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XEROPHILIC MOULDS ISOLATED FROM SPICES USED IN MEAT INDUSTRY AS POTENTIAL PRODUCERS OF MYCOTOXINS

ABSTRACT: Spices are often considered as one of the possible sources of meat products contamination with toxigenic moulds. Genera *Aspergillus*, *Eurotium* and *Penicillium* are most frequent xerophilic storage moulds that contaminate spices. Because spices are possible source of contamination of the final product and potential producers of mycotoxins, it is necessary to estimate the degree of moulds contamination and their ability to produce secondary metabolites – mycotoxins.

Mycological analysis was carried out on five samples of oregano and clove respectively. Presence of moulds was determined by parallel usage of Sabouraud maltose agar (SMA) and the medium that stimulates the growth of xerophilic species: malt – yeast extract agar with 50% of glucose (MY50G).

Isolated moulds were classified into five genera (*Aspergillus*, *Alternaria*, *Cladosporium*, *Rhizopus* and *Penicillium*) and 9 species.

Mycotoxins determination was carried out using ELISA test (commercial kits Tecna, Italy) for the presence of aflatoxin B1, ochratoxin, A and zearalenone.

The results showed the presence of aflatoxin B1, ochratoxin A, and zearalenone in almost all samples, except one sample of oregano and one clove sample.

We can conclude that it is necessary to introduce mandatory mycotoxins determination (aflatoxin B1, ochratoxin A), in raw material for meat industry, especially spices. These secondary metabolites are known as extremely toxic and are classified in group I of human carcinogens.

KEY WORDS: ELISA, moulds, mycotoxins, selective medium, spices

INTRODUCTION

Spices and herbs are valued for their distinctive flavors, colors, and aromas and are among the most versatile and widely used ingredients in food preparation

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and processing all over the world. As is the case with many other agricultural products, spices and herbs may be exposed to a wide range of microbial contamination during pre – and post – harvest (H a s h e m and A l a m r i, 2010). Such contamination may occur during processing, storage, distribution, sale and/or use (M c K e e, 1995).

Modern meat industry cannot be imagined without utilization of spices. However, spices, together with all other dried material of herbal origin, are never sterile. In most cases, they contain sporogenic bacteria and moulds. These microorganisms can cause spoilage of the product by their metabolic activity, consequently resulting in significant economic losses.

Presence of moulds in spices and later in sausages or other meat products can result in production of toxic metabolites – mycotoxins, independently of contamination degree (K o c i ć - T a n a c k o v et al., 2007). In addition, changes in odor and other undesirable sensory changes can also occur in contaminated products. Mycotoxins are fungal secondary metabolites identified in many agricultural products screened for toxicogenic moulds (C l e v s r t o n and L j u n g g r e n, 1985; CAST, 2003). Mycotoxins have been reported to be carcinogenic, teratogenic, tremorogenic, hemorrhagic, and dermatitic to a wide range of organisms, and known to cause hepatic carcinoma in man in humans and animals (F r i s v a d et al., 2005; Z i n e d i n e et al., 2006).

It has been reported that principal contaminants of spices are xerophilic moulds from the genera *Eurotium*, *Aspergillus,* and *Penicillium* (D i m i ć and Š k r i n j a r, 1995; D i m i ć et al., 2000; R o m a g n o l i et al., 2007).

Production of toxins primarily depends on genetic factors; however, environmental conditions at the site of moulds growth (temperature, water activity, matrix composition, moisture content, pH of the medium, contamination and physical destruction of the substrate, antifungal properties and other factors) are considered highly significant.

The authors Š k r i n j a r and B o l d o c k y (1994) determined the presence of 8 moulds species isolated from mixtures of spices intended for meat industry; *Aspergillus* species were dominant, while garlic had the highest degree of molds contamination, but only with two species (*Eutotium herbariorum* and *Penicillium granulatum*).

D i m i ć et al. (2000) established that about 46% of spices mixtures, 29% of black pepper and 25% of paprika was contaminated by moulds. According to this author, main sources of contamination were *Eutotium herbariorum*, *Aspergillus versicolor*, *A. sydowii* and *A. flavus*, *Penicillium auratiogriseum* and *P. chrysogenum*.

The same authors report that among 45 identified species responsible for fungal contamination, 55% was potentially toxigenic. Two samples of spices mixture contained high concentrations of ochratoxin A (32.00μg/kg in mixture of spices for frankfurters production), as well as zearalenone in three samples of black pepper (192.00μg/kg – 288.00μg/kg).

Considering that spices are possible source of contamination of final product, the aim of this paper was to determine contamination degree of final product with xerophilic moulds by using various selective cultivating media,

resulting in recommendation of most suitable medium, and to determine ability of isolated moulds to produce mycotoxins using semiquantitative immunoassays.

2. MATERIALS AND METHODS

2.1. Sampling

Samples of spices used in meat industry (oregano and clove) were investigated. Presence and enumeration of moulds, their determination and investigation of ability to produce mycotoxins were carried out.

2.2. Mycological procedures

2.2.1. Mould isolation

Enumeration of moulds in samples of spices (cfu/g) was carried out using dilution technique by Koch (H a r r i g a n, 1998). The enumeration of moulds was performed using 16-cm Petri dishes on both Sabouraud malt agar (SMA, Merck) with the addition of antibiotic (1ml of chloramphenicol, Sigma/100ml of medium) and the medium that stimulates the growth of xerophilic species – malt yeast extract agar with 50% of glucose (MY50G). SMA composition is as follows: peptone 10 g, D(+)glucose 40 g, agar-agar 15 g; distilled water ad 1000 mL. pH after sterilization should be 5.6±0.2.

The composition of the other selective medium for detection of xerophilic moulds species, MY50G, is: malt extract, Difco 0186, 10 g; yeast extract, Difco 0127, 2.5 g; glucose, 50 g; distilled water, 500 ml; agar 10 g. pH value after sterilization should be 5.7. The inoculated agar media were incubated for 7 days in dark at 25±1 °C and inspected for genus identification using macro and microscopic morphological characteristics.

2.2.2. Mould identification

Moulds determination included recultivation of grown colonies on media used for determination (Czapek and malt agar). Macroscopic and microscopic morphological characters were used in the identification process. Colony color, texture and diameter, the production of diffusible pigments, and exudates were among macroscopic features, while conidia and conidiophore arrangements were the microscopic.

All the isolates were identified according to S a m s o n and v a n R e e n e n - H o e k s t r a (1988), S a m s o n and P i t t (2000), S a m s o n et al. (2004) and P i t t and H o c k i n g (1997).

2.2.3. Mycotoxicological investigations

Presence of aflatoxin B1, ochratoxin A, and zearalenone was determined simultaneously with enumeration and identification of moulds from oregano and clove samples. Mycotoxins were determined using semiquantitative test – ELISA. Commercial kits Tecna, Italy were used. Sample preparation was carried out according to internal protocol for each mycotoxin. The results were calculated based on calibration curve obtained during measurements. The results were expressed as $\mu g/kg^{-1}$.

3. RESULTS AND DISCUSSION

Table 1 shows the results of mycological analyses – enumeration of molds in samples of oregano and clove using different selective media, SMA, and MY50G. These media have different composition of added sugars and water activity (aw). SMA contains maltose and aw is in the range 0.98-0.99, while MY50G contains glucose and aw is 0.89.

The media that are traditionally used in isolation and enumeration of moulds (SMA, potato dextrose agar, Czapek agar, and other) have high water activity (about 0.99). Numerous xerophilic moulds, which are carriers of spices contamination, have optional aw below 0.90. Collaborative investigations with SMA and MY50G provide more accurate perspective on micropopulation and contamination of examined samples.

Tab. 1. – Total viable count (TVC) per 1g of oregano and clove

Sample	Oregano		Clove	
	SMA (TVC)	**MY 50 G** (TVC)	**SMA** (TVC)	**MY 50 G** (TVC)
1	40	1.6×10^2	2.4×10^3	7×10^3
2	15	1.2×10^3	3.2×10^2	0
3	10	4.5×10^2	2.5×10^2	0
4	40	1.0×10^2	2.0×10^2	0
5	15	1.0×10^3	2.3×10^2	0

All investigated samples showed presence of moulds on both SMA and MY50G media (Table 1). However, significant differences were observed in enumeration of moulds grown on SMA and MY50G. Number of colonies was significantly higher on MY50G (from 1.0×10^2 to1.2×10^3 cfu/g), compared to SMA (from 10 to 40cfu/g).

The results of clove analysis showed somewhat different aspect (Table 1). The growth was determined in all samples cultivated on SMA, while no growth was observed in four samples cultivated on MY50G. Total count of moulds was between 2.0×10^2 (sample 4) and 2.4×10^3 (sample 1), while only one sample (number 1) showed moulds contamination using MY50G (7×10^3). Therefore, in this series of investigations, SMA was more appropriate for qualitative and quantitative determination of moulds.

These results pointed to the fact that media with limited amount of free water suppressed the growth of moulds that are not extremely xerophilic in nature. It is also known that xerophilic moulds can be divided into fast-growing and slow-growing forms (P i t t and H o c k i n g, 1985). Slow-growing moulds, even under optimal conditions can be overgrown by fast-growing xerophiles.

After monocultivation of different colonies formed on SAM and MY50G media and examination of their morphological and other properties, seven different species were identified. The majority belonged to genus *Aspergillus,*

in oregano samples (57.14%) and clove samples (28.57%), respectively. Other identified species in oregano samples were *Alternaria alternata* (14.28%), *Rhizopus stolonifer* (14.28%), and *Penicillium* sp. (14.28%).

Even percentage share (28.57%) was recorded in moulds from genus *Aspergillus* and *Penicillium* in clove samples. Other identified moulds were from genera *Alternaria (A. alternata),* 14.28%, *Rhizopus* (*R. stolonifer*)*,* 14.28%, *Penicillium* (*P. aurantiogriseum, Penicillium* sp.), 28.57% and *Cladosporium* (*Cladosporium* sp.) with the share of 14.28%.

Tables 2 and 3 show the percentage of moulds species in oregano and clove samples.

Tab. 2. – Presence of moulds species in oregano samples

Genus	Species	Share %%
Aspergillus	*A. flavus*	57.14
	A. niger	
	A. rubrum	
	A. candidus	
Alternaria	*A. alternata*	14.28
Rhizopus	*R. stolonifer*	14.28
Penicillium	*P.* sp.	14.28

Tab. 3. – Presence of moulds species in clove samples

Genus	Species	Share, %
Aspergillus	*A. flavus*	28.57
	A. rubrum	
Cladosporium	*Cladosporium* sp.	14.28
Penicillium	*P. aurantiogriseum*	28.57
	Penicillium sp.	
Alternaria	*A. alternata*	14.28
Rhizopus	*R. stolonifer*	14.28

The emergence of *Aspergilli*, *Penicillia*, and *Rhizopus* species on the three different media greatly indicated the presence of these moulds as the dominant mycoflora of different spices. This observation was greatly in agreement with other authors who studied mycoflora of spices and medicinal herbs (El-Kady et al., 1995; Dimić et al., 2008). Early Takatori et al. (1977) and Ayres et al. (1980) found the *Aspergillus* and *Penicillium* spp. the main mycopopulation of cardamom, cinnamon, fennel, coriander, cumin, black cumin and white pepper all of which are common in the food industry. The contamination with fungal species resulted from neutral extraneous contamination by dust following storage in humid conditions (Domsch et al., 1981). Moulds fall into two ecological categories, e.g., field and storage moulds. Field moulds were observed to invade development or mature seeds while it is on the plant, the major field moulds genera being *Alternaria*, *Fusarium* and

Cladosporium. On the other hand, storage moulds are those encountered on plants at moisture conditions routinely found in stored products. These moulds principally belong to species *Aspergillus* and *Penicillium* (A b o u D o n i a, 2008). The spices can undergo fungal contamination mainly during spice processing, storage and transport (D i m i ć et al., 2008).

In clove samples (Table 4), besides aflatoxicogenic moulds from genus *Aspergillus*, *Penicillium aurantiogriseum* has been determined as dominant species. This microorganism belongs to ochratoxin A producers (Š k r i n j a r and H o r v a t - S k e n d e r o v i ć, 1992).

Original medium, MY50G has shown better efficiency in determination of *Aspergillus* species, based on results of micropopulation quantitation in oregano samples. The frequency of *Aspergillus* species in overall population was 100% on MY50G, while frequency was only 51% on SMA (Figure 1). The highest frequency of *Penicillium* genera was determined on SMA medium (14.5%). On MY50G medium, this value was 3.3% (Figure 2).

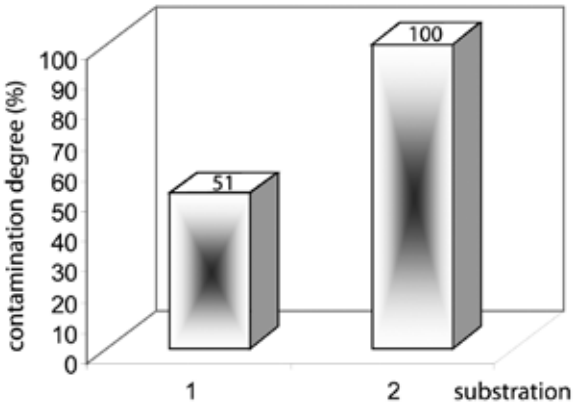

Fig. 1. – Frequency of *Aspergillus* spp. on SMA (1) and MY50G (2)

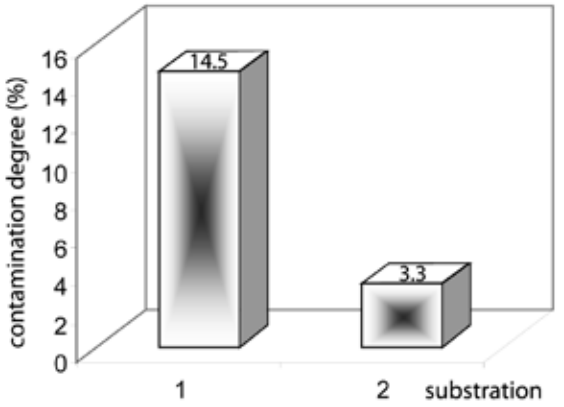

Fig. 2. – Frequency of *Penicillium* spp. on SMA (1) and MY50G (2)

The variation in frequency of mycopopulation of oregano and clove cultivated on SMA and MG50Y media is most probably related to the strain type within one species. Environmental factors also have significant effect and can induce the growth of mycopopulation on lower aw values (optimal temperature and type of nutritive components in the medium). Xerophiles, especially selective ones tend to be very sensitive on environmental conditions.

A s k u n et al. (2007) used Rose-Bengal Chloramphenicol Agar (Oxoid, CM 549) and Dichloran-Glycerol (DG18) Agar (Oxoid, CM 729) for determination of xerophilic moulds. Other media can also be used such as Dichloran-Glycerol (DG18) Agar Base (P i t t and H o c k i n g, 1985), MY70FG and MY50FG (B e u c h a t and H o c k i n g, 1990), MY50S and MY40S (B e u c h a t, 1998).

Presence of mycotoxins was determined in examined samples (5 oregano and 5 clove samples) in 80% of cases (Table 4).

Tab. 4. – The results of mycotoxins determination in samples of oregano and clove

Oregano			Clove		
Aflatoxin B_1 (μg/kg)	**Ochratoxin A** (μg/kg)	**Zearalenone** (μg/kg)	**Aflatoxin B_1** (μg/kg)	**Ochratoxin A** (μg/kg)	**Zearalenone** (μg/kg)
7.5	22.4	10	Not detected	3.2	8
17	10	4.5	31.5	26.9	11.5
10	12	7.6	6.8	11.2	7.4
Not detected	4	11.2	4	7	5.9
5.3	5.7	3.2	12	5	4.9

The origin of mycotoxins in spices samples can be the result of their previous synthesis during storage of these products. However, there is the possibility of their production by moulds that contaminated the spices during storage. This ability is characteristic for moulds belonging to *Penicillium* genus since they can reproduce at lower temperatures (around +5°C), and in some cases even produce toxins in these environmental conditions (Š k r i n j a r et al., 1998).

The risk of contamination by mycotoxins is an important food safety concern for grains and other field crops. Mycotoxins affecting groundnuts/peanuts, cereals (maize, rice, sorghum, wheat, barley and oats), spices (black pepper, ginger and nutmeg) and chili are considered to be of greater significance for human beings (B h a t and V a s a n t h i, 2003; CAST, 2003; B r y d e n, 2007).

Over the last two decades various international evaluation on maximum residue limits and regulations for mycotoxins were published. A study by the United Nations' Food and Agriculture Organization (FAO) on worldwide regulations for mycotoxins revealed that at least 77 countries now have specific regulations for mycotoxins (FAO, 2004). In the Republic of Serbia, maximum residue limits for mycotoxins in spices are set to 30 μg/kg for total aflatoxins and 10 μg/kg for ochratoxin A.

CONCLUSION

Based on obtained results, it can be concluded that it is necessary to use selective media adjusted to specific requirements of xerophiles in order to achieve proper isolation and accurate contamination degree of spices by xerophilic moulds. Utilization of selective media enables acquiring representative insight in spices mycopopulation.

Spices are potential source of mycotoxins, hence the necessity of regular mycotoxicological analysis of these products with the aim of consumers' protection, prevention of food spoilage and consequent significant economic losses. For this reason, such analyses should be mandatory in evaluation of food safety parameters.

The ELISA test was used as an initial screening procedure in order to determine the presence of ochratoxin A and other mycotoxins.

REFERENCES

A b o u D o n i a, M. A. (2008): Microbiological quality and aflatoxinogenesis of Egyptian spices and medicinal plants. Global Vet. 2 (4): 175–181.

A s k u n, T., E l t e m, R., E l t e m, E. (2007): Comparison of Rose-Bengal Chloramphenicol Agar and Dichloran Glycerol Agar (DG18) for Enumeration and Isolation of Moulds from Raisins. Journal of Applied Biological Sciences 1 (2): 71–75.

A y r e s, G. I., M u n d, T. I., S o n d i n, E. W. (1980): Microbiology of Food Spices and Condiments. A Series of Books in Food and Nutrition. Schmeigert, 249.

B e u c h a t, L. R. (1998): Progress in conventional methods for detection and enumeration of foodborne yeasts. Food Tech.Biotech., 36: 267–272.

B e u c h a t, L. R. and H o c k i n g, A. D. (1990): Some considerations when analysing foods for the presence of xerophilic fungi. J. Food Protect., 53: 984–989.

B h a t, R. V., V a s a n t h i, S. (2003): Food Safety in Food Security and Food Trade: Mycotoxin Food Safety Risk in Developing Countries. Vision 2020 for Food, Agriculture, and the Environment, Focus 10 /http://www.ifpri.org/2020/focus/focus10/focus10_03.pdfS.

B r y d e n, W. L. (2007): Mycotoxins in the food chain: human health implications. Asia Pac. J. Clin. Nutr. 16: 95–101.

CAST (Council for Agricultural Science and Technology), (2003): Mycotoxins: Risks in Plant, Animal, and Human Systems. Ames, IA, Task Force Report 139.

C l e v s r t o n, G., L j u n g g r e n, H. (1985): Aflatoxin formation and the dual phenomenen. Mycopathologia 92:129–139.

D i m i ć, G., Š k r i n j a r, M. (1995): Toksigene plesni i mikotoksini u začinskim smešama i biberu u zrnu korisćenim u industriji mesa. Tehnologija mesa 5: 302–305.

D i m i ć, G., Š k r i n j a r, M., D o š e n - B o g i ć e v i ć, V. (2000): Plesni, potencijalni proizvođač sterigmatocistina u začinima. Tehnologija mesa 41(4–6), 131–137.

D i m i ć, G. R., K o c i ć - T a n a c k o v, S. D., T e p i ć, A. N., V u j i č i ć, B. L., Š u m i ć, Z. M. (2008): Mycopopulation of spices. BIBLID 39, 1–9.

D o m s c h, K. H., G a m s, W., A n d e r s o n, T. H. (1981). In: Compendium of Soil Fungi, vols. 1 and 2. Academic Press, London.

El-Kady, I. A., El-Maraghy, S. M., Mostafa, M. E. (1995): Natural occurrence of mycotoxins in different spices in Egypt. Folia Microbiologica 40 (3): 297–300.

FAO (Food and Agriculture Organization) (2004): Worldwide regulations for mycotoxins in food and feed in 2003. FAO Food and Nutrition Paper no. 81. FAO, Rome, Italy, 1728–3264.

Frisvad, C. J., Skouboe, P., Samson, A. R. (2005): Taxonomic comparison of three different groups of aflatoxin producers and new efficient producer of aflatoxin B1, sterigmatocystine and 3-Omethylsterigmatocystine, *Aspergillus rumbelli* sp. nov.. Syst. Appl. Microbiol. 28: 442–453.

Harrigan, W. (1998): Laboratory Methods in Food Microbiology. Academic Press, San Diego, 359–375.

Hashem, M., Alamri, S. (2010): Contamination of common spices in Saudi Arabia markets with potential mycotoxin-producing fungi. Saudi Journal of Biological Sciences:17, 167–175.

Kocić-Tanackov, S. D., Dimić, G. R., Karali, D. (2007): Contamination of spices with moulds potential producers of sterigmatocystine. APTEFF 38: 29–35.

McKee, L. H. (1995): Microbial contamination of spices and herbs: a review. Lebensm. Wiss. Technol. 28: 1–11.

Pitt, J. I., Hocking, A. D. (1985): Fungi anf food spoilage. CSIRO Division of Food Research, Academic Press, Sidney.

Pitt, J. I., Hocking, A. D. (1997): Fungi and Food Spoilage. Blackie Academic & Professional.

Romagnoli, B., Menna, V., Gruppioni, N., Bergamini, C. (2007): Aflatoxins in spices, aromatic herbs, herbs–teas and medicinal plants marketed in Italy. Food Control 18: 697–701.

Samson, R. A., van Reenen-Hoekstra, E. S. (1988): Introduction to food-borne fungi, Centraalbureau voor Schimmelculturs, Baarn Delft, The Netherlands.

Samson, R. A., Hoekstra, E. S., Frisvad, J. C., Filtenborg, O. (2004): Introduction to Food and Airborne Fungi, 7th ed. Centraalbureau Voor Schimmelcultures, Wageningen, The Netherlands.

Samson, R. A., Pitt, J. I. (2000): Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* Classification. Harwood Academic Publisher, Amsterdam.

Škinjar M., Boldocky, K. (1994): Neka iskustva u ispitivanju mikološkog i mikotoksikološkog kvaliteta usitnjenog mesa za proizvodnju trajne kobasice, Tehnologija mesa 4–5, 166–168.

Škrinjar M., Horvat-Skenderović, T. (1992): Ochratoxin A prodiuction in dry sausage by Penicillium verrucosum var. cyclopium, 38th International Congress of Meat Science and Technology, Clermon, Ferrand, France, August 23–28, Proc. 4, 831–834.

Škrinjar M., Milašinović M., Došen-Bogićević V., Dimić, G., Maričić, D. (1998): Zastupljenost toksigenih plesni, ohratoksina A i aflatoksina u nekim polutrajnim kobasicama pakovanim u kutijama, Tehnologija mesa, 2: 52–55.

Takatori, K., Watanabe, K., Udagawa, S., Kurata, H. (1977): Mycoflora of imported spices and inhibitory effects of the spices on the growth of some fungi. Proc. Jpn. Assoc. Mycotoxicol. 9: 36–38.

Zinedine, A., Brera, C., Elakhdari, S., Catano, C., Debegnach, F., Angelini, S., De Santis, B., Faid, M., Benlemlih, M., Minardi, V., Miraglia, M. (2006): Natural occurrence of mycotoxins in cereals and spices commercialized in Morocco. Food Control 17: 868–874.

КСЕРОФИЛНЕ ПЛЕСНИ ИЗОЛОВАНЕ ИЗ ЗАЧИНА КОЈИ СЕ КОРИСТЕ У ИНДУСТРИЈИ МЕСА КАО ПОТЕНЦИЈАЛНИ ПРОДУЦЕНТИ МИКОТОКСИНА

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Резиме

Као један од могућих извора контаминације производа од меса токсигеним плеснима често се наводе зачини. Врсте које се појављују као контаминенти зачина су ксерофилне, складишне плесни, најчешће из родова *Aspergillus*, *Eurotium* и *Penicillium*. Узимајући у обзир чињеницу да зачини представљају могућ извор контаминације финалног производа, као и да су потенцијални продуценти микотоксина, неопходно је уочити степен контаминације плеснима и њихово потенцијално физиолошко својство да продукују секундарне метаболите – микотоксине.

Миколошким анализама обухваћено је по пет узорака оригана и каранфилића. Присуство плесни испитано је паралеленим коришћењем Sabouraud малтозног агара (SMA) и подлоге која фаворизује раст ксерофилних врста – сладни квашчев екстракт агар са 50% глукозе (MY50G).

Изоловане плесни сврстане су у пет родова (*Aspergillus*, *Alternaria*, *Cladosporium*, *Rhizopus* и *Penicillium*) и 9 врста.

Микотоксиколошка испитивања обухватила су утврђивање присуства афлатоксина Б1, охратоксина А и зеараленона. Испитивања су вршена употребом ELISA теста и комерцијалних китова произвођача Тецна, Италија.

Резултати испитивања су показали присуство афлатоксина Б1, охратоксина А и зеараленона у готово свим узорцима, осим у једном узорку оригана и у једном узорку каранфилића.

Као закључак наведених испитивања намеће се потреба за обавезним микотоксиколошким испитивањима сировина намењених изради производа од меса, првенствено зачина. Нарочито обавезним сматрају се испитивања присуства афлатоксина Б1 и охратоксина А. Наведени секундарни метаболити познати су као веома токсични микотоксини који су уврштени у групу 1 хуманих карциногена.

КЉУЧНЕ РЕЧИ: ELISA, зачини, микотоксини, плесни, селективне подлоге

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MITIGATING ABIOTIC STRESS IN CROP PLANTS BY MICROORGANISMS

ABSTRACT: Microorganisms could play an important role in adaptation strategies and increase of tolerance to abiotic stresses in agricultural plants. Plant-growth-promoting rhizobacteria (PGPR) mitigate most effectively the impact of abiotic stresses (drought, low temperature, salinity, metal toxicity, and high temperatures) on plants through the production of exopolysaccharates and biofilm formation. PGPR mitigate the impact of drought on plants through a process so-called *induced systemic tolerance* (IST), which includes: a) bacterial production of cytokinins, b) production of antioxidants and c) degradation of the ethylene precursor ACC by bacterial ACC deaminase. Symbiotic fungi (arbuscular mycorrhizal fungi) and dual symbiotic systems (endophytic rhizospheric bacteria and symbiotic fungi) also tend to mitigate the abiotic stress in plants.

KEY WORDS: adaptation, microorganisms, plant, soil, stress

INTRODUCTION

Abiotic stresses affect the productivity of agricultural crops as well as the microbial activity in soil. Extreme conditions such as prolonged drought, intense rains flooding, high temperatures, frost and low temperatures, which are expected to intensify in the future due to climate changes, will significantly affect plants and soil microorganisms.

Microorganisms could play an important role in adaptation strategies and increase of tolerance to abiotic stresses in agricultural plants. Plant-growth--promoting rhizobacteria (PGPR) are associated with plant roots and mitigate most effectively the impact of abiotic stresses (drought, low temperature, salinity, metal toxicity, and high temperatures) on plants through the production of exopolysaccharates and biofilm formation. When plants are exposed to stress conditions, rhizospheric microorganisms affect plant cells by different mechanisms like induction of osmoprotectors and heat shock proteins.

During the crop production, microorganisms can be used for (a) monitoring of biological activity in soil (microbial number, enzymatic activity and biodiver-

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sity); (b) as indicators of soil health/quality; (c) for mitigation of negative stress caused in plants by abiotic factors; and (d) as beneficial and effective microorganisms as inoculants (G r o v e r et al., 2010, K a s t o r i et al., 2006, M i l o s e v i c et al., 2008).

ADAPTATION OF MICROORGANISMS AS A RESPONSE TO ABIOTIC STRESSES

A large number of environmental factors affect the microbial communities in soil. Some factors are referred to as *modulators* (B a s l e r et al., 2001), in contrast to the *resources* needed for the growth of microbial communities (e.g., carbon, nitrogen). For example, soil temperature, pH, salinity, and water potential are considered as modulators. Plant and microbial communities change in response to stress conditions and there develop new, tolerant communities, adapted through complex regulatory processes involving many genes (M i l o š e v i ć and M a r i n k o v i ć, 2011).

Soil microbial communities consist of many populations, each with a characteristic response curve to a particular environmental factor, indicating the community's physiological flexibility. Changes in the environment may change the composition and biomass of a microbial community. All microorganisms have a set of optimal environmental conditions, which secure their optimal growth (P e t t e r s o n, 2004).

When exposed to stress (drought, excess moisture, high and low temperatures, metal toxicity), most microorganisms have the ability to survive in the soil in an inactive state, but their activity is restored under favorable conditions. Poor and/or degraded soils are inhabited by a narrow range of microbial genera and species, which is reflected on soil fertility and the growth of plants.

Prolonged exposure to stress and the impact of recurring stress factors (stress on stress) impacts the number of microbes in the soil, but not necessarily their metabolic activity (G r i f f i t h s et al., 2000). Each bacterial species has specific growth dynamics which is highly sensitive to environmental factors and it is a more reliable indicator of stress than metabolic activity (B l o e m and B r e u r, 2003; R a j a p a k s h a et al., 2004). Experiments have shown that respiration may increase or decrease in response to stress (T o b o r - - K a p ł o n et al., 2006), indicating that it is not a reliable stress indicator. In a study of R a j a p a k s h a et al. (2004), addition of 128 mg of Zn/kg of soil reduced microbial respiration by 30% and microbial growth by 90%. Reduced presence of azotobacters and reduced dehydrogenase activity was registered in soils with a nickel content of 23 to 75 mg/kg of soil. However, a high lead content in the soil inhibited the growth of azotobacters but it did not inhibit soil dehydrogenase activity (M i l o š e v i ć et al., 2008).

Microorganisms are capable of surviving high temperatures caused by fire depending on its duration and intensity. Fires develop high temperatures and cause a rapid loss of water (especially in surface soil layers), changing the soil microclimate, and indirectly affecting the soil microbial community. Most

biological reactions are temperature dependent. Exposure to high temperature increases the rates of nutrient decomposition and release. Burning of crop residues in a wheat-soybean rotation did not affect the total number of bacteria and the number of nitrogen-fixing bacteria in soil (H a r r i s et al., 1995). A study of V á z q u e z et al. (1993) showed that, one month after burning of vegetation cover, the bacterial population was 25 times lower and the number of fungi decreased by about 5% compared with a soil that was not subjected to burning. The population of fungi was reduced in the soils periodically subjected to burning over a period of 10 years (K l o p a t e k et al., 1994, cit. F i t e s - K a u f m a n et al., 2006).

Microbial adaptation to stress is a complex regulatory process in which a number of genes are involved (T o b o r - K a p ł o n et al., 2008; G r o v e r et al., 2010). Certain microbial species live in extreme habitats (thermophiles and halophytes) and they use different mechanisms to reduce stress (M a d i g en, 1999; cit. G r o v e r et al., 2010). When subjected to stress conditions, most rhizobacteria produce osmoprotectors (K+, glutamate, trehalose, proline, glycine, and polysaccharates).

MICROORGANISMS: ALLEVIATION OF ABIOTIC STRESSES ON PLANTS

Investigations has shown that certain microbial species and/or strains enhance plant tolerance to abiotic stresses such as drought, salinity, nutrient deficiency or excess (Y a n g et al., 2008), and high contents of heavy metals (R a j a p a k s h a et al., 2004; G r o v e r et al., 2010; M i l o š e v i ć and M a r i n k o v i ć, 2011). Specifically, rhizospheric microorganisms have the greatest impact on the tolerance of agricultural plants to abiotic stresses. When near plant roots, soil microorganisms trigger different mechanisms that affect plant tolerance to stress. They produce indole acetic acid, gibberellins, and other substances that promote the growth of root hairs and increase total root area, which in their turn facilitate nutrients uptake by plants. Plant-growth-promoting rhizobacteria (PGPR), which live in association with plant roots, elicit the largest influence on plants, affecting their productivity and immunity. PGPR inhabit the rhizosphere of many agricultural plants and they take part in increasing plant growth and reducing diseases caused by pathogenic fungi, bacteria, viruses, and nematodes (K l o p p e r et al., 2004). Y a n g et al. (2008) introduced the term ‘induced systemic tolerance’ (IST) that is caused by PGPRs. According to these authors, the mechanism of IST causes physical and chemical changes in plants, which result in plant tolerance to abiotic stresses.

The most important mechanism in many bacteria that directly stimulates plant growth is the production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Under stress conditions, the bacterial enzyme facilitates the growth of plants by decomposing plant ACC (ethylene precursor in plants). S a l e e m et al. (2007) described the role of ACC deaminase-containing

PGPRs in crop production. By reducing the level of ethylene, the plant becomes more resistant to stress conditions in the environment (G l i k, 1999).

AM fungi alleviate the effects drought and salinity stresses, osmoregulation and proline accumulation. *Glomus intraradices* increases the tolerance of *Pterocarpus officinalis* to excessive moisture (G r o v e r et al., 2010). In addition, dual symbiotic systems tend to mitigate the effect of abiotic stress on plants. The endophytic fungus *Cuvularia* sp. has been isolated from *Dichathelium lanuginosum* growing on a geothermal soil and showing to be thermotolerant to temperatures of 50ºC to 65ºC (R e d m a n et al., 2002). When the plant and the fungus grow separately, they do not tolerate temperatures above 38ºC.

Drought / excessive moisture

Drought stress limits crop growth and productivity, especially in arid and semi-arid regions. Some microbial species and/or strains that inhabit plant rhizosphere use different mechanisms to mitigate negative effects of drought on plants (Table 1). According to G r o v e r et al. (2010), certain microbial types may mitigate the impact of soil drought through production of exopolysaccharates, induction of resistance genes, increased circulation of water in the plant, and the synthesis of ACC-deaminase, indole-acetic acid and proline.

Crop inoculation (with e.g. *Bacillus amylolequifaciens*) leads to the production of polysaccharates (EPS) which tends to improve soil structure by facilitating the formation of macroaggregates. This in turn increases plant resistance to stress due to water shortage. Soils with a high content of small aggregates contain more nutrients in the form available for plants and microorganisms (NO_3, P_2O_5, K_2O), as indicated by high values of dehydrogenase (M i l o š e v i ć et al., 2002a). However, a high portion of small aggregates causes poor aeration and evacuation of water from soil pores, which leads to a decline in soil fertility in the long run. Macroaggregates are guardians of soil fertility, because they maintain a balance between aerobic and anaerobic conditions and ensure a gradual uptake of nutrients from soil reserves. Inoculation of wheat and sunflower with different species and/or strains of EPS-producing bacteria tended to alleviate drought stress (Table 1).

Tab. 1. – Effect of microorganisms on drought mitigation in crops

Microorganism	Crop	Mechanism
Pantoea agglomerans	Wheat	Production of EPS which affects the structure of rhizospheric soil
Rhizobium sp.	Sunflower	Production of EPS which affects the structure of rhizospheric soil
Pseudomonas putida -P45	Sunflower	Production of EPS which affects the structure of rhizospheric soil
Azospirilum sp.	Wheat	Increased water circulation
Achromobacter piechaudii ARV8	Tomato Pepper	Synthesis of ACC-deaminase

Variovorax paradoxus	Pea	Synthesis of ACC-deaminase
Pseudomonas sp.	Pea	Decreased ethylene production
AM fungi	Sorghum	Increased water circulation
Brome mosaic virus	Rice	Unknown
Pseudomonas mendocina and *Glomus intraradices*	Lettuce	Increased antioxidative status
Bacillus megaterium and *Glomus* sp.	Clover	Production of indole acetic acid and proline

Source: G r o v e r et al. (2010)

PGPR mitigate the impact of drought on plants through a process so-called *induced systemic tolerance* (IST) which includes: a) production of cytokinins, b) production of antioxidants and c) degradation of the ethylene precursor ACC by bacterial ACC deaminase. (a) The production of cytokinins causes the accumulation of abscisic acid (ABA) in leaves, which in its turn results in the closing of stomata (F i g u e i r e d o et al., 2008; C o w n et al., 1999; cit. Y a n g et al., 2009). (b) The production of antioxidants (e.g., the enzyme catalase) causes the degradation of reactive forms of oxygen. (c) The bacterial-produced ACC deaminase degrades the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) (Y a n g et al., 2009).

Oxygen is essential for the life on Earth. It is used by all aerobic organisms for the production of energy by the process of respiration. In the course of respiration, oxygen is reduced to water while complex organic molecules (lipids, carbohydrates, proteins) are subject to oxidative degradation. Of the total amount of oxygen in cells, only a small portion (2%-3%) is transformed into toxic forms that are referred to as reactive oxygen species (ROS). Homeostasis in plant cells is maintained for as long as there is a balance between the production of ROS and antioxidants. When exposed to drought stress, some rhizobacteria produce antioxidants which neutralize the toxic effects of ROS in plant cells, reducing damage to cells and biomolecules to a minimum (G o v e r et al., 2010).

Plant inoculation with ACC-deaminase-containing rhizobacteria causes root elongation and water uptake from deeper soil layers, which is reflected on plant growth and development especially under drought conditions (Z a h i r et al., 2008; cit. G o v e r et al., 2010).

Our investigation on the effect of soybean seed inoculation with five *Bradyrhizobium japonicum* strains under three drought levels of conditions showed that differences existed in the reduction of dry matter in plants (Table 2). The soybean plants inoculated with the strains D 216 and 2b plants were most tolerant to soil drought. On average for all three drought levels, the lowest dry weight reduction was registered in the plants inoculated with the strain D 216 (10.05%).

Soybean seed inoculation with five *Bradyrhizobium japonicum* strains under three drought levels resulted in uneven reduction of nitrogen in the aboveground plant parts (Table 3). On average for all three drought levels, the lowest nitrogen reduction in the aboveground plant parts was recorded in the

Tab. 2. – Effect of *Bradyrhizobium japonicum* inoculation on dry matter weight (g) in soybean plants grown under three drought levels (M i l o š e v i ć and M a r i n k o v i ć, 2011)

Drought intensity	*Bradyrhizobium japonicum* (strain)									
	D 216		518		511		2b		1b	
	g	%	g	%	g	%	g	%	g	%
Ø	7.316	100	7.304	100	6.007	100	7.089	100	7.520	100
V 2	7.113	97.15	6.996	95.60	5..617	93.06	6.509	91.09	6.781	89.10
V 3	6.454	86.64	6.334	84.69	5.333	87.36	6.379	88.87	6.539	85.00
V 4	6.421	86.06	6.241	82.97	4.892	77.21	6.254	86.65	6.431	83.07
AVERAGE V2-V4	6.663	89.95	6.524	87.75	5.281	85.88	6.381	88.87	6.584	85.72

plants inoculated with the strains 1 b and 511 (3% and 7%, respectively), as compared with the control variant. The results presented in Tables 2 and 3 indicate the possibility of selection and application of microbial strains in the production of soybean under drought conditions.

Tab. 3. – Effect of *Bradyrhizobium japonicum* inoculation on nitrogen content (%) in soybean plants grown under three drought levels

Drought intensity	*Bradyrhizobium japonicum* (strain)									
	D 216		518		511		2b		1b	
	% N	%	% N	%	% N	%	% N	%	% N	%
Ø	2.188	100	2.128	100	1.268	100	2.214	100	2.124	100
V 2	1.948	89	1.840	86	1.256	99	1.957	88	2.001	94
V 3	1.995	91	2.020	95	1.149	90	1985	90	2.096	98
V 4	2.040	93	1.990	93	1.160	91	2.007	91	2.090	98
AVERAGE V2-V4	1.994	91	1.950	91	1.188	93	1.983	90	2.062	97

Under conditions of excessive moisture, microorganisms take up the available oxygen while toxic substances accumulate in the soil. In such conditions, plants reduce the permeability of roots, water absorption and nutrients uptake, which reduce the growth of aboveground plant parts and roots. Provoked by excessive moisture, roots release large quantities of aminocyclopropane carboxylate-1 (ACC) into the soil. Some groups of bacteria degrade ACC and reduce its concentration in the soil by secreting the enzyme ACC-deaminase. In excessively moist soil, bacteria such as Enterobacter cloacae and Pseudomonas putida predominate over fungi and actinomycetes (G r i c h k o and G l i c k, 2001).

Mycorrhizal fungi mitigate the stress caused in plants by excessive moisture (S a i n t - E t i e n n e et al., 2006, cit. G r o v e r et al., 2010). It is hypothesized that, under conditions of excessive moisture, the accumulation of acetaldehyde and the high toxicity of ethanol intermediates in roots are responsible for damage to sensitive plant species.

Temperatures

High temperature promotes plant growth and development, while low temperature is the most important limiting factor to the productivity and geographic distribution of agricultural crops.

Some bacterial species and strains affect plant tolerance to high temperature (G r o v e r et al., 2010). So, *Pseudomonas* sp. strain NBRI0987 causes thermotolerance in sorghum seedlings, which consequently synthesize high molecular weight proteins in leaves thus increasing the plant biomass. The bacterium Burkholderia phytofirmans PSJN colonizes grapevine residues and protects the plant against heat and frost through increases in the levels of starch, and proline and phenols. Inoculation of wheat seeds with Serratia marscescens, strain SRM, and Pantoea dispesa, strain 1A increases the seedlings biomass and nutrients uptake at low temperatures.

Salinity

Microorganisms use different mechanisms to alleviate the salinity stress in agricultural crops (Tab. 4). Some rhizobacterial strains (PGPR) affect the growth and development of tomatoes, peppers, beans, and lettuce grown in saline environments (G r o v e r et al., 2010; Y i l d i r i u m and T a y l o r, 2005). Inoculation of wheat seedlings with bacteria that produce exopolysaccharates (EPS) affect the restriction of sodium uptake and stimulation of plant growth under conditions of stress caused by high salinity (A s h r a f et al., 2004, cit. G r o v e r et al., 2010). Corn, beans and clover inoculated with AM fungi improved their osmoregulation and increased proline accumulation which resulted in salinity resistance (F e n g et al., 2002, cit. G r o v e r et al., 2010).

Tab. 4. – Effect of microorganisms on mitigation of salinity stress in agricultural crops

Microorganism	Crop	Mechanism
Achromobacter piechaudii	Tomato	Synthesis of ACC-deaminase
Piriformaspora indica	Barley	Increased antioxidative capacity
AM fungi	Sorghum Corn Clover	Increased water circulation Improved osmoregulation and proline accumulation
B. amylolequifaciens	When	Restricted Na+ uptake
Rhizobium and *Pseudomonas*	Wheat	Restricted Na+ uptake

Source: G r o v e r et al. (2010)

Heavy metals

Heavy metals affect the soil microbial population, their effects depending on the element in question and its concentration on one side and the bacterial

species/strain on the other. Some heavy metals are essential micronutrients that are required in small quantities for the growth of microorganisms and plants. Microorganisms bind soluble heavy metals in three ways (biosorption, bioaccumulation, and the binding by metabolic products), which indirectly reduce the negative impact of heavy metals on plants (G o v e d a r i c a et al., 1997).

Studies have shown that the effect of nickel on the microbiological soil properties depended on the microbial group and agricultural plant species (K a s t o r i et al., 2006, M i l o š e v i c et al., 2002). *Methylobacterium oryzae* and *Burkholderia* sp. reduce nickel and cadmium stress in tomato by reducing their uptake and translocation (M a r q u e z et al., 2007; M a d h i y a n et al., 2007). Inoculation with rhizobacteria alleviates abiotic stresses to plants caused by drought, salinity and metal toxicity (D i m k p a et al., 2009). These authors pointed out that the bacteria that are used as biofertilizers are at the same time plant bioprotectants against stress. This interaction between plants and rhizobacteria (e.g., *Bacillus*) mitigates stress conditions. Heavy metals such as Cd, Ni, and Pb disrupt the water regimen in plants. Proline accumulation in plant cells is a biomarker for stress induced by heavy metals.

The symbiotic associations between *Rhizobium*/*Bradyrhizobium* and leguminous plants are sensitive to the presence of heavy metals in soil (G o v e d a r i c a et al., 1997). Heavy metals tend to inhibit nodulation. i.e., they interrupt the rate of symbiosis between plants and mikrosymbionts depends on heavy metals concentration in soil.

CONCLUSION

Microorganisms help agricultural plants to increase their tolerance and adaptation to abiotic stresses. The complex and dynamic interactions between microorganisms and plant roots under conditions of abiotic stress affect not only the plants but also the physical, chemical, and structural properties of soil. The possibility of mitigation of abiotic stresses in plants opens a new chapter in the application of microorganisms in agriculture. Some microbial species and strains could play an important role for understanding plant tolerance to stress, adaptation to stress, and mechanisms that develop in plants under stress conditions. Selection of microorganisms from stressed ecosystems may contribute to the concept of biotechnology application in agriculture.

LITERATURE

A n d e r s s o n, M., M i c h e l s e n, A., J e n s e n, M., Annelise K j ø l l e r A. (2004): Tropical savannah woodland: effects of experimental fire on soil microorganisms and soil emissions of carbon dioxide. Soil Biology and Biochemistry 36: 849–858.

B a l s e r, T. C., K i n z i g, A. P., F i r e s t o n e, M. K. (2001): Linking soil microbial communities and ecosystem functioning. In: A. P. Kinzig, S. W. Pacal, D. Tilman, Eds., The Func-

tional Consequences of Biodiversity: Empirical Progress and Theoretical Extensions, Princeton University Press. Princeton, NJ, 265–293.

Bloem, J., Breure, A. M. (2003): Microbial indicators. In: B. A. Markert, A. M. Breure, H. G. Zechmeister, Eds., Bioindicators and Biomonitors, Elsevier Science Ltd Amsterdam, Boston, London, New York, Oxford, Paris, San Diego, San Francisco, Singapore, Sydney, Tokyo, 259–282.

Dimkpa, C., Weinand, T., Asch, F. (2009): Plant-rhizobacteria interactions alleviate abiotic conditions. Plant Cell Environ. 32: 1682–1694.

Fites-Kaufman, J., Bradey, AF., Merrill, A. G. (2006): Plant and fire interactions. In: N. G. Sugihara, J. W. van Wagtenonk, K. E. Shaffer, J. Fites-Kaufman, A. E. Thode, Eds., Fire in California's Ecosystems, University of California Press Ltd, 94–118.

Glick, B. R. (2005): Modulation of plant ethylene levels by the enzyme ACC deaminase. FEMS Microbiol. Lett. 252: 1–7.

Govedarica, M., Milošević, N., Jarak, M. (1997): Teški metali i mikroorganizmi zemljišta. U: R. Kastori, Ed., Teški metali u životnoj sredini, Naučni institut za ratarstvo i povrtarstvo, Novi Sad 153–194.

Grichko, V. P., Glick, B. R. (2001): Flooding tolerance of transgenic tomato plants expressing the bacterial enzyme ACC deaminase controlled by the 35*S*, *rolD* or PRB-1*b* promoter. Plant Physiol. Biochem. 39: 19–25.

Griffiths, B. S., Ritz, K., Bardgett, R. D., Cook, R., Christensen, S., Ekelund, F., Sørensen, S. J., Bååth, E., Bloem, J., De Ruiter, P. C., Dolfing, J., Nicolardot, B. (2000): Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: An examination of the biodiversity-ecosystem function relationship. Oikos 90: 279–294.

Grover, M., Ali, S. Z., Sandhya, V., Rasul, A., Venkateswarlu, B. (2010): Role of microorganisms in adaptation of agriculture crops to abiotic stres. World J. Microbiol. Biotechnol.

Harris, P. A., Schomberg, H. H., Banks, P. A., Giddens, J. (1995): Burning, tillage and herbicide effects on the soil microflora in a wheat-soybean double-crop system. Soil Biology and Biochemistry 27(2): 153–156.

Kastori, R., Kadar, I., Sekulić, P., Bogdanović, D., Milošević, N., Pucarević, M. (2006): Uzorkovanje zemljišta i biljaka nezagađenih i zagađenih staništa. Naučni institut za ratarstvo i povrtarstvo, Novi Sad.

Madhaiyan, M., Poonguzhali, S., Sa, T. (2007): Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum* L.). Chemosphere 69: 220–228.

Marquez, L. M., Redman, R. S., Rodriguez, R. J., Roosinck, M. J. (2007): A virus in a fungus in a plant: Three-way symbiosis required for thermal tolerance. Science 315 (5811): 513–515.

Milošević, N., Govedarica, M., Kastori, R., Petrović, N. (2002): Effect of nickel on wheat plants, soil microorganisms and enzymes. Biologia: XLVII: 177–181.

Milošević, N., Govedarica, M., Jarak, M., Hađžić, V., Belić, M. (1996): Effect of Compaction on Soil Structure, Microbial Populations and Enzyme Activities, Biologia: XII: 79–84.

Milošević, N. (2008): Mikroorganizmi – bioindikatori zdravlja / kvaliteta zemljišta. Zbornik radova Instituta za ratarstvo i povrtarstvo 45: 505–515.
Pettersson, M. (2004): Factors Affecting Rate of Change in Soil Bacterial Communities. Doctoral thesis, Lund University, Gothorvm. Caroline, Sweden. http://www.lub.lu.se/luft/diss/sci_649/sci_649.pdf od 29.05.2011.
Rajapaksha, R. M., Tobor-Kapłon, M. A., Bååth, E. (2004): Metal toxicity affects fungal and bacterial activities in soil differently. Appl. Environ. Microbiol. 70: 2966–2973.
Redman, R. S., Sheehan, K. B., Stout, R .G., Rodriguez, R. J., Henson, J. M. (2002): Thermotolerance generated by plant/fungal symbiosis. Science 298: 1581.
Saleem, M., Arshad, M., Hussain, S., Bhatti, A. S. (2007): Perspective of plant growth promoting rhizobacteria (PGPR) containing AC deaminase in stress agriculture. J. Ind. Microbiol. Biotechnol. 34: 635–648.
Tobor-Kapłon, M. A., Bloem, J., De Ruiter, P. C. (2006): Functional stability of microbial communities from long-term stressed soils to additional disturbance. Environ. Toxicol. Chem. 25: 110–125.
Vázquez, F. J., Acea, M. J., Carballas, T. (1993): Soil microbial population after wildfare. FEMS Microbiology Ecology, 1393–104.
Yang, J., Kloepper, J., Ryu, C. M. (2009): Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci. 14: 1–4.
Yildirium, E., Taylor, A. G. (2005): Effect of biological treatment on growth of bean plants under salt stress. Ann. Rep. Bean Improv. Coop. 48: 176–177.

УТИЦАЈ МИКРООРГАНИЗАМА НА УБЛАЖАВАЊЕ ПОСЛЕДИЦА АБИОТИЧКОГ СТРЕСА КОД ПОЉОПРИВРЕДНИХ КУЛТУРА

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Резиме

Микроорганизми могу имати значајну улогу у стратегијама адаптација и повећању толерантности пољопривредних биљних врста на абиотичке стресове. Највећи утицај ублажавања абиотичких стресова на биљку (суша, ниске температуре, салинитет, токсичност метала и високе температуре) имају микроорганизми који насељавају ризосферно земљиште, а промотери су биљног раста (ПГПР), кроз продукцију егзополисахарида и формирањем биофилма. ПГПР ублажавају утицај суше на биљке *индукованим системом толеранције* (ИСТ): а) продукцијом бактеријског цитокинина б) продукцијом антиоксиданата и ц) деградацијом етилен прекурсора АЦЦ бактеријским АЦЦ-деаминазом. Такође и симбиозне гљиве (abscular mycorrhizal fungi) и дуал симбиозни системи (rhizosphere, endophytic bacteria и symbiotic fungi) утичу на ублажавање абиотичких стресова у биљкама.

КЉУЧНЕ РЕЧИ: адаптација, биљка, земљиште, микроорганизми, стрес

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MICROBIOLOGICAL TRANSFORMATIONS OF PHOSPHORUS AND SULPHUR COMPOUNDS IN ACID SOILS

ABSTRACT: The dynamics of phosphorus and sulphur in soil is closely related to the dynamics of the biological cycle in which microorganisms play a central role. There is not much microbiological activity in acid soils because aerobes are scarce, rhizosphere is restricted to the shallow surface layer, and the biomass of microorganisms decreases with higher acidity. The aim of the research was to investigate the number of microorganisms, which decompose organic and inorganic phosphorus compounds and organic sulphur compounds in calcocambisol, luvisol, and pseudogley.

The following parameters were determined in the soil samples: pH in H_2O and in 1MKCl; the content of $CaCO_3$ (%); humus content (%), nitrogen content (%); the content of physiologically active phosphorus and potassium (mg P_2O_5/100g of soil; mg K_2O/100g of soil). The number of microorganisms was determined by the method of agar plates on appropriate nutrient media: the number of microorganisms solubilizing phosphates on a medium by Muramcov; the number of microorganisms that decompose organic phosphorus compounds on a medium with lecithin; and the number of microorganisms that transform organic sulphur compounds on a medium by Baar.

All three types of soil are acid non-carbonate soils with a low level of available phosphorus and a more favorable amount of potassium, nitrogen, and humus. The largest number of bacteria, which transform organic phosphorus compounds, was found in calcocambisol. The largest number of phosphate solubilizing bacteria was recorded in pseudogley, whereas the largest number of phosphate solubilizing fungi was recorded in calcocambisol. The largest number of bacteria, which transform organic sulphur compounds, was recorded in pseudogley.

KEY WORDS: acid soil, microorganisms, phosphorus, sulphur

INTRODUCTION

Soil microorganisms play a major role in the decomposition of plant residues, creation of humus and maintenance of stable soil structure. They also participate in the cycles of the most important macro and microelements such as phosphorus and sulphur (C a i r n e y, 2000; K l i r o n o m o s et al., 2000).

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The dynamics of phosphorus in soil is closely related to the dynamics of the biological cycle in which microorganisms play a central role (Wakelin et al., 2004; Vassilev et al., 2006). Microorganisms affect the amount of phosphorus accessible to plants by means of mineralization of organic phosphorus compounds, immobilization of available phosphorus and solubilization of non-soluble phosphorus minerals such as tricalcium phosphate (Chen et al., 2006; Kang et al., 2002; Pradhan and Sukla, 2005). Organic phosphorus compounds in soil are an important supply of this plant nutrient but only after they have been mineralized (Gyaneshwar et al., 2002). Mineralization is catalyzed by microbiological enzymes phosphatases (Oberson et al., 2001). Organic phosphorus mineralization in soil depends on temperature, pH, soil moisture, degree of aeration (Dalal, 1977). According to Wittmann et al. (2003a), optimum pH value for phosphatase activity is 4-5 and optimum temperature 35º C.

Sulphur is one of the essential plant nutrients contributing to yield and quality of crops. There are two main forms of sulphur in soil, inorganic and organic sulphur (Landers et al., 1983). In soil, sulphur is mainly found in organic form (90%). Organic sulphur is present in three forms, ester sulfate-S, C-bonded S and non-reducible organic sulphur (Freney et al., 1975). Sulphur transformations in soil are considered to result primarily from microbial activity, which involves processes of mineralization, immobilization, oxidation, and reduction. Mineralization of organic sulphur compounds and transformation into forms accessible to plants is catalyzed by enzyme sulphatase (Eivazi and Bayan, 1996; Hayes et al., 2000). Sulphatase activity has been noticed even in soils with extremely low values of pH (pH 3-5), but its effects in such extreme conditions are unknown (Kahkonen et al., 2002).

As acid soils are found in many areas in our country, the aim of the research was to investigate the number of microorganisms that transform inaccessible phosphorus and sulphur compounds into forms accessible to plants.

MATERIAL AND METHODS

Acid soil samples were taken from the slopes of Golija mountain (calcocambisol), Radočevo (luvisol), and Kraljevačka valley (pseudogley).

The following chemical properties of soil were determined: pH in H_2O and in 1MKCl; the content of $CaCO_3$ (%); humus content (%); total nitrogen content (%); the content of physiologically active phosphorus and potassium (mg P_2O_5/100g of soil; mg K_2O/100g of soil).

The number of microorganisms was determined by the method of agar plates (Trolldenier, 1996) on appropriate nutrient media (Hi Media Laboratories Pvt. Limited Mumbai, India): the number of bacteria decomposing organic phosphorus compounds on a medium with lecithin, the number of phosphate solubilizing bacteria and fungi on a medium with aluminum phosphate, and the number of microorganisms transforming organic sulphur compounds on a medium by Baar. All groups of microorganisms were introduced into Petri

dishes with 0.5 ml soil suspension from 10^{-4} dilution. The microorganisms were incubated at the constant temperature of 28º C. The incubation of microorganisms, which transform phosphorus compounds, lasted 2-10 days, whereas the incubation of microorganisms, which transform sulphur compounds, lasted 7-10 days.

Statistical data analysis was performed using STATISTICA 10 software.

The significance of differences in the number of microorganisms between different soil types was determined upon the least significant difference (LSD).

RESULTS AND DISCUSSION

The investigated soil types are characterized by the chemical properties shown in Table 1.

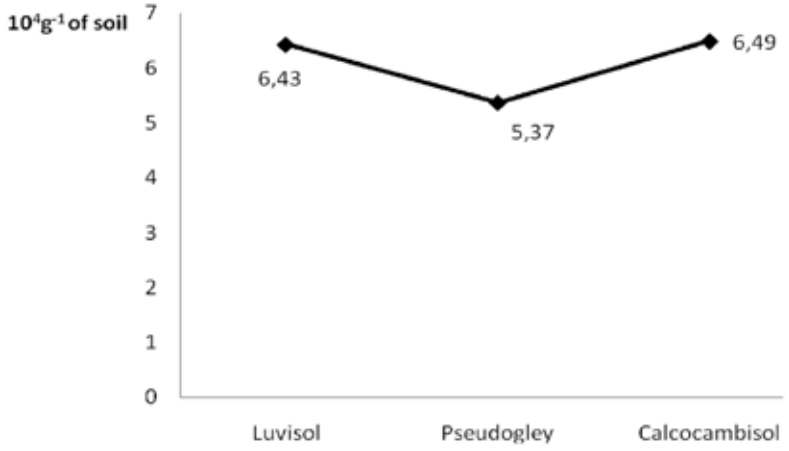
Tab. 1. – Chemical properties of the investigated soil types

Soil	pH		Humus %	N %	$CaCO_3$ %	mgP_2O_5 u 100 g	mgK_2O u 100 g
	In H_2O	In 1M KCl					
Calcocambisol	6.2	4. 8	3.2	0.26	0.26	4.9	12.25
Pseudogley	5.1	3.7	2.4	0.18	0.46	4.3	14.3
Luvisol	4.0	3.6	3.7	0.43	0	2.1	15.7

Calcocambisol is a low carbonate soil. It abounds in nitrogen and has high humus content. The amount physiologically active phosphorus in it is minimal whereas the amount of potassium is greater. Pseudogley is an extremely acid type of soil. It has a medium amount of nitrogen and humus. It is characterized by a very low amount of phosphorus and calcium carbonate and a medium amount of potassium. Luvisol is an extremely acid non-carbonate soil type. It is well- provided with nitrogen and humus. The amount of phosphorus in it is low whereas the amount of potassium is somewhat greater.

Microbial communities are important in soils because of their key function in ecosystems processes such as decomposition, nutrient cycling, and plant symbioses (N a n n i p i e r i et al., 2003). All these processes enable mutual relations of microorganisms, soil, and plants. Soil pH is one of the most important soil properties related to the composition of microbial communities (B a a t h and A n d e r s o n, 2003; N i l s s o n et al., 2007; W u et al., 2009; B r a d y and W e i l, 2002).

Three types of acid soils were used in this research, which certainly affected the number of microorganisms. The number of bacteria decomposing organic phosphorus compounds ranged from 5.37 to 6.49 x 10^4 g^{-1} of absolutely dry soil (Graph 1). The differences were not statistically significant (LSD for 5% =5.42).



Graph 1. – The number of bacteria transforming organic phosphorus compounds (10^4 g^{-1} of soil)

T a r a f d a r and C l a a s e n (1988) documented that almost half of the microorganisms in soils and on plant roots were able to mineralize organic P through the phosphatase action. Organic phosphorus compounds are mainly phospholipids, phytin, and nucleoproteins. A large number of bacteria are able to use phospholipids and nucleoproteins transforming them into phosphate anions. In this research, a large number of bacteria decomposing organic phosphorus compounds could provide plants with a significant amount of this nutrient. However, in acid soils, phosphate anions form bonds aluminum and iron cations and become available to plants only after the activity of microorganisms. In acid soils, fungi are more active solubilizers of insoluble phosphates, whereas in neutral soils, bacteria are more active. In this research, the number of fungi solubilizing phosphates inaccessible to plants was similar in all three types of soil, ranging from 1.41 to $1.73x10^4$ g^{-1} of absolutely dry soil (Graph 2). The differences in the number of this group of microorganisms in the investigated types of soil were not statistically significant (LSD for 5% = 1.21).

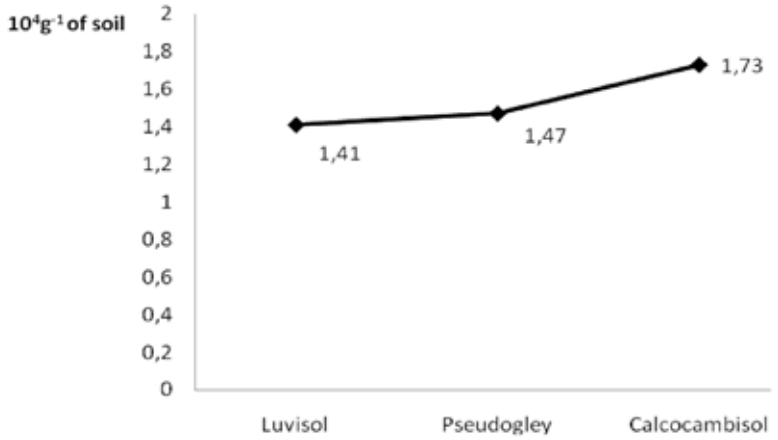
Phosphate solubilizing bacteria were not found in calcocambisol, whereas in the other two types of soil their number ranged from 0.49 x 10^4 g^{-1} to 8.81 x 10^4 g^{-1} of absolutely dry soil (Graph 3). A statistically significant difference in the number of bacteria was recorded between pseudogley and the other two soil types and it was on the level $P< 0.05$ (LSD for 5%= 7.09).

Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein, 1986). It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms (H a l d e r, 1990; L e y v a l, 1989; S a l i h, 1989). Among them, gluconic acid seems to be the most frequent agent of mineral phosphate solubilization. Another organic acid identified in strains with phosphate-solubilizing ability is 2-ketogluconic acid (H a l d e r, 1990, 1993). Bacteria are more effective in

phosphorus solubilization than fungi (A l a m et al., 2002). Among the whole microbial population in soil, phosphorus-solubilizing bacteria constitute 1 to 50 %, while phosphorus-solubilizing fungi are only 0.1 to 0.5 % in P solubilization potential (C h e n et al., 2006).

Unlike these results, in this research the number of phosphate solubilizing fungi was larger than the number of bacteria apart from pseudogley where the number of bacteria was larger. This could be the result of unfavorable conditions for microorganism activity in the investigated soil types. The investigated soil types are characterized by heavy soil texture, unfavorable water-air ratio, and acidity (M i l j k o v i ć, 1996). Our results confirmed the results of Y a h y a and A z a w a i (1998) who concluded that phosphate solubilizing fungi in infertile soils were more numerous than bacteria.

The number of bacteria, which transform organic sulphur compounds, amounted to thousands (Graph 4). Their smallest number was recorded in luvisol (12.16 x $10^4 g^{-1}$), whereas their largest number was recorded in pseudogley (28.01 x 104 g^{-1}). The differences in the number between the investigated soil types were not statistically significant (LSD for 5% = 20.45).



Graph 2. – The number of phosphate solubilizing fungi (10^4 g^{-1})

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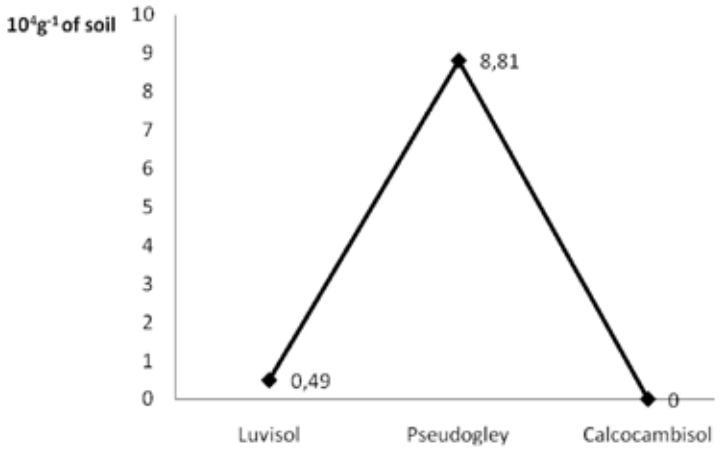

Graph 3. – The number of phosphate solubilizing bacteria (10^4 g^{-1} of soil)

acid seems to be the most frequent agent of mineral phosphate solubilization. Another organic acid identified in strains with phosphate-solubilizing ability is 2-ketogluconic acid (H a l d e r, 1990, 1993). Bacteria are more effective in phosphorus solubilization than fungi (A l a m et al., 2002). Among the whole microbial population in soil, phosphorus-solubilizing bacteria constitute 1 to 50 %, while phosphorus-solubilizing fungi are only 0.1 to 0.5 % in P solubilization potential (C h e n et al., 2006).

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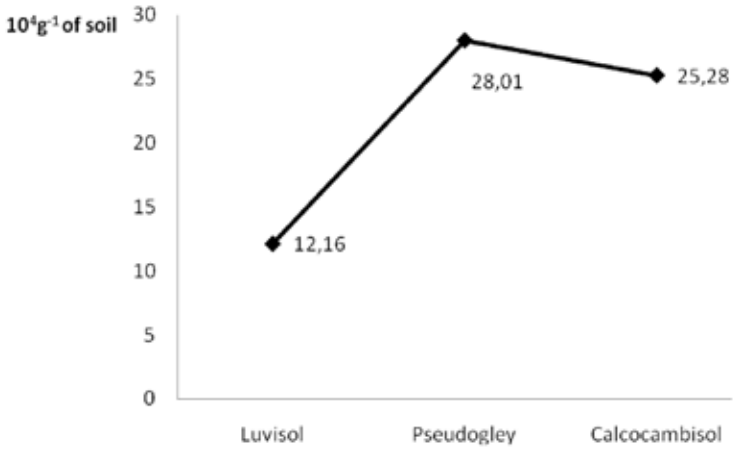
The number of bacteria, which transform organic sulphur compounds, amounted to thousands (Graph 4). Their smallest number was recorded in luvisol (12.16 x $10^4 g^{-1}$), whereas their largest number was recorded in pseudogley (28.01 x 104 g^{-1}). The differences in the number between the investigated soil types were not statistically significant (LSD for 5% = 20.45).

Sulphur is one of the essential plant nutrients classified as secondary nutrient. Sulphur transformations in soil are considered to result primarily from microbial activity which involves processes of mineralization, immobilization, oxidation and reduction (V i d y a l a k s h m i, 2009). So far, a great deal of attention has been paid to the isolation and identification of Sulphur Oxidizing Bacteria (C h a p m a n, 1990; W o o d, 1991; J o h n s o n, 1992), whereas very little attention has been devoted to investigating their number and activity in soil. A relatively large number of bacteria, which transform



Graph 4. – The number of bacteria that transform organic sulphur compounds (104 g^{-1} of soil)

organic sulphur compounds in the investigated soils, confirms the results of W i t t m a n et al. (2003b) who stated that the optimum pH value for sulphatase is 4-5, which indicates that the hydrolitic activity of the enzyme is adapted to acid soils.

CONCLUSION

All three types of soil belong to the group of acid, non-carbonate soils with a low level of accessible phosphorus and a more favorable content of potassium, nitrogen, and humus.

The number of bacteria decomposing organic phosphorus compounds ranged from 5.37 x 10^4 g^{-1} to 6.49 x 10^4 g^{-1} of absolutely dry soil.

The number of phosphate solubilizing fungi was similar in all three types of soil, ranging from 1.41 x 10^4 g^{-1} to 1.73 x $10^4 g^{-1}$ of absolutely dry soil.

Phosphate solubilizing bacteria were not found in calcocambisol, whereas in the other two soil types their number ranged from 0.49 x 10^4 g^{-1} to 8.81 x 10^4 g^{-1} of absolutely dry soil.

The number of bacteria that transform organic sulphur compounds amounted to thousands. The smallest number was recorded in luvisol (12.16 x 10^4 g^{-1}), whereas the largest number was recorded in pseudogley (28 x 10^4 g^{-1}).

REFERENCES

A l a m, S., K h a l i l, S., A y u b, N., R a s h i d, M. (2002): In vitro solubilization of inorganic phosphate by phosphate solubilizing microorganism (PSM) from maize rhizosphere. Intl. J. Agric. Biol, 4, pp. 454–458.

Baath, E., Anderson, T. H. (2003): Comparison of soil funga/bacterial ratios in a pH gradient using phisiological and PLFA-based tehniques. Soil Biology and Biochemistry 35, pp. 955–963.
Brady, N. C., Weil, R. R. (2002): The Nature and Properties of Soil (13th ed.), Springer Netherlands, pp. 249.
Cairney, J. W. G. (2000): Evolution of mycorriza systems, Naturwisenscha 87, pp. 467-475.
Chapman, S. J. (1990): Thiobacillus population in some agricultural soils. Soil Biol. Biochem., 22, pp. 479–482.
Chen, Y. P., Rekha, P. D., Arunshen, A. B., Lai, W. A., Young, C. C. (2006): Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl. Soil Ecol., 34, pp. 33–41.
Dalal, R. C. (1977): Soil organic phosphorus. Adv. Agron. 29, pp. 85-117.
Freney, J. R., Melville, G. E., Williams, C. H. (1975): Soil organic matter fractions as sources of plant-available sulphur Soil Biol. Biochem., 7, pp. 217–221.
Goldstein, A. H. (1986): Bacterial solubilization of mineral phosphates: historical perspective and future prospects, Am J Altern Agri, 1, pp. 51–70.
Gyaneshwar, P., Kumar, G. N., Parekh, L. J., Poole, P. S. (2002): Role of soil microorganisms in improving P nutrition of plants. Plant Soil, 245, pp. 83–93.
Halder, A. K., Mishra, A. K., Bhattacharyya, P., Chakrabartty, P. K. (1990): Solubilization of rock phosphate by Rhizobium and Bradyrhizobium. J Gen Appl Microbiol, 36, pp. 81–92.
Halder, A. K., Chakrabartty, P. K. (1993): Solubilization of inorganic phosphate by Rhizobium. Folia Microbiol, 38, pp. 325–30.
Hayes, J. E., Richardson, A. E., Simpson, R. J. (2000): Components of organic phosphorus in soil extracts that are hidrolized by phytase and acid phosphatase. Biology and Fertility of soil 32, pp. 279–286.
Jhonshon, B. D., Pal Ghauri, M. A., Mazinf, S. (1992): Isolation and characterisation of an acidophilic, heterotrophic bacterium capable of oxidizing ferrous ion. Appl. Environ. Microbiol., 58, pp. 1423–1428.
Kahkonen, M. A., Wittmann, C., Kurola, J., Iilvsniemi, H., Salkinoja-Salonen, M. S. (2002): Microbial activity in boreal forest soil in cold climate. Boreal Environment Research 6, pp. 19–28.
Landers, D. H., David, M. B., Mitchell, M. J. (1983): Analysis of organic and inorganic sulfur constituents in sediments, soils and water Int. J. Environ. Anal. Chem., 14, pp. 245–256.
Leyval, C., Berthelin, J. (1989): Interaction between Laccaria laccata, Agrobacterium radiobacter and beech roots: influence on P, K, Mg and Fe mobilization from minerals and plant growth. Plant Soil vol. 117., p.p. 103–110.
Miljković, N. S. (1996): Osnovi pedologije, Prirodno-matematički fakultet, Institut za geografiju, Novi Sad.
Nannipieri, P., Ascher, J., Ceccherini, M. T., Landi, L., Pietramellara, G., Renella, G. (2003): Microbial diversity and soil functions Eur. J. Soil Sci., 54, pp. 655–670.
Nilsson, L. O., Baath, E., Falkengren-Grerup, U., Wallander, H. (2007): Growth of ectomycorrhizal mycelia and composition of soil microbial communities in oak forest soils along a nitrogen deposition gradient Oecologia, 153, pp. 375–384.

Oberson, A., Friesen, D. K. K., Rao, I. M., Buechler, S., Frossard, E. (2001): Phosphorus transformations in an Oxisol under contrasting land-use systems: the role of the soil microbial biomass, Plant Soil 237, pp. 197–210.

Pradhan, N. and Sukla, L. B. (2005): Solubilization of inorganic phosphate by fungi isolated from agriculture soil. African J. Biotechnol. 5, pp. 850–854.

Tarafdar, J. C. and Claasen, N. (1988): Organic phosphorus compounds as a phosphorus source for higher plants through the activity of phosphatases produced by plant roots and microorganisms. Biol. Fert. Soils 5:308–312.

Trolldenier, G. (1996): Plate Count Technique. In Methods in Soil Biology. Ed. Franz Schinner, Ellen Kandeler, Richard Ohlinger, Rosa Margesin. Springer – Verlag berlin Heildeberg., pp. 20–26.

Vassilev, M., Vassileva, N., Nikolaeva, I. (2006): Simultaneous P-solubilizing and biocontrol activity of microorganisms: potentials and future trends Applied Microbiology and Biotechnology, 71, pp. 137–144.

Vidyalakshmi, R., Paranthaman, R., Bhakyaraj, R. (2009): Sulphur Oxidizing Bacteria and Pulse Nutrition – A Review World Journal of Agricultural Sciences, vol. 5., No 3, pp. 270–278.

Wakelin, S. A., Warren, R. A., Harvey, P. R., Ryder, M. H. P. (2004): Phosphate solubilization by Penicillium spp. closely associated with wheat roots Biology and Fertility of Soils, 40, pp. 36–43.

Wittmann, K., Kahkonen, A. M., Iilvesniemi, H., Kurola, J., Salkinoja-Salonen, M. S. (2003a): Areal activities and stratification of hydrolytic enzymes involved in the biochemical cycles of carbon, nitrogen, sulphur and phosphorus in podsolized boreal forest soils, Soil Biology and Biochemistry, Vol. 36, Issue 3, pp. 425–433.

Wittmann, K., Suominen, K., Salkinoja-Salonen, M. S. (2003b): Evaluation of ecological disturbance and intrinsic bioremediation potential of pulp mill contaminated lake sediment using key enzymes as probes. Environmental Pollution, 107, pp. 255–261.

Wood, A. P, Kelly, D. P. (1991): Isolation and characterization of Thiobacillus halophilus sp. nov., a sulphur oxidizing autotrophic eubacterium from a Western Australian hypersaline lake. Arch. Microbiol., 156, pp. 277–280.

Wu, Y. P., Zhou, B. Ma., Wang, H. Z., Xu, J. M., Kemmitt, S., Brookes, P. (2009): Changes in the soil microbial community structure with latitude in eastern China, based on phospholipid fatty acid analysis Applied Soil Ecology, 43, pp. 234–240.

Yahya, A., Azawi, S. K. A. (1998): Occurrence of phosphate solubilizing bacteria in some Iranian soils. Plant Soil 117, pp. 135–141.

МИКРОБИОЛОШКЕ ТРАНСФОРМАЦИЈЕ ЈЕДИЊЕЊА ФОСФОРА И СУМПОРА У КИСЕЛИМ ЗЕМЉИШТИМА

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Резиме

Динамика фосфора и сумпора у земљишту тесно је повезана са динамиком биолошког циклуса у којем микроорганизми имају централну улогу. Микробиолошка активност у киселим земљиштима није велика, јер су потиснути аероби, ризосфера је ограничена на плитку површинску зону, а са повећањем киселости смањује се и биомаса микроорганизама. Циљ ових истраживања је био да се испита заступљеност микроорганизама који разлажу органска и неорганска једињења фосфора и органска једињења сумпора у калкокамбисолу, лувисолу и псеудоглеју.

У узорцима земљишта одређени су следећи параметри: реакција земљишта (pH) у H_2O и у 1MKCl; садржај $CaCO_3$ (%); садржај хумуса (%); садржај азота (%); садржај физиолошки активног фосфора и калијума (mg P_2O_5/100g земљишта; mg K_2O/100g земљишта). Број микроорганизама одређиван је методом агарних плоча на одговарајућим селективним хранљивим подлогама: број микроорганизама који разлажу фосфате на подлози по: Мурамцов, број микроорганизама који разлажу органска фосфорна једињења на подлози са лецитином, а број микроорганизама који трансформишу органска једињења сумпора на подлози по Baar-у.

Сва три типа земљишта спадају у групу киселих бескарбонатних земљишта, са ниским садржајем приступачног фосфора и повољнијим садржајем калијума, азота и хумуса. Највећи број бактерија које трансформишу органска једињења фосфора утврђен је у калкокамбисолу. Највећи број бактерија које разлажу фосфате утврђен је у псеудоглеју, а највећи број гљива које разлажу фосфате у калкокамбисолу. Највећи број бактерија које трансформишу органска једињења сумпора утврђен је у псеудоглеју.

КЉУЧНЕ РЕЧИ: кисело земљиште, микроорганизми, сумпор, фосфор

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PRELIMINARY CHECKLIST OF MYXOMYCOTA AND ASCOMYCOTA FROM FRUŠKA GORA MOUNTAIN

ABSTRACT: Fruška Gora mountain represents very important source of natural and semi-natural forest ecosystems in the northern part of the Republic of Serbia and therefore it is important source of habitats for different groups of fungi. As opposed to coordinated inventory and monitoring projects of fungi established around Europe long ago, mycological researches in Serbia are still sporadic and insufficiently coordinated by authorities and experts. In accordance with that, available data concerning the state of fungi in Serbia are scarce. The aim of this work was to collect all relevant unpublished data considering fungi in Fruška Gora and to present checklist of two fungal phyla: Myxomycota and Ascomycota. In the presented checklist, 23 recorded species of Myxomycota (known as fungal analogues) were distributed in 2 classes, 5 orders, and 7 families. The first class (Protosteliomycetes) contained only one species – *Ceratiomyxa fruticulosa* (fam. Ceratiomyxaceae). The largest order was Trichiales (9 species), while the dominant families were Stemonitidaceae and Trichiaceae, each with 6 species recorded. The most abundant species was *Lycogala epidendrum* (fam. Reticulariaceae), with 13 records. Phylum Ascomycota was represented with 95 species belonging to 6 classes, 12 orders and 29 families. The most highly represented classes were Leotiomycetes (32 species) and Pezizomycetes (31 species). The most abundant species were: *Xylaria polymorpha* (17 records), *Xylaria hypoxylon* (14 records), and *Sarcoscypha coccinea* (14 records).

KEY WORDS: Ascomycota, Checklist, Database, Fruška Gora mountain, Fungi, Myxomycota

INTRODUCTION

Despite recommendations of mycological experts and institutions (EC-CF-European Council for the Conservation of Fungi, IMA-International Mycological Association, EMA-European Mycological Association, BMS-British Mycological Society, etc.) for coordinated inventory and monitoring of fungi worldwide and global efforts of mycological society in this task, mycological researches in Serbia are still sporadic, uncoordinated and mostly neglected by

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experts (Ivančević, 1995; Karaman, 1997). Although some important but individual and unofficial projects for inventory and mapping of fungi in Serbia exist, they are mostly done by mycological societies (Lukić, 2008; Ivančević, 2010b) and no checklists or databases have been unveiled recently. Some of the most comprehensive works concerning fungi in Serbia were published by Ivančević (1995, 2002) and Uzelac (2009).

Surrounded by flat, mostly agricultural land, Fruška Gora mountain represents very important source of natural and semi-natural forest ecosystems, and thus represents a main center of interest for the investigation of fungi in the northern part of the Republic of Serbia. It was proclaimed a National Park in December 1960, with active protection area of 25.525 ha. Fruška Gora is on the border of continental climate, but the influence of height gradient and forest cover gives it the characteristics of subcontinental climate. The base of ecosystem is represented by the sessile oak forest with butcher's-broom (Aculenro-euerco Carpinentat serbicum Jov.) (Stevanović, 1995). Diversity of forest vegetation, specific geological substrate, and climate influenced the rich biodiversity, with fungi as an essential component.

Research of fungi in Fruška Gora has been represented only in a few papers (Ranković, 1955; Lisiewska and Jelić, 1971; Karaman, 1997, 2005). Cooperation between National park Fruška Gora and researchers from Department of Biology and Ecology (University of Novi Sad) has resulted in the creation of a *Database of Fungi of Fruška Gora mountain*, which represents summary of findings available to the authors from previous unpublished data. Therefore, the aim of this work was to present in the form of checklist a part of this database, concerning two divisions – Myxomycota and Ascomycota.

Slime molds, although no longer included in the kingdom Fungi but placed inside the kingdom Protozoa, are still being studied by some mycologists as protozoan fungal analogues, reaching the number of 1165 taxa (Kirk et al., 2008). As opposed to Fungi, slime molds have phagotrophic nutrition and somatic stages in the form of protoplasts bounded by plasma membranes, without cell walls (Mehrotra and Aneja, 1990). Since there are only two published works about slime molds in Serbia (Ing and Ivančević, 2000; Ivančević, 2010a), we included them in this checklist, as well.

Ascomycota represent the largest group of Fungi, accounting approximately 75% of all described fungi (Taylor et al., 2012), including lichenized forms and most of the fungi that lack evident sexual stage. In presented checklist, only non-lichenized part of Ascomycota is given.

MATERIAL AND METHODS

Database of Fungi in Fruška Gora mountain contains a list of species recorded by numerous authors in two time periods: 1983-1996 and 2004-2011, from 28 localities (Fig. 1), nine of which (number 3, 7, 8, 13, 15, 16, 24, 25, 28) covers the areas with the first degree of protected regime in the National park.

The first period of investigation comprised data gathered from private lists with the courtesy of legators, while records made in the second investigation period were confirmed by mycological group of Department of Biology and Ecology in Novi Sad. Determination was conducted on the basis of morphological and anatomical characteristics of the fruiting bodies, microscopic characteristics of spores and other relevant structures (Olympus BX51, Japan) and the specific chemical reactions. Literature of Department's Library, on-line books, keys (D e n i s, 1986), and specialized mycological sites were consulted during determination and systematic representation. Some of the photos made during fieldwork are available on the website by Dagiša Savić: http://www.naturefg.com/. In the presented checklist, names of legators were abbreviated (full names in Results), while localities were marked with numbers and presented on map (Fig. 1). Taxa determined to the level of genus were also included. Each species in the checklist was placed within relevant higher ranks (phylum, class, order, and family) and presented with the following data: year of finding, locality, and collector (legator). Classification and nomenclature was done in accordance with *Species 2000 & ITIS Catalogue of Life: 2011 Annual Checklist* (B i s b y et al., 2011) and *Index Fungorum* (K i r k, 2012).

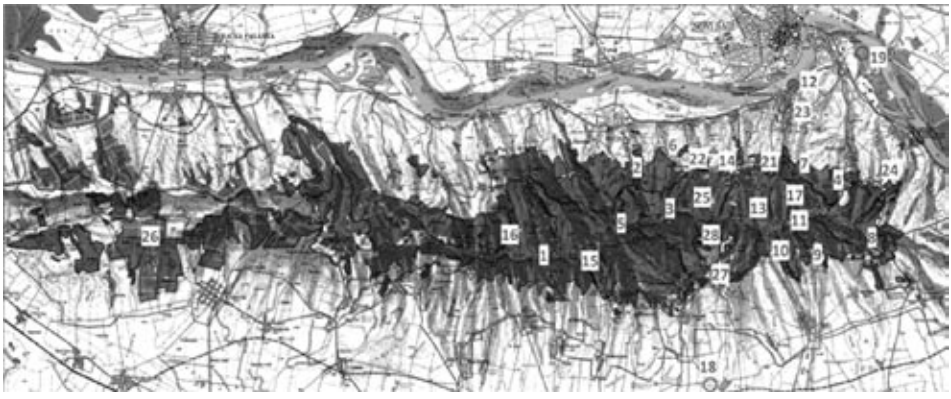

Fig. 1. – Map of Fruška Gora mountain with localities of fungal recording:
1. Andrevlje, 2. Beočin, 3. Brankovac, 4. Bukovac, 5. Crveni čot, 6. Dumbovo, 7. Glavica, 8. Grgeteg, 9. Hopovo, 10. Iriški venac, 11. Iriški venac – predajnik, 12. Kamenički park, 13. Kraljeve stolice, 14. Ledinci, 15. Letenka, 16. Papratski do, 17. Paragovo, 18. Pavlovci, 19. Petrovaradinski rit, 20. Popov čot, 21. Popovica, 22. Rakovac, 23. Sr. Kamenica, 24. Stražilovo, 25. Veliki Gradac, 26. Vorovo, 27. Vrdnik, 28. Zmajevac

RESULTS

Part of the database presented in this work contains 2 phyla (Myxomycota and Ascomycota) with 9 classes, 17 orders, 36 families, 78 genera, and 118 species (Tab. 1).

Twenty-three species in the checklist belonged to the phylum Myxomycota/Mycetozoa. They were distributed in 2 classes (Protosteliomycetes,

Tab. 1. – Number of taxa (Myxomycota and Ascomycota) recorded on the Fruška Gora Mountain

Phyla	Class	Order	Family	Genus	Species
Myxomycota	2	5	7	15	**23**
Ascomycota	6	12 (+ 1*) incert)	29 (+3*)	63	**95**
Total	**8**	**17**	**36**	**78**	**118**

* Incertae sedis

Myxomycetes), 5 orders (Protosteliales, Liceales, Physarales, Stemonitales, Trichiales), 7 families (Ceratiomyxaceae, Reticulariaceae, Didymiaceae, Physaraceae, Stemonitidaceae, Arcyriaceae, Trichiaceae), and 15 genera. Class *Protosteliomycetes* had only one family – Ceratiomyxaceae, with one species *Ceratiomyxa fruticulosa*. The largest order of Myxomycetes was Trichiales, with 9 species. Dominant families were Stemonitidaceae and Trichiaceae. *Lycogala epidendrum* was the most abundant species, with 13 records.

In the phylum Ascomycota, 95 non-lichenized species were recorded, belonging to 6 classes (Dothideomycetes, Pezizomycetes, Leotiomycetes, Orbiliomycetes, Sordariomycetes, Taphrinomycetes), 12 orders, 29 families, and 63 genera. The most represented classes were Leotiomycetes (32 species) and Pezizomycetes (31 species), with Pezizales being the most represented order. Dominant families in the phylum Ascomycota were Erysiphaceae (11), Xylariaceae (9), Pyronemataceae (7), Morchelaceae (7), and Helotiaceae (6). The most abundant species were *Xylaria polymorpha* (17 records), *Xylaria hypoxylon* (14 records), and *Sarcoscypha coccinea* (14 records).

LIST OF LEGATORS/COLLECTORS

SD – Savić Dragiša, JM/KM – Jarić/Karaman Maja, MM – Matavulj Milan, RD – Radnović Dragan, RP – Radišić Predrag, TA – Tepavčević Andrea, MM & MA – Maksimović M. & Mihajlović A, KI – Kadar Irenka, SA – Sopka Ana, VD – Vranješ D, KK – Kujundžić Kristina

CHECKLIST OF SPECIES

MYXOMYCOTA
PROTOSTELIOMYCETES
Protosteliales
Ceratiomyxaceae
1. *Ceratiomyxa fruticulosa* (O. F. Müll.) T. Macbr. – 17 (RD, 1995)
MYXOMYCETES
Liceales
Reticulariaceae
2. *Dictydiaethalium plumbeum* (Schumach.) Rostaf. ex Lister – 16 (SD, 2007)
3. *Lycogala epidendrum* (J. C. Buxb. ex L.) Fr. – 3 (RD, 1996), 6 (SD, 2004), 7 (MM & MA, 1996. x2), 10 (KI, 1989), 24 (JM, 1996; JM & SA, 1996), 16

(MM & JM, 1996), 17 (TA, 1993; TA, 1995; SD, 2002), 21 (RD & MM, 1995. x2)

4. *Lycogala flavofuscum* (Ehrenb.) Rostaf. – 28 (JM, 1996)
5. *Reticularia lycoperdon* Bull. – 16 (SD, 2007)

Physarales

Didymiaceae

6. *Didymium* sp. – 23 (SD, 2007)

Physaraceae

7. *Physarum* sp. – 26 (SD, 2007)
8. *Fuligo septica* (L.) F. H. Wigg. – 7 (RD, 1995), 17 (TA, 1994)

Stemonitales

Stemonitidaceae

9. *Comatricha laxa* Rost. – 11 (RD, 1996)
10. *Comatricha nigra* (Pers.) J. Schröt. – 17 (RD, 2007)
11. *Lamproderma* sp. – 22 (SD, 2008)
12. *Stemonitis axifera* (Bull.) T. Macbr – 7 (RD, 1995), 16 (SD, 2007)
13. *Stemonitis fusca* Roth – 7 (MM & MM, 1996)
14. *Stemonitis herbatica* Peck – 11 (RD, 1996)

Trichiales

Arcyriaceae

15. *Arcyria cinerea* (Bull.) Pers. – 10 (SD, 2008)
16. *Arcyria denudata* (L.) Wettst. – 6 (SD, 2008)
17. *Arcyria pomiformis* (Leers) Rostaf. – 10 (SD, 2007)

Trichiaceae

18. *Hemitrichia* sp. – 17 (SD, 2008)
19. *Hemitrichia serpula* (Scop.) Rostaf. – 17 (SD, 2010)
20. *Metatrichia vesparium* (Batsch) Nann.-Bremek. Ex G.W. Martin & Alexop. – 10 (SD, 2008)
21. *Trichia botrytis* (J. F. Gmel.) Pers. – 10 (SD, 2006), 17 (RD, 1995)
22. *Trichia favoginea* (Batsch) Pers. – 17 (SD, 2007)
23. *Tubifera ferruginosa* (Barsch) J. F. Gmel. – 27 (MM & MM, 1996)

ASCOMYCOTA (non-lichenized)

DOTHIDEOMYCETES

Capnodiales

Mycosphaerellaceae

24. *Mycosphaerella millegrana* (Cooke) J. Schrot. – 10 (SD, 2011)
25. *Ramularia rubella* (Bonord.) Nannf. – 24 (SD, 2011)
26. *Septoria urticae* Roberge ex Desm. – 24 (SD, 2011)

Incertae sedis

Polystomellaceae

27. *Dothidella ulmi* (C.-J. Duval) G. Winter – 23 (SD, 2011)

Incertae sedis

28. *Catinella olivacea* (Batsch.) Boud. – 4 (SD, 2008)

Pleosporales

Montagnulaceae

29. *Microsphaeropsis hellebori* (Cooke & Massee) Aa – 1 (SD, 2011)

Pleosporaceae

30. *Alternaria alternata* (Fr.) Keissl. – 18 (SD, 2011)

PEZIZOMYCETES

Pezizales

Discinaceae

31. *Gyromitra esculenta* (Pers.) Fr. – 17 (RP, 1984)
32. *Gyromitra infula* (Schaeff.) Quél. – 15 (SD, 2004)

Helvellaceae

33. *Helvella acetabulum* (L.) Quél. – 4 (SD, 2004), 7 (MM & MA, 1996. 2x)
34. *Helvella crispa* (Scop.) Fr. – 7 (MM & MA, 1996. 2x), 14 (SD, 2002; SD, 2005), 17 (TA, 1994; RP, 1996; SD, 2003), 23 (IA & RP, 1996)
35. *Helvella elastica* Bull. – 17 (TA, 1994; RP, 1996; SD, 2003)
36. *Helvella lacunosa* Afzel. – 7 (MM & MA, 1996. 2x), 23 (RP, 1996), 27 (RP, 1996)
37. *Helvella monachella* (Scop.) Fr. – 17 (TA, 1995)

Morchellaceae

38. *Disciotis venosa* (Pers.) Arnould – 10 (RP, 1990), 17 (SD, 2003), 12 (TA, 1995)
39. *Mitrophora semilibera* (DC.) Lév. – 24 (SD, 2004)
40. *Morchella costata* (Vent.) Pers. – 23 (SD, 2010)
41. *Morchella crassipes* (Vent.) Pers. – 4 (SD, 2006)
42. *Morchella vulgaris* (Pers.) Boud. – 19 (RP, 1984.; RP, 1985)
43. *Ptychoverpa bohemica* (Krombh.) Boud. – 21 (TA, 1994)
44. *Verpa conica* (O.F. Müll.) Sw. – 19 (RP, 1985)

Pezizaceae

45. *Peziza badia* Pers. – 7 (MM & MA, 1996), 10 (RP, 1990; KI, 1990)
46. *Peziza cerea* Sowerby – 21 (JM & RD, 1995)
47. *Peziza echinospora* P. Karst. – 10 (RP, 1990)
48. *Peziza repanda* Wahlenb. – 4 (SD, 2004)
49. *Peziza vesiculosa* Bull. – 10 (RP, 1990), 23 (RP, 1989)

Pyronemataceae

50. *Aleuria aurantia* (Pers.) Fuckel – 10 (SD, 2003)
51. *Humaria hemisphaerica* (F. H. Wigg.) Fuckel – 17 (TA, 1995; SD, 2005)
52. *Otidea alutacea* (Pers.) Massee – 8 (SD, 2005)
53. *Otidea onotica* (Pers.) Fuckel – 17 (TA, 1993; SD, 2003)
54. *Scutellinia scutellata* (L.) Lambotte – 6 (SD, 2002), 7 (MM & MA, 1996), 10 (KI, 1990), 15 (SD, 2005), 17 (TA, 1994; SD, 2004), 27 (MM & MA, 1996)
55. *Scutellinia umbrorum* (Fr.) Lambotte – 24 (JM & SA, 1996)
56. *Tarzetta catinus* (Holmsk.) Korf & J. K. Rogers – 24 (DM, 2001)

Sarcoscyphaceae

57. *Sarcoscypha coccinea* (Jacq.) Boud. – 6 (SD, 2002), 7 (JM & KK, 1997), 10 (KI, 1989, 1989, 1990. x2, RP, 1996), 16 (MM & JM, 1996. x2), 17 (TA, 1993; SA & VD, 1996; SD, 2002), 21 (JM & SA, 1997), 24 (JM & SA, 1996)

Sarcosomataceae

58. *Urnula craterium* (Schwein.) Fr. – 26 (SD, 2007)

Tuberaceae

59. *Tuber aestivum* Vittad.

60. *Tuber brumale* Vittad.
61. *Tuber melanosporum* Vittad.

LEOTIOMYCETES

Erysiphales

Erysiphaceae

62. *Erysiphe aquilegiae* DC. (host: *Ranunculus repens*) – 10 (SD, 2011)
63. *Erysiphe cichoracearum* var. *cichoracearum* DC. – 23 (SD, 2011) (host: *Sonchus oleraceus*), 10 (SD, 2011) (host: *Sonchus arvensis*), 10 (SD, 2011) (host: *Cirsium arvense*)
64. *Erysiphe convolvuli* var. *convolvuli* DC. – 23 (SD, 2011) (host: *Convolvulus arvensis*)
65. *Erysiphe depressa* (Wallr.) Schltdl. – 10 (SD, 2011) (host: *Arctium lappa*)
66. *Erysiphe platani* (Howe) U. Braun & S. Takam. – 23 (SD, 2011) (host: *Platanus occidentalis*)
67. *Erysiphe polygoni* DC. (host: *Polygonum aviculare*) – 23 (SD, 2011)
68. *Microsphaera berberidis* (DC.) Lév. (host: *Berberis thunbergii*) – 23 (SD, 2011)
69. *Microsphaera trifolii* (Grev.) U. Braun (host: *Galega officinalis*) – 1 (SD, 2011)
70. *Neoerysiphe galeopsidis* (DC.) U. Braun – 1 (SD, 2011) (host: *Glechoma hirsuta*), 1 (SD, 2011) (host: *Stachys silvatica*)
71. *Phyllactinia guttata* (Wallr.) Lév. – 23 (SD, 2011) (host: *Syringa vulgaris*)
72. *Sawadaea bicornis* (Wallr.) Homma (host: *Acer platanooides*) – 10 (SD, 2011)

Helotiales

Dermataceae

73. *Marssonina brunnea* (Ellis & Everh.) Magnus – 2 (SD, 2011)
74. *Marssonina salicina* Tehon – 2 (SD, 2011)
75. *Mollisia cinerea* (Batsch) P. Karst. – 17 (SD, 2007)

Bulgariaceae

76. *Bulgaria inquinans* (Pers.) Fr. – 7 (MM & MA, 1996), 17 (TA, 1994; RP, 1996), 24 (JM & SA, 1996), 26 (JM, 1996)
77. *Holwaya mucida* (Schulzer) Korf & Abawi – 16 (SD, 2007), 10 (SD, 2008)

Helotiaceae

78. *Hymenoscyphus calyculus* (Sowerby) W. Phillips – 10 (KI, 1989)
79. *Hymenoscyphus fructigenus* (Bull.) Gray – 14 (SD, 2005)

Hyaloscyphaceae

80. *Arachnopeziza aurata* Fuckel – 10 (SD, 2007)
81. *Lachnellula subtilissima* (Cooke) Dennis – 13 (SD, 2007)
82. *Lachnum virgineum* (Batsch) P. Karst. – 17 (SD, 2007), 5 (SD, 2007)
83. *Pezizella alniella* (Nyl.) Dennis – 17 (SD, 2009)

Incertae sedis

84. *Ascocoryne cylichnium* (Tul.) Korf – 6 (SD, 2008)
85. *Ascocoryne sarcoides* (Jacq.) J. V. Groves & D. E. Wilson – 10 (KI, 1989), 17 (SD, 2002)

86. *Bisporella citrina* (Batsch) Korf & S. E. Carp. – 4 (SD, 2003), 6 (SD, 2002; SD, 2004), 10 (KI, 1990; RD, 1995), 16 (MM & JM, 1996), 24 (JM & SA, 1996)
87. *Chlorociboria aeruginascens* (Nyl.) Kan. Ex Ram., Korf & L.R. Batra – 1 (SD, 2007. x2)

Leotiaceae

88. *Leotia lubrica* (Scop.) Pers. – 17 (RP, 1996), 19 (TA, 1994)

Rutstroemiaceae

89. *Rutstroemia bolaris* (Batsch) Rehm – 17 (SD, 2009)
90. *Rutstroemia firma* (Pers.) P. Karst. – 14 (SD, 2005)

Sclerotiniaceae

91. *Dumontinia tuberosa* (Bull.) L. M. Kohn – 10 (SD, 2007)

Rhytismatales

Rhytismataceae

92. *Propolis farinosa* (Pers.) Fr. – 5 (SD, 2007)
93. *Rhytisma acerinum* (Pers.) Fr. – 10 (SD, 2011)

ORBILIOMYCETES

Orbiliales

Orbiliaceae

94. *Orbilia sp.* – 17 (SD, 2007)

SORDARIOMYCETES

Coronophorales

Chaetosphaerellaceae

95. *Chaetosphaerella phaeostroma* (Durieu & Mont.) E. Müll. & C. Booth – 25 (SD, 2007)

Hypocreales

Clavicipitaceae

96. *Claviceps purpurea* (Fr.) Tul. – 24 (SD, 2008)
97. *Elaphocordyceps ophioglossoides* (Ehrh.) G. H. Sung, J. M. Sung & Spatafora – 16 (MM & JM, 1996)
98. *Epichloë typhina* (Pers.) Tul. & C. Tul. – 17 (SD, 2009)

Nectriaceae

99. *Nectria cinnabarina* (Tode) Fr. – 7 (MM & MA, 1996; JM & KK, 1996), 16 (MM & JM, 1996)
100. *Nectria coccinea* (Pers.) Fr. – 10 (SD, 2007)
101. *Nectria peziza* (Tode) Fr. – 12 (SD, 2007)

Incertae sedis

102. *Stilbella byssiseda* (Pers.) Seifert – 21 (SD, 2008)

Xylariales

Diatrypaceae

103. *Eutypella scoparia* (Schwein.) Ellis & Everh. – 17 (SD, 2007)
104. *Diatrype disciformis* (Hoffm.) Fr. – 1 (SD, 2005), 16 (MM & JM, 1996), 28 (JM, 1996)
105. *Diatrypella quercina* (Pers.) Cooke – 17 (SD, 2010)

Xylariaceae

106. *Biscogniauxia nummularia* (Bull.) Kuntze – 7 (JM & KK, 1996), 16 (MM & JM, 1996; SD, 2005), 21 (MM & RD, 1995; JM & SA, 1997), 28 (JM, 1996)

107. *Daldinia concentrica* (Bolton) Ces. & De Not. – 6 (SD, 2002)
108. *Hypoxylon fragiforme* (Pers.) J. Kickx f. – 10 (KI, 1990; KI, 1990), 16 (MM & JM, 1996), 17 (SA & VD, 1996; SD, 2002), 21 (JM & SA, 1997), 28 (JM, 1996)
109. *Hypoxylon howeanum* Peck – 16 (SD, 2007)
110. *Kretzschmaria deusta* (Hoffm.) P. M. D. Martin – 10 (KI, 1990; KI, 1990; RP, 1990), 21 (MM & RD, 1995. x2)
111. *Xylaria carpophila* (Pers.) Fr. – 17 (TA, 1994), 28 (JM, 1996)
112. *Xylaria hypoxylon* (L.) Grev. – 6 (Savić, D., 19.10.2004.), 10 (KI, 1989. x2, 1990. x3; RP, 1990; MM & JM, 1996), 15 (SD, 2002), 16 (MM & JM, 1996), 17 (TA, 1994; SA & VD, 1996), 21 (JM & SA, 1997), 24 (JM & SA, 1996)
113. *Xylaria longipes* Nitschke – 10 (KI, 1990; KI, 1990. x2), 16 (SD, 2007), 24 (JM & SA, 1996), 28 (JM, 1996)
114. *Xylaria polymorpha* (Pers.) Grev. – 6 (SD, 2002), 7 (MM & MA, 28.06. 1996. x2), 9 (RD, 1995), 10 (RP, 1988; KI, 1989. x2; KI, 1990. x4; RD, 1995), 17 (TA, 1994; RP, 1996), 12 (JM, 1995; RP, 1988), 26 (JM, 1996)

Diaporthales

Gnomoniaceae

115. *Asteroma impressum* Fuckel – 24 (SD, 2011)
116. *Gloeosporium carpini* (Lib.) Desm. – 24 (SD, 2011)

TAPHRINOMYCETES

Taphrinales

Taphrinaceae

117. *Taphrina deformans* (Berk.) Tul. – 20 (SD, 2011)
118. *Taphrina pruni* Tul. – 10 (SD, 2011)

DISCUSSION

Species presented in this checklist were collected from 28 different localities (Fig. 1). The most thoroughly investigated localities were Iriški venac (33 species recorded) and Paragovo (30), Paparatski do (15), Glavica (14), Stražilovo (14), Sr. Kamenica (12), and Popovica (11).

List of Myxomycota known in Serbia, compiled by Ivančević (2010), contains 92 species collected mostly in central and southern parts of country. Our contribution to the knowledge of Myxomycota diversity in Serbia, with 23 species listed in this paper is modest but valuable. Additional significance lies in the fact that so far no data were published for the region of Fruška Gora. Furthermore, two species (*Stemonitis herbatica* and *Arcyria pomiformis*) were new in Serbia when compared to Ivančević records (2010).

The only data from surrounding countries available to us (concerning Myxomycota) were those from Austria, Bulgaria, and Turkey (http://www.austria.mykodata.net; Fakirova et al., 2000; Sesli and Denchev, 2009). When we compared recorded Myxomycota species of Fruška Gora with those in Austrian Fungi Database, only three were common to both lists: *Dictydiaethalium plumbeum* with 15 records, *Hemitrichia serpula* with 76 records,

and *Tubifera ferruginosa*, (syn: *Stemonitis ferruginea* Ehrenb) with only one record. In the published checklist of Bulgarian side of mountain Stara planina (Central Balkan Mountain) 18 species and 7 genera of Myxomycota were listed. Like in our records, *Lycogala epidendrum* was the most abundant, which is predictable since this species has cosmopolitan distribution (S t e p h e n s o n et al., 2000). In the Turkish national fungal checklist 222 species of Myxomycota are noted within only 41 genera. Diversity of genera of Myxomycota in our checklist (15) was high when compared to the number of recorded species (23). Following of our data were in accordance with the Turkish checklist: *Ceratiomyxa fruticulosa* was the only species in the class Protosteliomycetes, *Lycogala epidendrum* was the most abundant species, and Stemonitidaceae was one of the dominant families. Species from our checklist which had no records in Turkey were *Dictydiaethalium plumbeum*, *Hemitrichia serpula,* and *Tubifera ferruginosa*. These species have wide distribution in both northern and southern hemisphere (http://data.gbif.org) hence these records could not be explained by the regional differences.

In the Database for Fruška Gora mountain, fungi belonging to the phylum Ascomycota are not as numerous as those of the phylum Basidiomycota – 108:443 (unpublished data). It seems that representatives of this large division of fungi are often overlooked during field investigations, due to their size or specific interest of investigators, and therefore neglected in checklists. In addition, Bulgarian checklist has 42 Ascomycota species and 319 Basidiomycota for the Central Balkan Mountain (F a k i r o v a et al., 2000). Turkish checklist contains 152 species of Ascomycota and 1822 species of Basidiomycota. In comparison with these data that relate to whole country territory, number of Ascomycota reported for the region of Fruška Gora mountain is significant. The most abundant species were *Xylaria polymorpha* (17 records), *Xylaria hypoxylon* (14 records), and *Sarcoscypha coccinea* (14 records); they are widely distributed and dominating species in others checklists and databases (I v a n č e v i ć, 1996; F a k i r o v a et al., 2000; D i m i t r o v a and G y o s h e v a, 2009; S e s l i and D e n c h e v, 2009; http://www.austria.mykodata.net).

Among the species of Ascomycota recorded on Fruška Gora mountain, *Morchella vulgaris* (morel) and *Tuber aestivum* (summer truffle) are on the list of wild species protected by law in Serbia (*The Regulation on designation and protection of strictly protected and protected wild species of plants, animals and fungi*); *Tuber aestivum* is also on the list of wild species protected from collecting activities, use, and trafficking (*The Regulation on putting under control the use and trade of wild flora and fauna*) (http://www.ekoplan.gov.rs/src/1-2-Pravilnici-288-document.htm). *Ptychoverpa bohemica* (DD) and *Verpa conica* (NT) are icluded in preliminary Red-list of Serbia (I v a n č e v i ć, 1998).

CONCLUSION

Database of Fungi in Fruška Gora Mountain includes 574 species of fungi (Ascomycota, Basidiomycota) and fungal analogues (Myxomycota), containing

over 2.000 records. Undoubtedly, these records represent just a part of total diversity of fungi in Fruška Gora and it is expected that the number of species should rise as the research continues and gets more organized. Many of the valuable, unknown, data are in the hands of hobbyists, enthusiasts from mycological societies and unknown individuals who spend the most time in nature and have a great passion, knowledge, and experience in field mycology. Future work on this Database should include them as well, of course with the guidance of professionals.

In conclusion, it should be stressed out that joint action and creation of integrated database of fungi is the first step on the way to better understanding of fungal distribution and population, which could result in a future protection of these unique organisms in Serbia and surrounding countries. Natural and semi-natural ecosystems are an indispensable basis for the conservation of biological diversity; it provides us with important data about living organisms that would be lost if these ecosystems were destroyed or converted to intensive use. This work supports and advocates further investigation of fungi in our region in the future.

REFERENCES

Austrian Mycological Society (2009): Database of fungi in Austria. Edited by Dämon, W., Hausknecht, A., Krisai-Greilhuber, I. – [http://www.austria.mykodata.net] [*Date of visit resp. access to database*].

Bisby, F. A., Roskov, Y. R., Orrell, T. M., Nicolson, D., Paglinawan, L. E., Bailly, N., Kirk, P. M., Bourgoin, T., Baillargeon, G., Ouvrard, D., eds (2011). Species 2000 & ITIS Catalogue of Life: 2011 Annual Checklist. Digital resource at: www.catalogueoflife.org/annual-checklist/2011/. Species 2000: Reading, UK.

Dennis, R. W. G. (1968): British Ascomycetes. Revised and enlarged edition of british cup fungi. Verlag von J. Cramer, by CHR. Belser, Stuttgart.

Dimitrova, E., Gyosheva, M. (2009): Bulgarian Pezizales: diversity, distribution and ecology. Phytologia Balcanica 15(1): 13–28.

Fakirova, V. I., Gyosheva, M. M., Denchev, C. M. (2000): Checklist of the macromycetes of Central Balkan Mountain (Bulgaria), 6. Simpozijum o flori jugoistočne Srbije i susednih regiona, Soko banja, 04.-07.2007., Srbija, 25–38.

Global Biodiversity Information Facility (GBIF). (n.d.). Available from: http://data.gbif.org

Gyosheva, M. M., Denchev, C. M., Dimitrova, E. G., Assyov, B., Petrova, R. D., Stoichev, G. T. (2006): Red list of fungi in Bulgaria. Mycologia Balcanica 3: 81–87.

Ing, B., Ivančević, B. (2000): A Checklist of Myxomycetes from the Former Yugoslavia. In: Nowotny W (ed.), Wolfsblut und Lohblüte. Lebensformen zwischen Tier und Pflanze, Stapfia 73, Landesmuseum, Linz.

Ivančević, B. (1995): Diverzitet makromiceta u Jugoslaviji sa pregledom vrsta od međunarodnog značaja. U: Stevanović, V., Vasić, V., (eds.), Biodiverzitet Jugoslavije sa pregledom vrsta od međunarodnog značaja, Biološki fakultet Univerziteta u Beogradu i Ecolibri, Beograd.

Ivančević, B. (1996): Mycological research on the mountain of Kopaonik. Ekologija 31(1): 45–53.
Ivančević, B. (1998): A preliminary Red List of the macromycetes of Yugoslavia. In: Perini, C. (ed.): conservation of fungi in Europe, pp. 57–61. Universita degli Studi, Siena.
Ivančević, B. (2002): Zabeležene vrste makromiceta u srbiji i Crnoj Gori do 1993. godine. World of Mushrooms 14: 7–11. Sr.
Ivančević, B. (2010a): Distribucija i ekologija plazmodijalnih gljiva (Myxomycota) u Srbiji. Magistarska teza, Biološki fakultet, Beograd. Sr.
Ivančević, B. (2010b): ECCF questionnaire for Serbia. European Council for the Conservation of Fungi, Newsletter 15: 22–23.
Karaman, M. (1997): Kartiranje gljiva Fruške Gore. Diplomski rad, Prirodno-matematički fakultet, Novi Sad. Sr.
Karaman, M., Matavulj, M., Novaković, M., Savić, D. (2005): Gljive Fruške gore. Zbornik apstrakata 8. Simpozijuma o flori jugoistočne Srbije i susednih regiona, Niš, 20.-24. 06.2005., Srbija, 22. Sr.
Kirk, P. (2012): Index Fungorum. CABI, CBS and Landcare Research. Available from: http://www.indexfungorum.org/
Kirk, P., Cannon, P. F., Minter, D. W., Stalpers, J. A. (2008): Ainsworth & Bisby's Dictionary of the Fungi, 10th edn. CAB International, Wallingford, UK.
Lisiewska, M., Jelić, M. B. (1971): Mycological investigations in the beech forests of some reservations in Serbia (Yugoslavia). Fragmenta Floristica et Geobotanica 8(1): 147–161.
Lukić, N. (2008): The distribution and diversity of Amanita genus in central Serbia. Kragujevac Journal of Science 30: 105–115.
Mehrotra, R. S., Aneja, K. R. (1990): An introduction to Mycology. John Wiley & Sons, Pte Ltd.
Ministarstvo životne sredine i prostornog planiranja. (2010). Pravilnik o proglašenju i zaštiti strogo zaštićenih i zaštićenih divljih vrsta; Pravilnik o prekograničnom prometu i trgovini zaštićenim vrstama. Dostupno na: http://www.ekoplan.gov.rs/src/1-2-Pravilnici-288-document.htm. Sr.
Ranković, B. (1955): Prilog poznavanju gljiva naših rezervata (Gljive Fruške gore). Zavod za zaštitu prirode i naučno proučavanje prirodnih retkosti Narodne Republike Srbije, Naučna knjiga, Beograd, 3–10. Sr.
Sesli, E., Denchev, C. M. (2009): Checklists of the myxomycetes, larger ascomycetes, and larger basidiomycetes in Turkey. Mycotaxon 106: 65–67.
Stephenson, S., Steven, L., Stempen, H. (2000): Myxcomycetes. A Handbook of Slime Molds, Timber Press, Portland. ISBN 0-88192-439-3.
Stevanović, V. (1995): Biogeografska podela teritorije Jugoslavije. U: Stevanović V, Vasić V, (eds.), Biodiverzitet Jugoslavije sa pregledom vrsta od međunarodnog značaja, Biološki fakultet Univerziteta u Beogradu i Ecolibri, Beograd. Sr.
Taylor, J. W., Spatafora, J., Berbee, M. (2012): Ascomycota. Sac Fungi. Version 09, October 2006 (under construction). In: The *Tree of Life Web Project*, Available from: http://tolweb.org/
Uzelac, B. (2009). Gljive Srbije i Zapadnog Balkana. BGV Logic, Beograd. Sr.

ПРЕЛИМИНАРНА ЛИСТА ГЉИВА ФРУШКЕ ГОРЕ – РАЗДЕЛИ MYXOMYCOTA И ASCOMYCOTA

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Резиме

Фрушка гора представља важан извор природних и полуприродних шумских екосистема у северном делу Србије, а самим тим и важан извор станишта за различите групе гљива. За разлику од добро организованих пројеката инвентаризације и мониторинга гљива који се већ дуго спроводе у већини европских земаља, миколошка истраживања у Србији су још увек спорадична и недовољно координисана од стране професионалних миколога. Циљ овог рада је био да се прикупе и у виду листе представе сви релевантни, до сада необјављени подаци о присуству гљива из раздела Myxomycota и Ascomycota на Фрушкој гори. У представљеној листи налазе се 23 врсте Myxomycota, распоређене у оквиру 2 класе, 5 редова и 7 породица. Прва класа (Protosteliomycetes) представљена је свега једном врстом – *Ceratiomyxa fruticulosa* (фам. Ceratiomyxaceae). Ред Trichiales је најзаступљенији (9 врста), а Stemonitidaceae и Trichiaceae су доминантне породице, свака са по 6 забележених врста. Најзаступљенија врста је *Lycogala epidendrum* (фам. Reticulariaceae), са 13 налаза. Раздео Ascomycota је представљен са 95 врста које су сврстане у 6 класа, 12 редова и 29 породица. Најзаступљеније класе су Leotiomycetes (са 32 врсте) и Pezizomycetes (са 31 врстом). Најзаступљеније врсте овог раздела су: *Xylaria polymorpha* (17 налаза), *Xylaria hypoxylon* (14 налаза) и *Sarcoscypha coccinea* (14 налаза).

КЉУЧНЕ РЕЧИ: Ascomycota, база података, гљиве, листа, Myxomycota, Фрушка гора

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FUNGI IN THE LEGISLATION OF THE REPUBLIC OF SERBIA

ABSTRACT: Conservation and protection of fungi have lately been considered as extremely important elements of the environmental conservation, and numerous environmental, scientific, medical, economic, cultural, ethical, and other reasons for such attitude exist today. This paper presents an overview of official regulations on the protection of fungi in the Republic of Serbia from the Act of Protection of 1991 until today. The paper lists and analyses the good and bad provisions of individual legal regulations. It registers the effects of the adopted regulations on the actual efficiency of protection of endangered species of fungi (macrofungi, mushrooms), and considers the impact of chronological development of legislation on the population of fungi in nature, and presents general measures to improve protection of mushrooms in the future. These measures primarily include reliable information and study of fungi as a basis for their effective protection based on scientific knowledge.

KEY WORDS: conservation, fungi, legal regulations, protection, Republic of Serbia

INTRODUCTION

The study of fungi and awareness of their unique position and ecological role in the environment came late as compared to plants and animals, although fungi comprise a very large and important group of organisms. It was not until the seventies of the twentieth century that it was finally accepted that they represented a separate group of organisms, taxonomically set aside in a separate Kingdom, and that they were substantially different from the plants with which they were usually grouped, as well as from the animals.

The ability to decompose dead organic matter and form symbiotic relations with a large number of vascular plants and parasitic species are dominant features of fungi that enable them to survive and to participate in their environment forming terrestrial ecosystems. Estimates indicate that between

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no less than 85% species (K i r k et al., 2001) and as many as 95% (B r u n - d r e t t, 1991) plant species form mycorrhiza with fungi. In the process of matter cycle, fungi are the dominant group capable of decomposing lignin and cellulose from plant residues, allowing the recycling of plant material and its re-usage in the biocenosis. Without the fungi in the forest, the masses of fallen leaves and dry branches would bury trees to the crowns in a relatively short time, and life would become impossible.

The notion that fungi were threatened, just as many other organisms on our planet, and that there was a risk of reduction of their numbers and disappearance of some of their species came late. It was only in the second half of the last century, during the 70-ies, that a trend of decrease in their numbers or even disappearance of some fungi species was noticed and reported, especially those related to complex and well-preserved ecosystems (J a n s e n & Ł a w r y - n o w i c z, 1991). Also observed at the time was a decrease in the number of species that were collected for food from nature. In the late eighties, after numerous reports of threats, international initiatives were launched to preserve mushrooms and subsequently the European Council for Conservation of Fungi – ECCF was formed. At that time, there already existed fully-fledged organizations and movements for protection of animal and plant species.

Generally, it is considered that the main reasons for categorization of mushrooms into the group of threatened organisms are the disappearance and contamination of their habitats, primarily due to human activities, such as pollution of the atmosphere, industrialized agriculture, unfavorable forestry practice, and anthropogenic alterations of large areas. All those issues lead to the degradation of fungal habitats. In addition, it is believed that uncontrolled and excessive commercial mass collecting of edible wild mushrooms in the limited space has long-term negative effects.

After substantial knowledge of vulnerability of macromycetes was collected, fungi slowly began to be incorporated into the programs of nature protection in the last decade of the twentieth century. A framework of actions that will address their conservation was becoming more and more formal and was recognized by some states to a greater or lesser extent. The need to introduce some kind of control because of massive collecting of edible mushrooms was also recognized in the Republic of Serbia around that time. The first regulations formally treating the collecting and trading of edible mushrooms in the territory of Serbia were adopted, attempting to regulate issues in this field. This was not conservation of fungi in the full sense of the word and it did not include what is now primarily considered as conservation and protection, relating essentially to the rare and threatened species that are vulnerable to a greater extent. Instead, this protection related to the commercial edible species that are usually very numerous. Nevertheless, the state administration recognized the need to establish some limitations on exploitation of at least a part of the population of mushrooms.

Because the preservation of fungi is an extremely important field, and there are numerous environmental, scientific, health, economic and other reasons that support this view, this paper presents an overview of legal provisions, especially

those regarding the protection of fungi (macromycetes) in nature. The main objective of this paper is a chronological review of regulations on the protection of fungi and the examination of the effects of enacted regulations on the population of mushrooms and improving their conservation in the future.

Other regulations which deal with macromycetes very indirectly, such as laws on forestry, national parks and similar, which govern nature conservation in general, but do not explicitly mention mushrooms, have not been considered. Neither was considered the laws governing other fields related to fungi, such as regulations in food industry related to mushrooms, regulations on the protection of materials, medical or pharmaceutical and related aspects, and similar.

RESEARCH ON FUNGI AS A BASIS OF THEIR PROTECTION IN SERBIA

The vital requirement for the preservation of fungi or any other organisms is the awareness of the existing problems, their thorough study, updated and satisfactory taxonomic inventories, and ecological and chorological research. Although mycological research data were collected for about a century in the Republic of Serbia, it was done randomly and non-systematically, as a result of individual enthusiasm rather than a result of systematically conducted researches and these data were not sufficient for making well-grounded decisions and regulations on the protection and preservation of mushrooms (I v a n č e v i ć, 1995).

Adequate protection of fungi can be established only when solid and reliable data are available, collected through systematic and long-term scientific studies. It is therefore necessary to make substantial investment in prior fundamental mycological research. Another necessary requirement for determining the state of endangered fungi is monitoring, specifically, monitoring of population size, their abundance, diversity, and distribution over a continued long period, using standardized methodology. Based on all data collected, a Red List of threatened fungi can be formed, preferably by using the generally accepted IUCN classification (IUCN, 2001). On the other hand, it is not wise to put off protective measures until such time as the optimum level of knowledge of mushrooms is reached (I v a n č e v i ć, 2001). Rather, general feasible measures ought to be taken based on general knowledge and experience from similar territories, relying on a greater experience and more researches (M a t a v u l j et al., 1998; M a t a v u l j and K a r a m a n, 2004).

Although the first data for a Red List of threatened mushrooms were published long ago (I v a n č e v i ć, 1993) and the first preliminary Red List of Serbian fungi was published by the end of the last century (I v a n č e v i ć, 1998), the opportunity to obtain for the Red List the status of an official, scientifically verified document that could be a basis for establishment and implementation of appropriate measures to preserve and protect fungi, has not been used in Serbia. In addition, a list of macromycetes was published that have the status of globally significant species in the territory of Serbia. The Serbian state has a special responsibility for these species, even though they are not endangered to a significant degree in the territory of Serbia (I v a n č e v i ć, 1995).

MATERIAL AND METHODS

For the overview of legal regulations on the protection of fungi in the Republic of Serbia, the legal provisions of the Republic of Serbia (laws and other regulations) relating to environmental protection were used:

- *Закон о заштити природе. Службени гласник Социјалистичке Републике Србије бр. 29, 1988*; [Nature Conservation Law, 1988]
- *Одлука о стављању под заштиту биљних врста као природних реткости. Службени гласник Социјалистичке Републике Србије 11, 17. 03. 1990*; [Decision on putting plant species under protection as natural rarities, 1990]
- *Одлука о изменама и допунама одлуке о стављању под заштиту биљних врста као природних реткости. Службени гласник СРС 49, 15. 08. 1991*; [Decision on amending the decision on putting plant species under protection as natural rarities, 1991]
- *Закон о заштити животне средине. Службени гласник Републике Србије 66/1991, 83/1992, 53/1993, 67/1993, 48/1994 и 53/1995*; [Environmental Protection Law, 1991]
- *Уредба о заштити природних реткости; Службени гласник Републике Србије 50, 09. 07. 1993*; [Regulation on the Protection of Natural Rarities, 1993]
- *Наредба о контроли коришћења и промета дивљих биљних и животињских врста. Службени гласник Републике Србије 50, 09. 07. 1993. и 36/1994*; [Directive on control of use and trade of wild plant and animal species, 1993]
- *Наредба о стављању под контролу коришћења и промета дивљих биљних и животињских врста. Службени гласник Републике Србије 16, 05. 04. 1996. и 44/1996;* [Directive on control of use and trade of wild plant and animal species, 1996]
- *Наредба о стављању под контролу коришћења и промета дивљих биљних и животињских врста. Службени гласник Републике Србије 17, 07. 04. 1999*; [Directive on control of use and trade of wild plant and animal species, 1999]
- *Закон о заштити животне средине. Службени гласник Републике Србије 135/2004 и 36/2009*; [Environmental Protection Law, 2004 – **Actual**]
- *Уредба о стављању под контролу коришћења и промета дивље флоре и фауне. Службени гласник Републике Србије 31/2005, 45/2005-испр., 22/2007, 38/2008, 9/2010*; [Regulation on putting the use and trade of wildlife under control, 2005 – **Actual**]
- Convention on the Conservation of European Wildlife and Natural Habitats – the Bern Convention (Republic of Serbia has signed and ratified this convention on 9 January 2008 and it began to be implemented from May 1 2008)

- *Закон о заштити природе. Службени гласник Републике Србије 36, 12.05.2009. i 88/2010*; [Nature Conservation Law, 2009 – **Actual**]
- *Правилник о проглашењу и заштити строго заштићених и заштићених дивљих врста биљака, животиња и гљива. Службени гласник Републике Србије 5, 05. 02. 2010*; [Regulation on the proclamation and protection of strictly protected and protected wild species of plants, animals and fungi, 2010 – **Actual**]

RESULTS AND DISCUSSION

LEGISLATIVE AND REGULATIVE ANALYSIS

Under the amendments to the Decision on putting plant species under protection as natural rarities (1990) based on Nature Conservation Law (1988), the following mushrooms have been listed as protected species since 1991: *Boletus edulis*, *Pleurotus ostreatus*, *Cantharellus cibarius*, all species of genera: *Morchella*, *Agaricus*, and *Lactarius*.

Environmental Protection Law (1991) does not include fungi. Furthermore, Regulation on the Protection of Natural Rarities (1993) does not include fungi, too.

Directive on control of use and trade of wild plant and animal species (1993) – mushroom species placed under control (protected): all species of *Morchella* and *Lactarius* genera, all edible species of the genus *Agaricus*, *Cantharellus cibarius*, *Craterellus cornucopioides*, *Boletus edulis*, *Amanita caesarea*, *Pleurotus ostreatus*, *Bovista nigrescens* and *Bovista plumbea*.

Directive on control of use and trade of wild plant and animal species (1996) – mushroom species placed under control (protected): all species of *Morchella* and *Lactarius* genera, all edible species of the genus *Agaricus*, *Cantharellus cibarius*, *Craterellus cornucopioides*, *Boletus edulis*, *Amanita caesarea*, *Pleurotus ostreatus*, *Bovista nigrescens* and *Bovista plumbea*.

Directive on control of use and trade of wild plant and animal species (1999) – the species of mushrooms placed under control (protected): *Agaricus* spp., *Boletus aereus*, *Boletus aestivalis*, *Boletus edulis*, *Boletus pinophilus*, *Bovista nigrescens*, *Bovista plumbea*, *Cantharellus cibarius*, *Craterellus cornucopioides*, *Lactarius deliciosus*, *Lactarius deterrimus*, *Lactarius salmonicolor*, *Lactarius sanguifluus*, *Lactarius semisanguifluus*, *Marasmius oreades* and *Pleurotus ostreatus*.

Environmental Protection Law (2004) and Regulation on putting the use and trade of wildlife under control (2005) – Under the latest amendments to this Regulation from 2010, the following species of mushrooms are protected by being placed under control: *Boletus aerreus, Boletus reticulatus, Boletus edulis, Boletus pinophilus, Cantharellus cibarius, Craterelluss cornucopioides, Lactarius deliciosus, Lactarius deterrimus, Lactarius salmonicolor, Lactarius sanguiifluus, Lactarius semisanguifuus, Marasmius oreades, Tuber magnatum* and *Tuber aestivum*.

Nature Conservation Law (2009) and Regulation on the proclamation and protection of strictly protected and protected wild species of plants, animals and fungi (2010) – list of strictly protected and protected fungal species:

Strictly protected fungal species:

Albatrellus ovinus
Amanita vittadinii
Battarrea phalloides
Boletus dupainii
Boletus impolitus
Boletus regius
Boletus rhodoxanthus
Boletus satanas
Catathelasma imperiale
Entoloma bloxamii
Fomitopsis rosea
Geastrum fornicatum
Geastrum melanocephalum
Geastrum schmidelii
Hapalopilus croceus
Hericium alpestre
Hericium cirrhatum
Hericium coralloides
Hericium erinaceus
Hygrocybe calyptriformis
Hygrocybe coccineocrenata
Hygrocybe punicea
Hygrophorus marzuolus
Leccinellum crocipodium
Leucopaxillus giganteus
Mutinus canninus
Myriostoma coliforme
Panaeolus semiovatus
Phallus hadriani
Phylloporus rhodoxanthus
Podoscypha multizonata
Polyporus umbellatus
Psilocybe serbica
Pycnoporellus alboluteus
Rhodotus palmatus
Sarcosphaera coronaria
Scutiger pes-caprae
Strobilomyces strobilaceus

Protected fungal species:

Amanita caesarea
Boletus aereus
Boletus edulis
Boletus pinophilus
Boletus reticulatus
Cantharellus amethysteus
Cantharellus cibarius
Cantharellus cinereus
Cantharellus friesii
Craterellus cornucopioides
Hydnum repandum
Hygrophorus russula
Lactarius deliciosus
Lactarius deterrimus
Lactarius salmonicolor
Lactarius sanguifluus
Lactarius semisanguifluus
Marasmius oreades
Morchella elata
Morchella esculenta
Morchella vulgaris
Russula cyanoxantha
Russula virescens
Tuber aestivum
Tuber macrosporum
Tuber magnatum

A review of the existing regulations may provide an insight into a few basic trends that have determined the approach to protection of fungi in Serbia. On one hand, the need to protect certain species of mushrooms was recognized in Serbia relatively early, already in the late eighties of the twentieth century. The rapid growth of interest in edible wild mushrooms led at that time to a significant increase of economic investments and financial flows related to the activities of organized collecting and purchase of wild mushrooms. For a while, the then Yugoslavia was the world's largest exporter of bolete mushrooms, a large share of which was collected in the territory of Serbia. There was a legitimate concern that the uncontrolled collecting of mushrooms in large quantities may lead to a decrease in their number and to their vulnerability.

On the other hand, the interest of the state administration was to place collecting of wild mushrooms under control in order to raise funds from the trade of wild mushrooms. Although there were expert draft proposals relating

primarily to fungi protection, that included, among other things, limiting the allowable amount that an individual can collect daily, mushroom pickers licensing, supervision of the amount of collected carpophores (fruiting bodies) and other measures that would enable monitoring of populations of macrofungi and their effective protection, they were not included in the adopted legislations. When they were eventually included, it was in a modified form or without tools that could enable control of their application. The role of the adopted measures was primarily to ensure a regular payment of taxes for the mushroom wholesale trade, and initially, to provide more favorable conditions of mushroom wholesale to the companies from Serbia by limiting administratively the maximum purchase price for the collected mushrooms. Thus the companies outside of Serbia were no longer able to offer a higher purchase price and thus obtain priority in wholesale. Allegedly, the low purchase price was supposed to make the picking of wild mushrooms unprofitable, and thus protect them from over-exploitation. Despite the early expressed concern for the protection of fungi, the precautionary protection measures turned out to be ineffective.

Based on the Nature Conservation Law from 1988, certain species of fungi were for the first time placed under protection in 1991, as "natural rarities threatened by exploitation and trade", under the Decision on amending the decision on putting plant species under protection as natural rarities (1991). In addition to a completely inadequate formulation of "natural rarities," comprising the species that were collected on a large scale for commercial purposes, mushrooms were considered as a plant species. The taxonomic nomenclature of these mushroom species contained grave mistakes. The regulation provided for two measures of fungi protection, including a ban on collecting young and underdeveloped fruiting bodies and a ban of harvesting more than 90% of a "total number" in the area of picking. In addition, it stipulated that mushroom collecting should not be performed at the waste dumping sites and near traffic junctions, which was supposed to protect the users of the collected mushrooms.

Measures aimed at control of mushroom collecting included an approval issued by the Serbian Institute for Environmental Protection subject to payment of an appropriate tax, and the obligation on part of the purchaser of wild mushrooms (legal or natural person) to submit data on purchased quantities of mushrooms to the Institute. Optionally, Article 4 of the Decision envisaged, in respect of the fungi listed as natural rarities, that "a program of protection and development will be adopted which will establish conditions for complete information and popularization of the protected natural rarities." It was not envisaged how to implement the control of two proposed measures of fungal protection, and expert proposals that involved additional measures of protection were not included in the regulations. However, even this flawed document was useful in terms of raising general awareness that mushrooms have the importance and place in the living world and that we cannot use them as an inexhaustible natural source without any restrictions.

After the Nature Conservation Law (1988), the Government of the Republic of Serbia adopted the Environmental Protection Law in 1991. This Law governed the protection of threatened plant and animal species that were still

designated as "natural rarities," which was an inadequate definition largely criticized by environmentalists and scientists who were experts on endangered species. Based on this Law from 1991, the Regulation on the Protection of Natural Rarities (1993) was adopted, but, unfortunately, the endangered species of fungi were not included, and their protection was omitted, although at that time there already existed data on the species of fungi that were endangered in Serbia (I v a n č e v i ć, 1993).

Mushrooms were still perceived in our public as a less important part of the plant kingdom and their unique and important role in nature was not understood. Based on Environmental Protection Law (1991), only the Directive on control of use and trade of wild plant and animal species (1993) was adopted, which included commercial species and largely reiterated the provisions of the previous Decisions on control of trade from 1991, perhaps otherwise phrased. Thus instead of the earlier ban on harvesting more than 90% of the existing specimens, the Directive provided that 10% of existing fruiting bodies was not allowed to be collected. The only novelty was Article 7, providing that the collecting may not be done in the same area every year and that a period of at least one year had to elapse before collecting may be resumed in the same area. However, the implementation of that provision was not mandatory if it was estimated that there was no need for such a measure. Unfortunately, there were no criteria and instruments for objective assessment. The list of included species was somewhat extended due to the interest to enable commercial collecting of species not covered by the previous Decision. Some of the errors in the nomenclature of these species were fixed but some still existed, which indicated a lack of cooperation of legislators and experts mycologists.

New Directive on control of use and trade of wild plant and animal species from 1996 brought nothing new and reiterated the earlier positions. The term "individual mushrooms" (in Serbian *"јединке гљива"*) was wrongly and consistently replaced by the term "identical mushrooms" (in Serbian *"једнаке гљиве"*) in several places, so Article 5. became confusing and meaningless. The provision about leaving a number of fruiting bodies in nature no longer specified the exact amount.

That was the time of the biggest disparity between the inadequate legal protection and the enormous pressure on nature and mushroom habitats, which became seriously endangered due to mass collecting of commercial species and numerous negative or indirect consequences of such collecting, including permanent removal of mushroom fruiting bodies from certain areas, soil compacting, intentional destruction of all other mushroom species, littering and pollution of the environment (I v a n č e v i ć, 1998b). The trade control included only fresh mushrooms while dried and processed mushrooms were not controlled and were exported to the Western markets in large quantities. Young immature specimens of bolete mushrooms, whose collecting was formally forbidden, were exported in brine. Table 1 shows quantities of some of the species that were traded in that period, based on the data from the Ministry for the environment.

Due to the alarming situation with the protection of fungi in Serbia, which was similar to that in some other countries of Southeast Europe, the European

Council for the Conservation of the Fungi expressed its concern at the meeting in 1997 in Vipiteno, Italy, and it was scheduled to hold an international scientific symposium ECCF at Tara Mountain in Serbia on 22-27 September 1998, with the participation of experts from Serbia. This meeting was cancelled at the last minute because of concerns of some participants because of the armed conflict in Kosovo, which escalated in that time. After the 1999 war, and the turbulent social upheaval that followed, ECCF offered an official advisory support to the Government of the Republic of Serbia in 2001, through the Directorate for Environmental Protection of the then Ministry of Health and the Environment (Anders Bohlin in lit.), but that offer was not accepted.

Tab. 1. – The quantities of mushrooms collected in the Republic of Serbia during 1993-1997

Year		The quantities of mushrooms purchased (in kg)					
		Boletus edulis	*Cantharellus cibarius*	*Craterellus cornucopioides*	*Morchella* spp.	*Lactarius* spp.	*Amanita caesarea*
	Requested	9 769 200	5 778 300	?	963 570	115 000	0
1993	Allowed	5 186 100	2 605 500	?	36 610	63 000	0
	Requested	15 688 600	6 545 700	167 500	127 900	82 000	17 000
1994	Allowed	1 212 981	631 004	18 800	1 800	40 000	0
	A priori	4 500 000	2 000 000	?	?	60 000	0
1995	Approval issued for	3 792 036	1 502 027	119 200	2 520	0	0
	A priori.	5 000 000	3 000 000	100 000	15 000	100 000	100 000
1996	Approval issued for	3 948 682	1 192 950	65 550	1 130	60000	5 000
1997	A priori	5 000 000	1 500 000	100 000	2 000	300 000	5 000

Legend: In 1993 and 1994, buyers applied for amounts of mushrooms for purchase ("Requested") and based on such applications they were allowed the maximum amount they could purchase from individual collectors ("Allowed"). The allowed amounts were determined based on the assessment after all applications were submitted. From 1995 onward, a competition was opened for the maximum amount of mushrooms that can be collected that year, determined in advance, at the very beginning of that year ("A priori"). The total amount that buyers were actually requesting was calculated at the end of the year ("Approval issued for"). Buyers were paying for the license for purchase regardless of whether or not they collected the requested amount of fungi. ? = Missing data.

In the meantime, because of many signals pointing to a bad situation of endangered mushrooms, in late 1998 in Serbia started work on new documents that were supposed to provide adequate protection for the commercial species of mushrooms, as well as for other species of fungi that were endangered. Therefore, some edible species that were relatively rare were planned to be included in the list of the endangered fungi, which, however, could be collected commercially, subject to a prior estimate and evaluation. As result, Directive on control of use and trade of wild plant and animal species (1999) was issued during the war and the devastating bombardments of Serbia by the NATO. That was the first document to list fungi separately from the plants. The nomenclature of species' names was corrected. Finally, some provisions

on how to protect endangered species were included in the text – the way of picking, keeping accurate records of the amounts of collected mushrooms. Unfortunately, it was not done in the form proposed by the consulted mycologists, thus Article 8 prescribed that the "…fruiting bodies should be collected in the container that allows ventilation for dissemination of the spores." Proper packaging serves for conserving the quality of harvested mushrooms, and dissemination of spores during transport is a phenomenon that, in our opinion, does not affect the protection of mushrooms*.

This provision was copied from the regulations of the countries in the region that were published at that time (P i r m a n, 1994), probably due to a lack of understanding of foreign experience on part of the lawgivers. Furthermore, the form of the approved quantities of wild mushrooms that were allowed to be collected was specified for the first time, *i.e.* whether they were fresh or dried mushrooms (weight ratio 10:1). The reports on the collected amounts were required for the first time to indicate the site where the mushrooms were picked and to keep track of collected quantities of protected species for monitoring purposes. Consequently, this regulation finally brought some positive changes, though not all that was needed. (Earlier, the purchaser had to provide general information on the amounts collected and sold). Picking more than 66% (two thirds) fruiting bodies in the area of collecting was prohibited. Members of the genus *Morchella* were no longer among the protected species, since they were intended to be covered by other regulations on endangered species. However, the state of war and subsequent social changes delayed the adoption of such regulations for a decade.

The actual Environmental Protection Law was adopted in 2004 and based on this Law a new Regulation on putting the use and trade of wildlife under control (2005) was passed. Positive innovations in this Regulation included the provisions on the procedures for collecting hypogeal species of mushrooms, as well as inclusion of two species of genus *Tuber* in the list of protected mushrooms. This Regulation without significant alterations applies even today. The unnecessary provision on packaging related to ventilation to enable spore dissemination still exists in the text, which shows how difficult it is for mycologists to exert influence on lawmakers.

The actual Nature Conservation Law, (2009), the first since 1988, was adopted in 2009. This Law introduced many new solutions, because of the desire to be aligned with the EU regulations. Article 59 defined which parts of that law, currently inactive, would begin to apply upon the accession of the Republic of Serbia to the European Union. Mushrooms were listed as a separate group of organisms, different from and on a par with plants and animals. Under Article 27, protected natural goods also included protected species, which could have the status of a protected or a strictly protected species. Protection measures for strictly protected species finally allowed inclusion of rare and endangered species of wild mushrooms, in addition to the commercial

* Packaging that allows dissemination of spores was referred to in the first version of Slovenian regulation on protection of wild mushrooms from 1994 (*Uradni list RS 38/94*) but was excluded from the text in the next version of 1998 (*Uradni list RS 57/98*).

species. A large number of Articles of the Law provided for the protection of species' habitats, as the necessary requirement of protection of the very species. This allowed introduction of new, more effective conservation measures. Regulation on the proclamation and protection of strictly protected and protected wild species of plants, animals and fungi (2010) was published in accordance with this Law. The list contained 38 strictly protected fungi and 26 protected fungal species. It was not ensured, in accordance with the Law, that the lists of protected species should be formed based on the Red List, or well-documented studies, instead, the species were defined arbitrarily, in a very short time, which later resulted in problems and criticism of experts for certain groups of organisms.

The Nature Conservation Law (2009) provided for the protection and preservation of nature, previously was governed by the still applicable Environmental Protection Law (2004). Therefore, with regard to wild mushrooms, this led to parallelisms and inconsistencies. The Regulation on the Control of Trade (2005) has a "senior" position and originates from an earlier period than the Regulation on Protected Species (2010), and provisions of these regulations do not refer one document to the other. The Environmental Protection Law (2004), which was used for preparing the Regulation on putting the use and trade of wildlife under control (2005), does not recognize the new Nature Conservation Law (2009) as it was accepted much earlier. The Nature Conservation Law (2009) does not include ordinances from the Regulation on putting the use and trade of wildlife under control (2005) which was prepared according to the older Environmental Protection Law (2004), so one subset of species protection is regulated according to the old Environmental Protection Law (2004) and another by the new Nature Conservation Law (2009). In this way both laws are broken by the same activity but the supervising inspection services are not having any evidence. The nomenclature of scientific names in these two laws is different, as well as some of the vernacular names used for the same species in the simultaneously applicable regulations prepared according to these different laws.

In addition to domestic legislation, there are obligations originating from the international conventions signed by Serbia that have obligatory character. The Bern Convention, which protects the flora, fauna and habitats of species in Europe, came into force in Serbia in mid 2008. Mushrooms have not yet been officially included in the lists of species covered by the Bern Convention, primarily for administrative and political reasons, and their protection under the provisions of the Bern Convention is not mandatory in Serbia. The list of fungal species that have been proposed for inclusion in the Bern Convention is now in the form of an official proposal confirmed by the Standing Committee of the Bern Convention. On this basis, the Council of Europe adopted a Recommendation on the conservation of wild mushrooms in Europe whose implementation by signatory countries is desirable (Recommendation 132; 2007).

The Recommendation invited the countries to define management and maintenance of habitats as a priority with the aim of protecting the European species of mushrooms; to take into account the Directive of the European Council to protect European macromycetes and to apply it when developing and

implementing their national policies to protect macromycetes; and to include those who have profit from wild mushrooms in the protection mushroom habitats. This presented a powerful tool for correction of national legislation, relating to the protection of the wild mushrooms. Unfortunately, the public, experts as well as competent authorities and institutions are poorly acquainted with this Recommendation that applies to the Republic of Serbia as well. In the first half of 2011, the Council of Europe demanded a national report on the implementation of this recommendation, and this was the first opportunity to analyze the contribution of and the possibilities of acting in accordance with the Recommendation in Serbia.

A project for making a revised version of the Red List of fungi, with a detailed evaluation of their vulnerability factors, was offered to the state authorities in 2007 (I v a n č e v i ć, et al., 2007), but its implementation has not been approved so far. Meanwhile, Article 36 of the Nature Conservation Law (2009) provided that: "The species that are or may become endangered shall be protected as strictly protected wildlife, or protected wildlife. The species protected under this law shall be determined on the basis of national and international Red Lists or Red Books, professional findings and scientific knowledge." The same Article provided that the Red Book or Red List may be adopted by the Ministry of Environmental Protection. Consistent application of these legal provisions, once they are enforced, should provide a scientific basis for protection measures and help align Serbian legislation with the legislation of the countries that have had more developments in this field.

CONCLUSION

The first regulations on the protection of fungi in Republic of Serbia were adopted in 1991. They were related to several edible wild species that were collected for commercial purposes. The aim of adopted measures was protection of wild mushrooms against excessive collecting and the threat that it might bring, but in practice, they secured collecting of revenue for the state from the use and trade in wild mushrooms. Subsequently, during the last twenty years, new regulations were adopted several times, but only with minor changes, while the basic purpose remained the same, and provisions that would ensure protection based on advanced experience of other countries and on scientific data were not incorporated in the legislation, although it was possible. The initial positive effect of such regulations, which showed to the public the threat to wild mushrooms, was lost over the years, and even turned into the opposite, based on the opinion that when something was paid for (tax for collecting wild mushrooms) then it may be fully disposed of without much regard. The effect of the prescribed measures on wild mushroom protection was not significant and did not prevent the removal of huge amounts of fruiting bodies from nature in certain territories, accompanied with habitat disturbance and a number of harmful side effects.

The first major changes occurred with the adoption of the Law on Nature Protection in 2009, which finally placed under protection the rare and endan-

gered species of fungi and their habitats, in addition to the commercial species. Owing to the provisions of this Law, the first study was drafted with the aim of protecting an area exactly because it was a habitat of strictly protected species of wild mushrooms. The proposed protected area, located on Ada Ciganlija near Belgrade, had the size of 21 ha. At the time of submission of this paper to print, the procedure for official declaration of protection was in the final stage. Only a formal final decision on declaration was missing, which would make the Republic of Serbia one of the first countries in Europe to protect a fungal habitat, in accordance with the recommendations of the Bern Convention. Therefore, the application of these legal provisions is expected to bring developments to the adequate protection of fungi in the Republic of Serbia and to have a positive effect on populations of endangered species.

When the actual Nature Conservation Law (2009) and bylaws were adopted, the existing errors and omissions were not removed, and the legal provisions on the election and proclamation of protected species were not fully observed, therefore it is necessary to do so in the future. Other regulations should also be amended, especially the Law on Environmental Protection, and other regulations dealing with the protection of fungi. They should also be brought in line with one another. The evolution of legislation concerning wild mushroom protection in Serbia has become closer to the stage when acceptable and more effective modes of protection are being prescribed, but it took unnecessarily too long, and changes that would allow the optimum state of affairs are yet to be undertaken.

REFERENCES

B r u n d r e t t, M. (1991): Mycorhizas in natural ecosystems. *Adv. Ecol. Res.* 21: 171–313.

I v a n č e v i ć, B. (1993): Recent status of investigation and conservation of fungi in Yugoslavia, including potential species for the Provisional red list of Yugoslav fungi. *Lectures on the III Meeting of European Council for Conservation of Fungi*, Neuchatel, Suisse.

I v a n č e v i ć, B. (1995): Diversity of macromycetes in Yugoslavia with an overview of species of international significance. In: Stevanović V and Vasić V (eds.): *Biodiversity of Yugoslavia with an overview of species of international importance.* Faculty of Biology and Ecolibri, Belgrade. (In Serbian)

I v a n č e v i ć, B. (1998a): A preliminary Red List of the macromycetes of Yugoslavia. In: Perini C (ed.): *Conservation of fungi in Europe*, Università degli Studi, Siena, pp. 57–61.

I v a n č e v i ć, B. (1998b): Conservation of fungi in Yugoslavia. In: Perini C (ed.): *Conservation of fungi in Europe*, Università degli Studi, Siena, pp. 51–56.

I v a n č e v i ć, B. (2001): Review and analysis of legal measures for the protection of fungi in Serbia. *Book of Abstracts of the Symposium on Protection of Fungi of South-eastern Europe*. Igalo. (in Serbian)

I v a n č e v i ć, B. (2005): Review of the Fungi Conservation in Serbia. *European Council for the Conservation of Fungi Newsletter,* 14: 39–40.

I v a n č e v i ć, B., S a v i ć, S., N i k š i ć, M. (2007): Predlog projekta izrade Crvene liste gljiva Srbije i evaluacija faktora njihove ugroženosti. The project proposal of the preparation of

Red List mushrooms of Serbia and evaluation of their vulnerability factors. *Ministry of Environment of the Republic of Serbia*. Belgrade. (in Serbian)

K i r k, P. M., C a n n o n, P. F., D a v i d, J. C., S t a l p e r s, J. A. [eds] (2001): *Ainsworth and Bisby's Dictionary of Fungi, Ninth Edition*. CABI Publishing, Wallingford.

IUCN (2001) IUCN Red List Categories and Criteria: Version 3.1. *IUCN Species Survival Commission. IUCN,* Gland, Switzerland and Cambridge, UK. II + 30 pp.

J a n s e n, A. E., Ł a w r y n o w i c z, M. [eds] (1991): Conservation of Fungi and Other Cryptogams in Europe. *Łódź Society of Sciences and Arts*, Łódź.

M a t a v u l j, M., K a r a m a n, M., R a d n o v i ć, D., B o k o r o v, M. (1998): Dugačka "Crvena lista ugroženih" ili kratka "Bela lista neugroženih" vrsta gljiva. Long "Red list of threatened" or short "White list of non-threatened" species of mushrooms. *Zaštita prirode – Nature protection,* 50:169–174. (in Serbian)

M a t a v u l j, M., K a r a m a n, M. (2004): "Crvena lista ugroženih" ili "Bela lista neugroženih" vrsta gljiva kao oruđe u ekološkom menadžmentu. "Red List of Threatened" or "white list of non-threatened" fungi as a tool in environmental management. *Proceedings of the First Conference "Systems Environmental Management"*, Novi Sad, «ZORA-XXI», 1: 148–154. (in Serbian)

P i r m a n, I. (1994): Ograničeno sabiranje pečuraka. Limited collecting mushrooms. *Galenika holding company*, Belgrade-Zemun, 38 (4): 11 (in Serbian).

ГЉИВЕ У ЗАКОНОДАВСТВУ РЕПУБЛИКЕ СРБИЈЕ

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Резиме

Очување и заштита гљива изузетно су важна област и за то постоје многобројни еколошки, научни, здравствени, економски, културни и други разлози. Приказан је преглед прописа који се баве заштитом гљива у Србији, почев од акта заштите из 1991. Наведене су и анализиране добре и лоше одредбе појединачних прописа. Сагледани су ефекти донетих прописа на стварну ефикасност заштите угрожених врста гљива, процењено је какав утицај хронолошки развој законодавства има на популације гљива у природи и размотрене су опште мере ради побољшања заштите гљива у будућности. Те мере пре свега подразумевају добро познавање и проучавање гљива као основу за њихову ефикасну заштиту утемељену на научним сазнањима.

КЉУЧНЕ РЕЧИ: гљиве, законска регулатива, заштита, очување, Република Србија

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MYRTUS COMMUNIS AND *EUCALYPTUS CAMALDULENSIS* CYTOTOXICITY ON BREAST CANCER CELLS

ABSTRACT: *In vitro* cytotoxicity of methanol, ethyl acetate, *n*-buthanol, and water extracts of *Myrtus communis* L. and *Eucalyptus camaldulensis* Dehnh. was examined against two human breast cancer cell lines (MCF 7 and MDA-MB-231) using MTT and SRB assays. The results showed significant cytotoxic potential of examined extracts, with IC50 values ranging from 7 to 138 μg/ml for *M. communis* and 3-250 μg/ml for *E. camaldulensis*. The two plants generally expressed similar activity, and no significant difference in cell line's sensitivity towards extracts was observed. The results indicate to *M. communis* and *E. camaldulensis* as candidates for thorough chemical analyses for identification of active compounds, and eventually for attention in the process of discovery of new natural products in the control of cancer.

KEY WORDS: cytotoxicity, *Eucalyptus camaldulensis* Dehnh., MCF 7, MDA-MB-231, *Myrtus communis* L.

INTRODUCTION

Molecules derived from natural sources, such as plants, play a dominant role in the discovery of conventional drugs for the treatment of most human diseases, and therefore represent a basis of modern medicine (Jones et al., 2006). A large number of plant extracts have been screened for cytotoxic effects against cancer cell lines over the last thirty years, which have resulted in some significant drugs being introduced. In addition, it was shown that some extracts are cytotoxic and selective, either between different cancer cell lines or between cancer and non-cancer cell lines, and act principally by inhibiting cell proliferation, but by different mechanisms (da Rocha et al., 2001).

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Myrtus communis L. (myrtle) and *Eucalyptus camaldulensis* Dehnh., both belonging to the Myrtaceae family, grow throughout the Mediterranean region (Grbović et al., 2010; Mimica-Dukić et al., 2010). Myrtle's leaves and fruit are traditionally used as antiseptic, disinfectant, and hypoglycemic agents (Elfellah et al., 1984). *M. communis* extracts have been confirmed to exhibit antimicrobial (Mansouri, 1999; Mansouri et al., 2001; Bugarin, 2010) and antioxidative effects (Bugarin, 2010; Tuberoso, 2010), while its essential oil showed considerable antioxidant and antimutagenic effects (Mimica-Dukić et al., 2010). It is prominent that myrtle plants could be a promising source of natural antioxidants, anti-genotoxic, antimutagenic and, perhaps, chemopreventive agents (Mimica-Dukić et al., 2010). Several species of *Eucalyptus* are also used in traditional medicine, and are reported as agents with analgesic, anti-inflammatory, and antimicrobial properties (Silva et al., 2003; Williams et al., 1998). Crude organic extract of *E. camaldulensis* has been proven to exhibit both antimicrobial and gastroprotective activities (Adeniyi et al., 2006), while its essential oil showed antibacterial, antioxidant and antimutagenic effects (Grbović et al., 2010). The presence of flavonoids and tannins, which are known to have antibacterial and antifungal properties, is confirmed in organic extracts of *E. globulus*, which in addition revealed antimicrobial activity (Egwaikhide et al., 2008). However, anticancer activity of both *Myrtus* and *Eucalyptus* species has been poorly investigated, and their complete toxicity profile has yet to be determined.

In this study, the *in vitro* cytotoxicity of different extracts of *M. communis* and *E. camaldulensis* was examined against MCF 7 and MDA-MB-231 human breast cancer cell lines using MTT and SRB cytotoxicity assays.

MATERIALS AND METHODS

Plant material and preparation of extracts: Plant extracts were prepared from dry leaves of the species *Myrtle communis* L. and *Eucalyptus camaldulensis* Dehnh., collected at the Montenegro coastline (Tivat) in August 2004. Air-dried, finely ground leaves samples were extracted by maceration with 70% methanol (8 ml per 1 g of drug) during 48h at room temperature. After filtration, solvent was evaporated in vacuum; crude residue was dissolved in hot distilled water and washed exhaustively with petrol ether to remove ballast compounds (lipids and pigments). A part of washed extract was concentrated in vacuum to dryness, and dissolved in DMSO giving final concentration of 10 mg/ml ("methanol extract"). The rest of the washed extract was partitioned with chloroform, ethyl acetate, and *n*-butanol, in order to obtain chloroform, ethyl acetate, *n*-butanol, and water soluble fractions. All of the fractions were concentrated in vacuum and dissolved in DMSO (10 mg/ml).

Cell culture and treatment: Plant extracts were evaluated for their *in vitro* cytotoxicity towards two human breast adenocarcinoma cell lines: MCF 7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative). Cell culturing and experimental procedure were conducted as previously de-

scribed (Jevtović et al., 2010; Karaman et al., 2009). The cells were grown in Dulbecco's Modified Eagle's Medium with 4.5% of glucose (DMEM, PAA Laboratories) supplemented with 10% fetal calf serum (FCS, PAA Laboratories) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded into 96-well microtiter plates at the density 5000 cells/0.1 ml/well to ensure their exponential growth throughout the experimental period. After 24h, cells were exposed in triplicates to serial of extracts' dilutions (50-1.56 μg/ml) dissolved in dimethyl sulfoxide (DMSO, Sigma) for 72h. The final concentration of DMSO did not exceed 0.5%, and did not cause any background response in the bioassay. Control and cell-free blank wells were included in each plate. Following incubation, cytotoxicity assays were conducted.

MTT test: The principle of MTT test is based on cellular reduction of the soluble yellow MTT tetrazolium salt (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) to a blue formazan product by mitochondrial dehydrogenases in viable cells. The intensity of the blue color formed by this reaction is a criterion for cell viability. Following incubation with plant extracts, the medium was removed and cells were incubated for 3h with 0.05mg/0.1ml/well of MTT (Sigma) dissolved in serum-free DMEM. After that, the medium was removed, formazan salts were dissolved in 0.1 ml/well of 0.04 M HCl in isopropanol, and light absorbtion was measured using a plate reader (Thermo-Labsystems) on 540 nm, with reference wavelength 690 nm.

SRB test: SRB test is a colorimetric assay, based on the ability of the protein dye sulforhodamine B (SRB) to bind to protein basic amino acid residues of trichloroacetic acid (TCA)-fixed cells. The incorporated dye can be solubilized for measurement, and the results are linear with cell number (Voigt, 2005). Following incubation with plant extracts, the medium was removed and the cells were fixed in TCA (0.025 ml of 50% w/v TCA per well) for 1 h at 4°C, washed five times with distilled water, air-dried, and stained with 0.05 ml of 0.4% SRB in 1% acetic acid for 30 min. Then, the cells were washed five times with 1% acetic acid to remove the unbound dye and air-dried. The incorporated dye was solubilized in 10 mM TRIS (pH 10.5) for 5 min, and light absorption was measured using a plate reader (ThermoLabsystems) on 492 nm, with reference wavelength 690 nm.

Calculation of cytotoxicity and IC50: In both tests, cell cytotoxicity was calculated as a percentage of corresponding control value (non-treated cells) obtained in a minimum of three independent experiments. The half-maximal inhibitory concentration values (IC50), defined as the concentration that inhibits 50% of cell growth, were calculated from concentration-response curves. IC50 values were expressed as the mean of a minimum of three repeated experiments performed for each plant extract.

RESULTS AND DISCUSSION

It has been previously reported that compounds exhibiting cytotoxic effects in cell lines can demonstrate distinct kinetic profiles that fit into three

categories: acute (< 1h to full toxicity), subacute (1–40h), and long term (>40h) (X i a et al., 2008). Guided by these findings, exposure time of 72h in our study has been selected to allow the expression of full cytotoxic potential of investigated plant extracts.

All *M. communis* and *E. camaldulensis* extracts exhibited cytotoxic effects on MCF 7 and MDA-MB-231 cell lines, and the results were confirmed both by MTT and SRB assay. At the highest tested concentration (50 μg/ml), cytotoxicity of organic extracts/fractions was in the range from 40% to as high as 98%, while for water fraction it was in the range of 30-70%. The effects were dose dependent and based on dose-response curves IC50 values were determined (Table 1). In the case of *M. communis*, ethyl acetate and *n*-butanol fractions exhibited the highest antiproliferative effect on both cell lines with IC50 values ranging from 7 to 25 μg/ml. Total methanol extract and water fraction induced less pronounced cytotoxicity, with IC50 values ranging from 14 to 138 μg/ml. Overall pattern of the results obtained for *E. camaldulensis* extracts also indicate to ethyl acetate and *n*-butanol fractions as the most cytotoxic (IC50 5-41 μg/ml). It can also be noticed that general level and pattern of cytotoxicity of all investigated extracts is rather similar for both tested plants.

Further commenting on obtained IC50 results and antiproliferative potency of tested plant extracts requires a precaution due to a noticeable difference between the IC50 values obtained by the two applied cytotoxicity assays. Namely, IC50 values determined by SRB assay are in all cases lower than ones calculated from MTT assay. This dissimilarity is especially pronounced in the case of treatment of MDA-MB-231 cells by *E. camaldulensis* extracts, with the range of difference from 7 to 78 times. MTT and SRB assays are basically used for the assessment of cellular chemosensitivity and are related to the total cell number, therefore giving a relative measure of survival (R i s t i ć - - F i r a et al., 2008). The reason for a discrepancy in our results probably lies in a difference in the sensitivity of applied cytotoxicity assays and targets which they reflect, since they measure distinct biological parameters in living cells. The same cell line could have poor capacity to reduce MTT, and at the same time could show high values of absorbance in the SRB assay, all because SRB assay does not depend on enzymatic activity but on protein content of the cells. In contrast to MTT testing, SRB staining is less sensitive to environmental fluctuation such as variations in pH or depletion of glucose, and is independent of intermediary metabolism, which are all the factors that may be influenced by the test substances (L i n et al., 1999). In addition, for the study of growth kinetics, the SRB assay is more suitable because of the higher sensitivity and better linearity with cell number (K e e p e r s et al., 1991).

In any case, when comparing our results with the findings from the similar studies, antiproliferative potency of *M. communis* and *E. camaldulensis* on the two human breast cancer cell lines is evident. Aqueous acetone extract of *E. camaldulensis* exhibited a dose-dependent growth inhibitory effect on MCF 7 cell line, with recorded IC50 value of 36.5 μg/ml (S i n g a b et al., 2011). Comparable effect on A2780 human ovarian cancer cell line was reported for methylene, hexane, and dichloromethane fruit extracts of *E. camaldulensis*, with

Tab. 1 – IC50 values (μg/ml) of different extracts of *Myrtus communis* and *Eucalyptus camaldulensis*, obtained by MTT test and SRB test on MCF 7 and MDA-MB-231 human breast cancer cell lines.

Extract/fraction	MCF 7 cell line		MDA-MB-231 cell line	
	MTT test	SRB test	MTT test	SRB test
Myrtus communis				
Methanol	40.1	16.0	55.0	18.7
Ethyl acetate	21.8	12.6	25.1	7.2
n-Butanol	22.4	15.5	23.5	6.9
Water	50.5	28.6	138.0	14.1
Eucalyptus camaldulensis				
Methanol	67.2	12.5	147.5	5.7
Ethyl acetate	26.7	7.9	34.4	4.9
n-Butanol	40.3	11.0	41.3	4.9
Water	86.5	21.6	250.7	3.2

The results are presented as the mean of three independent experiments performed in triplicate for each cytotoxicity assay.

IC50 values of 17.2-17.5 μg/ml, while leaf extract had IC50 value of 19.3 μg/ml (Topçu et al., 2011). Ethanolic extract of plants used in Thai traditional medicine showed high cytotoxic activity against MCF 7 cells (IC50 = 31-35 μg/ml), but water extract showed no cytotoxic effect (Sakpakdeejaroen and Itharat, 2009). Methanolic extract of *E. camaldulensis* also revealed marked toxicity (IC50 = 20.7 μg/ml) on human bladder carcinoma cell line as determined by MTT test (Al-Fatimi et al., 2005). On the other hand, leaves extract of another plant from *Myrtaceae* family expressed very low cytotoxic potency on MCF 7 cells (IC50 = 820±190 μg/ml), and were no cytotoxic to MDA-MB-231 cells as determined by MTT test (Kaileh et al., 2007).

Chemical constituents responsible for the cytotoxic activities detected in our study might be speculated. Among the investigated plant extracts, total methanol extract of both *M. communis* and *E. camaldulensis* was chemically analyzed. The results revealed high levels of plant phenols, especially flavonoids, flavonol-3-O-glycosides, flavonol-7-O-dyglicosides, phenol carbolic acids and their derivatives, rutin, elagic acid and their derivatives, etc. (Bugarin, 2010; Grbović, 2010). It is shown that some flavonoids exert cytotoxic effects towards human lung embryonic fibroblasts and human umbilical vein endothelial cells by increasing level of intracellular reactive oxygen species (Matsuo et al., 2005). In addition, structure-cytostatic activity relationship was shown after treatment of Rhesus monkey kidney and rat glial tumor cells by various flavonoids (Sánchez et al., 2001). All these results indicate that the compounds identified in methanol extract from our study might be responsible for its cytotoxicity. Other plant extracts were not chemically analyzed, but certain conclusions about type of compounds responsible for their effect might be presumed according to the results of comparable studies. In one of them, the cytotoxic activity of leaf organic extracts of *E. camaldulensis* was attributed to

triterpenoids, mostly urosolic and oleanolic acids (T o p ç u et al., 2011). Globulocin A and eucaglobin isolated from leaves of *E. globules* had antioxidant, anti-inflammatory, and anti-melanogenesis activity (H a s e g a w a et al., 2008). Moreover, it was shown that myrtucommulone, a unique nonprenylated acylphloroglucinol contained in the leaves of *M. communis*, acts as a strong inducer of apoptosis selectively for cancer cells with lower cytotoxicity for normal non-transformed cells (T r e t i a k o v a et al., 2008). In addition, all these, or structurally similar substances, could also present active compounds in extracts from our study.

It is known that *in vitro* cytotoxicity can be cell-type specific (X i a et al., 2008; S á n c h e z et al., 2001). Regarding sensibility of the two cell lines (MCF 7 and MDA-MB-231) towards investigated plant extracts, our results did not reveal significant difference in a cellular response. Only in the case of *E. camaldulensis* extracts, results of SRB test could indicate a greater sensitivity of MDA-MB-231 cell line. However, since this finding is not confirmed by MTT test, no conclusions on difference in sensitivity could be drawn. The same cell lines were used in testing of medicinal plant extracts in other study, and they exhibited different sensitivity towards the plant extracts (S t e e n k a m p and G o u w s, 2006). There is also an example where MCF 7 and MDA-MB-231 cell lines, depending on a tested plant extracts, had either very similar or a different sensitivity as determined by MTT test (K a i l e h et al., 2007).

CONCLUSION

In conclusion, examined extracts of *M. communis* and *E. camaldulensis* exhibit considerable cytotoxic potential for MCF 7 and MDA-MB-231 human breast cancer cell lines. The two plants can be recommended for thorough chemical analyses for identification of active compounds and eventually for attention in the process of discovery of new natural products in the control of cancer.

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REFERENCES

A d e n i y i, B. A., L a w a l, T. O., O l a l e y e, S. B. (2006): *Antimicrobial and gastroprotective activities of Eucalyptus camaldulensis (Myrtaceae) crude extracts*, J. Biol. Sci. 6: 1141–1145.

Al-Fatimi, M., Friedrich, U., Jenett-Siems, K. (2005): *Cytotoxicity of plants used in traditional medicine in Yemen*, Fitoterapia 76: 355–358.

Bugarin, D. (2010): *Antioxidant, antimicrobial and antimutagenic potential of the Myrtus communis L*. PhD dissertation, University of Novi Sad, Novi Sad.

da Rocha, A. B., Lopes, R. M., Schwartsmann, G. (2001): *Natural products in anticancer therapy*, Curr. Opin. Pharmacol. 1: 364–369.

Egwaikhide, P. A., Okeniyi, S. O., Akporhonor, E. E., Emua, S. O. (2008): *Studies on bioactive metabolites constituents and antimicrobial evaluation of leaf extracts of Eucalyptus globulus*, Agricultural Journal 3: 42–45.

Elfellah, M. S., Akhter, M. H., Khan, M. T. (1984): *Anti-hyperglycemic effect of an extract of Myrtus communis in streptozotocin-induced diabetes in mice*, J. Ethnopharmacol. 11: 275–281.

Grbović, S. (2010): *Chemical features and biological activities of the E. camaldulensis Dehnh. and E. gunnii Hook. from Montenegro*. PhD dissertation, University of Novi Sad, Novi Sad.

Grbović, S., Orčić, D., Couladis, M., Jovin, E., Bugarin, D., Balog, K., Mimica-Dukić, N. (2010): *Variation of essential oil composition of Eucalyptus camaldulensis (Myrtaceae) from Montenegro coastline*, APTEFF 41: 151–158.

Hasegawa, T., Takano, F., Takata, T., Niiyama, M., Ohta, T. (2008): *Bioactive monoterpene glycosides conjugated with gallic acid from leaves of Eucalyptus globulus*, Phytochemistry 69: 747–753.

Jevtović, V. S., Pelosi, G., Ianelli, S., Kovačević, R. Z., Kaišarević, S. (2010): *Synthesis, structural studies and biological activity of a dioxovanadium(V) complex with pyridoxal semicarbazone*, Acta. Chim. Slov. 57: 363–369.

Jones, W. P., Chin, Y. W., Kinghorn, A. D. (2006): *The role of pharmacognosy in modern medicine and pharmacy*, Curr. Drug Targets 7: 247–264.

Kaileh, M., Berghe, W. V., Boone, E., Essawi, T., Haegeman, G. (2007): *Screening of indigenous Palestinian medicinal plants for potential anti-inflamatory and cytotoxic activity*, J. Ethnopharmacol. 113, 510–516.

Karaman, M., Kaišarević, S., Somborski, J., Kebert, M., Matavulj, M. (2009): *Biological activities of the lignicolous fungus Meripilus giganteus (Pers.: Pers.) Karst*, Arch. Biol. Sci. 61: 853–861.

Keepers, Y. P., Pizao, P. E., Peters, G. J., Van Ark-Otte, J., Winograd, B., Pinedo, H. M. (1991): *Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for in vitro chemosensitivity testing*, Eur. J.Cancer 7: 897–900.

Lin, Z. X., Hoult, J. R. S., Raman, A. (1999): *Sulphorhodamine B assay for measuring proliferation of a pigmented melanocyte cell line and its application to the evaluation of crude drugs used in the treatment of vitiligo*, J. Ethnopharmacol. 2: 141–150.

Mansouri, S. (1999): *Inhibition of Staphylococcus aureus mediated by extracts from Iranian plants*, Pharm. Biol. 5: 375–377.

Mansouri, S., Foroumadi, A., Ghaneie, T., Najar, A. G. (2001): *Antibacterial activity of the crude extracts and fractionated constituents of Myrtus communis*, Pharm. Biol. 39: 399–401.

Matsuo, M., Sasaki, N., Saga, K., Kaneko, T. (2005): *Cytotoxicity of flavonoids toward cultured normal human cells*, Biol. Pharm. Bull. 28: 253–259.

Mimica-Dukić, N., Bugarin, D., Grbović, S., Mitić-Ćulafić, D., Vuković--Gacić, B., Orčić, D., Jovin, E., Couladis, M. (2010): *Essential oil of Myrtus communis L. as a potential antioxidant and antimutagenic agents*, Molecules 15: 2759–2770.

Ristić-Fira, A. M., Petrović, I. M., Korićanac, L. B., Valastro, L. M., Privitera, G., Cuttone, G. (2008): *Assessment of the inhibitory effects of different radiation qualities of chemotherapeutic agents on a human melanoma cell line*, Phys. Medica 24: 187–195.

Sakpakdeejaroen, I., Itharat, A. (2009): *Cytotoxic compounds against breast adenocarcinoma cells (MCF-7) from Pikutbenjakul*, J. Health Res. 23: 71–76.

Sánchez, I., Calendrón, J., Ruiz, B., Tellez, J., Caldaza, L., Taboada, J. (2001): *In vitro cytotoxicity of flavonoids against MK2 and C6 tumour cells*, Phytother. Res. 15: 290–293.

Silva, J., Abebe, W., Sousa, S. M., Duarte, V. G., Machado, M. I. L., Matos, F. J. A. (2003): *Analgesic and anti-inflammatory effects of essential oils of Eucalyptus*, J. Ethnopharmacol. 89: 277–283.

Singab, A. N., Al-Sayed, E., Martiskainen, O., Sinkkonen, J., Pihlaja, K. (2011): *Phenolic constituents of Eucalyptus camaldulensis Dehnh., with potential antioxidant and cytotoxic activities*, Rec. Nat. Prod. 5: 271–280.

Steenkamp, V., Gouws, M. C. (2006): *Cytotoxicity of six South African medicinal plant extracts used in the treatment of cancer*, S. Afr. J. Bot. 72: 630–633.

Topçu, G., Yapar, G., Türkmen, Z., Gören, A. C., Öksüz, S., Schilling, J. K., Kingston, D. G. I. (2011): *Ovarian antiproliferative activity directed isolation of triterpenoids from fruits of Eucalyptus camaldulensis Dehnh*., Phytochem. Lett. 4: 421–425.

Tretiakova, I., Blaesius, D., Maxia, L., Wesselborg, S., Schulze-Osthoff, K., Cinatl, Jr J., Michaelis, M., Werz, O. (2008): *Myrtucommulone from Myrtus communis induces apoptosis in cancer cells via mitochondrial pathway involving caspase-9*, Apoptosis 13: 119–131.

Tuberoso, C. I. G., Rosa, A., Bifulco, E., Melis, M. P., Atzeri, A., Pirisi, F. M., Desse, M. A. (2010): *Chemical composition and antioxidant activities of Myrtus communis L. berries extracts*, Food Chem. 123: 1242–1251.

Voigt, W. (2005): *Sulforhodamine B assay and chemosensitivity*, Methods Mol. Med. 110: 39–48.

Williams, L. R., Stockley, J. K., Yan, W., Home, V. N. (1998): *Essential oils with high antimicrobial activity for therapeutic use*, Int. J. Aromather. 4: 30–40.

Xia, M., Huang, R., Witt, K. L., Southall, N., Fostel, J., Cho, M-H., Jadhav, A., Smith, C. S., Inglese, J., Portier, C. J., Tice, R. R., Austin, C. P. (2008): *Compound Cytotoxicity Profiling Using Quantitative High-Throughput Screening*, Environ. Health. Perspect. 116: 284–291.

ЦИТОТОКСИЧНОСТ *MYRTUS COMMUNIS* И *EUCALYPTUS CAMALDULENSIS* НА ЋЕЛИЈАМА КАНЦЕРА ДОЈКЕ

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Резиме

Испитивана је *in vitro* цитотоксичност метанолског, етил-ацетатног, *n*-бутанолског и воденог екстракта *Myrtus communis* and *Eucalyptus camaldulensis* на две хумане ћелијске линије канцера дојке (MCF 7 и MDA-MB-231) помоћу два теста цитотоксичности (MTT и SRB). Резултати су показали значајан цитотоксичан потенцијал испитиваних екстраката, са IC50 вредношћу у опсегу од 7 до 138 μg/ml за екстракте *M. communis* и 3-250 μg/ml за екстракт *E. camaldulensis*. Обе биљке су показале сличну активност, а није показана ни значајна разлика у сензитивности две ћелијске линије на испитане биљне екстракте. На основу добијених резултата, *M. communis* and *E. camaldulensis* се могу препоручити за темељну хемијску анализу у циљу идентификације активних једињења, а такође и као биљке које би могле имати значај у изучавању нових природних продуката у контроли канцера.

КЉУЧНЕ РЕЧИ: *Eucalyptus camaldulensis* Dehnh., MCF 7, MDA-MB-231, *Myrtus communis* L., цитотоксичност

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EFFECT OF LEAD CONTAMINATION OF MAIZE SEED ON ITS BIOLOGICAL PROPERTIES

ABSTRACT: Effect of treatment of seed with various lead concentrations (0, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} mol/dm^3) on accumulation and distribution of lead (Pb) in seedling, seed germination, seedling growth, and mobilization of mineral matter during seed germination was investigated.

Content of Pb in the root and the shoot indicates that seeds imbibed in solutions of various Pb concentrations took up Pb intensively. Content of Pb in the root and the shoot increased with increase of Pb concentration and it was much larger in the root than in the shoot. Contrary to this, the accumulation coefficient was greater in the shoot than in the root.

Treatment of seed with Pb did not significantly affect its biological properties. Increase of Pb concentration decreased germination ability, germination energy, and percentage of typical seedlings, while increasing the number of atypical seedlings and non-germinated seeds.

Contamination of seed by Pb did not affect the dry matter mass and the growth of young plants shoots, while the length of the primary root, the mesocotyl root as well as the root mass at the highest Pb concentration, significantly decreased.

Translocation of mobilized mineral matter from the seed during germination and growth of young plants into the root and shoot was specific, depending on elements. Only the implementation of the highest implemented Pb concentration affected mobilization and translocation of some elements.

Based on the obtained results, it can be concluded that maize is characterized by significant tolerance to Pb contamination during seed germination and growth of seedlings.

KEY WORDS: germination, growth, lead imbibition of grain, maize, translocation of mineral elements

INTRODUCTION

Lead (Pb), unlike some other heavy metals, is not essential for higher plants and other organisms. At higher concentrations, Pb is highly toxic to man, animals and plants, which is why it is considered a dangerous pollutant. Pb pollution sources are in the first place transportation means, industry and its products, which use Pb in production technology. This heavy metal enters the

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food chain mainly through plants, which is why it is very important to understand its accumulation and distribution in plants and its effect on physiological and biochemical processes in plants (K a b a t a - P e n d i a s and P e n d i a s, 2000). Even more so, since Pb remains in biological systems for a long period, which significantly increases its toxicity and probability of entering the food chain.

Number of experiments established unfavorable effects of high Pb concentrations on life processes in plants. It was determined that Pb inhibits seedling growth (L a n e and M a r t i n, 1977), cell elongation (L a n e et al., 1978) and photosynthesis (Kastori et al., 1998), causes interruption of mitosis (W i e r z b i c k a, 1988), affects the water regime (K a s t o r i et al., 1996) and mineral nutrition of plants (C s e h et al., 2000). Toxicity of heavy metals arises, among other things, from their ability to bond with enzymes, which alters their activity (V a s n A s s h e and C l i j s t r e s, 1990). During germination enzyme activity is very intensive, complex organic compounds are being degraded and at the same time new compounds are being synthesized and mineral matter mobilized. Having all this in mind, we saw the need to examine effect of contamination of seed by Pb on its biological properties, seed germination, growth of young plants and mineral matter mobilization during germination.

MATERIAL AND METHODS

The experiment was carried out using the seed of NS 7016 maize hybrid. Enrichment of seeds with Pb was completed by their imbibition in solutions with various concentrations of $PbCl_2$ (0, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} mol Pb/dm^3) at 22^{o}C during 24 hours. Following of the water uptake dynamics showed that at 22^{o}C during 24 hours the water content in the seed reaches 37%, which is sufficient for germination and enables maximal introduction of Pb into the seed tissues. After imbibition, seeds were rinsed with distilled water. Seeds were kept in thermostat for germination for 4 days at 25^{o}C. Afterwards, biological value of seed was examined, according to the standard procedure published in Official Gazette of SFRY (Službeni list SFRJ 47/87). Investigated properties were germination ability, germination energy, representation of typical and atypical seedlings and share of non-germinated seeds. After seven days, we measured length of the shoot, primary root and the mesocotyl root. The shown values represent the average of 20 measurements.

Plant material was rinsed with deionized water before drying. Drying was performed to constant mass, after which it was determined the dry matter mass of the shoot and the root. Afterwards, the plant material was homogenized and grinded to powder, prepared for the analysis. Plant material was digested using cc HNO_3 + cc H_2O_2. The content of mineral matter was determined by ICP. The nitrogen content was determined by Kjeldahl method.

Distribution of Pb represents the ratio of Pb in the shoot and the root and its total content in the young plant. The accumulation coefficient was determined according to Duvigneaud (L a r c h e r, 1995).

Test results were statistically processed by calculating the smallest difference between arithmetic means.

RESULTS AND DISCUSSION

The maize seed took up Pb very intensively, which is verified by its high content in the root and the shoot of young plants (Tab. 1). The obtained results show that Pb ions go more or less undisturbed through the seed coat and the semi-permeable membranes of the seed cells. The Pb content in dry matter in unpolluted conditions span from 0.1 to 10 μg/g of dry matter, with an average of 2 μg/g (C a n n o n, 1976). S i l l a n p a a and J a n s s o n (1992) investigated the Pb content in different edaphic conditions in 30 countries in young wheat and maize plants, and they determined that its content spans from 0.2 to <1 μg/g of dry matter, while in some countries it was >1 μg/g. According to K a b a - t a - P e n d i a s and K a b a t a (2000), the Pb content in cereal grain in different countries spanned from 0.01 to 2.28 μg/g of dry matter. The Pb content in the root and the shoot of untreated seed determined by these investigations confirm findings of the mentioned authors, while the Pb content in the root was much higher than in the shoot. In the root, Pb mostly accumulates in the apoplast. The xylem sap is characterized by high Pb content, where it is bonded to organic acids, in the first place citric acid. In the root, Pb is also intensively bonded to functional groups of xylem elements. There is a high positive correlation between Pb content in the nutrient medium and plants (K a b a t a - P e n d i a s and W l a c e k, 1985), which is also confirmed by these results. With increase of Pb concentration, its content in the root and the shoot also increased significantly, especially at the highest Pb concentration seed treatment.

Tab. 1 – Content, distribution, and accumulation coefficient of lead in young maize plants at various treatments of seed with lead

Organ	Treatment (mol Pb/dm^3)				
	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}
	Content (μg Pb/g DM)				
Shoot	0.29	2.07	2.00	9.31**	33.59**
Roots	3.76	4.54	5.40**	19.34**	182.67**
	Distribution (%)				
Shoot	9.15	35.10	34.21	38.40	18.80
Roots	90.85	64.90	66.79	61.60	81.20
	Coefficient of accumulation				
Shoot	0.00	7.13	6.89	32.10	115.82
Roots	0.00	1.21	1.44	5.14	48.58

With mobilization of nutrient compounds of the seed during germination, there was also intensive translocation of Pb into formed organs. Taking up and translocation of Pb also depends on its form. Taking up Pb is more intensive from nitrate than from sulphate or carbonate form. At implementation of an organic compound (Pb-tetraalkyl), translocation in the spring wheat grain was very intensive. The Pb content was 20 μg/g of dry matter (D i e h l et al., 1983). In this paper Pb was implemented in inorganic form, as a highly solu-

ble salt $PbCl_2$ that enabled intensive taking up of Pb by the treated seed and its translocation during germination into newly formed organs. It was observed that when Pb was uptaken through the root from the nutrient medium, its accumulation in the root was much higher than in the above-ground part (Lane and Martin, 1977, Janjatović et al., 1991, Kastori et al., 1998). Zimdahl (1975) states that only 3% of Pb is translocated from the root to the above-ground parts. Reasons for limited translocation of Pb from the root to the above-ground parts were investigated by several authors in the past. Malone et al. (1974) state that in maize plants Pb is primarily accumulated in dictyosome vesicles, which merge and close the Pb deposition. Afterwards, the vesicles move from the cytoplasm to the outer side of the plasmalemma, bond to the cell wall, where Pb is than being accumulated. The Pb accumulation in the root is explained, among other, by its anatomic built. It was noticed that Pb is accumulated around endodermis, which is why it is considered a barrier to apoplastic Pb transport. This barrier is not, however, total but partial, to which points the fact that closer to the root top in the protophloem there is more Pb than in the same tissue at a larger distance from the root top (Lane and Martin, 1977). Due to the specific built of the endodermis, according to some authors, there is a noticeable difference in Pb accumulation between monocotyledonous and dicotyledonous plants (Miller, 1977).

Significantly higher accumulation of Pb in the root, established in this test, cannot be explained only by its built or accumulation in the cell wall, since Pb arrived to the root and the shoot of young plants by its mobilization from the seed. During germination, products of decomposition of organic and mineral matter from the seed partially go to the environment, which creates possibility for the root of the young plant to uptake Pb from the environment, which could partially explain a higher accumulation of Pb in the root. Plants are characterized by various mechanisms of protection from the unfavorable effects of high concentrations of heavy metals (Arsenijević-Maksimović et al., 2001). It is possible that accumulation of Pb in the root is a form of protection mechanism from accumulation in the above-ground parts of the plant.

Treatment of seed by Pb did not significantly affect its biological value (Tab. 2). With the increase of Pb concentration, a tendency of decrease of germination ability, germination energy and typical seedlings is observed, as well as and increase of share of atypical seedlings and non-germinated seeds. However, the obtained differences in the mentioned parameters were not statistically significant. It could be because Pb mostly remained in the peripheral parts of the seed and did not reach the germ, causing its effect to be expressed later, to which point the results given in the Tab. 3.

With the increase of Pb concentration, the length of the primary root, mesocotyl root and the root dry mass decreased, while it did not significantly affect the seedling (Tab. 3). This difference in effect on certain organs can be explained by the difference in Pb accumulation, which was higher in the root than in the shoot. Generally, it can be said that toxic concentrations of heavy metals decrease the growth of the root to a greater extent than the growth of the aboveground parts. The decrease of the root length can be explained by the

Tab. 2 – Biological properties of the seed treated with various concentrations of lead

Parameters	Concentration of Pb (mol/dm^3)				
	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Typical seedling (%)	96.75	95.50	95.00	94.75	93.75
Atypical seedling (%)	2.25	2.75	4.50	4.00	4.75
Non-germinated seed (%)	1.00	1.75	0.50	1.25	1.25
Germination energy (%)	97.00	95.00	95.00	95.00	94.00
Germination ability (%)	97.00	95.00	95.00	95.00	94.00

effect of this element on cell elongation and cell division (L a n e and M a r t i n, 1977, L a n e et al., 1978). Sensitivity of certain plant species to presence of higher Pb concentrations varies. According to tests performed by D i e h l et al. (1983), in wheat the grain yield is significantly decreased when Pb concentration in straw reaches 45 mg/kg of dry matter, while in spinach which is considered a species very sensitive to Pb, already at concentration of 10-15 mg/kg of dry matter the yield of leaves decreases (J u d e l and S t e l t e, 1977). Reaction of certain plant species to higher concentrations of Pb depends on the Pb/Ca concentration ratio in the environment. Having our results in mind, it may be concluded that maize in early phases of growth is characterized by high tolerance to toxic concentrations of Pb. Higher concentrations of heavy metals, among which Pb, can induce protein synthesis, phytochelatin, which was found in maize seedlings as well (S z i g e t i, 1998). In the tissue culture there was detected forming of peptides with the sequence $(\gamma\text{-Glu-Cys})_{24}$-Gly. It is assumed that peptides containing SH-groups bond Pb in the cell and therefore protect the enzymes containing similar functional groups. Lead is not essential for higher plants. There is, however, data that indicates that lower concentrations of Pb can show stimulating effect on plant growth (L a n g e r w e r f f et al., 1973). B r o y e r et al. (1972) state that if plants need Pb, its sufficient concentration is 2 to 6 ppb. It has been established that treating seed with high Ni concentration reduces biological value of the seed, as well as the growth of the primary root (D o r o g h á z i et al., 2010).

Lower applied concentrations of Pb have not significantly affected mobilization of mineral matter during seed germination, which is why only the values obtained using higher concentrations will be shown (Tab. 4). Content of all investigated elements except P and S in the root, and N, P, K and Mo in

Tab. 3 – Effects of seed treatment with various lead concentrations on growth and mass of young maize plants

Parameters	Concentration of Pb (mol/dm^3)				
	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Shoot length (cm)	10.13	10.26	9.90	10.49*	9.85
Primary root length (cm)	12.11	11.80	11.70	11.91	11.20*
Mesocotyl root length (cm)	9.20	9.03	8.89	9.37	8.61**
Shoot dry mass (mg)	28.30	27.00	28.10	27.80	28.70
Root dry mass (mg)	36.50	33.10	39.50	36.50	29.90**

the shoot, in Pb treated seeds was lower than in the control. Presence of higher concentration of Pb in the seed reduced to a greater extent only mobilization of Ca, Na, and Cu. Lead can decrease and in some cases also increase uptake and accumulation of certain elements. In young cucumber plants, uptake of K, Ca and Fe significantly decreased in presence of $PbCl_2$ (S z i g e t i, 1998).

Tab. 4 – Effects of seed treatment with lead on concentration and distribution of macro- and microelements in shoots and roots of maize

Macro--nutrients (mg/kg DM)	Plant part	Treatment Pb (mol/dm^3)		Micro--nutrients (µg/g DM)	Plant part	Treatment Pb (mol/dm^3)	
		0	10^{-2}			0	10^{-2}
N	Sh	3610.1	3880.0	Fe	Sh	164.10	163.30
	R	1940.0	1930.0		R	240.30	226.25
P	Sh	617.0	634.2	Zn	Sh	47.51	42.57
	R	314.9	328.4		R	52.72	50.82
K	Sh	1323.8	1419.8	Cu	Sh	5.73	3.38
	R	825.5	823.8		R	7.52	5.70
S	Sh	263.4	235.8	Mn	Sh	14.10	12.73
	R	216.0	220.6		R	57.92	53.33
Mg	Sh	114.6	119.0	Mo	Sh	0.19	0.23
	R	277.8	266.4		R	1.26	1.20
Ca	Sh	141.8	132.4				
	R	1042.0	955.4				
Na	Sh	63.4	53.4				
	R	504.7	479.1				

Sh-Shoot; R-Roots

Remobilization of elements from certain parts of the seed during germination varies. Out of the total content of mineral matter in the seed, the seed coat contains 55-57%, except for S, which is around 40% (M o u s s a v i - N i k et al., 1997).

Distribution of certain elements during seed germination was specific. Elements that constitute organic matter, N, P, S, and K that does not yield organic compounds, accumulated more significantly in the shoot than in the root. Other investigated elements, Cu, Zn and especially Ca, Mg, Fe, Na, Man and Mo were present in the root at a much greater extent than in the shoot. Intensive remobilization of K to the shoot during germination was also established by M o u s s a v i - N i k et al. (1997).

REFERENCES

A r s e n i j e v i ć - M a k s i m o v i ć, I., J e l i ć, M., L o m o v i ć, S., K a s t o r i, R. (2001): Genetic and physiological bases of plant tolerance to high concentrations of heavy metals and aluminium. In: Quarrie S et al. (ed.) Genetic and Breeding of Small Grains. Agricultural Research Institute Serbia. Beograd, 377–405.

Broyer, T. C., Johnson, C. N., Paull, R. E. (1972): Some aspects of lead in plant nutrition. Plant Soil 36: 301–308.
Cannon, H. L. (1976): Lead in vegetation. In: Lovering TG (ed.) Lead in the Environment. U.S. Geol. Surv. Prof. Pap. 957: 23–35.
Cseh, E., Fodor, F., Varga, A., Záray, G. (2000): Effect of lead treatment on the distribution of essential elements in cucumber. J. Plant Nutr. 23: 1095–1105.
Diehl, K. H., Rosopulo, W., Kreuzer, W., Judel, G. K. (1983): Das Verhalten von Bleitetraalkylen in Boden und deren Aufnahme durch die Pflanze. Z. Pflanzenernähr. Bodenkunde 146: 551–559.
Dorogházi, O., Kastori, R., Maksimović, I. (2010): Nickel translocation from seed during germination and growth of young maize plants. Proc. Nat. Sci, Matica Srpska Novi Sad 119: 17–25.
Janjatović, V., Kastori, R., Petrović, N., Knežević, A., Kabić, D. (1991): Uticaj olova na morfološko-anatomsku građu biljaka kukuruza (*Zea mays* L). Zbornik Matice srpske za prirodne nauke 80: 121–129.
Judel, G. K., Stelte, W. (1977): Gefaesversuche mit Gemüsepflanzen zur Frage der Bleiaufnahme aus dem Boden. Z. Pflanzenernähr. Bodenkunde 140: 421–429.
Kabata-Pendias, A., Pendias, H. (2000): Trace Elements in Soil and Plants. CRC Press, Boca Raton.
Kabata-Pendias, A., Wlacek, K. (1985): Excessive uptake of heavy metals by plants from contaminated soil. Soil. Sci. Ann. 36: 33–37.
Kastori, R., Petrović, N., Gašić, O., Janjatović, V. (1991): Uticaj olova na akumulaciju i distribuciju mineralnih materija u soji (*Glycine max.* (L.) Merr.). Proc. Nat. Sci, Matica Srpska 80: 55–65.
Kastori, R., Petrović, N., Petrović, M. (1996): Effect of lead on water relations, proline concentration and nitrate reductase activity in sunflower plants**.** Acta Agron. Hung. 44: 21–28.
Kastori, R., Plesničar, M., Sakač, Z., Panković, D., Arsenijević-Maksimović, I. (1998): Effect of Excess Lead on Sunflower Growth and Photosynthesis. J. Plant Nutr. 21: 75–85.
Lane, S. D., Martin, E. S. (1977): A histochemical investigation of lead uptake in *Raphanus sativus*. New Phytol. 79: 281–286.
Lane, S. D., Martin, E. S., Garrod, J. F. (1978): Lead toxicity effects on indol-3-qcetic acid induced cell elongation. Planta 144: 79–91.
Langerwerff, I. V., Armiger, W. H., Soecht, A. W. (1973): Uptake of lead alfalfa and corn from soil and air. Soil Sci. 115: 455–460.
Larcher, W. (1995): Physiological Plant Ecology. Springer-Verlag, Berlin, Heidelberg, New York.
Malone, C., Koeppe, D. E., Miller, R. J. (1974): Localization of lead accumulated in corn plants. Plant Physiol. 53: 388–394.
Miller, R. J., Koeppe, D. E. (1977): Accumulation and physiological effects of lead in corn. Proc**.** Fourth Ann. Conf. Trace Sub. Environ. Health Univ. Missouri.
Moussavi-Nik, M., Rengel, Z., Pearson, J. N., Hollamby, G. (1997): Dynamics of nutrient remobilization from seed of wheat genotypes during imbibition, germination and early seedling growth. Plant Soil 197: 271–280.

Petrović, N., Arsenijević-Maksimović, I. (1996): Effect of Different Lead Concentrations on Accumulation and distribution of essential mineral elements in sunflower (*Helianthus annus* L.). J. Sci. Agric. Res. 57: 21–32.

Sillanpaa, M., Jansson, H. (1992): Status of cadmium, lead, cobalt and selenium in soils and plants of thirthy countries. FAO, Soil Bull. Rome 65: 195–201.

Szigeti, Z. (1998): Növények és a stressz. In: Láng, F. (ed.) Növényélettan – A növényi anyagcsere. ELTE Eötvös Kiadó, Budapest: 915–984.

Van Asshe, F., Clijasters, H. (1990): Effects of metals on enzyme activity in plants. Plant Cell Environ. 13: 195–206.

Wierzbicka, M. (1988): Mitotic disturbance induces by low doses of inorganic lead. Caryologia 41: 143–160.

Zimdahl, R. L. (1975): Entery and movement in vegetation of lead derived from air and soil sources. 68th Annu. Meeting of Air Pollution Control Association, Boston.

УТИЦАЈ КОНТАМИНАЦИЈЕ СЕМЕНА КУКУРУЗА ОЛОВОМ НА ЊЕГОВА БИОЛОШКА СВОЈСТВА

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Резиме

Испитано је дејство третирања семена кукуруза различитим концентрацијама олова (10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} mol/dm^3) на накупљање и дистрибуцију олова (Pb) у клијанцу, клијање семена, пораст поника и мобилизацију минералних материја у току клијања.

Садржај Pb у корену и изданку поника указује да семе потопљено у растворе различитих концентрација Pb интензивно усваја Pb. Садржај Pb у корену и изданку повећао се са повећањем примењених концентрација Pb и био је значајно већи у корену него у изданку. Насупрот томе, коефицијент акумулације био је већи у изданку него у корену.

Третирање семена Pb није у већој мери утицало на његова биолошка својства. Са повећањем концентрације Pb смањили су се клијавост семена и удео типичних поника, а повећао се број атипичних поника и непроклијалих семена.

На масу суве материје и раст изданка младих биљака контаминација семена Pb није испољила дејство, док су се дужина примарног корена, корена мезокотила као и маса корена значајно смањили при третирању семена највећом концентрацијом Pb.

Транслокација мобилизираних минералних материја из семена у току клијања и раста младих биљка у корен и изданак била је специфична, зависна од елемента. Само је примена највеће испитиване концентрације Pb испољила утицај на мобилизацију и транслокацију неких елемената.

На основу добијених резултата може се закључити да се кукуруз у фази клијања семена и пораста поника одликује толерантношћу према контаминацији Pb.

КЉУЧНЕ РЕЧИ: клијавост, кукуруз, мобилизација минералних елемената, раст, третирање оловом

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GENETIC, CHEMICAL, AND PHYSICAL PREDISPOSITIONS OF NEW MAIZE INBRED LINES AND HYBRIDS WITH EFFICIENT PHOTOSYNTHESIS**

ABstract: This study confirmed our hypothesis that new maize inbred lines and hybrids derived from them had a dominant property of an efficient photosynthetic model. This property is successfully used in breeding programmes, modern technologies of the seed, and commercial maize production. This statement is supported by the results displayed on the erect position of the top leaves of new maize inbred lines and photosynthetic and florescence parameters: the change of the delayed chlorophyll fluorescence intensity during its course and dynamics, the Arrhenius criterion for the determination of critical temperatures (phase transition temperatures) and the activation energies, as a measure of conformational changes in chloroplasts and the thylakoid membrane. Furthermore, a grain structure including its physical and chemical parameters of new maize inbred lines and hybrids was analysed in the present study. In addition, relevant breeding, seed production and technological traits, properties and parameters of new maize inbred lines and maize hybrids were observed in the present study. The overall presented results show that properties of new inbred lines and maize hybrids are based on the nature of conformational and functional changes that occur in their chloroplasts and thylakoid membranes, as well as, on progressive effects in modern breeding, contemporary hybrid seed production, and the commercial maize production.

KEY WORDS: delayed chlorophyll fluorescence, grain, hybrid, inbred, leaf, photosynthetic model, thylakoid membrane, *Zea mays* L.

INTRODUCTION

The complex and interdependent processes in fundamental, multidisciplinary, and applied sciences are frequently interrelated. This manuscript presents

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** Authors devote this manuscript to the memory of Professor Dr. Milorad Piper, the first and a long-time Director of the Maize Research Institute. This is the 36th anniversary of his death. This manuscript presents implementation of his ideas on activities related to maize

the bonds of interrelated studies carried out within breeding, photosynthesis, fluorescence, biophysical chemistry and seed production in new maize inbred lines and hybrids with efficient photosynthetic functions. Maize breeding and seed production have been intensively developing for the last 65 years. As a result of such activity, over 1400 grain and silage hybrids have been derived. Modern equipment and technical and technological prerequisites were provided for carrying out the process of breeding and hybrid and commercial maize seed production (Duvick, 1977, 1984; Sprague, 1984; Trifunović, 1986; Dumanović, 1986; Hallauer, 1988; Ivanović et al., 1995; Radenović et al., 2000).

Since 1978, the number of plants per area unit (plant density) has been significantly increasing, which resulted in the significant increase in grain yields of both, maize hybrids and commercial maize (Radenović et al., 1978, 2001 a, b; Kojić and Ivanović, 1986). At the same time, a programme on breeding and the seed production of maize hybrids that included inbreds with erect top leaves has been developed (Radenović et al., 1978, 2003, b, 2004a, b, 2007, 2008; Felner et al., 2006). According to our hypothesis, new maize inbred lines with erect leaves are the closest to the assumptive maize photosynthetic model (Radenović and Grodzinskij, 1998).

The studies on maize photosynthesis carried out in the previous period did not have a more important application in breeding and the production of maize hybrid seed. It was almost impossible to present practical results and a clear and direct interrelationship among photosynthesis, breeding and the production of maize hybrid seed by an old and traditional approach. The way out was found in the functional connection of interdependence of photosynthetic functions and fluorescence (Radenović et al., 2000, 2001a, b, 2004a, b, 2007, 2010).

During the last 40 years, new and significant studies within the field of bioluminescence and fluorescence phenomena and processes within the plant systems, including maize, have been carried out (Barber and Neumann, 1974; Bukhov et al., 1989; Dzhibladze et al., 1988; Govindjee and Papageorgiou, 1971; Govindjee et al., 1990; Haveman and Lavorel, 1975; Hipkins and Barber, 1974; Holzapfel and Haug, 1974; Jurisnic, 1986; Jurisnic and Govindjee, 1982; Krause and Weis, 1991; Lichtenthaler and Rinderle, 1988; Mccauley and Rubby, 1981; Papageorgiou, 1975; Veselovski and Veselova, 1990; Marković et al., 1987, 1993, 1999; Radenović, 1994; Radenović et al., 1994 a, b; Radenović and Jeremić, 1996). The direct dependence of the delayed chlorophyll fluorescence (DF) intensity on changes of photosynthetic processes in chloroplasts and thylakoid membranes of maize intact leaves was determined (Radenović, 1994 a, b; Radenović and Jeremić, 1996). S Conditions that provided monitoring of complex photosynthetic processes in the intact leaf of maize inbreds by parameters of a photosynthetic and fluorescence model in the form of chlorophyll DF were developed (Radenović et al., 2000, 2001a, b, 2010).

Research methods within the field of biophysical chemistry contributed to diversified binding of studies on photosynthetic and transport processes in

chloroplasts and the thylakoid membrane and different chemical structures of grain with processes of fluorescence spectroscopy and chemical kinetics (R a d e n o v i ć, 1994; R a d e n o v i ć et al., 2007, 2008, 2010; R u b i n et al., 1988).

The objective of the present study was to show that new inbred lines that are included into new high yielding maize hybrids could be an efficient photosynthetic model and could contribute to the functional connection of breeding, photosynthesis and florescence, and thereby to a greater extent to progress of maize breeding and the modern production of hybrid seed and commercial maize.

MATERIAL AND METHODS

Plant material – The studies were performed with the following two new maize inbred lines: ZPPL 218 and ZPPL 318 and the hybrids developed from them: ZP 600, ZP 606, and ZP 666. The observed inbreds and maize hybrids belong to the collection of the Maize Research Institute, Zemun Polje, Belgrade, Serbia.

As these are new inbred lines and maize hybrids, their traits will be presented in this manuscript. Figure 1 shows the actual appearance of new maize inbred lines with erect top leaves: ZPPL 218 and ZPPL 318 and prospective maize hybrids: ZP 600, ZP 606, and ZP 666 with their ears.

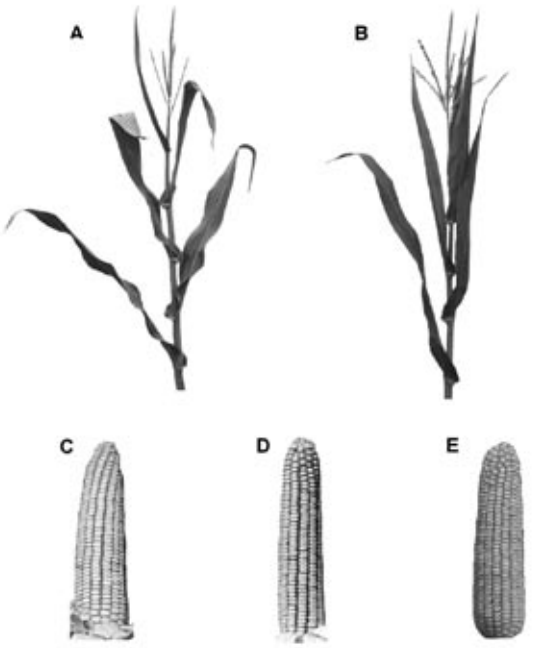

Fig. 1. – Actual appearance of new inbred lines with erect top leaves: ZPPL 218 (A), ZPPL 318 (B), and of prospective hybrids with their ears: ZP 600 (C), ZP 606 (D) and ZP 666 (E)

Methods – Overall studies of the stated new inbred lines and prospective maize hybrids developed from them with erect top leaves encompassed several series of experiments in which new and conventional methodological procedures were applied.

1. *The measure of an angle and leaf area* – The first series of experiments was related to studying the erect position of top leaves. A specially designed protractor was used to measure the angle between lines of the position of the above-ear leaf and the position of the plant stalk of new maize inbred lines. The leaf area was measured by using the portable area meter (model LI-3000). Measures of the angle between the above-ear leaf and the stalk and the leaf areas were carried out on 218 plants for each inbred line during the three-year period. These methodical procedures were described in previously published papers (R a d e n o v i ć et al., 2003, 2004a, b, 2007).

2. *Photosynthetic and fluorescence measurements* – The second series of the experiments was related to photosynthetic-fluorescence measurements, including thermal processes of DF, critical phase transition temperatures and activation energies. The test maize inbreds grown in the experimental field of the Maize Research Institute, Zemun Polje, were brought to the laboratory between 7 a.m. and 8 a.m. Plants sampled in the field were transversally cut in the ground internode. In the laboratory, plants were internode lengthwise placed in water. Prior to the fluorescence experiment, all plants were kept under the black ball glass for two hours. Segments of intact above ear leaves were taken from such plants and placed into a chamber of the phosphoroscope. The intact leaf segments were kept in the chamber (in the dark) for at least 15 minutes, and then thermal processes of delayed chlorophyll fluorescence were measured. These tests were performed on 168 plants of each maize inbred line.

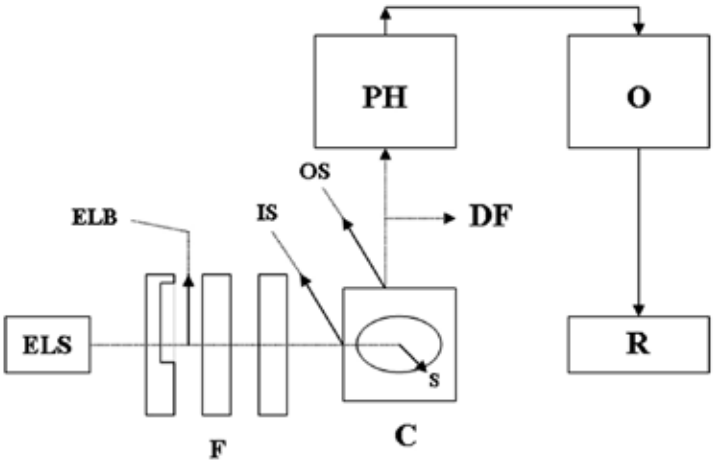

Fig. 2. – Experimental setup of the photosynthetic fluorescence method and the measuring equipment for delayed chlorophyll fluorescence: C – dark chamber with a sample stand; S – sample (intact leaf segment), ELS – excitation light source, PH – photo-multiplier; O – oscilloscope, R – printer, ELB – excitation light beam, DF – luminescent light (delayed fluorescence), IS – input dark chamber slot, OS – output dark chamber slot, F – filters

The improved non-invasive photosynthetic-fluorescence method used to measure DF is schematically presented in Figure 2. This method, developed at the Maize Research Institute, Zemun Polje, has been improved several times. Photosynthetic-fluorescence measurements were performed after a method that had been described in previously published papers (R a d e n o v i ć, 1994; M a r k o v i ć et al., 1996; R a d e n o v i ć et al., 2001a, b, 2002, 2004a, b, 2007, 2008, 2010).

3. *Functional dependence of the yield of prospective maize hybrids for various locations in Serbia* – Functional dependence of the yield of new and prospective maize hybrids ZP 600, ZP 606 and ZP 666 was observed in eight different locations in Serbia with the application of standard methods for a contemporary maize production (R a d e n o v i ć et al., 2010).

4. *Broader presentation of breeding and seed production properties of new inbred lines and maize hybrids with erect top leaves* – As new maize inbred lines, with erect top leaves, and prospective hybrids were observed a broader presentation of relevant breeding, seed production and technological traits, properties and parameters gained by use of standard methods is given.

5. *Chemical composition, physical properties and a structure of grain of prospective maize hybrids with efficient photosynthetic functions* – Methods used for the determination of the chemical composition, physical properties and a structure of grain of prospective maize hybrids with erect top leaves were fully described in previously published papers (R a d o s a v l j e v i ć et al., 2000; R a d e n o v i ć et al., 2010).

RESULTS

1. The measure of the angle and the area of the above-ear leaf – Results on the measures of angles between the above-ear leaf and the stalk and the average leaf areas are presented in Table 1. Based on obtained results on the measures of angles it can be stated that the observed new maize inbred lines belong to the group of 10-15 recently developed inbred lines with erect top leaves.

Tab. 1. – The angle of the above-ear leaf and the leaf area of new maize inbred lines with efficient photosynthesis

Inbred line	FAO maturity group	Heterotic origin of the inbred*	Angle of the above-ear leaf in degrees		Area of the above-ear leaf (x 10^3 cm^2)	
			$\bar{x}$	σ	$\bar{x}$	Σ
ZPPL 218	650	Zemun Polje – Lancaster	22.1º	1.36	3.91	0.41
ZPPL 318	600	Zemun Polje – BSSS	21.2 º	1.15	3.58	0.39

*Studied new maize inbred lines represent good heterotic pairs, they are characterised as good general combiners for grain yield, they increase well and they are high yielding inbreds

2. Empirical procedure for photosynthetic fluorescence studies on the above-ear leaf – The detailed studies on thermal processes of DF of observed new maize inbred lines with erect top leaves were performed. The thermal curve is a curve that shows the dynamics of changes in the stationary DF level intensity in dependence on a temperature. The trend of its establishment is most often analogous to changes in the duration in seconds of segments marked with **a, b, c, d, e, f,** and **g**, Figure 3, which was determined by the empirical procedure (R a d e n o v i ć et al., 2008, 2009, 2010). Monitoring the course of the thermal curve and the analysis of the duration of certain segments provided data on the existence of a greater number of critical temperatures (phase transition temperatures) at which greater or smaller structural and functional changes occurred in chloroplasts and the thylakoid membrane of observed new maize inbred lines with erect top leaves.

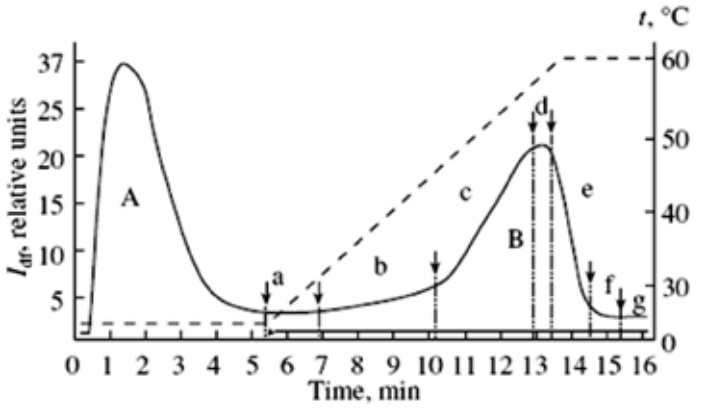

Fig. 3. – Schematic presentation of the empirical procedure for typical changes in DF intensities ($\mathbf{I_{df}}$) on the intact above-ear leaf of the observed new maize inbred lines (solid line) and changes in temperatures (dashed line): Curve **A** indicates induction processes of DF, while curve **B** encompasses photosynthetic fluorescence thermal processes of DF. Typical temporal segments (**a**, **b**, **c**, **d**, **e**, **f** and **g**) on the thermal curve **B** correspond to dynamics of $\mathbf{I_{df}}$ changes at the time of a DF formation. Conformational and functional changes in chloroplasts and the thylakoid membrane of observed new maize inbred lines with erect top leaves occur at the interception points of typical temporal segments

3. The exact temperature dependence of the delayed chlorophyll fluorescence intensity for the thylakoid membrane of new maize inbred lines with erect top leaves – The experimental measures of changes in the stationary DF level in dependence on the temperature, ranging from 25 to 60 ºC, were performed. The dynamics of temperature dependence for observed new maize inbred lines with erect top leaves is presented in Figure 4A and B.

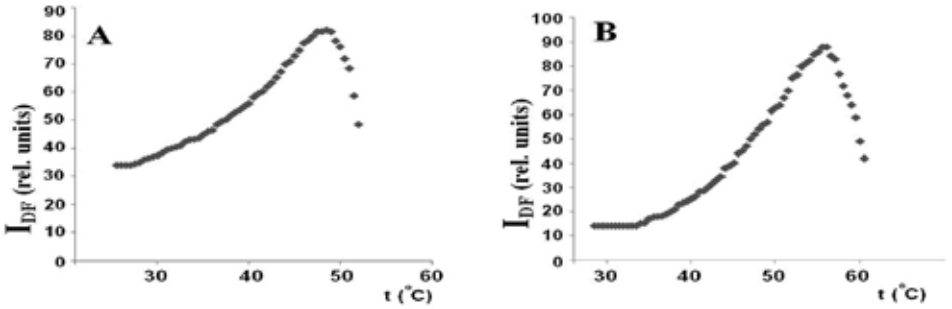

Fig. 4. – A, B. Changes in the intensity of the delayed chlorophyll fluorescence (I_{DF}) of thermal processes in dependence on the effects of temperatures in chloroplasts and the thylakoid membrane of the intact above-ear leaf of new maize inbred lines with erect top leaves: ZPPL 218 (A), and ZPPL 318 (B)

4. The Arrhenius plot for the determination of critical temperatures and conformational changes in chloroplasts and the thylakoid membrane of the new maize inbred lines with erect top leaves – The Arrhenius plot is based on the linearization of the exact DF temperature dependence of observed new maize inbred lines. Critical temperatures (phase transition temperatures) at which conformational changes occur in chloroplasts and the thylakoid membrane are determined by the application of the Arrhenius plot. Results of the Arrhenius plot application to new maize inbred lines with erect top leaves are presented in Figure 5A, B.

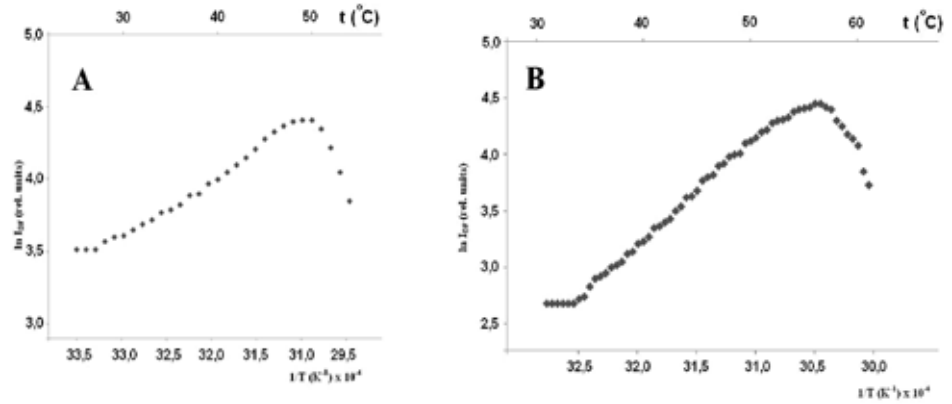

Fig. 5. – A, B. The Arrhenius plot for the determination of critical temperatures (T, ºK) and conformational changes in chloroplasts and the thylakoid membrane of the above-ear leaf of observed new maize inbred lines with erect top leaves: ZPPL 218 (A) and ZPPL 318 (B)

5. Activation energy and critical temperatures in the thylakoid membrane of the observed new maize inbred lines with erect top leaves – Detailed studies on the thermal processes of DF, and especially on the analysis

of experimental thermal curve, encompassed not only the temperature dependence and the Arrhenius plot, but also the estimation of values of activation energies (Ea) for critical temperatures (phase transition temperatures) in chloroplasts and the thylakoid membranes of the observed new maize inbreds with erect top leaves: ZPPL 218 and ZPPL 318. Obtained results are shown in Table 2.

Tab. 2. – Changes in activation energies (Ea) and critical temperatures (tºC) in the course of thermal processes in the thylakoid membrane of the intact above-ear leaf of studied new maize inbred lines with erect top leaves

ZPPL 218		ZPPL 318	
Ea, kJ/mol	t, ºC	Ea, kJ/mol	t, ºC
–	27.0	–	33.5
43.1	29.0	40	38
27.3	36.9	77.23	53.5
37.0	43.5	26.09	56.5
42.5	47.8	50.51	59.3
51.1	49.9	227.52	–

6. Functional dependence of the yield of new maize hybrids for different locations in Serbia – New and prospective maize hybrids: ZP 600, ZP 606 and ZP 666, are mainly indented for the cultivation in Banat, Srem, Bačka, Mačva, and alongside riverbanks in Serbia. The preliminary results on yields of the stated maize hybrids are presented in Table 3.

Tab. 3. – Yields of new and prospective maize hybrids (t ha^{-1}) with efficient photosynthesis in eight locations of Serbia*

Hybrid	Locations in Serbia**								
	1 $\overline{x}$	2 $\overline{x}$	3 $\overline{x}$	4 $\overline{x}$	5 $\overline{x}$	6 $\overline{x}$	7 $\overline{x}$	8 $\overline{x}$	Average $\overline{x}$
ZP 600	12.8	11.0	11.2	12.9	11.8	10.6	10.8	13.5	11.8
ZP 606	12.9	11.6	10.5	12.5	10.9	10.7	10.4	12.2	11.6
ZP 666	12.7	10.9	9.5	11.6	10.6	10.0	10.3	12.5	11.0

*Results were obtained during 2009, 2010 and 2011
**Locations in Serbia by the ordinal number: 1 – Loznica, western Serbia; 2 – Sakule, southern Banat; 3 – Smederevo, the Danube region; 4 – Zmajevo, southern Bačka; 5 – Žarkovac, eastern Srem; 6 – Batoš, mid Banat; 7 – Divoš, northern Srem; 8 – Bečej, eastern Bačka

7. Brief survey of breeding and seed production traits of new maize inbred lines and maize hybrids with efficient photosynthetic functions – Observed new maize inbred lines ZPPL 218 and ZPPL 318 have been included in breeding for the last 2-3 years. Due to it, relevant observations of their total traits, performances, and parameters are presented in Table 4.

Tab. 4. – Relevant breeding and seed production traits of new maize inbred lines with efficient photosynthesis

Ord. no.	Name and defining of traits	Brief description of breeding, seed production and technological traits of new maize inbred lines	
		ZPPL 218	ZPPL 318
1	Heterotic origin	Zemun Polje – Lancaster	Zemun Polje – BSSS
2	FAO maturity group	650	600
3	Grain yield ha^{-1} in kg at 14% moisture		
	a) dry land farming	3220±204	4056±265
	b) irrigation	4186±255	6045±330
4	Number of plants ha^{-1} at harvest		
	a) dry land farming	65000	71500
	b) irrigation	71500	79400
5	Inbred tolerance to stress factors such as drought, high temperature and the like	Inbred has a good tolerance to drought and high temperatures	Inbred has a good tolerance to drought and high temperatures
6	Grain properties	Kernel of this inbred belongs to dent type and has plenty of anthocyanins on its flanks	Yellow-orange kernel of this inbred belongs to semi-dent type
7	% moisture in grain at harvest	Inbred is harvested at 18.00%	Inbred is harvested at 22.00%
8	Is grain suitable for nutrition of ruminants and nonruminants	Inbred has high quality grain and hybrids developed from this inbred have also high quality grain suitable for nutrition of ruminants and nonruminants.	Inbred has high quality grain and hybrids developed from this inbred have also high quality grain suitable for nutrition of ruminants and nonruminants.
9	Is the inbred suitable for the development of silage hybrids?	Inbred is very suitable for the development of silage hybrids	Inbred is very suitable for the development of silage hybrids
10	Grain digestibility (%)	81.6	80.44

In the same way, new and prospective maize hybrids ZP 600, ZP 606, and ZP 666 have already caught attention of experts; hence, it is necessary to study their overall traits. Results on studied traits of observed new and prospective hybrids are presented in Table 5.

8. Chemical composition and physical properties of new inbred lines and prospective maize hybrids with efficient photosynthetic functions – Results on studies of chemical composition and physical properties of new inbred lines (ZPPL 218 and ZPPL 318) and prospective maize hybrids with erect top leaves (ZP 600, ZP 606 and ZP 666) are presented in Tables 6 and 7.

Tab. 5. – Relevant breeding and seed production traits of prospective maize hybrids with efficient photosynthesis

Ord. no.	Name and defining of traits	Brief description of breeding, seed production and technological traits of prospective maize hybrids		
		ZP 600	ZP 606	ZP 666
1	Grain yield ha^{-1} in kg at 14% moisture	11930	11830	11750
2	Optimum sowing density and affinity of hybrids to densities (ha)	58-62000	58-62000	60-65000
3	Regional distribution of hybrids according to agroecological characteristics of the region	Very adaptable, tolerant to drought and various growing conditions of the region	Very tolerant to drought and high temperatures. Hybrid has high yields under conditions of Banat.	Very adaptable, resistant to precipitation distribution and high temperatures
4	FAO maturity group	FAO 580-600	FAO 640-660	FAO 580-600
5	Description of the essential hybrid stalk traits	Medium tall, slender, elastic and very firm	Medium tall, slender, elastic and very firm	Medium short, slender, elastic and very firm
6	General data on the type and quality of grain	Yellow kernel belongs to a dent type, is of high quality. 1000-kernel mass is 488.7 g	Orange kernel belongs to a dent type. 1000-kernel weight is 474.6 g. Protein content up to 12%.	Orange kernel is deeply set. 1000-kernel weight is 356.2 g. Oil content up to 6%.
7	Dates of sowing and data on emergence and early growth	Tolerates early sowing, has good emergence and early growth	Tolerates early sowing. Emerges excellently under conditions of positive temperatures. Has a good early growth.	Tolerates early sowing. It prefers a slightly deeper sowing. Has a good emergence and early growth.
8	Tolerance, resistance and adaptability	Very adaptable to soil and conditions of cropping practices. Tolerant to drought and high temperatures.	It prefers more fertile soils and intensive cropping practices. Extremely tolerant to drought.	Very tolerant and adaptable to growing conditions. Tolerant to drought.
9	Stay green and suitability for silage	Grain filling period is long and dry down is good. Expresses stay green trait. It is excellent for silage production.	It has grain of high quality with protein content up to 12%. It is suitable for ruminants and nonruminants.	It has extremely pronounced stay green trait. Stalk is short and slender hence silage mass yield is low.
10	Grain suitability for the nutrition of domestic animals	Proportion of horny floury endosperm is greater. Grain is healthy and of high quality. It suitable for ruminants and nonruminants.	It has grain of high quality with protein content up to 12%. It is suitable for ruminants and nonruminants.	Grain protein, i.e. oil content amount to 10-11%, i.e. 6%, respectively. It is suitable for ruminants and nonruminants.
11	Grain digestibility (%)	92.47	92.08	96.65

Tab. 6. – Chemical composition of new maize inbred lines and hybrids with efficient photosynthesis

Inbred	Starch (%)	Proteins (%)	Oil (%)	Crude fibre (%)
ZPPL 218	69.10	9.60	5.79	2.18
ZPPL 318	71.27	10.31	4.91	2.39
Hybrid				
ZP 600	73.01	7.76	5.76	1.91
ZP 606	73.48	9.84	5.06	2.23
ZP 666	74.32	9.61	6.10	2.42

Tab. 7. – Physical properties of grain of new maize inbred lines and hybrids with efficient photosynthesis

Inbreds	TKW*	TW	D	FI	MR	HEF	SEF
ZPPL 218	341.5	844.1	1.29	23.28	10.5	58.1	41.9
ZPPL 318	316	811.7	1.28	24.31	12.2	62.3	37.7
Hybrids							
ZP 600	488.0	788.2	1.27	34.3	12.7	55.8	44.2
ZP 606	474.0	777.0	1.26	48.2	11.3	57.9	59.1
ZP 666	356.0	806.2	1.28	24.5	10.9	42.7	40.0

* TKW = 1000-kernel weight (g), TW = test weight (kg m^{-3}), D – density (g cm^{-3}), FI – floatation index (%), MR – milling response (s), HEF – hard endosperm fraction, (%), SEF – soft endosperm fraction (%)

DISCUSSION

The second half of the 20th and the first decade of the 21st century are characterised by a great success achieved in maize breeding and the production of fundamental maize seed, hybrid maize seed of high quality and of commercial maize. The number of plants per area unit has been increasing since 1978. This programme was referred to as a "plant density" programme and it further directly affected the yield increase of high quality fundamental and hybrid maize seed (Radenović et al., 1978, 2001 a, b). In addition, a programme on the development of maize inbred lines with erect top leaves was established at the same time as the "plant density" programme. It was considered that inbreds with the erect top leaves were the closest to the proposed efficient photosynthetic model (Radenović et al., 1978; Radenović and Grodzinski, 1998; Radenović et al. 2000, 2001a, c, 2003, 2004a). The complementary and mass implementation of these programmes led to very important results in both, maize breeding and the hybrid seed production (Ivanović et al., 1995; Trifunović, 1986; Trifunović et al., 2000; Dumanović, 1986; Kojić and Ivanović, 1986). New and numerous hybrids for grain and silage were d eveloped and grown on large areas due to their high yielding potential and the appropriate quality of the plant and the grain (Duvick, 1984; Russell, 1986; Dumanović, 1986; Hallauer, 1988; Kojić and Ivanović, 1986; Ivanović et al., 1995).

The special contemporary breeding studies have been performed on top leaves of maize inbred lines. In recent times, the ear leaves have been particularly observed, but also other top leaves up to the tassel. The most efficient and the longest photosynthetic processes necessary for the maize plant have been achieved by these leaves (R a d e n o v i ć and G r o d z i n s k i, 1998). According to the stated, a hypothesis that top leaves (above-ear leaves) achieving the efficient photosynthesis has been proposed.

This study was an attempt to answer the following questions by using different interdependent studies and analyses: (1) were there reliable and dominant traits of maize inbred lines with erect top leaves by which planned and satisfactory progress in maize breeding and the high-quality hybrid seed maize production could be achieved? and (2) which traits should such maize inbred lines have?

The gained results of experimental studies can offer at least a partial answer to asked questions. The first series of experiments included the measure of the angle and the leaf area of observed new maize inbred lines with erect top leaves. The results obtained on these traits (Table 1) classify them into important breeding and seed production traits (R a d e n o v i ć et al., 2003, 2004a, b, 2007, 2008, 2010). The second series of experiments encompassed photosynthetic fluorescence studies on conformational and functional changes in chloroplasts and the thylakoid membrane of the intact above-ear leaf of new maize inbred lines. The temperature dependence of thermal processes of DF for the studied maize inbred lines is presented in a form of the empirical procedure (Figure 3). However, the exact results of the temperature dependence of DF for all new maize inbred lines with erect top leaves are presented in Figure 4A, B. The presented results show that the temperature dependence of DF in each of new maize inbred lines with erect top leaves is characterised by typical intersection points of two segments on the thermal curve (Figures 3 and 4A, B). The first typical point occurred on the intersection of the segment **a** and the segment **b** and it represented the lowest critical temperature at which the initial change in the DF intensity was observed. The second typical point occurred on the intersection of the segment **b** and the segment **c** and it was related to a linear monotony with the angle of the increasing part of the DF intensity curve. Evident changes in the structure of the thylakoid membrane occurred in this region. The third typical point reflected a smaller or a greater rotundity of DF intensity peaks. The breaking "conformational" changes occurred in two intersection points of the segments **c** and **d** and the segments **d** and **e**. The fourth typical point was related to the linear monotony and the inclination angle of the declining part of the DF intensity curve. This segment of the thermal curve bore the last conformational changes that had occurred in chloroplasts and the thylakoid membrane. These changes can hardly be described as characters of functioning of a living leaf. The typical intersection points designated as **f** and **g** almost had no physiological role. The analysed typical intersection points, Figures 3 and 4A, B, can be considered the points characterising new maize inbred lines with erect top leaves, as these points are precisely the points of conformational and functional changes in the thylakoid membrane (R a d e n o v i ć et al., 2003, b, 2004a, b, 2007, 2008, 2010).

All critical temperatures (phase transition temperatures) at which even the slightest conformational changes had occurred in chloroplasts and the thylakoid membranes of new maize inbred lines with erect top leaves were determined by the Arrhenius criterion and the linearisation of the DF temperature dependence. The values of critical temperatures in ºC, their frequency and intermediate distance characterise observed new maize inbred lines with erect top leaves in relation to their tolerance, resistance, flexibility and adaptability not only to increased and high temperatures, but also to drought (R a d e n o v i ć et al., 2001a, b, c, 2002, 2003). The Arrhenius criterion is based on the existence of straight lines. Each Arrhenius straight line represents its activation energy (Ea). The intersection point of two straight lines is designated by a critical temperature. Results of the Ea values in the inclining and declining part of the thermal curve are explained by lesser or greater conformational changes that occur in the molecules of pigments (chlorophyll) in the thylakoid membrane with the temperature increase. Due to such changes, these molecules become more reactive and thereby gain the additional energy that is used in the recombining process of the DF occurrence (Table 2) (R a d e n o v ić, 1994; R a d e n o v i ć et al., 2001c, 2004a, b).

Presented photosynthetic fluorescence traits of studied new maize inbred lines with erect top leaves can contribute to more exact, rational and expeditious proceedings of breeding processes and the production of high-quality hybrid maize seed and commercial maize, which makes these maize inbred lines exceptionally important.

Achieved results on yields of new and prospective maize hybrids (Table 3) should be considered as preliminary ones. According to the description of breeding, seed production and technological traits, properties and parameters (Table 5), it is obvious that these are stable hybrids with high quality grains. However, it is necessary to find appropriate locations for such hybrids (Banat, Bačka, Srem, Mačva, river valleys...) in which their full genetic potential of the yield can be used.

Gained results (Tables 6 and 7) present physical traits and the chemical composition that especially indicate grain quality of new maize inbred lines and prospective hybrids with efficient photosynthetic functions (R a d o s a v l j e v i ć et al., 2000; R a d e n o v i ć et al., 2010).

A brief survey of breeding, seed production and technological traits of new inbred lines and prospective maize hybrids with efficient photosynthetic functions (Tables 4 and 5) completes above presented results and contributes to the improvement of modern programmes of both, breeding and current hybrid seed and commercial maize productions.

CONCLUSION

According to obtained results, it can be concluded that the non-invasive photosynthetic fluorescence method can be used in breeding and the production of maize hybrid seed and thereby the estimation of new maize inbred

lines for tolerance, resistance, flexibility and adaptability to increased and high temperatures, as well as, to drought can be performed. The application of the stated non-invasive method resulted in the determination of numerous traits, properties and parameters of the photosynthetic apparatus of new inbred lines and maize hybrids with efficient photosynthetic functions, such as:

- Temperature dependence observed within the range of 24°C to 60°C,
- The critical temperatures at which greater or smaller structural and functional changes occur in chloroplasts the thylakoid membrane were determined,
- Values of activation energy (Ea, kJ/mol) alongside straight lines before and after occurrence of critical temperatures in the thermal chlorophyll DF process were determined,
- Different monotonies of the intensity in the inclining part of the thermal curve was present; these monotonies point out to unequal tolerance, resistance, flexibility, stability and adaptability of new maize inbred lines to increased and high temperatures, as well as, to drought,
- It was shown that observed, new inbred lines maize hybrids have a dominant property of efficient photosynthetic functions,
- A numerous relevant breeding, seed production and technological traits of new maize inbred lines and hybrids with efficient photosynthetic functions were presented,
- A functional dependence of yields of prospective maize hybrids for eight locations in Serbia was established,
- It was established that commercial maize of studied new and prospective hybrids is of high quality that provides its diverse utilisation.

REFERENCES

Barber, J., Neumann, J. (1974): An energy conservation site between H_2O and DBMIB: evidence from msec delayed light and chlorophyll fluorescence studies in chloroplasts. FEBS Lett. 40: 186–189.

Bukhov, N. G., Rakhiberdyeva, M. G., Karapetyan, N. V. (1989): Nature of slow transient phenomena of variable and delayed fluorescence in leaves. Soviet Plant Physiol. 36: 1045–1054 (in Russian).

Dumanović, J. (1986): Modern maize breeding programmes pp. 77-94. In: Proc. Genetics and breeding of Maize. December 11-12 1986., (Ed. M. Penčić). Maize Research Institute, Zemun Polje, Belgrade, Yugoslavia.

Duvick, D. N. (1977): Genetic rates of grain in hybrid maize yields during the past 40 years. Maydica 22: 187–196.

Duvick, D. N. (1984): Genetic Contribution to Yield Gains of U.S. Hybrid Maize, 1930 to 1980. In: Genetic Contributions to Yield Gains of Five Major Crop Plants, ed. W. R. Fehr, CSSA, Special Publication 7, Crop Science Society of America, American Society of Agronomy, Madison, WI, USA, pp. 15–47.

Dzhibladze, T. G., Bukhov, N. G., Karapetyan, N. V. (1998): Relations between kinetic curves of variable fluorescence and decisecond component of delayed fluorescence in plant leaves. Biofizika 33: 121–125 (in Russian).

Felner, M., Ford, E. D., Van Volkenburgh, E. (2006): Development of erect leaves in a modern maize hybrids is associated with reduced responsiveness to auxin and light of young seedlings in vitro. Plants Signalling and Behaviour 1 (4): 201–211.

Govindjee, Papageorgiou, G. (1971): Chlorophyll Fluorescence and Photosynthesis: Fluorescence Transients. In: Photophysiology, ed. A.C. Giese, Academic Press, NY, USA, 6: 1–46.

Govindjee, Van Der Ven, M., Preston, C., Seibert, M., Gratton, E. (1990): Chlorophyll a fluorescence lifetime distribution in open and closed photo system II reaction centre preparation: Analysis by multifrequency phase fluorometry. Biochim. Biophys. Acta 1015: 173–179.

Hallauer, A. R. (1988): Modern methods in maize breeding. (1988), Book of Proceedings of the Workshop on Maize Breeding and Maize Production EUROMAIZE '88, October 6-8,1988, Belgrade, Yugoslavia, pp. 1–20.

Haveman, J., Lavorel, J. (1975): Identification of the 120 msec phase in the decay of delayed fluorescence in spinach chloroplasts and aubchloroplasts particles as the intrinsic back reaction. The dependence of the level of this phase on the thylakoids internal pH. Biochim. Biophys. Acta 408: 269–283.

Hipkins, M. F., Barber, J. (1974): Estimation of the activation energy for millisecond delayed fluorescence from uncoupled chloroplasts. FEBS Lett. 42: 289–292.

Holzapfel, C., Haug, A. (1974): Time course of microsecond delayed light emission from Scenedesmus obliques. Biochim. Biophys. Acta 333: 52–58.

Ivanović, M., Petrović, R., Drinić, G., Trifunović, V. (1995): Fifty years of ZP hybrids breeding. pp 3-16. *In:* Proc. Breeding, Production and Maize Utilization. 50 Years of Mize Researc Institute "Zemun Polje", September 28-29, 1995 Ed. Maize Research Institute, Zemun Polje, Belgrade.

Jurisnic, P. (1986): Delayed Fluorescence: Current Concepts and Status. In: Light Emission by Plants and Bacteria, ed. Govindjee, Amesz & Fork CD, Academic Press, Orlando, FL, USA, 291–328.

Jursinic, P., Govindjee (1982): Effects of hydroxylamine and silicomolybdate on the decay in delayed light emission in the 6-100 microsecond range after a single 10 ns flash in pea thylakoids. Photosynth. Res. 3: 161–177.

Kojić, L., Ivanović, M. (1986): Long-term maize breeding programms. pp. 57-75. In: Proc. Genetics and breeding of Maize. December 11-12 1986, Ed. Maize Research Institute, Zemun Polje, Belgrade.

Krause, G. H., Weis, E. (1991): Chlorophyll fluorescence and photosynthesis: The basic. Ann. Rev. Plant Physiol. Plant Mol. Biol. 42: 313–349.

Lichtenthaler, H. K., Rinderle, U. (1988): The role of chlorophyll fluorescence in the detection of stress conditions in plants. CRC Crit. Rev. Anal. Chem. 19 (Suppl. I): S29–S85.

Marković, D., Jeremić, M., Radenović, Č. (1996): Zakasnela fluorescencija hlorofila. U: Savremena biofizika, (Editor B. Beleslin, D. Vučelić, Č. Radenović). Izdavač "Velarta", Beograd.

Marković, D., Jeremić, M., Radenović, Č., Schara, M. (1993): Irreversible structural changes in thylakoid membranes at high temperatures detection by luminescence and EPR. Gen. Physiol. Biophys. 12: 37–47.

Marković, D., Jeremić, M., Radenović, Č., Vučinić, Ž. (1987) A study of temperature induced structural changes in photosynthetic system using delayed fluorescence. Journal Serb. Chem. Soc. 52: 331–336.

Marković, D., Radenović, Č., Rafailović, L., Žerajić, S., Jeremić, M., Marković, M. (1999): Temperature dependence of delayed fluorescence induction curve transient. Gen. Physiol. Biophysics 18: 257–267.

McCauley, S. W., Rubby, R. H. (1981): Delayed fluorescence induction in chloroplasts. Irradiation dependence. Bichim. Biophys. Acta 638: 268–274.

Papageorgiou, G. (1975): Chlorophyll fluorescence: intrinsic probe of photosynthesis. In: ed. Govindjee, Bioenergetics of Photosynthesis, Academic Press, New York, pp. 319–371.

Radenović, Č. (1994): A study of delayed fluorescence in plant models: Photosynthetic transportation and membrane processes. J. Serb. Chem. Soc. 59: 595–617.

Radenović, Č., Babić, M., Delić, N., Hojka, Z., Stanković, G., Trifunović, B. V., Ristanović, D., Selaković, D. (2003): Photosynthetic properties of erect leaf maize inbred lines as the efficient photo-model in breeding and seed production. Genetika 35 (2): 85–97.

Radenović, Č., Babić, M., Delić, N., Ristanović, D. (2001c): Effects of changes in thylakoid membranes – a measure for evaluation of resistance and adaptability of maize inbred lines to high temperature. Matica Srpska. Proceedings for Nat. Sci. 101: 59–69.

Radenović, Č., Filipović, M., Anđelković, V., Babić, M., Radojčić, A. (2009): Significant breeding properties of inbred maize lines as the basis for creating new, higher yielding hybrids. Russian Agricultural Sciences 35 (6): 374–377.

Radenović, Č., Filipović, M., Babić, M., Stanković, G., Radojčić, A., Sečanski, M., Pavlov, J., Branković Radojčić, D., Selaković, D. (2008): Actual prestigious properties of maize inbred lines – a good initial basis for the efficient development of new and yielding maize hybrids. Genetika 40 (2): 121–133.

Radenović, Č., Grodzinskij, D. (1998): Erect leaf maize inbred lines – an efficient photosynthetic model. – A wrtitten communication Belgrade-Kiev and vice versa. pp 1–2.

Radenović, Č., Grodzinskij, D., Filipović, M., Radosavljević, M., Videnović, Ž., Denić, M., Čamdžija, Z. (2010). The prestigious maize inbred lines and hybrids with erect top leaves are characerised by a property of an efficient photosynthetic model and a satisfactory base for the further progress in breeding and selection**.** Physiology and Biochemistry of Cultivated Plants 42 (3): 187–201.

Radenović, Č., Hojka, Z., Selaković, D., Mišović, M. M., Pavlov, M., Sečanski, M. (2004b): Photosynthetic properties of elite erect leaf maize inbred lines and their contribution to seed production improvement. Matica Srpska. Proceedings for Nat. Sci. 106: 45–56.

Radenović, Č., Jeremić, M. (1996): The study of delayed light emission in a plant models. Arch. Biol. Sci. 48: 1–18.

Radenović, Č., Jeremić, M., Marković, D. (1994a): Delayed chlorophyll fluorescence in plants models. Photosynthetica 30: 1–24.

Radenović, Č., Konstantinov, K., Delić, N., Stanković, G. (2007): Photosynthetic and bioluminiscence properties of maize inbred lines with upright leaves. Maydica 52 (3): 347–356.

Radenović, Č., Ristanović, D., Trifunović, V. (1978): The theoretical and the development programme on the increase of the plant number per area unit for the development of erect leaf maize lines and for their more effective application in breeding. The internal note, Maize Research Institute, Zemun Polje, Belgrade, pp. 1–3.

Radenović, Č., Šatarić, I., Husić, I., Mišović, M. M., Filipović, M., Kojić, L. (2000): A study of functioning of thylakoid membranes in inbred lines of maize (*Zea mays* L.). Genetika 32 (3): 377–386.

Radenović, Č., Šatarić, I., Ivanović, M., Husić, I., Kojić, L. (2001b): Conformational and functional changes in thylakoid membranes – parameters for evaluation of maize inbred lines resistance to temperatures and drought. J. Sci. Agric. Research 62 (216-217): 5–20.

Раденович, Ч., Бабич, М., Делич, Н., Шатарич, И., Койич, Л. (2002): Новый фото-синтетическо-биолюминесцентный метод в селекции кукурузы. Кукуруза и сорго 4: 21–24.

Раденович, Ч., Бабич, М., Хојка, З., Станкович, Г., Трифунович, Б. В., Ристановић, Д., Делич, Н., Селакович, Д. (2004a): Характеристика инбредных линий кукурузы с вертикально стоящими листьями для эффективного испольозования в селекции. Доклады Российской академии сельскохозяйственных наук 2: 7–9.

Раденович, Ч., Еремич, М., Маркович, Д. (1994b): Фотоиндуцированная биолуминисценция растений: фотосинтетические, транспортние и мембраные процесы. Физиология и биохимия культурных растения 26 (5): 419–433.

Раденович, Ч., Шатарич, И., Иванович, М., Койич, Л. (2001a): Биолюминесцентный отзыв инбредных линий кукурузы (*Zea mays* L.) на температуру и засуху. Доклады Российской академии сельскохозяйственных наук 4: 13–16.

Radosavljević, M., Bekrić, V., Božović, I., Jakovljević, J. (2000): Physical and chemical properties of various corn genotipes as a criterion of technological quality. Genetika 32 (3): 319–329.

Rubin, A. B., Focht, A. A., Venediktov, P. S. (1988): Some kinetic properties of electron – transfer processes on the primary photosynthesis reaction. Transaction of the Moscow Society of Naturalists 28: 172–184.

Russell, W. A. (1986): Contributions of breeding to maize improvement in United States, 1920s-1980s. Iowa State J. Res. 61:5–34.

Sprague, G. F. (1984): Organization of breeding programs. 20th Ann. Illinois Corn Breeding School (Urbana, USA) 20, 20.

Trifunović, B. V., Stanković, G., Rošulj, M., Trifunović, V. (2000): Multiple regression analysis of prolificacy and effects on grain yield in synthetic populations of maize (*Zea mays* L.). Genetika 32 (3): 355–362.

Trifunović, V. (1986): Forty years of modern maize breeding in Yugoslavia. pp. 5-46. In: Proc. Genetics and breeding of maize. December 11-12 1986, (Ed. M. Penčić), Maize Research Institute, Zemun Polje, Belgrade.

Veselovski, V. A., Veselova, T. V. (1990): Luminescent Characteristic of Plants Photosynthetic Apparatus. In: Luminescence of Plants, "Nauka", pp 8–78, Moscow, Russia.

ГЕНЕТИЧКЕ И ХЕМИЈСКО-ФИЗИЧКЕ ПРЕДИСПОЗИЦИЈЕ НОВИХ ЛИНИЈА И ХИБРИДА КУКУРУЗА СА ЕФИКАСНИМ ФОТОСИНТЕЗОМ

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Резиме

Проучаване су две нове инбред линије кукуруза: ZPPL 218 i ZPPL 318 и са њима створени перспективни хибриди ZP 600, ZP 606 и ZP 666 за које је доказано да поседују доминантно својство ефикасног фотосинтетичног модела што се успешно користи у оплемењивању, савременим технологијама за производњу хибридног семена и меркантилног кукуруза. Овој констатацији иду у прилог изложени резултати о усправном положају вршних листова нових инбред линија кукуруза и о фотосинтетично-флуоресцентним показатељима: промени интензитета закаснеле флуоресценције хлорофила у њеном току и динамици, Аренијусовом критеријуму за одређивање критичних температура (температуре фазних прелаза) и о енергији активације као мери структурних промена у хлоропластима и тилакоидној мембрани. У раду се анализира структура зрна укључујући и његове физичке и хемијске показатеље нових инбред линија и хибрида кукуруза. Исто тако, у раду се разматрају релевантна селекционарска, семенарска и технолошка својства, карактеристике и параметри нових инбред линија и хибрида кукуруза. Укупно изложени резултати показују да су својства нових инбред линија и хибрида кукуруза заснована на природи структурних и функционалних промена, које се одигравају у хлоропластима и тилакоидној мембрани као и на прогресивним ефектима у модерном оплемењивању, савременој производњи хибридног семена и меркантилног кукуруза.

КЉУЧНЕ РЕЧИ: закаснела флуоресценција хлорофила, *Zea mays* L., зрно, линија, лист, тилакоидна мембрана, фотосинтетични модел, хибрид

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VARIABLE MECHANISMS OF ACTION OF LITHIUM DURING GENERATING OF MEMBRANE POTENTIAL OSCILLATIONS ACROSS THE EXCITABLE MEMBRANE OF THE *NITTELA* CELL[**]

ABSTRACT: This study presents results on variable mechanisms of lithium transport processes during generating of membrane potential oscillations across the very excitable membrane of the *Nittela* cell. Generating of several classes of oscillations, single and local impulses of the membrane potential, were presented in dependence on effects of a high LiCl concentration (10 mM), with which the cell membrane is very excited. Results on membrane potential oscillations are presented, and then some of oscillogram parameters were displayed. The assertion is that oscillations of the membrane potential are caused by total oscillatory transport processes: Li^+, K^+, Na^+ and Cl^- across the very excitable cell membrane. The paper presents the hypothesis on mechanisms of oscillatory transport processes of ions (Li, Na, K and Cl) expressed over different classes of oscillations, single and local impulses of the membrane potential across the excitable membrane of the *Nittela* cell.

KEY WORDS: excitable membrane, lithium, membrane potential, *Nittela* cell, oscillation parameters, oscillatory transport

INTRODUCTION

General information on lithium – Lithium is alkaline earth metal, which occurs in nature in the form of different minerals or ions in minerals or sea water (140-270 ppb, parts per billion) (Crichton, 2008). Lithium is found in trace amounts in biological systems lithium. Furthermore, lithium in low concentrations (69-5760 ppb) is found in plants, planktons and inverte-

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brates. Almost all tissues and tissue fluids of vertebrates contain lithium (21-763 ppb). Marine organisms have tendency to accumulate lithium in greater concentrations (Chassard-Bouchaud et al., 1984). Its role within biological systems and under physiological conditions has not been yet sufficiently clarified. Recent nutritional studies on mammals show that the presence of lithium in the rate of 1 mg per day affects health of organisms, which suggests that lithium could be classified as an essential biomicroelement. It was observed that a low-dose lithium uptake promotes longevity in humans and metazoans (Zarse et al., 2011). In medicine, lithium in the form of Li-carbonate or Li-citrate, is used to treat bipolar disorder (Ghasemi et al., 2008, 2009, 2010, Baldessarini et al., 2006). In industry, lithium is used in the manufacture of ceramics and glass resistant to high temperatures, and in the production of lubricants resistant to high temperatures, as well as, in the production of Li-ion batteries (Ebensperger et al., 2005).

Lithium-oscillations of the membrane potential – The method of recording biopotentials by means of microelectrodes is being used to study a variable mechanism of action of lithium during generating of membrane potential oscillations, and indirectly during the inducement of the oscillatory lithium transport across the excitable membrane of the *Nittela* cell (Vorobiev et al, 1967, 1968). A rhythmic fluctuation of the membrane potential was recorded by the minimum improvement of this method (Radenović et al., 1968). However, only in 1976, some bioelectric responses to the membrane of the *Nittela* cell (change in ψ_m, membrane oscillations and single impulses) induced by lithium concentrations were registered for the first time (Radenović, 1976). Somewhat later, typical oscillations ψ_m were registered (Radenović and Penčić, 1970), and then oscillations ψ_m caused by monovalent cations, among which Li^+ had also been present, were registered (Radenović et al., 1977). The *Nitella* and *Chara* (freshwater green algae of the family *Characeae*) were used as the object of studies on membrane potential oscillations induced by lithium. The greatest number of experiments with actions of lithium was performed on the *Nitella mucronata* cells. These cells are large (diameter: 0.6-1.0 mm, length: 40-80 mm) and they are suitable for bioelectrochemical and electrophysiological studies. Today, the stated objects are considered the conventional model object for studies of complex membrane-transport processes (Vuksanović et al., 1998, Radenović, 2001, Radenović et al., 2006).

Results obtained in scarce previous studies are not sufficient to develop a complete and complex idea of the oscillatory transport of Li^+ across the very excitable membrane of the *Nitella* cell. Some new issues related to oscillatory membrane processes arose. It was assumed that Li^+ was their inevitable inducer, but not the only (Damjanović and Radenović, 1971, Radenović and Vučinić, 1976, 1985, Radenović, 1985a). New issues are primarily related to various oscillations of transport processes caused by effects of shocking levels of lithium ions (Radenović et al., 1977, 2006).

The special attention in this study was paid to variable mechanisms of lithium transport processes during generating oscillations ψ_m across the very excitable membrane of the *Nitella* cell.

MATERIAL AND METHODS

Plant objects – The experiments were performed on cells of freshwater alga *Nitella mucronata*. These cells are large (diameter: 0.6-1.0 mm, length: 40-80 mm) and they are suitable for bioelectrochemical and electrophysiological studies. Growing conditions, object preparations, treatment prior and during measuring of ψ_m were described in previously published papers (R a d e n o v i ć and P e n č i ć, 1970, R a d e n o v i ć, 1974, R a d e n o v i ć and V u č i n i ć, 1976, R a d e n o v i ć et al., 1977). It is generally accepted that these cells represent a classical model system for diverse studying on membrane-transport processes (R a d e n o v i ć, 1982, 1985a, 1985b, 2001, V u k s a n o v i ć et al., 1998, R a d e n o v i ć et al., 2005, 2006).

Method – The measurement of rhythmic and membrane bioelectric signals: single impulses, sequences of impulses and different forms of membrane potential oscillation (ψ_m, mV) was performed after the method with a microelectrode technique, which was previously described in principle and details in studies carried out by R a d e n o v i ć and P e n č i ć, 1970, R a d e n o v i ć, 1974, R a d e n o v i ć and V u č i n i ć, 1976, R a d e n o v i ć et al., 1977, (Fig. 1).

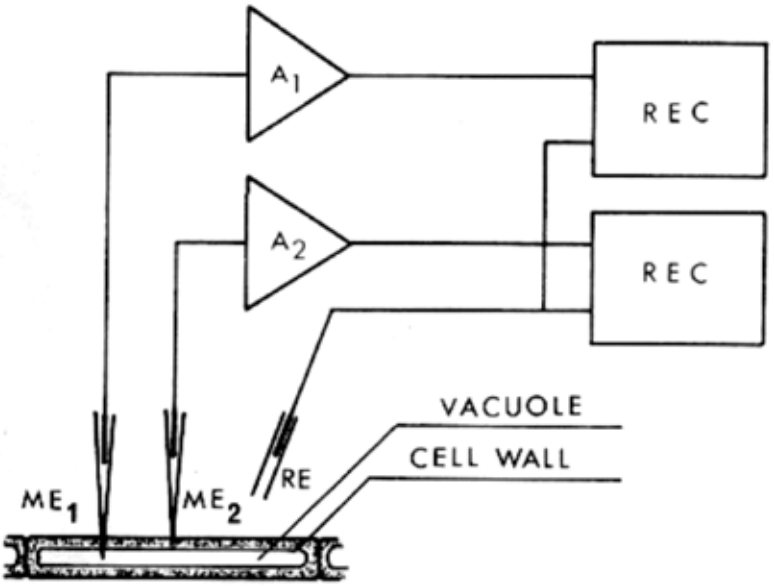

Fig. 1. – Schematic diagram of the method of measurement of the membrane potential across the *Nitella* cell applying the microelectrode technique: ME_1 – microelectrode in the vacuole, ME_2 – microelectrode in the cell wall, RE – reference electrode, A_1 and A_2 – amplifiers, REC – recorders.

RESULTS

The initial measurement of the equilibrium resting membrane potential (ψ_m, mV) is generally accepted as a rule, for bioelectric (bioelectrochemical and electrophysiological) measurements across membranes of the *Nitella* cell. If its value ranges from -80 mV to -150mV the experiment on the membrane of the *Nitella* cell can be continued. It should be known that the value of the uniform resting membrane potential in the *Nitella* cell depends on a physiological state of the cell, growing conditions, age, as well as, on the season (R a d e n o v i ć, 1974).

There is a possibility to observe different classes of membrane potential oscillations, single and local impulses within numerous bioelectric studies (R a d e n o v i ć, 1985b). In order to recognise the oscillatory response easily the following is given:

- Local impulse can occur in the initial part of the oscillation, but it can be clearly pronounced and easily registered.
- Single impulse can be pronounced more often and more regularly and can simply be registered.
- Sequences of single impulses occur often and they are simply registered. In literature, they are referred to as membrane potential oscillations.
- It is shown that membranes of the *Nitelle* cell are capable, under effects of selected stimuli, to generate local and single impulses and repetitive membrane potential oscillations (R a d e n o v i ć and P e n č i ć, 1970, R a d e n o v i ć and V u č i n i ć, 1976, R a d e n o v i ć et al., 1977).

This paper presents four examples of Li-oscillations of the membrane potential.

1. Instantaneous generation of lithium-oscillations in the direction of membrane potential depolarisation

Generation of lithium-oscillations in the direction of depolarisation of the membrane potential is given in the form of six different classes (Fig. 2.1-2.6). Their generation is explained by the effects of the concentration gradient of lithium (10 mM), sodium (1 mM), and potassium (0.1 mM). Furthermore, the electrochemical potential gradient and the electric potential gradient also affect the generation of lithium oscillations. They induce the formation of a strong electric field that pulls out the ions (Li, Na and K) and in such a way, their transport is provided. The intensity and dynamics of the overall transport processes of the ions (Li, Na, and K) are significantly affected by the nature of movements of active molecules (proteins, lipids and pigments): rotational, flip-flop and lateral. When the membrane is very excited, the mentioned types of movements of active molecules and the effects of stated gradients establish the interdependence of processes that affect six different classes of oscillations. Therefore, the interdependence of processes of competitiveness of ions (Li, Na, and K) in the overall transport processes, the dominance of certain types of movements of active molecules and the very excitable membrane, determine parameters and form of six classes of membrane potential oscillations.

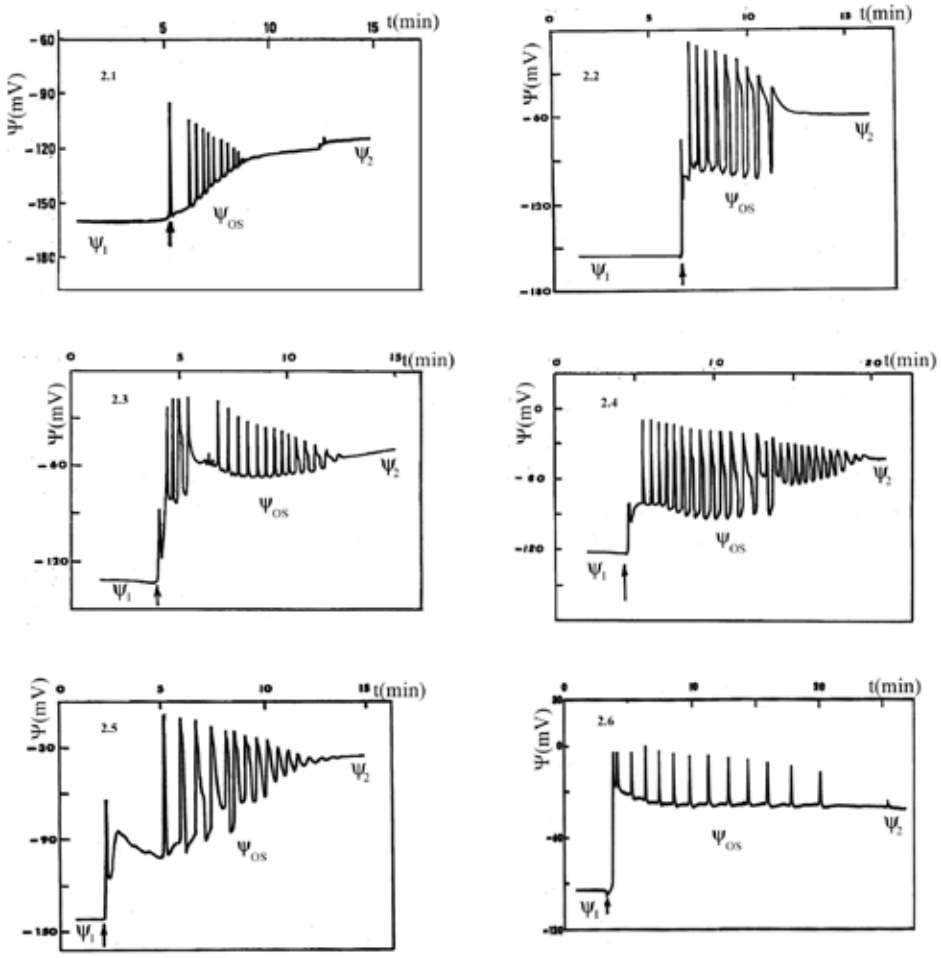

Fig. 2.1-2.6. – Instantaneous generation of lithium-oscillations in the direction of membrane potential depolarisation triggered off by the exchange of the standard solution for the LiCl solution of the shocking concentration of 10 mM.
Symbols: standard solution (SR: 0.1 mM KCl + 1.0 mM NaCl), ψ_1 – equilibrium membrane potential prior to oscillating, ψ_2 – equilibrium membrane potential generated by effects of Li after oscillating, ψ_{os} – class of membrane potential oscillations established by effects of shocking LiCl concentration, arrows – indicate the moment when SR was replaced with LiCl solution of the shocking concentration. This Fig. shows six different classes of membrane potential oscillations.

The stated classes of Li-oscillations of the membrane potential are characterised by non-standard parameters (Tab. 1).

Tab. 1. – Non-standard parameters of lithium-oscillations in the direction of membrane potential depolarisation

Figures designations	Ψ_1	Ψ_2	Non-standard parameters Ψ_{os}		
			Number of impulses	Duration of oscillation	Type of oscillation
Fig. 2.1.	-155	-110	10	shorter than standard	symmetric damped
Fig. 2.2.	-160	-60	10	shorter than standard	unsymmetric damped
Fig. 2.2.	-135	-55	18	within limits of standard	symmetric / unsymmetric damped
Fig. 2.4.	-120	-40	28	longer than standard	unsymmetric damped
Fig. 2.5.	-150	-30	16	somewhat shorter than standard	irregularly – symmetric dumped
Fig. 2.6.	-90	-30	14	somewhat longer than standard	differently damped

2. Delayed generation of lithium-oscillations in the direction of membrane potential depolarisation

Delayed generation of lithium-oscillations in the direction of membrane potential depolarisation is presented in the form of three different classes (Fig. 3.1-3.3). They are affected by concentration gradients of competitive ions (Li, Na, and K) in transport processes. The certain classes of membrane potential oscillations ($\psi_{m,}$ mV), (Fig. 3.1-3.3) appear when dominance of particular types of movements of active molecules (protein, lipids and pigments) occur. A gradual generation of the equilibrium membrane potential (ψ_1) in the direction of its repolarisation (Fig. 3.3) precedes the occurrence of membrane potential oscillations. It is believed that Na^+ causes such generation of ψ_1. However, ψ_{os} oscillations are different from all three classes of membrane potential oscillations ($\psi_{m,}$ mV), (Fig. 3.1-3.3).

These classes of lithium-oscillations of the membrane potential (Fig. 3.1-3.3) can be analysed through non-standard parameters (Tab. 2).

Tab. 2. – Non-standard parameters of delayed generation of lithium-oscillations

Figures designations	Ψ_1	Ψ_2	Non-standard parameters Ψ_{os}		
			Number of impulses	Duration of oscillation, (min)	Type of oscillation
Fig. 3.1.	-95	-60	16	8	irregularly – unsymmetric dumped
Fig. 3.2.	-120	-55	20	15	unsymmetric dumped
Fig. 3.3.	-100	-60	8	10	irregularly – unsymmetric dumped

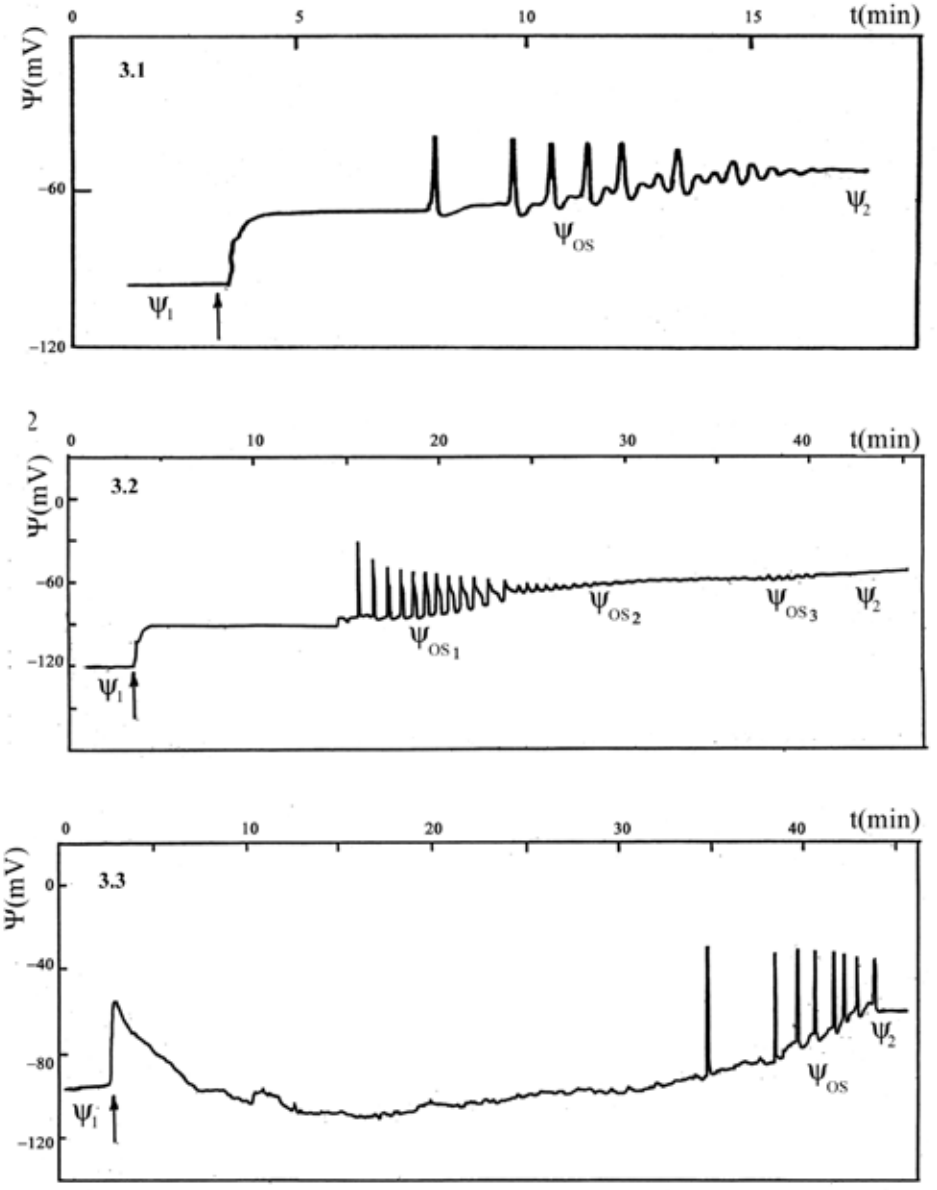

Fig. 3.1-3.3. – Delayed generation of lithium-oscillations in the direction of membrane potential depolarisation triggered off by the exchange of SR for the LiCl solution of shocking concentration (10 mM).
Symbols are the same as in Fig. 2.1-2.6. This Fig. shows three different classes of membrane potential oscillations.

3. Instantaneous generation of lithium-oscillations in the direction of membrane potential repolarisation

Generation of lithium-oscillations in the direction of membrane potential repolarisation rarely occurs and it is presented in Fig. 4. Basically, the explanation of this class of membrane potential oscillations is identical as in Fig. 2.1-2.6 and Fig. 3.1-3.3. Different physical and chemical conditions occurring in the very excitable membrane lead, not so infrequently, to interdependence of various processes that move, some one way and some in the opposite direction. Such a state of interdependent processes refers to both, transport processes of ions (Li, Na, and K) and frequent changes in types of movements of active molecules, first of all proteins. Bearing in mind herein stated, it is possible to understand "anomalies" occurring during generation of ψ_2, and in the case of specific oscillation ψ_{os} presented in Fig. 4.

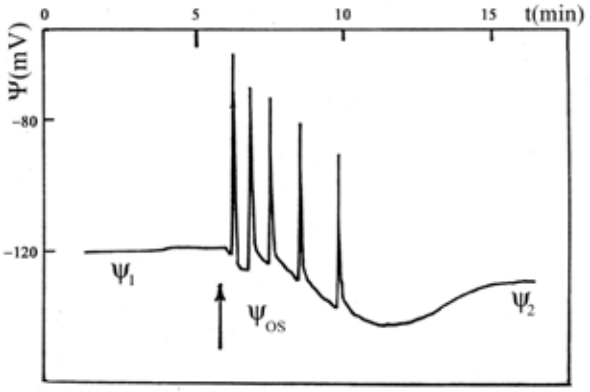

Fig. 4. – Instantaneous generation of lithium-oscillations in the direction of membrane potential repolarisation triggered off by the exchange of SR for the LiCl solution of the shocking concentration of 10 mM.
Symbols are the same as in Fig. 2.1-2.6. This Fig. shows one class of membrane potential oscillations.

Lithium-oscillations in the direction of membrane potential repolarisation (Fig. 4) have the following non-standard parameters: ψ_1 = -120 mV, ψ_2 = -130 mV, number of impulses = 5, duration of lithium-oscillation = 7 min and the type of lithium-oscillation is irregular, unsymmetric and undamped.

4. Instantaneous generation of lithium with the unaltered level of membrane potential prior to and after oscillating

Generation of lithium with the unaltered level of membrane potential prior to and after oscillating extremely rarely occurs and it is presented in Fig. 5.

Lithium-oscillations with the unaltered level of membrane potential prior to and after oscillating (Fig. 5) was triggered off by the exchange of SR for the LiCl solution of a shocking concentration and has the following non-standard parameters: ψ_1 = -100 mV, ψ_2 = -100 mV, number of impulses = 8, duration of lithium-oscillation = 4 min and the type of lithium-oscillation is less regular, unsymmetric and damped.

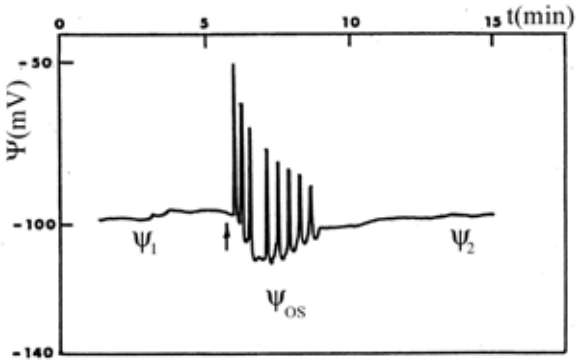

Fig. 5. – Instantaneous generation of lithium with the unaltered level of the equilibrium membrane potential prior to and after oscillating triggered off by the exchange of SR for the LiCl solution of the shocking concentration of 10 mM.
Symbols are the same as in Fig. 2.1-2.6. This Fig. shows one class of membrane potential oscillations.

DISCUSSION

Discussion on general information on lithium – A molecular mechanisms of effects of lithium are still insufficiently clarified (K l e i n and M e l t o n, 1996). According to its ionic radius Li^+ is the most similar to Mg ion, which suggests its possible competition with the activities of Mg ion. It is considered that Li ion can affect inactivation of enzyme GSK3β, which can cause resetting of the circadian clock in the brain (Y i n et al., 2006). Recently it has been suggested that lithium could interfere with NO regulatory pathway, which has a key role in the nervous system (G h a s e m i et al, 2008.). It was also shown that lithium could interfere with inositol phosphatases, i.e. could inhibit inositol monophosphatase (E i n a t et al., 1998.). Besides, it is considered that the Li ion interferes with the transmembrane transport of monovalent and bivalent cations of nerve cells due to its similarity with them (Na, K and Mg, Tab. 3) (L a s s a l l e s et al., 1981).

Tab. 3. – Physical and chemical characteristics of Li, K, Na and Mg

Characteristics	Li	K	Na	Mg
Atomic radius (Å)	1.33	2.03	1.57	1.36
Ionic radius (Å)	0.60	1.33	0.95	0.65
Hydrated radius (Å)	3.40	2.32	2.76	4.67
Polarizing power (z/r^2)	2.80	0.56	1.12	2.05
Electronegativity	1.0	0.80	0.9	1.0

Discussion on studies presented in this paper. – Results obtained on oscillatory bioelectric signal (local impulses, isolated single impulses, sequence of local and single impulses and typical oscillation of the membrane

potential), presented in this paper, are only a smaller part of our long-term studies on total membrane potential oscillations, and indirectly on oscillatory transport of ions (K, Na, Ca, Li, and Cl) across excitable cell membrane (R a d e n o v i ć, 1982, 1985a, V u č i n i ć et al., 1987, V u l e t i ć et al., 1985, 1987). This is especially true for lithium-oscillations of the membrane potential, which are very specific and as such give the possibility to analyse a number of questions to which answers are not yet known in detail.

Some parameters of lithium-oscillations of the membrane potential have already been studied (R a d e n o v i ć et al., 1977, R a d e n o v i ć, 1982, 1985a, 1985b). However, they should be mentioned considering that there is a possibility to establish the analogy between oscillations in physics and biology (V u k s a n o v i ć et al., 1998, K o l j s et al., 1993). These parameters are: basic level of the membrane potential oscillation, impulse spike potential (the level up to which a membrane is depolarised during generating single or successive impulses and the complete oscillation), amplitude of single or successive impulses generated during the membrane potential oscillation, the relationship of the amplitude of one impulse with the amplitude of the following or previous impulse in the selected membrane potential oscillation, impulse interval (the duration between two successive impulses) and other standard parameters given in Tab. 4 (R a d e n o v i ć et al., 1977).

Tab. 4. – Standard parameters of membrane potential oscillations induced by effects of standard concentrations of Li, Na and K on the membrane of the *Nitella* cell

Ions	Oscillation duration (min)	Number of impulses	Impulse amplitude (mV)	Frequency (imp/min)	Damping factor
Li^+	11.7	13	39±14	1.44±0.6	1.195
K^+	1.9	6	39±19	3.62±1.4	2.153
Na^+	24.1	24	54±16	1.04±0.5	1.081

In addition, attention should be paid to issues such as the kinetics of single impulses and the kinetics of the complete oscillation of the membrane potential. The important issues are the character of occurrence and behaviour of rhythms of bioelectric signals (D a m j a n o v i ć and R a d e n o v i ć, 1971, R a d e n o v i ć and V u č i n i ć, 1976, V u č i n i ć et al., 1987), but also effects of concentrations of selected ions on generating membrane potential oscillations (V u l e t i ć et al., 1985, 1987). The above-mentioned issues and parameters characterising membrane potential oscillations are directly dependent on transport processes occurring across the very excitable cell membrane (R a d e n o v i ć, 1998).

As it is known, systems with one or two degrees of freedom are the basis for studying the mechanism of the membrane potential. Furthermore, different types of movements of lipids, proteins, pigments and other complex-bound structures contribute to the mechanism of the total transport processes across the very excitable cell membrane (R a d e n o v i ć, 1998). These types of movements within the very excitable membrane can be as follows: lateral movement

(typical for lipids and proteins), rotational movement (typical for proteins specialised for the ion transport) and so-called flip-flop movement (typical for lipids and proteins that regulate the ion transport from one side of excitable cell membrane to other).

When the degree of excitability of the cell membrane is higher, then the variable types of movements of active molecules (lipids, proteins and other molecules) are more significant in their intensity, dynamics and diversity, which affects the total ion transport processes (K o l j s et al., 1993, R a d e n o v i ć, 1998), especially lithium transport processes (R a d e n o v i ć, 1976, R a d e n o v i ć et al., 1977, 2005, 2006).

As it is known, the transport of ions (including lithium) across the very excitable cell membrane is characterised by passive and active ion transport processes. Diffusion is considered to be a dominant bearer of passive transport processes in the very excitable membrane. It is expressed as a simple, limited, and facilitated diffusion. It is clear that there are at least three promoters of the passive ion transport: concentration gradient, electrochemical potential gradient, and electric potential gradient.

Obtained results, presented in this paper, indicate that lithium-oscillations in the direction of membrane potential depolarisation occurs under particular conditions (Fig. 2.1-2.6, Tab. 1). Moreover, delayed generation of lithium-oscillations in the direction of membrane potential depolarisation occurs (Fig. 3.1-3.3.; Tab. 2). Generation of lithium-oscillations in the direction of membrane potential repolarisation also occurs (Fig. 4). It is interesting to mention that generation of lithium with the unaltered level of the membrane potential also occurs prior to and after oscillating (Fig. 5).

CONCLUSION

Based on the gained results and the discussion, as well as, our overall information on oscillatory processes induced by Li, K, Na, NH_4 and Ca, we present the following hypothesis:

• Lithium-oscillations (local and single impulses and other classes of oscillations) of the membrane potential occur when the cell membrane is very excited. Such a membrane, as a rule, is accompanied by the activities of ions K^+, Na^+, Li^+ and Cl^-, which are not constant under such conditions in subcellular components (vacuole, cytoplasm, and cell wall).

• The usual ion transport processes are disturbed under effects of lithium: first, diffusion (concentration gradient is altered), electrodiffusion (electrochemical potential gradient is changed), biocurrents (electric potential gradient is altered), and fluid flow (hydrostatic pressure gradient is modified). The mentioned dynamic states determine the degree of excitability of the very excitable cell membrane. Hence, when the cell membrane is very excited, then local, single and complete membrane potential oscillations inevitably occur. These oscillations occur in the form of certain classes, but also in the form of different irregularities (chaos). At the same time and under such conditions,

oscillating of active proteins starts in the cell membrane, and they rhythmically, regularly, irregularly (state of chaos) induce the transport of ions, Na, K and Li, across the very excitable membrane, which takes an oscillatory regime. In such a state, transport processes of ions, K, Na and Li, adopt a co-operative character, which induce conformational changes of active ion channels that stretch and contract within the oscillatory regime, and thereby rhythmically modify transport ability of the excitable cell membrane for ions of K, Na and Li.

• Under such conditions, oscillatory changes occur in cell supplying, and thereby in supplying the very excitable membrane with energy: electric, osmotic and chemical.

• Moreover, the bonds between membrane transport processes and metabolism are disturbed, i.e. weakened. This is particularly related to weakening of the self-regulation of the matter within each cell.

REFERENCES

Baldessarini, R. J., Tondo, L., Davis, P., Pompili, M., Goodwin, F. K., Hennen, J. (2006): Decreased risk of suicides and attempts during long-term lithium treatment: A meta-analytic review. Bipolar Disord. 8 (5pt2): 625–39.

Chassard-Bouchaud, C., Galle, P., Escaig, F., Miyawaki, M. (1984): Bioaccumulation of lithium by marine organisms in European, American and Asian coastal zones: microanalytic study using secondary ion emission. C R Acad Sci III 299 (18): 719–724.

Crichton, R. (2008): Some Facts about Lithium. ENC Labs. http://www.enclabs.com/lithium.html. Retrieved 2010-10-15.

Damjanović, Z., Radenović, Č. (1971): Some implications of ringing bioelectrical response in Nitella cels. In: Membranes, Transport, ed. Verlog der Winer Medizinischen Academie, wiena, pp. 441–445.

Einat, H., Kofman, O., Itkin, O., Lewitan, R. J., Belmaker, R. H. (1998): Augmentation of lithium's behavioral effect by inositol uptake inhibitors. J Neural Transm 105 (1): 31–38.

Ebensperger, A., Maxwell, P., Moscoso, C. (2005): The lithium industry: Its recent evolution and future prospects. Resour. Policy 30 (3): 218–231.

Ghasemi, M., Raza, M., Dehpour, A. R. (2010): NMDA receptor antagonists augment antidepressant-like effects of lithium in the mouse forced swimming test. J. Psychopharmacol 24 (4): 585–594.

Ghasemi, M., Sadeghipour, H., Mosleh, A., Sadeghipour, H. R., Mani, A. R., Dehpour, A. R. (2008): Nitric oxide involvement in the antidepressant-like effects of acute lithium administration in the mouse forced swimming test. Eur Neuropsychopharmacol 18 (5): 323–332.

Ghasemi, M., Sadeghipour, H., Poorheidari, G., Dehpour, A. R. (2009): A role for nitrergic system in the antidepressant-like effects of chronic lithium treatment in the mouse forced swimming test. Behav. Brain Res. 200 (1): 76–82.

Klein, P. S., Melton, D. A. (1996): A molecular mechanism for the effect of lithium on development Proc. Natl. Acad. Sci. U.S.A. 93 (16): 8455–8459.

K o l j s, O. R., M a k s i m o v, G. V., R a d e n o v i ć, C. N. (1993): Biofizika ritmiceskovo vozbuzdenja, izdateljstvo Moskovskovo univerziteta, Moskva.
L a s s a l l e s, J. P., H a r t m a n n, A., T h e l l i e r, M. (1980): Oscillation of the electric potential of frog skin under the effect of Li+: experimental approach. J. Membr. Biol. 56 (2): 107–119.
R a d e n o v i ć, Č. (1974): Bioelektrične pojave biljnih sistema, izd. Nolit, Beograd, str. 7-161.
R a d e n o v i ć, Č. (1976): Some models of bioelectric response (changes in membrane potential, membrane oscillations and individual impulses) caused by lithium concentrations in the membrane of the living plant cells. Book of Abstracts, VII Yugoslav Symposium on Biophysics, November 11-13, 1976, Bled, Slovenia, Yugoslavia.
R a d e n o v i ć, Č. (1982): Ritmični bioelektrični signali kod biljaka. U: Multidisciplinarne nauke i njihova uloga u naučnotehničkom progresu, izd. Centar za multidisciplinarne studije, Beograd, str. 89–103.
R a d e n o v i ć, Č. (1985a): Ритмические электрические сигналы у растений. Физиол. биох. культурних растений 7 (6): 523–529.
R a d e n o v i ć, Č. (1985b): Karakteristike nekih klasa oscilacija membranskog potencijala. Zb. rad. IX Jugoslovenskog simpozijuma o elektrohemiji, Dubrovnik, Jugoslavija, str. 78–81.
R a d e n o v i ć, Č. (1998): Transportni procesi kroz membranu. U: Savremena biofizika, izd. Velarta, Beograd, str. 3–90.
R a d e n o v i ć, Č. (2001): The mechanism of oscillatory ion transport and biopotential across the excited cell membrane. Proc. Nat. Sci. Matica Srpska 100: 91–104.
R a d e n o v i ć, Č. N., B e l j a n s k i, M. V., M a k s i m o v, G. V., K a l a u z i, A. A., D r a ž i ć, M. D. (2005): The mechanism of the NH_4 ion oscillatory transport across the excitable cell membrane. Proc. Nat. Sci. Matica Srpska 109: 5–19.
R a d e n o v i ć, Č., B e l j a n s k i, M., M a k s i m o v a, N., M a k s i m o v, G. V. (2006): Characteristics of lithium oscillatory transport trough excited biomembrane. Book of Proceedings of Physical Chemistry 2006, Belgrade pp. 276–278.
R a d e n o v i ć, Č. N., C a r e v a, L. A., S i n j u h i n, A. M. (1968): O različnoj prirode ritmičeskih kolebanij potencijalov citoplazmi I obolochki rastiteljnih kletok. Biofizika 13: 270–281.
R a d e n o v i ć, Č. N., P e n č i ć, M. (1970): Oscillations bioelectriques dans des membranes de Nitella. Physiol. plant. 23: 697–703.
R a d e n o v i ć, Č., V u č i n i ć, Ž. (1976): Simultaneous measurement of the cell wall and the vacuoles during the oscilltory response to the Nitella cell. Physiol. plant. 37: 207–212.
R a d e n o v i ć, Č., V u č i n i ć, Ž. (1985): Oscillations of the membrane potential. Period. biol. 87 (2): 161–165.
R a d e n o v i ć, Č., V u č i n i ć, Ž., D a m j a n o v i ć, Z. (1977): Oscillations of the bioelectric potential across the membranes of Nittela triggered by monovalent cations. In: Electrical phenomena at the Biological Membrane Level, Roux E. ed. Elsevier Psci. Pub. Co., Amsterdam.
V o r o b l j e v, L. N., R a d e n o v i ć, Č., K h y t r o v i, Y. A., Y a g l o v a, L. G. (1967): Исследование скачков биопотенциалов при введении микроэлектрода в вакуоль клеток Nittela. Биофизика 12 (6): 1016–1021.
V o r o b l j e v, L. N., S u - j u n j, L., R a d e n o v i ć, Č. (1968): Регулирование ионного состава и биоэлектрических потенциалов клеток при измерении их внешней и внутренней

среды. В: Физико-химические основы авторегуляции в клетках, изд. “Наука”, Москва, СССР, стр. 143–154.
Vučinić, Ž., Radenović, Č., Damjanović, Z. (1987): Oscillations of the vacuolar potential in Nitella. Physiol. Plant. 44: 181–186.
Vuksanović, V., Radenović, Č., Beleslin, B. (1998): Oscillatory phenomena, processes and mechanisms – physical and biological analogy. Yugoslav. Physiol. Pharmacol. Acta 34: 247–258.
Vuletić, M., Radenović, Č., Vučinić, Ž. (1987): The role of calcium in the generation of membrane potential oscillations in Nittela cells. Gen. Physiol. Biophys. 6: 203–207.
Vuletić, M., Vučinić, Ž., Radenović, Č. (1985): Electrogenic proton pump in Nittela and the effect of calcium. Gen. Physiol. Biophys. 4: 195–200.
Yin, L., Wang, J., Klein, P. S., Lazar, M. A. (2006): Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock. Science 311 (5763): 1002–5.
Zarse, K., Terao, T., Tian, J., Iwata, N., Ishii, N., Ristow, M. (2011). Low-dose lithium uptake promotes longevity in humans and metazoans. Eur. J. Nutr. 50 (5): 387–389.

ПРОМЕНЉИВИ МЕХАНИЗМИ ДЕЛОВАЊА ЛИТИЈУМА ПРИ НАСТАЈАЊУ ОСЦИЛАЦИЈА МЕМБРАНСКОГ ПОТЕНЦИЈАЛА НА ПОБУЂЕНОЈ ЋЕЛИЈСКОЈ МЕМБРАНИ NITELLE

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Резиме

У овом раду су изложени резултати проучавања промењивог механизма транспортних процеса литијума, при настајању осцилација мембранског потенцијала, на екстремно побуђеној мембрани ћелије Nitelle. Показано је настајање неколико класа осцилација, појединачних и локалних импулса мембранског потенцијала, у зависности од деловања шокантне концентрације (10 mM) LiCl, са којом се ћелијска мембрана екстремно побуђује. Дају се резултати осциловања мембранског потенцијала, а затим се излажу неки од параметара осцилограма. Тврди се да је осциловање мембранског потенцијала условљено укупним осцилаторним транспортним процесима: Li^+, K^+, Na^+ и Cl^- кроз екстремно побуђену ћелијску мембрану. Изложена је хипотеза о механизмима осцилаторних транспортних процеса јона (Li, Na, K и Cl) изражена преко различитих класа осцилација, појединачних и локалних импулса мембранског потенцијала кроз екстремно побуђену мембрану ћелије Nitelle.

КЉУЧНЕ РЕЧИ: литијум, мембрански потенцијал, осцилаторни транспорт, параметри осциловања, побуђена мембрана, ћелија Nitelle

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INVESTIGATION OF SAND IN PIPING

ABSTRACT: For the investigation of the grain size distribution of the material washed out from the piping, we used 20 samples originating from different places on the Danube and the Tisza rivers. The grading characteristics of these samples were investigated based on selected grain sizes and the uniformity of gradients. Based on the investigations it has become possible to identify which grain size fractions are likely to be washed out, and how to characterize those fractions. Based on the grain size distribution curves it has been made possible to define the boundaries of the zone susceptible to piping.

The zone limits of granular soils liquefied by earthquakes and the zone limits of the soil out washed from piping are very similar. This apparent correspondence already formerly raised the hypothetic question of whether piping occurring during high flood can be simulated by shape to similar surface liquefaction phenomena experienced during earthquakes, as in both cases a volcanic cone is formed through the crater of which water is constantly issuing, dragging away solid particles.

KEY WORDS: coefficient of uniformity, grain size distribution, hydraulic failure, piping

INTRODUCTION

This paper places particular emphasis on a special feature in the protection against piping directing attention to some important aspects of flood defense and to the need for going into a deeper study of certain details of the phenomenon of piping. Undoubtedly, we know a great deal more about piping than we did just 30 years ago, nevertheless investigations need be continued. Research has been pursued in various fields in an attempt to find answers to some peculiar but very real problems, as follows:

- In the case of recorded actual piping's the mean hydraulic gradient only reached a value that was as low as less than one fifth of the allowable value, yet failure conditions did occur (Nagy et al., 1994).
- Grading entropy shed light on soils prone to piping from the theoretical side (Lőrincz, 1986, 1993, Lőrincz et al., 2004, Imre et al., 2008), but the practical approach revealed that in the vicinity of all the piping's

a particular layer prone to piping failure invariably occurred in the stratified soil (L ő r i n c z, N a g y, 1995, 2010).

- The giant piping on the Tisza's (Fig. 1) in year 2000 raised a number of questions since piping occurred in the embankment (and not in the subsoil). This phenomenon formerly not heard of is quite possible provided that the material of the embankment is sufficiently loose and the fill contains a layer prone to piping.
- Parallel to what was mentioned above a remarkable development can be observed in international experience, in numerical modeling and in the study of transient phenomena of discreet particles to provide theoretical backing for flood phenomena.

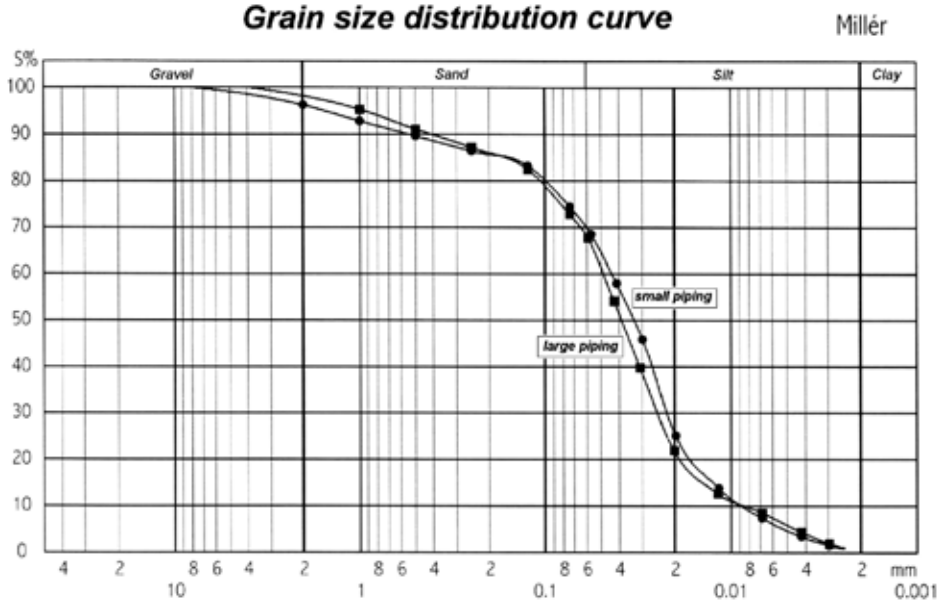
Fig. 1. – The giant piping on the Tisza's in year 2000

Formerly little attention was paid to the investigation of material washed out of a piping (N a g y, 2000). The grain size distribution of the washed out material was not tested, nor was it compared to the grading of surrounding soil layers. Now, an important question arises: Is it the entire mass of a soil layer or only certain fractions of the soil that are washed out? During the high flood on the Danube in 2006, samples were taken from a number of piping. Tests on these samples form the backbone of the present paper.

INVESTIGATION SITES

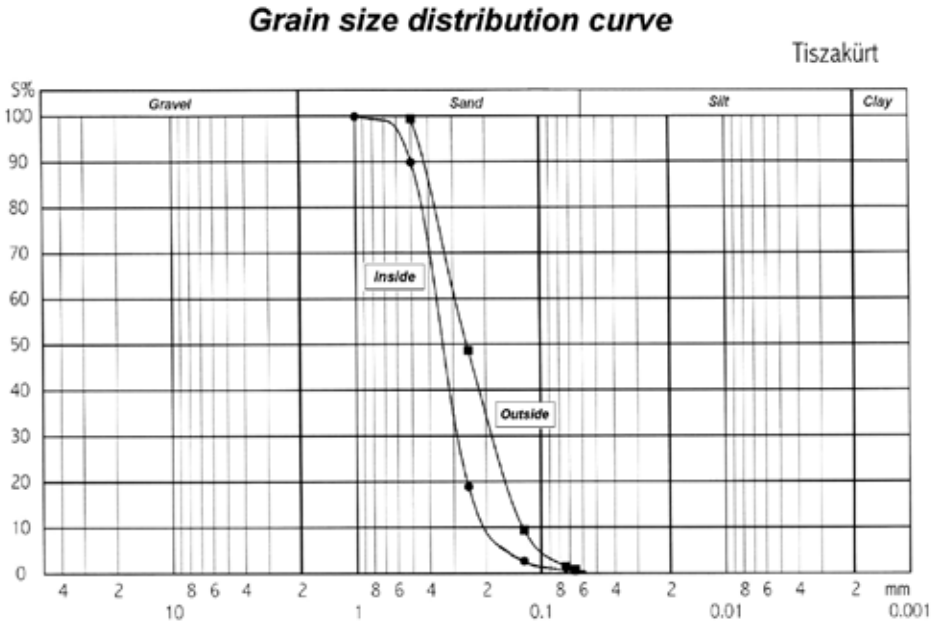
The summary of investigated sites grouped according to the rivers and to the years of occurrence is contained in Table 1. Materials obtained from 12 piping by the river Tisza, 7 by the Danube and 1 by the river Sajó were tested. Based on these samples the effect of a number of factors has also been revealed at the various sites.

Do grading curves of samples taken from piping maximum a few meters away from each other (see e.g. Fig. 3) show a different picture? Piping located close nearby (samples 5 – 8 and 17 – 18 in Table 1) are supposed to give identical grading curves; yet testing experience indicates a difference in the shape of the grain size distribution curves (Graph 1).



Graph 1. – Grain size distribution of two pipings located close to each other (Soils No. 17-18 in Tables 1 and 2)

Another question also arises as to what differences, if any, can be between the grain size diagrams of two samples taken from different parts of the same piping (Samples No. 2-3, 5-6, 14-15 and 19-20 respectively in Table 1). It can be said that a difference does exist. Finer particles are conveyed and then deposited by the water farther away from the piping. Therefore, at the central part where water is issuing in concentrated flow sedimentation of the coarsest grains is expected, while finer grains settle at gradually increasing distances. As can be seen in Graph 2, the difference between the grading curves can be relatively great.



Graph 2. – Grain size distribution curves of two samples taken from different parts of the same piping (Soils No. 19–20 in Tables 1 and 2)

Fig. 2. – Piping (partly behind the car) at the edge of an ox-bow near Bölcske (2006), with a difference in elevation of minimum two meters between ground level at the ox-bow and ground level at the adjacent high bank (Areas No. 9–10 in Tables 1 and 2)

It is a common premise that any test is worth as much as the reliability of the underlying data. In geotechnics, determination of the grain size distribution is a routine test and as such can be relied upon for correctness of test results. Yet, any theoretical conclusion is of no use if the determination of the grain size distribution curve is unreliable. The laboratory tests referred to in this set of tests were carried out at different laboratories, though the majority of the samples were tested at the Geotechnical Laboratory of the Budapest University of Technology.

Tab. 1. – Sites of piping investigations

Number	Year	River, location	Remarks	References
1	1998	Tisza, right bank	Tivadar, inner side	Nagy (1999)
2	1998	Tisza, right bank	Tivadar, outer side	Nagy (1999)
3	1998	Tisza, right bank	Dombrád	Nagy (2003)
4	2006	Duna, right bank 12+150	Abda	
5	2006	Duna, right bank. 41+206	Dombor, small piping	
6	2006	Duna, right bank 41+206	Dombor, small piping, crater	
7	2006	Duna, right bank 41+206	Dombor, big piping	
8	2006	Duna, right bank 41+206	Dombor, big piping, crater	
9	2006	Duna, right bank 79+420	Bölcske, ox-bow	
10	2006	Duna, right bank 79+420	Bölcske, ox-bow	
11	2006	Tisza, right bank 61+075		
12	2006	Tisza, right bank 71+300		
13	2006	Tisza, left bank 13+250	Tiszasas, marshy bushland	
14	2006	Tisza, left bank 13+580	Tiszasas, edge of crater	
15	2006	Tisz, left bank 13+580	Tiszasas, centre of crater	
16	2010	Sajó, left bank 6+266		
17	2010	Tisza, Millér	Small piping	
18	2010	Tisza, Millér	Large piping	
19	2010	Tisza, Tiszakürt	Outer part of crater	
20	2010	Tisza, Tiszakürt	Inner part of crater	

SHAPE OF THE GRAIN SIZE DISTRIBUTION CURVE

The grain size distribution curves of the material ejected from piping have continuous smooth shape characteristic of natural soils. When the grain size distribution curves of all the samples tested are plotted in the same graph, they clearly define a distinct zone. The types of soil located within this zone (silty sand, fine sand, sand) exhibit no considerable cohesion, and at the same time the mass of their individual soil particles is small enough allowing them to be readily removed from their position by seeping water (Graph 3.). It should be noted that under sufficiently high hydraulic gradients any type of soil (or even rock) could be washed out by piping. What is significant in this respect

is that for soils within the domain in Graph 3 the lowest hydraulic gradient is necessary.

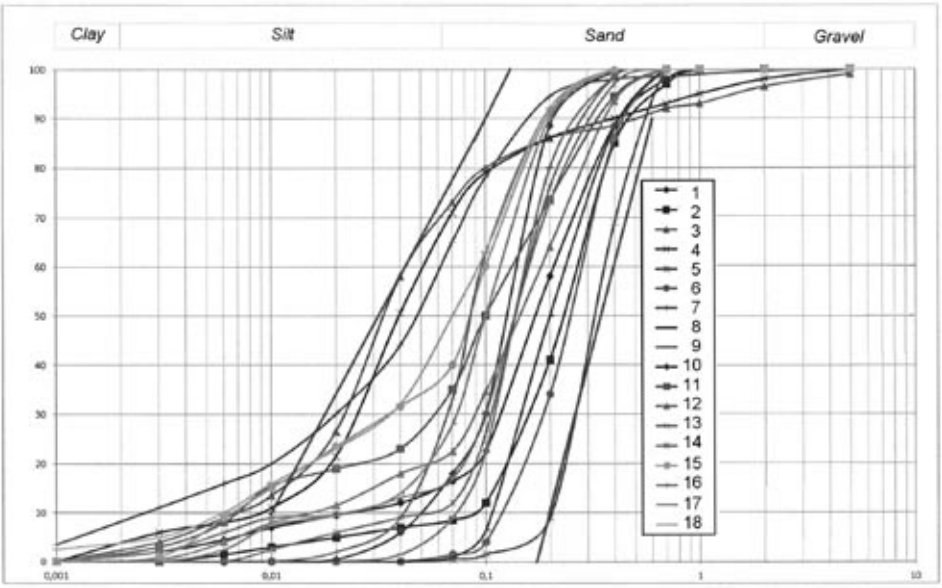
Fig. 3. – Piping at Dombor as seen from the crest of the dyke. Washing out of soil first occurred some 20 meters away from the toe of the embankment (Areas No. 5-8 in Tables 1 and 2)

The zone of grain size distribution curves of all the soils tested is shown again in Graph 4. This zone shows a striking similarity to the zone representing the limits of granular soils liquefied by earthquakes. The grading limits shown in Graph 2 are copied onto a graph presented in Smoltczyk's book (2002) defining various degrees of hazard for liquefaction due to earthquake. (Zone 1: moderately susceptible, Zone 2: highly susceptible). This apparent correspondence already formerly raised the hypothetic question of whether piping occurring during high flood can be simulated by shape to similar surface liquefaction phenomena experienced during earthquakes, as in both cases a volcanic cone is formed through the crater of which water is constantly issuing, dragging away solid particles. The apparent similarity of the zones of grain size distribution, curves in the two cases (Graph 3) strongly suggest that the two phenomena should indeed be closely related.

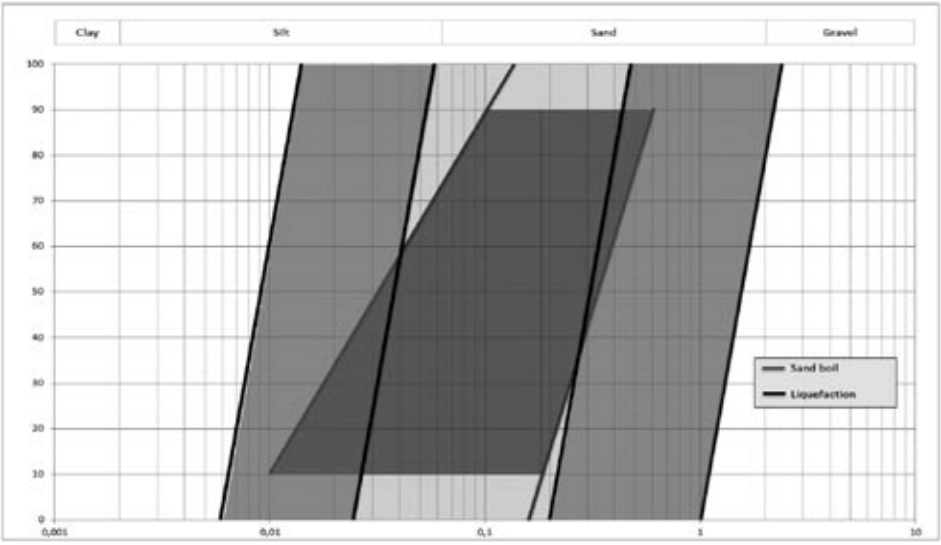
Following the reasoning one cannot help raising a question concerning the similarity of surface phenomena observed in both cases and the similarity of grading: i.e. **whether piping constitutes a pseudo-static liquefaction or liquefaction (e.g. one triggered off by earthquake) constitutes a dynamic piping**.

It should be noted that several expert's reports have recently been prepared dealing with failure of tailing dams where breach of the dam was judged

to have been caused by liquefaction but in none of those cases was failure attributable to earthquake effect. This means that liquefaction may also occur under static loading conditions.



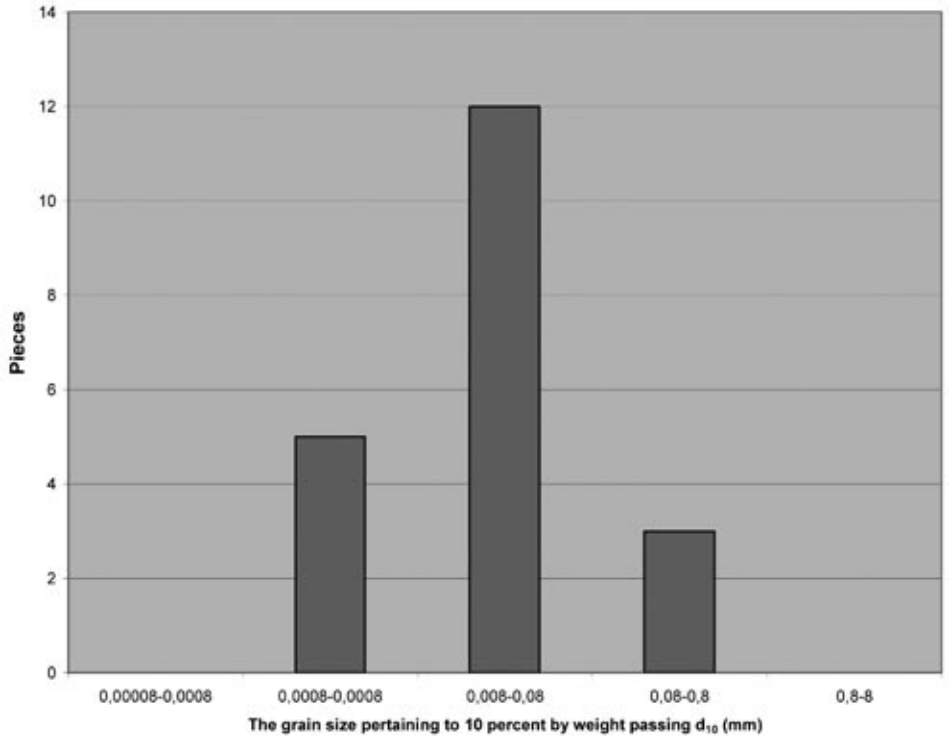
Graph 3. – Grain size distribution curves and limiting envelops of the critical zone



Graph 4. – Zones of soils most susceptible to liquefaction (Smoltczyk, 2002) with limiting lines for the zone of piping added

THE GRAIN SIZE PERTAINING TO 10 PERCENT PASSING

In the study of the grain size distribution a crucial point is the determination of the grain size pertaining to 10 percent passing (see Table 2). This grain size is determinant in respect of seepage phenomena and also in assessing the uniformity of grading. As can be seen in Graph 5, in none of the tests on material washed out of the piping was grain sizes $d_{10} > 0.33$ or $d_{10} < 0.0033$ identified. This means that a domain of grain sizes d_{10} spanning over two orders of magnitude is affected in respect of washing out by piping. Frequency values should normally decrease towards both sides of the histogram but probably because of the relatively small number of elements and the few number of categories this tendency does not appear here.



Graph 5. – Frequency distribution of d_{10} values in the material washed out by piping

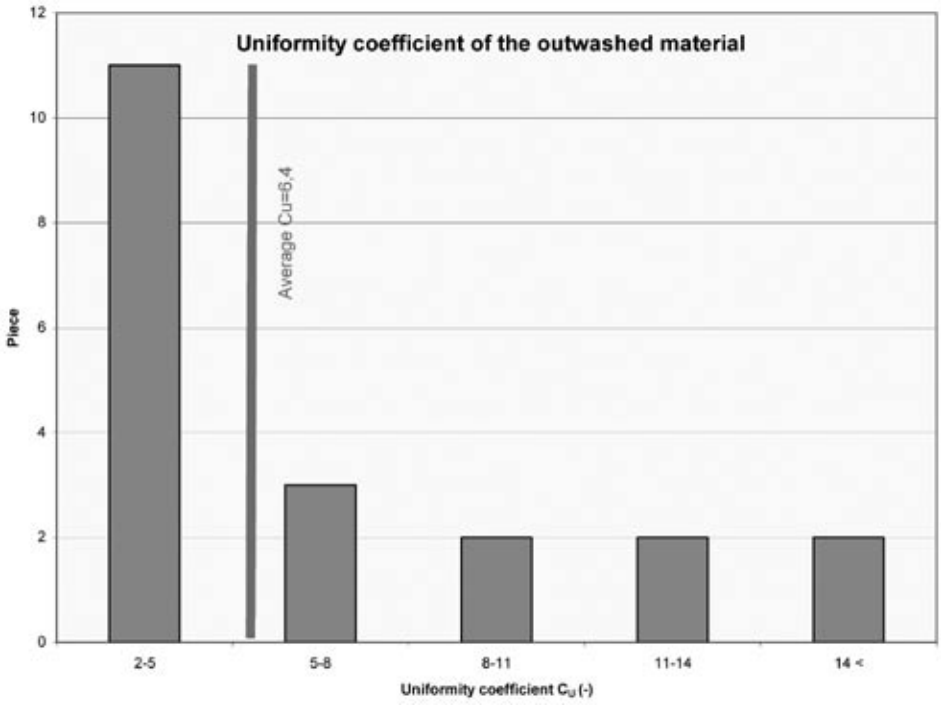
Tab. 2. – Some characteristics of the material washed out by piping

Number	d_{10}	Uniformity coefficient (C_U)	Description of soil
1	0.08	3.25	Fine sand
2	0.025	8.4	Silty sand
3	0.036	4.6	Silty sand
4	0.0071	17.8	Silty sand
5	0.007	13.9	Silty sand
6	0.041	2.4	Sand
7	0.026	4.3	Sand
8	0.006	15.2	Silty sand
9	0.026	6.5	Silty sand
10	0.016	10.8	Silty sand
11	0.056	2.1	Fine sand
12	0.049	3.5	Silty sand
13	0.106	2.2	Fine sand
14	0.073	2.3	Fine sand
15	0.051	2.6	Fine sand
16	0.007	12.6	Silty sand
17	0.007	6.1	Silty sand
18	0.0083	5.9	Silty sand
19	0.17	2.2	Sand
20	0.13	2.3	Sand

Fig. 4. – Piping at Abda (2006), some 20 m away from the dyke toe
(Area No. 4 in Tables 1 and 2)

THE UNIFORMITY COEFFICIENT

Uniformity coefficient (C_U) values of the washed out soils are shown in Table 2. The highest value was $C_U = 17.8$ and the mean value was $C_U = 6.4$. No soil with $C_U < 2.0$ was identified. The frequency distribution of the uniformity coefficients can be seen in Graph 6, where the category of $C_U = 2\text{-}5$ is the most populous, containing more than half of the samples tested. Fine-grained soils with low coefficient of uniformity are the ones that can be most readily washed out or removed from their position, since they have no cohesion and the mass of their grains is small.

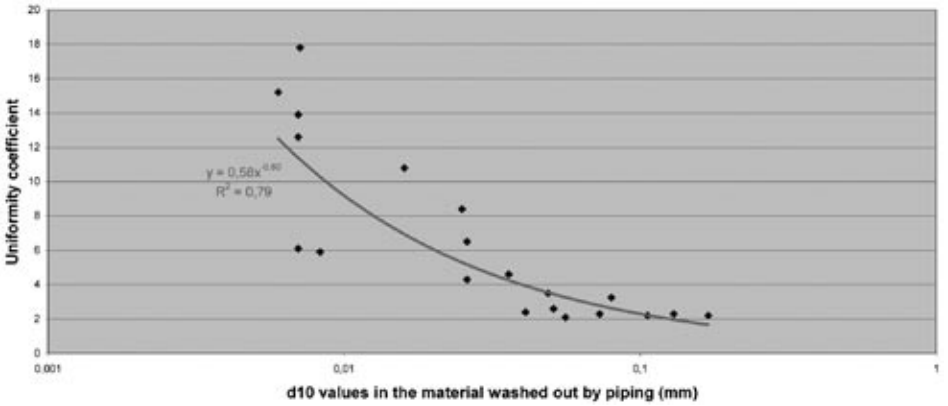


Graph 6. – Frequency distribution of uniformity coefficients in the material washed out by piping

CORRELATION BETWEEN UNIFORMITY COEFFICIENT AND GRAIN SIZE D_{10}

An evaluation of the relationship between the uniformity coefficient (C_U) and the grain size pertaining to 10 percent by weight passing leads to an inverse relation that is the value of C_U tends to decrease with the increase in d_{10}. In other words, the more course-grained the washed out soils, the more closely

they are to a perfectly uniform single-grained soil. The most astonishing fact is the very tight correlation giving a value of nearly R = 0.9 !! (See Graph 6) In spite of the fact that the samples originated, from various regions of the country, and were tested in several laboratories.



Graph 7. – The relationship between the uniformity coefficients and the grain size pertaining to 10 percent by weight passing

SUMMARY

Piping's are the most spectacular phenomena preceding and ultimately leading to breach of flood protection dykes. A review of historic data showed that 1 in every 12 to 13 embankment failures was the consequence of piping (N a g y, 2002). Flood defense counter measures to conquer piping are well established and proven, (P é c h, 1892, T á p a y, S z a l a i, 1954, P o l g á r et al., 1974, N a g y, 2009), while theoretical treatment of ground failure due to piping is not satisfactorily profound. It is an undisputable fact that we know a great deal more about piping than we did say 30 years ago, but continued research must go on even by resorting to practical experience if necessary. Neither hydraulic criteria, nor structural criteria of piping are known deeply enough. We are surely aware of certain parameters that contribute to the build-up of piping, but their effect cannot yet be quantified. In the case of fully developed piping's the average hydraulic gradient normally has a value hardly reaching one fifth of the allowable threshold value, yet ground failure does occur. Density conditions have not been properly dealt with, and also little attention has been paid to the testing of material ejected by piping.

This paper looks at the process of piping from the aspect of material structure by focusing on the grain size distribution of the material washed out from the piping. Using 20 samples originating from different regions of Hungary, the grading characteristics of these samples were investigated based on some selected grain sizes and the uniformity gradients. Based on these inves-

tigations it has become possible to identify which grain size fractions are likely to be washed out, and to characterize those fractions whose washing out is not expected. Based on the grain size distribution curves it has been made possible to define the boundaries of the zone susceptible to piping. The investigations provided useful results concerning values of the uniformity gradient and the grain size pertaining to 10 per cent and the relationship between them.

In order to obtain a deeper understanding of the process of piping the question whether the washed out material consists of the entire mass of a layer or only of a grain size fraction within the layer needs be investigated. To this end a more profound knowledge of the environment of the piping would be very important.

Fig. 5. – Piping at the initial section of a trench drain near Tiszakürt in 2010
(Areas No. 19–20 in Tables 1 and 2)

REFERENCES

I m r e, E., R ó z s a, P., L ő r i n c z, J. (2008): Characterization of some sand mixtures. Proc. of the 12th Int. Con. of Int. Association for Computer Methods and Advances in Geomechanics (IACMAG) pp: 2064–2075, Goa, India.

L ő r i n c z, J. (1986): Soils grading entropy. Dr. Univ. dissertation. BME Építőmérnöki kar, Geotechnikai Tanszék. In Hungarian.

L ő r i n c z, J. (1993): On particle migration with the help of grading entropy. Proc., Conf. on Filter sin Geotechnical and Hydraulic Engineering, Brauns, Heibaum and Schuler, eds., pp. 63–65, Balkema, Rotterdam, the Netherlands.
L ő r i n c z, J., N a g y, L. (1995): Subsoil piping investigation of flood dikes with the help of grading entropy R+D report. In Hungarian.
L ő r i n c z, J., I m r e, E., G á l o s, M., T r a n g, Q. P., T e l e k e s, G., R a j k a i, K. (2004): Grading entropy variation due to soil crushing. Second International Conference from Experimental Evidence Towards Numerical Modeling of Unsaturated Soils. September 18-19, 2003 Bauhaus-university, pp: 215-231. Weimar, Germany.
L ő r i n c z, J., N a g y, L. (2010): Practical characterization of piping with the help of grading entropy Hidrológiai Közlöny, 90. évfolyam, Vol. 3., pp. 59–64. In Hungarian.
N a g y, L. et. al. (1994): Engineering Geophysical Method for Risk Mapping of Flood Protection Sub-catchment Defended by Levees. 7th Int. IAEG Congress, pp. 617–641, Lisboa
N a g y, L. (1999): The Tivadar piping. 17. MHT Vándorgyűlés Miskolc, Vol. 1., pp. 130–146. Budapest.
N a g y, L. (1999): Investigation of the Tivadar buzgár at 1998. november flood. Hidrológiai Közlöny, 79. évf., Vol. 4., pp. 217–222. In Hungarian.
N a g y, L. (2000): Geotechnical problems of dikes. Vízügyi Közlemények, LXXXII. évf., Vol. 1., pp. 121–146. In Hungarian.
N a g y, L. (2002): Years Dike Failures in the Carpathian Basin. Proceedings of the 2nd International Symposium on Flood Defense (ISFD'2002), Beijing, Balkema Publishers, pp. 786–793, Rotterdam, the Netherlands
N a g y, L. (2003): Investigation of the Dombrád piping. Vízügyi Közlemények Vol. 1., pp. 205–215. In Hungarian.
N a g y, L., S z l á v i k, L. ed. (2004): Flood control in the practice. KTVM Vízügyi Hivatala. In Hungarian.
N a g y, L. (2009): Flood control at the villages. Innova-Print Kft. ISBN 978-963-06-7458-4. In Hungarian.
P é c h, J. (1892): Dike defense, Practical guideline for water engineers and flood control experts. In Hungarian.
P o l g á r et al. ed. (1974): Flood defense handbook. VÍZDOK, In Hungarian.
S c m o l t c z y k, U. ed. (2002): Geotechnical Engineering Handbook, Hardcover: 2175 pp., Publisher: Wiley-VCH; 1 edition (June 9, 2003) ISBN-10: 3433014523, ISBN-13: 978-3433014523
S z l á v i k, L., N a g y, L. (1997): The Surány phenomenon. Dangerous dike failures during the 1997 year summer flood. Víztükör, XXXVIII. évf., Vol. 4., p. 35. In Hungarian.
T á p a y, L., S z a l a i, M. (1954): Flood control handbook. In Hungarian.

ПРОУЧАВАЊЕ ПЕСКА У ОДЛИВАЊУ

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Резиме

За проучавање дистрибуције величине честица материјала који се излива током одливања, користили смо 20 узорака који потичу са различитих места на рекама Дунав и Тиса. Градијентне карактеристике тих узорака проучене су на основу одабраних величина честица и униформности градијената. На основу тих проучавања било је могуће идентификовати која величина честице ће се вероватно излити и установити како да се окарактеришу ти делићи честица. На основу криве о дистрибуцији величине честица било је могуће дефинисати границе зоне подложне одливању.

Границе зоне грануларних врста земљишта које су се навлажиле због земљотреса и границе зоне земљишта изливеног током одливања, врло су сличне. Та очита кореспондентност већ раније је довела до хипотетичког питања да ли се одливање које се дешава током високих поплава може симулирати обликом који личи на појаву влажења површине током земљотреса, јер се у оба случаја формира купа кроз чији отвор вода стално истиче, повлачећи чврсте честице.

КЉУЧНЕ РЕЧИ: дистрибуција величине честица, коефицијент униформности, одливање, хидраулички поремећај

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

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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 and enclosed with the author's written consent for the publishing of the manuscript.

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

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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.

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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 документ на адресу: zmspn@maticasrpska.org.rs, уз обавезну потписану изјаву аутора у вези са пријавом рада за штампу.

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2. Припрема рукописа

2.1. Текст рада пише се електронски на страни A4 (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, Ре-

* Ово упутство важи од 2012. године од броја часописа 122.

зултати 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 абецедним редом и одвојити зарезима.

2.7. Апстракт на енглеском и резиме на српском треба да представљају 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 запослени 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 абецедним 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: Навод „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 целини 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 Engineerings Index, Inc.,).

5.5. Референце се не преводе 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.

MATICA SRPSKA
Department of natural Sciences
NOVI SAD
Matica Srpska, Matica Srpska St., No. 1

THE 5th INTERNATIONAL SCIENTIFIC MEETING

MYCOLOGY, MYCOTOXICOLOGY AND MYCOSES

Organizer	Matica Srpska, *Department of Natural Sciences*
Venue	Novi Sad, Matica Srpska, Matica Srpska St., No. 1
Date	**April 17th-19th, 2013**
Topics	I Mycology II Mycotoxins in food and feed III Human and animal mycotoxicosis IV Human, animal and plant mycosis
Participation	▪ Plenary lectures ▪ Oral presentations ▪ Posters ▪ Company presentations: producers/distributors of laboratory equipments, chemicals, microbiological media and other commodities for microbiological/mycotoxicological laboratories.
Title and abstract submission deadline	**November 15th, 2012**
Manuscript	prepared for press in accordance with *Matica Srpska Proceedings for Natural Sciences* instructions, submission deadline: **December 15th, 2012**
Company presentation submission deadline	**February 28th, 2013**
Application and manuscripts should be sent to the address	Matica Srpska, 21000 Novi Sad, Matica Srpska St. № 1 The 5th international scientific meeting *Mycology, mycotoxicology and mycoses* E-mail: mzrnic@maticasrpska.org.rs

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MYCOLOGY, MYCOTOXICOLOGY AND MYCOSES

Novi Sad, April 17-19, 2013.

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