.

## REFERENCE

- Bilai, V. I. (1989): *Osnovi obšće mikologii*, Kiev: Visšaja škola, 375.
- Bilai, V. I., Pidopličko, N. M. (1970): *Toksinoobrazujuščie mikroskopičeskie gribi*, Kiev: Naukova Dumka, 273.
- Christensen, M. A. (1989): *View of fungal ecology*, Micologia, 81(1): 1-19.
- Domsch, K. H., Gams, W., Andersen, T. H. (1993): *Compendium of soil fungi*. London: Acad. Press., V, 1, 859.
- Đukić, D., Mandić, L., Božarić, L., Pešaković, M., Stanojković, A. (2011): *Mikrobiološki indikatori sanitarnog stanja gradskog zemljišta*, XVI savetovanje o biotehnologiji, 16 (18): 527-532.
- Đukić, D., Mandić, L., Marijana, P., Božarić, L. (2009 b): *Perzistencija salmonela u rizosfernom zemljištu i biljkama*, XIV Savetovanje o biotehnologiji, Zbornik radova, 15: 27-30.
- Đukić, D., Mandić, L., Marijana, P., Novosel, P. (2009 a): *Kolonizacija biljaka sa E. coli u uslovima zagađenog zemljišta*, XIV Savetovanje o biotehnologiji, Zbornik radova, 15: 23-26.

- Emmons, C. W., (1951): *Isolation of Cryptococcus neoformans from soil*, J. Bacteriol., 62: 685-690.
- Hedayati, M. H., Mohseni-Bandpi, A., Moradi, S. (2005): *A survey on the pathogenic fungi in soil samples of potted plants from Sari hospitals*, Journal of Hospital Infection, 58: 59-62.
- Hoog, de G. S., Guarro, J., Gene, J., Figueras, M. J. (2000): *Atlas of clinic fungi*, Central Bureau voor Schimmelcultures. Universitat Rovira i Virgili, 1126.
- Islam, M., Morgan, J., Doyle, P. M., Phatak, C., Millner, P., Jiang, X. (2004): *Fate of Salmonella enterica serovar Typhimurium on Carrots and Radishes grown in Fields Treated with Contaminated Manure Compost or Irrigation water*, Applied and Environmental Microbiology, 70 (4): 2497-2502.
- Kul'ko, A. B., Marfenina, O. E. (1998): *Characterization of the species composition of microscopic fungi in the urban snow cover*, Microbiology (translated from Mikrobiologiya), 67 (4): 569-572.
- Kurup, P. V., Schmitt, J. A. (1970): *Human-pathogenic fungi in the soils of Central Ohio*, The Ohio Journal of Science, 70 (5): 291-295.
- Litvin, V. Ju. (1999): *Prirodnootčagovje infekcii: ključevje voprosi i novje pozicii*, Žurnal mikrobiologij, 5: 26-33.
- Marfenina, O. E. (1996): *Is there an Increase of Health Risks due to the Impact of Environmental pollution on Outdoor Microfungal growth?* Zbt. Bakt., 285, p. 5-10.
- Marfenina, O. E. (2000): *Mycological properties of urban soils*, First International Conference on Soils of Urban, Industrial, Traffic and Maining Areas. Preceedings, University of Essen, V, III, 677-681.
- McGinnis, M. R. (2004): *Pathogenesis in indoor fungal diseases*, Medical Mycology, 42 (2): 107-117.
- Mirčnik, T. G. (1988): *Počvennaja mikologija*, Moskva: MGU.
- Schata, M., Jorde, W., Elixman, J. H., Linskens, H. F. (1989): *Allergies to molds caused by fungal spores in air conditioning equipment*, Environ. Int. 15: 177-179.
- Vanbreuseghen, R. (1952): *Technique biologique pour l'isolement des dermatophytes du sol*, Ann. Soc. Belge Med. Trop., 32: 173-178.
- Zdanova, N. N., Vasil'evskaya, A. I., Artyshkova, L. V. (1994): *Changes in micromycete communities in soil in response to pollution by long-lived radionuclides emitted in the Chernobyl accident*, Mycol. Res., 98: 789-795.
- Zvagincev, D. G. (1999): *Strukturno-funkcionaljnaja rolj počv v biosfere*, Moskva: GEOS, 101-112.

## ПОТЕНЦИЈАЛНО ПАТОГЕНЕ, ПАТОГЕНЕ И АЛЕРГЕНЕ ПЛЕСНИ УРБАНИХ ЗЕМЉИШТА

Драгутин А. Ђукић<sup>1</sup>, Лека Г. Мандић<sup>1</sup>, Лидија Божарић<sup>2</sup>, Бојана Д. Трифуновић<sup>3</sup>,  
Маријана И. Пешаковић<sup>4</sup>

<sup>1</sup> Агрономски факултет, Цара Душана 34, 32000 Чачак, Србија

<sup>2</sup> Специјалистичка ветеринарска лабораторија Подгорица, Булевар Џорџа  
Вашингтона бб, Црна Гора

<sup>3</sup> Градска управа за урбанизам града Чачка, Жупана Страцимира 2, 32000 Чачак,  
Србија

<sup>4</sup> Институт за воћарство, Краља Петра I 9, 32000 Чачак, Србија

### Резиме

Ради проучавања динамике бројности земљишних плесни, које могу компромитовати имунолошки систем човека, одабране су огледне парцеле на различитој удаљености (100, 300, 500, 700, 900 m) од основног извора загађења – КАП (Комбинат алуминијума Подгорица). Узорци земљишта су узимани у јулу и октобру 2008. године са три различита места огледних парцела на дубини 0–10 cm. Одређивана је бројност потенцијално патогених, кератинолизних и алергених (меланиногених) плесни, што може бити значајан допринос дијагностици и профилакси.

Бројност медицински значајних плесни знатно је већа у урбаном, него у незагађеном (контролном) земљишту. Њихова бројност се готово закономерно смањивала са удаљеношћу од основног извора загађења (КАП), док је њихово учешће у земљишту било знатно више у јесен него у пролеће.



## INSTRUCTION TO AUTHORS

([www.maticasrpska.org.rs](http://www.maticasrpska.org.rs))

This version of Instruction to Authors is valid starting from the year 2012 and the journal number 122

### 1. General remarks

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

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

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

### 2. Planning and preparing of the manuscript

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

2.2. First name, middle initial and last name should be given for all authors of the manuscript and their institutional affiliations, institution name, and mailing address. In complex organizations, a full hierarchy should be mentioned (e.g. University of Novi Sad, Faculty of Sciences – Department of Biology and Ecology). The institution of employment of each author should be

stated below the author's name. The position and academic degrees should not be cited. If there is more than one author, indicate separately institutional affiliation for each of the authors. Put the name and mailing address (postal or e-mail address) of the author responsible for correspondence at the bottom of the first page. If there is more than one author, write the address of only one author, usually the first one.

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

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

2.5. The authors should submit the title of the article with last name and the initials of the first author.

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

2.6. List up to 10 key words using words and phrases that describe the content of the article in the best way and that allow indexing and electronic searching of the paper. List the key words alphabetically and divided by commas.

2.7. The Abstract in English language and Summary in Serbian language should be a short and informative presentation of the article. Depending on the length of the article, the Abstract may have from 100 to 250 words. Summary written in Serbian language can be 1/10 length of the article and should contain the title of the article, first, middle initial, and last names of the authors, authors' institutional affiliation and address, and key words.

2.8. Write the information about financial support, advices, and other forms of assistance, if necessary, at the end of the article under the Acknowledgement. Financial support acknowledgement should contain the name and the number of the project, i.e. the name of the program from which the article originated, and the name of the institution that provided the financial support. In case of other forms of assistance the author should submit the first name, middle initial, last name, institutional affiliation, and the address of the person providing the assistance or the full name and the address of the assisting institution.

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

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



## 5. References

### 5.1. List the References alphabetically. Examples:

- (a) Articles from journals: Last name CD, Last name CD (2009): Title of the article. Title of the journal (abbreviated form) 135: 122-129.
- (b) Chapters in the book: Last name ED, Last name AS, Last name IP (2011): Title of the pertinent part from the book. In: Last name CA, last name IF (eds.), Title of the book, Vol.4, Publisher, City
- (c) Books: Last name VG, Last name CS (2009): Title of the cited book. Publisher, City
- (d) Dissertations: Last name VA (2009): Title of the thesis. Doctoral dissertation, University, City
- (e) Unpublished articles: designation "in press" should be used only for papers accepted for publishing. Unpublished articles should be cited in the same way as published articles except that instead of journal volume and page numbers should write "in press" information.
- (f) Articles reported at scientific meetings and published *in extenso* or in a summary form: Last name FR (2011): Proceedings, Name of the meeting, Meeting organizers, Venue, Country, 24-29
- (g) World Wide Web Sites and other electronic sources: Author's last name, Author's initial. (Date of publication or revision). Title, In: *source in Italics*, Date of access, Available from: <Available URL>. Use n.d. (no date) where no publication date is available. Where no author is available, transfer the organization behind the website or the title to the author space.

5.2. References in the text should include author's last name and the year of publishing. When there are two authors both should be cited, but in case of three or more authors, cite the first author only and follow with et al.

5.3. If two or more articles of the same author or authors published in the same year are cited, designate the publishing years with letters a, b, c, etc., both in text and reference list.

5.4. The names of the periodicals should be abbreviated according the instructions in the *Bibliographic Guide for Authors and Editors* (BIOSIS, Chemical Abstracts Service, and Engineering Index, Inc.).

5.5. Do not translate references to the language of the article. Write the names of cited national periodicals in their original, shortened form. For example, for the reference in Serbian language, put (Sr) at the end of the reference.

## 6. Units, names, abbreviations, and formulas

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

6.2. Write the names of living organisms using *Italics* font style.

6.3. Abbreviated form of a term should be put into parenthesis after the full name of the term first time it appears in the text.

6.4. Chemical formulas and complex equations should be drawn and prepared for photographic reproduction.

## 7. Figures

7.1. Authors may use black-and-white photographs and good quality drawings.

7.2. A caption with the explanation should be put below each figure.

## 8. Tables

8.1. Type tables on separate sheet of papers and enclosed them at the end of the manuscript.

8.2. Number the tables using Arabic numerals.

8.3. Above each table, write a capture with table explanation.

8.4. On the left margin, indicate the place of the tables in the text.

## 9. Electronic copy of the article

9.1. After the acceptance of the article, send a CD with final version of the manuscript and a printed copy to facilitate technical processing of the text. Articles should be written in Microsoft Word format and sent to the Editorial office of the *Matica Srpska Proceedings for Natural Sciences, Matica Srpska*, Matica Srpska Street, 21000 Novi Sad (Uredništvo Zbornika Matice srpske za prirodne nauke, Matica srpska ul., 21000 Novi Sad).

9.2. Before printing, the manuscripts shall be sent to the authors for the approval of final version. Corrections of the text prepared for printing should be restricted to misspelling and printing errors as much as possible. For major changes of the text, a fee will be charged. Corrected manuscript should be returned to the Editorial office as soon as possible.

9.3. Authors are entitled to ten reprints.

## УПУТСТВО АУТОРИМА ([www.maticasrpska.org.rs](http://www.maticasrpska.org.rs))

Ово упутство важи од 2012. године од броја часописа 122

### 1. Опште напомене

1.1 Зборник Матице српске за природне науке / Matica Srpska Proceedings for Natural Sciences (скраћени наслов: Proc. Nat. Sci. Matica Srpska) објављује оригиналне научне радове и прегледне чланке као и кратка саопштења из свих области које обухвата назив часописа. Прегледни радови се објављују само на позив редакције. Радови који су већ објављени у целости или у деловима или су понуђени другом часопису не могу бити прихваћени. Часопис објављује два броја годишње.

1.2. Прихватају се рукописи писани на енглеском језику. Језик мора бити исправан у погледу граматике и стила. Рукопис се доставља електронском поштом као посебан докуменат на адресу: [zmospn@maticasrpska.org.rs](mailto:zmospn@maticasrpska.org.rs), уз обавезну потписану изјаву аутора у вези са пријавом рада за штампу.

1.3. По примању рукописа, аутор ће добити шифру свог рада, коју треба увек наводити у даљој преписци. Уредништво ће обавестити аутора о приспећу рукописа у року од седам дана, а о мишљењу рецензената у року од два месеца од пријема. Сваки рад се рецензира и лекторише.

### 2. Припрема рукописа

2.1. Текст рада пише се електронски на страни А4 (21x29,5 cm), с маргинама од 2,5 cm, увлачењем првог реда новог пасуса, и размаком међу редовима 1,5. Текст треба писати у фонту *Times New Roman* словима величине 12 а сажетак, кључне речи, резиме и подножне напомене словима величине 10 pt.

2.2. Наводе се име, средње слово и презиме свих аутора рада као и назив установе (без скраћеница) у којој су аутори запослени, заједно са пуном поштанском адресом. У сложеним организацијама наводи се укупна хијерархија (на пример: Универзитет у Новом Саду, Природно-математички факултет – Департман за биологију и екологију). Место запослења наводи се непосредно испод имена аутора. Функције и звања

аутора се не наводе. Ако је аутора више, мора се, посебним ознакама, назначити из које од наведених установа потиче сваки од наведених аутора. Контакт адреса аутора (поштанска или електронска) даје се у напомени при дну прве странице чланка. Ако је аутора више, даје се само адреса једног, обично првог аутора.

2.3. Рукопис оригиналног научног рада треба поделити на: Сажетак, Кључне речи, Увод, Материјал или Метод или Материјал и метод, Резултати или Резултати и дискусија, Дискусија, Закључак, Литература, Сажетак и Кључне речи на српском језику и Захвалност (уколико за то постоји потреба). Оригинални научни радови не смеју бити дужи од 10 страна, укључујући литературу, табеле, легенде и слике.

2.4. Наслов рада треба да буде информативан, али не дужи од десет речи. У интересу је часописа и аутора да се користе речи прикладне за индексирање и претраживање.

2.5. Аутори треба да доставе и текући наслов који треба да садржи презиме и иницијале првог аутора (ако је аутора више, преостали се означавају са “et al.”) и наслов рада у скраћеном облику, не више од пет речи.

2.6. За кључне речи треба користити термине или фразе које најбоље описују садржај чланка за потребе индексирања и претраживања. Број кључних речи не може бити већи од 10. Треба их навести абecedним редом и одвојити зарезима.

2.7. Апстракт, на енглеском и резиме на српском, треба да представља кратак информативни приказ чланка. Апстракт у зависности од дужине чланка треба да има од 100 до 250 речи. Резиме на српском језику може бити до 1/10 дужине чланка и треба да садржи наслов рада, имена аутора, средње слово и презимена, назив и место у којима су аутори за послени и кључне речи.

2.8. Податке о финансијској помоћи, саветима и другим врстама помоћи, уколико за то постоји потреба, треба навести на крају рада, под насловом Захвалност. У захвалници за финансијску помоћ треба навести назив и број пројекта, односно назив програма у оквиру којег је чланак настао, као и назив институције која је финансирала пројекат или програм. У случају других видова помоћи треба навести име, средње слово и презиме, установу и седиште лица које је пружало помоћ, а ако је помоћ пружала установа пун назив и адресу.

3. Прегледни рад треба да садржи: Апстракт, Кључне речи, Закључак, Литературу, као и Резиме и Кључне речи на српском. Прегледни радови не смеју бити дужи од 12 страна, укључујући литературу, табеле, легенде и слике.

4. Кратко саопштење се пише по упутствима за оригиналан научни рад, али не сме да буде дуже од 5 страна.

## 5. Литература

5.1. Литературне наводе треба сложити абecedним редом на следећи начин:

- а. Чланци из часописа: Презиме CD, Презиме SP (2009): Назив рада. Име часописа (скраћени облик) 135: 122-129.
- б. Поглавља у књизи: Презиме ED, Презиме AS, Презиме, IP (2011): Наслов цитираног дела у књизи. In: Презиме CA, Презиме IF (eds.), Назив књиге, Вол. 4, Издавач, Град, 224-256.
- в. Књиге: Презиме VG, Презиме CS (2009): Наслов цитиране књиге. Издавач, Град.
- г. Дисертације: Презиме VA (2009): Назив тезе. Докторска дисертација, Универзитет, Град.
- д. Необјављени радови: Наводи „у штампи” треба да се односи само на радове прихваћене за штампу. Необјављени радови: цитирати као да се ради о објављеном раду осим што се уместо волумена часописа и броја страна наводи „у штампи”.
- ђ. Радови саопштени на научним скуповима штампани у целине или у изводу: Презиме FR. (2011): Зборник, Назив скупа, Организатор скупа, Место одржавања, Држава, 24-29.
- е. Електронски извори:

### World Wide Web Sites and Other Electronic Sources

Author last name, Author initial. (Date of publication or revision). Title, In: *source in Italics*, Date of access, Available from: <Available URL>

Use n.d. (no date) where no publication date is available.

Where no author is available, transfer the organisation behind the website, or the title, to the author space.

5.2. Референце у тексту треба да укључе презиме аутора и годину издања. Ако има два аутора, треба навести обојицу, а у случају три или више аутора треба навести првог аутора и назначити “et al.”.

5.3. Ако се наводе два или више радова истог или истих аутора, објављених у истој години, потребно је у тексту и списку литературе ставити а, б, ц, итд. иза године објављивања.

5.4. Имена часописа треба скраћивати према “Bibliographic Guide for Authors and Editors” (BIOSIS, Chemical Abstracts Service and Engineering Index, Inc., ).

5.5. Референце се не превode на језик рада. Наслови цитираних домаћих часописа дају се у оригиналном, скраћеном облику. Ако је референца нпр. на српском језику на крају се стави (Sr).

## 6. Јединице, имена, скраћенице и формуле

6.1. Треба користити SI ознаке за јединице (SI Systeme International d’Un.), изузетно се могу користити и друге званично прихваћене јединице.

6.2. Називе живих организама на латинском треба писати италиком.

6.3. При коришћењу скраћеница у тексту, пун термин треба навести приликом првог спомињања, а скраћеницу додати у загради.

6.4. Хемијске структурне формуле и сложене једначине треба нацртати и припремити за фотографску репродукцију.

## 7. Илустрације

7.1. За илустрације могу се користити црно беле фотографије и цртежи доброг квалитета.

7.2. Свака илустрација треба да има текст (легенду) који објашњава садржај прилога. (испод слике).

## 8. Табеле

8.1. Табеле треба куцати на одвојеним страницама и приложити их на крају рада.

8.2. Табеле се означавају арапским бројевима.

8.3. Свака табела треба да почне насловом који објашњава њен садржај (изнад табеле).

8.4. Места табела у тексту треба означити на левој маргини.

## 9. Копија рада у електронској форми

9.1. После прихватања рада потребно је доставити CD са коначном верзијом рада. Приложити и једну копију одштампаног рада ради лакше техничке обраде. Рукопис треба слати на адресу: Уредништво Зборника Матице српске за природне науке, Матица српска, Ул. Матице српске, 21000 Нови Сад. Рукописи се шаљу у Word формату.

9.2. Пре уласка рада у штампу ауторима се доставља рукопис за коначну ревизију. Исправљање текста припремљеног за штампу треба ограничити на штампарске грешке. Значајне промене текста ће се наплаћивати. Кориговани текст треба вратити Уредништву у најкраћем могућем року.

9.3. Аутори добијају 10 бесплатних примерака сепарата.

*Зборник Матице српске за природне науке* издаје Матица српска  
Излази двапут годишње  
Уредништво и администрација: Нови Сад, Улица Матице српске 1  
Телефон: 021/420-199  
e-mail: [zmspn@maticasrpska.org.rs](mailto:zmspn@maticasrpska.org.rs)  
[www.maticasrpska.org.rs](http://www.maticasrpska.org.rs)  
*Proceedings for Natural Sciences* published by Matica Srpska  
Published twice a year  
Editorial and publishing office: Novi Sad, Ul. Matice Srpske 1  
21000 Novi Sad, Serbia  
Phone: +381-21/420-199

---

The editors of the Matica Srpska *Proceedings for Natural Sciences*  
Completed the selection for Issue 121/2011 on Juli 15, 2011  
Editorial Staff Secretary: Julkica Boarov  
Managing editor: Dr. Slavka Gajin  
English text proof-reader: Stanka Matejašev and Vera Vasilić  
Technical design: Vukica Tucakov  
Published in December 2011  
Computer set: Vladimir Vatić, GRAFIT, Novi Sad  
Printed by: BUDUĆNOST

---

Публиковање овог броја помогли су  
Министарство за просвету и науку Републике Србије и  
Покрајински секретаријат за науку и технолошки развој АП Војводине  
Publication of this volume was supported by the Ministry of Education and Science  
of Republic of Serbia and The Provincial Secretariat of Science and Tehnological  
Development of The Autonomous Province of Vojvodina

CIP – Каталогизација у публикацији  
Библиотека Матице српске, Нови Сад  
5/6(082)

**ЗБОРНИК Матице српске за природне науке** = Matica  
Srpska proceedings for natural sciences / главни и одговорни  
уредник Рудолф Кастори. – 1984, св. 66– . – Нови Сад : Ма-  
тица српска, Одељење за природне науке, 1984– . – 24 cm  
Два пута годишње. – Наставак публикације: Зборник за  
природне науке. – Текст на енг. језику, резимеи на енг. и на  
срп. језику.

ISSN 0352-4906

COBISS.SR-ID 5845250