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IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF *Fusarium* sp. FIESC3 THE CAUSAL AGENT OF SEED ROT IN ONION (*Allium cepa* L.)

ABSTRACT: Onion (*Allium cepa* L.) is one of the most important vegetable crops in Serbia, where it is grown on an approximate surface of 20,000 ha. During the routine quality control analysis of onion seed in 2014, fungal infection was observed in an average of 28% of the seed. The objective of this paper was to isolate, determine, and identify *Fusarium* sp. based on the pathogen's morphological and molecular characteristics. Onion seed samples were collected from different localities in the region of Vojvodina. To obtain a DNA sequence-based identification, a total DNA of the 25 isolates was extracted directly from the mycelium (~ 100 mg wet weight), with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Following DNA extraction, the translation elongation factor 1-alpha region was amplified by PCR with the primer pair EF1 and EF2. An amplicon of 700 bp was amplified in 25 tested isolates. Identification of one isolate was performed by sequencing the translation elongation factor *EF-1α* gene, which was deposited in the NCBI GenBank database under accession number KP658211 (*Fusarium* sp. FIESC3).

KEYWORDS: FIESC3, *Fusarium* sp., onion, *EF-1α* gene, seed, sequencing

INTRODUCTION

Onion (*Allium cepa* L.) is one of the economically most important vegetable crop in Serbia. During the routine quality control analysis of onion seed in 2014 in Serbia, *Fusarium* fungal infection was observed on an average of 28% of the seed. Species of *Fusarium oxysporum*, *F. proliferatum*, and *F. equiseti* have been described as the most common seed-borne fungi which attack and

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can be transmitted by vegetable seeds. Seed health is a critical component for a successful crop production, while reduction in seed germination and growing energy occurres due to seed-borne diseases (Jasnić *et al*., 2005). Lević *et al*. (2009) indicate that *Fusarium* species periodically cause significant diseases, especially wilt of onion, garlic, and tomato, but there is a lack of information about *Fusarium* species associated with onion seed (*Allium cepa* L.) in Serbia. The significance of *Fusarium* basal rot of onion has been increasing in Serbia, not only in the production of green onion, but also in the production of onion sets and bulbs (Lević *et al*., 2009). The most frequent species isolated from onion sets and seedlings, as well as from the soil in Serbia are *Fusarium oxysporum* Schlecht. f. sp. *cepae* (Hanz) Snyd. & Hans, *F. oxysporum*, *F. moniliforme*, *F. solani*, *F. equiseti*, and *F. accuminatum* (Klokočar-Šmit *et al*., 1990; Lević 2008, Lević *et al*., 2009). The identification of *Fusarium* species traditionally relies on morphological and physiological characteristics, which is the most difficult step in the process of identification (Rahjoo *et al*., 2008), considering that *Fusarium* species can produce three types of asexual spores (Agrios, 1988). This is especially evident among very closely related *Fusarium* species, such as members of FIESC, which represents a complex of morphologically similar species (*F. equiseti*/*F. semitectum*/*F. incarnatum*) (O' Donnell *et al*., 2009, 2012). Recently, a multilocus sequence typing scheme analysis has been applied to members of FIESC and revealed that FIESC comprises 30 phylogenetically distinct species (O'Donnell *et al*., 2009, 2012). DNA sequence-based identification of some unknown isolates can be achieved using the translation-elongation factor *1-α TEF* gene region which has become the marker of choice as a single-locus identification tool in *Fusarium* (Geiser *et al*., 2004). Moreover, members of FIESC have been reported to produce type A and B trichothecene mycotoxins that cause toxicosis in humans and animals (O'Donnell *et al*., 2009).

The objective of this paper was isolation, morphological and molecular DNA sequence-based identification of *Fusarium* sp. isolates from onion seed samples collected from storage and warehouses at different localities in the region of Vojvodina, Serbia.

MATERIALS AND METHODS

Isolation and morphological characteristics

Blotter method was used for seed incubation during the routine seed health analysis (Mathur and Kongsdal, 2003). Onion seeds were immersed in NaOCl solution (1% available chlorine) for 3 min, washed in sterile water and drained. A set of three filter papers were immersed in sterile water, and placed completely wet in a Petri dish (ø9mm). Plating of 18 samples (400 seeds per sample) was done aseptically, a maximum of 10 seeds per plate were placed onto a blotter surface of each plate (Mathur and Kongsdal, 2003). Plates were incubated at 22 ºC for 7 days in alternating cycles of 12 hours light (NUV) and 12 hours darkness. After incubation, fungi which developed on each seed were examined under different magnifications of a stereomicroscope and identified.

Infected seeds were transferred to a potato dextrose agar (PDA), and incubated for seven days at 25 °C in alternating cycles of 12 hours light and 12 hours darkness, in order to induce sporulation and pigmentation in culture (Burgess *et al*., 1994). Light source consisted of three neon tubes measuring 40 W and a black light tube (Philips TLD 36W/08). For morphological identification, 25 isolates were single-spored and sub-cultured on both PDA and Carnation leaf agar (CLA) (Leslie and Summerell, 2006). Incubation lasted 7–10 days at 25 °C, in alternating cycles of 12 hours light and 12 hours darkness. Colony morphology was recorded from cultures grown on PDA and CLA.

Pathogenicity test

Pathogenicity test of 25 isolates was conducted using Knop agar slants, in controlled conditions in the laboratory (Tuite, 1969). A piece of mycelium (approximately 2-3 mm) of each isolate, grown on PDA for 7 days, was placed at the bottom of each test tube. Onion seeds were disinfested in 1% NaOCl for 2 to 3 min, rinsed with sterillized distilled water three times, and then dried on a sterile filter paper under aseptic conditions. Seeds were carefully placed and slightly pressed, approximately 2 cm above the inoculum. As a positive control, determined isolate designated as FE-3 (*Fusarium equiseti*) from our collection, was used. Onion seeds placed on a solid agar without mycelia were used as a negative control. Tubes were kept in the laboratory for two weeks, in a vertical position at room temperature (21−25 ºC) with day/night shift.

Sequencing and phylogenetic analysis

To obtain a DNA sequence, a total DNA of the 25 investigated isolates and one positive control FE-3 was extracted directly from the 7 days old mycelium (~ 100 mg wet weight), with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Following DNA extraction, the translation elongation factor *1-alpha* gene region was amplified by PCR with the primer pair EF1 (forward primer: 5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and EF2 (reverse primer: 5'- GGA(G/A)GTACCAGT(G/C)ATCATGTT-3') (Geiser *et al*., 2004). The amplification was performed in Eppendorf Mastercycler PCR device, using the modified program by Abdel-Satar *et al*. (2003): following 35 repeated cycles: 94 °C – 1 min, 53 °C – 1 min, 72 °C – 2 min. The PCR mixture with a total volume of 25 µl consisted of 2x Eppendorf Master Mix (Taq DNA polymerase 1.25 U, 30mM Tris-HCl, 50mM KCl, 1.5mM MgCl2; 0.1% Igepal-CA630; 0.2 mM dNTP); 0.6 µM of each primer, and 1µl of fungal DNA. Amplification fragments were determined using electrophoresis on 1% agarose gel containing ethidium bromide (0.5 µg/mL). The expected size of the amplified fragments was estimated by comparison with O'RangeRuler^{TM} 100 bp DNA Ladder (SM0623), ready-to-use (Fermentas, Lithuania). The agarose gel was visualised on UV transilluminator, and the images were captured with DOC PRINT system (Vilbert Lourmat, USA).

Identification of one isolate was performed by sequencing the translation elongation factor *EF-1α* gene. Purification and sequencing of the amplified fragments were done in the biotechnology company MACROGEN in Seoul, South Korea (http://dna.macrogen.com, Korea). Sequences were analyzed using the program FinchTV Version 1.4.0. Sequence of Serbian L1 isolate was compared with the previously reported isolates available in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/BLAST) and the *Fusarium* ID-database (Geiser *et al*., 2004), using the ClustalW program (Thompson *et al*., 1994) and MEGA5 software (Tamura *et al*., 2011). Manual corrections of aligned database, phylogenetic and molecular evolutionary analyses were conducted using MEGA 6 software package (Tamura *et al*., 2013). These gene sequences were assembled and edited using FINCHTV v.1.4.0 (http://www.geospiza.com). Multiple alignments and comparisons with reference strains for each of the genes were performed using CLUSTALW integrated into MEGA 6 software (Tamura *et al*., 2013). The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the analyzed taxa. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2011) and are presented as units of the number of base substitutions per site.

RESULTS

Morphological characteristics

Onion seeds placed on the blotter surface were covered with white, cottony mycelium, reddish to purple pigmentation observed under the seeds. The presence of macroconidia typical for *Fusarium* sp. on the infected onion seed was confirmed by microscopic examination on an average of 28% of the seed. The development of mycelium of 25 isolates on PDA occurred at medium mycelial growth rate and formed a colony 6 cm in diameter after seven days of incubation in darkness at 25 °C. A total of 25 isolates (L1-L25) formed whitish to pale salmon colonies, and the colour was uniform throughout the entire colony. The orange pigmentation developed on the reverse surface of the colony. On CLA, isolates formed hyaline, thin-walled, slightly curved, fusoid macroconidia, with 4–6 septae (23–40×3.5–6 μm). Microconidia and chlamydospores were not observed.

Pathogenicity test

Pathogenicity of onion seedlings under *in vitro* conditions, in a test tube on a Knop agar, was confirmed in 25 tested isolates. Five days after the inoculation, isolates caused a change in tissue colour and emergence of necrotic spots on the roots and shoot basis, spreading to the upper part of the shoot. The roots were completely infected by fungal mycelia after 10 days. After 14 days fungal mycelia of 25 isolates completely covered the seedlings, causing root

necrosis and seedling decay. No symptoms were observed on seedlings in the tubes which were used as negative control. Pathogen was reisolated and morphological identity was confirmed on PDA and CLA.

Sequencing and phylogenetic analysis

Primers EF1/ EF2 successfully detected the presence of *Fusarium* sp. in all tested isolates and amplified DNA fragments of predicted size. One clear band of 700 bp was visible in all tested isolates as well as in positive control. No amplicon was recorded in negative control. Identification of one isolate was performed by sequencing the translation elongation factor *EF-1α* gene, which was deposited in the NCBI GenBank database under accession number KP658211. BLASTn queries of GenBank and the *Fusarium* ID-database (Geiser *et al*., 2004), showed 100% identity to accessions GQ505648.1 (NRRL36323), and GQ505646.1 (NRRL36318) from an unnamed phylogenetic species within the *Fusarium incarnatum-equiseti* species complex designated FIESC3 (O'Donnell *et al*., 2009). TEF partial gene sequence of *F. proliferatum* was analysed to conduct a phylogenetic tree. Sequences generated in this study were added to the sequences of different *Fusarium* species selected from a BLAST search in NCBI GenBank for better understanding of their phylogenetic relationship (Geiser *et al*., 2004). A NJ tree constructed showed that the onion isolates were grouped together with *Fusarium equiseti* (KF754798, KP881270) and *Fusarium incarnatum* (JN092338) (Figure 1) strains from database:

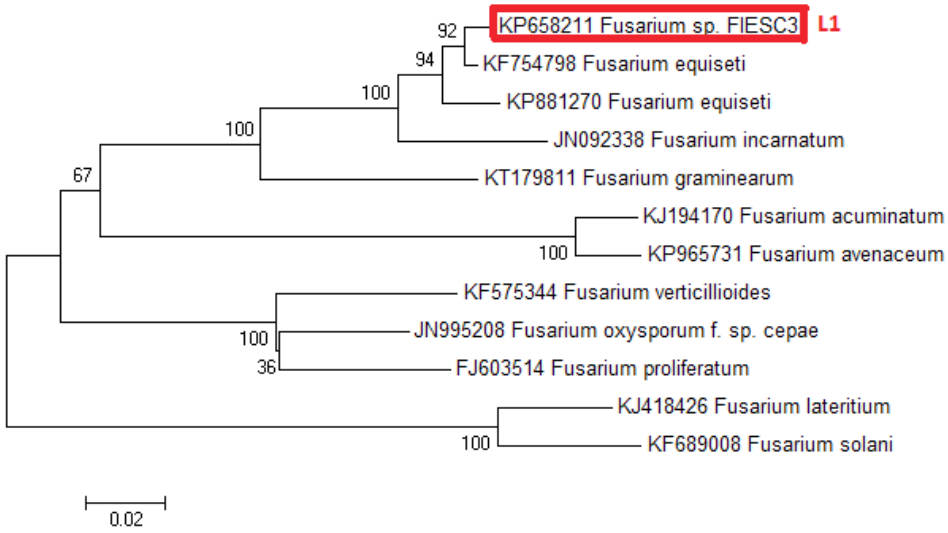

Figure 1. Phylogenetic tree based on Neighbour-Joining (NJ) analysis of TEF gene sequences for L1 isolate from onion and other Fusarium reference strains from NCBI database. Bar – estimated nucleotide substitutions per site is 0.02.

DISCUSSION

Fusarium species, members of FIESC, represent a complex of morphologically similar species (*F. equiseti*/*F. semitectum*/*F. inarnatum*) that comprises 30 phylogenetically distinct species (O'Donnell *et al.,* 2009, 2012). The correct identification of these species is therefore very important together with morphological identification. Using primers EF1 and EF2 to amplify translation elongation factor *1-alpha* gene region a specific band at 700 bp was obtained by PCR for 25 isolates in this study. This part of genome sequence is considered a highly significant information on species level for the entire *Fusarium* genus (Summerell *et al*., 2003; Geiser *et al*., 2004; Kristensen *et al*., 2005). Identification of one isolate was performed by sequencing the translation elongation factor *EF-1α* gene, which was deposited in the NCBI GenBank database under accession number KP658211. In our study, we recovered only one phylogenetic species, designated as FIESC 3. BLASTn queries of GenBank and the *Fusarium* ID-database (Geiser *et al*., 2004) showed 100% identity with accessions GQ505648.1 (NRRL36323), and GQ505646.1 (NRRL36318) from an unnamed phylogenetic species within the *Fusarium incarnatum-equiseti* species complex designated FIESC3 (O'Donnell *et al*., 2009). Castella and Cabanes (2014) used this tool for the analysis of phylogenetic diversity of *Fusarium incarnatum-equiseti* species (FIESC) complex of 51 strains isolated from Spanish wheat. Pathogens of genus *Fusarium* are well known as seed-borne as well as soil borne species able to produce various mycotoxins. Geographic area and climate are the most important factors that influence the occurrence of *Fusarium* and pattern of infestation by various *Fusarium* species (Castella and Cabanes, 2014). Our results showed that causal agent of seed rot in onion is a new species in Serbia designated as *Fusarium* sp. FIESC3. This is not unexpected considering that recent studies in Northern Europe have shown that the predicted climate changes towards 2050 are expected to change the *Fusarium* species composition in world (Parikka *et al*., 2012). Infection of seed results in reduced germination and *Fusarium* rot are hard to control being seed-borne, long persistant, capable of infection and spread in field and storage (Özer & Köycü, 2004; Klokočar-Šmit *et al*., 2008). Previously, Jasnić *et al*. (2005) showed that the decrease in seed germination and plant emergence of soybean is due to seed infection by *Fusarium* sp. in agroecological conditions of Serbia. Morphological identification is time consuming, because these species have small morphological differences and it is difficult to distinguish them from each other. On CLA, all tested isolates formed hyaline, thin-walled, slightly curved, fusoid macroconidia. Microconidia and chlamydospores were not observed. According to Nelson *et al*. (1983) and Burgess *et al*. (1994), morphology of macroconidia, microconidia, and chlamydospore can be assessed in cultures grown on CLA.

CONCLUSION

Knowledge of the composition of populations of *Fusarium* species transmitted by onion seed is of great importance for the establishment of appropriate measures for protection of seeds and seedlings. Based on the completion of Koch's postulates and sequence analysis, investigated isolates belong to *Fusarium* sp. FIESC3, a causal agent of pre-emergence dumping off, decay and rot of onion seed in Serbia. Due to its ability to reduce seed germination, the presence of this pathogen could significantly impact onion production in Serbia. These findings will provide the base to develop the effective disease management strategies.

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REFERENCES

Abdel-Satar MA, Khalil MS, Mohmed IN, Abd-Elsalam KA, Verreet JA (2003): Molecular phylogeny of Fusarium species by AFLP fingerprint. *Afr. J. Biotechnol.* 2: 51–56.

Agrios GN (1988): *Plant Pathology*, 3rd. ed. Academic Press, Inc. New York, pp 803.

Burgess LW, Summerell BA, Bullock S, Gott KP, Backhouse D (1994): *Laboratory Manual for Fusarium Research*. Third edition, Sydney.

Castella G, Cabanes FJ (2014): Phylogenetic diversity of Fusarium incarnatum-equiseti species complex isolated from Spanish wheat. *Antonie van Leeuwenhoek* 106: 309–317.

Geiser DM, Del Mar Jiménez-Gasco M, Kang S, Makalowska I, Narayanan N, Ward TJ, Zhang N, Kuldau GA, O'Donnell K (2004): *FUSARIUM*-ID v. 1.0: A DNA Sequence Database for Identifying *Fusarium*. *Eur. J. Plant. Pathol.* 110: 473–479.

Jasnić S, Vidić M, Bagi F, Đorđević V (2005): Pathogenicity of Fusarium species in soybean. *Matica Srpska J. Nat. Sci.* 109: 113–121.

Klokočar-Šmit Z, Jandrić-Ševar M, Kovačev D, Petrov M (1990): *Proučavanje fuzariozne truleži luka i efikasnosti hemijskog tretiranja pred uskladištenje*. Jugoslovenski simpozij, Intenzivno gajenje povrća i proizvodnja u zaštićenom prostoru. Ohrid: 133–140.

Klokočar-Šmit ZD, Lević J, Maširević S, Gvozdanović-Varga J, Vasić M, Aleksić S (2008): *Fusarium* rot of onion and possible use of bioproduct. *Matica Srpska J. Nat. Sci.* 114: 135–148.

Kristensen R, Torp M, Kosiak B, Holst-Jensen A (2005): Phylogeny and toxigenic potential is correlated in *Fusarium* species as reveald by partial translation elongation factor *1 alpha* gene sequences. *Mycol. Res.* 109: 173–186.

Leslie JF, Summerell BA (2006). *The Fusarium Laboratory Manual*, Blackwell: Iowa, pp. 388.

Lević J (2008): *Vrste roda Fusarium u oblasti poljoprivrede, veterinarske i humane medicine*. Beograd: Cicero, pp. 1226.

Lević J, Stanković S, Krnjaja V, Bočarov-Stančić A (2009): *Fusarium* species: The occurrence and the importance in agricultural of Serbia. *Matica Srpska J. Nat. Sci.* 116: 33–48.

Mathur SB, Kongsdal O (2003): *Common Laboratory Seed Health Testing Methods for Detecting Fungi*, ISTA, Switzerland.

Nelson PE, Tousson TA, Marasas WFO (1983): *Fusarium* species. An illustrated manual for identification. The Pennsylvania State University Press, University Park, Pennsylvania.

O'Donnell K, SuttonDA, Rinaldi MG, Gueidan C, Crous PW, Geiser DM (2009): Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-equiseti* and *F. chalmydosporum* species complexes within the United States. *J. Clin. Microbiol.* 47: 3851–3861.

O'Donnell K, Humber RA, Geiser DM, Kang S, Park B, Robert VA, Crous PW, Johnston PR, Aoki T, Rooney AP, Rehner SA (2012): Phylogenetic diversity of insecticolous fusaria inferred from multilocus DNA sequence data and their molecular identification via *FUSARIUM*-ID and *Fusarium* MLST. *Mycologia* 104: 427–445.

Özer N, Köycü D (2004): Seed-borne Fungal Diseases of Onion, and their control Fruit and Vegetable Diseases. *Disease Management of Fruits and Vegetables* 1: 281–306.

Parikka P, Hakala K, Tiilikkala K (2012): Expected shifts in *Fusarium* species composition on cereal grain in Northern Europe due to climatic change. *Food Addit. Contam.* 29: 1543–1555.

Rahjoo V, Zad J, Javan-Nikkhah M, Mirzadi Gohari A, Okhovvat SM, Bihamta MR (2008): Morphological and molecular identification of Fusarium isolated from maize ears. *Iran. J. Plant Pathol.* 90: 463–468.

Summerell BA, Salleh B, Leslie JF (2003): A utilitarian approach to *Fusarium* identification. *Plant dis.* 87: 117–128.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011): MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731–2739.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* 30: 2725–2729.

Thompson JD, Higgins DG, Gibson TJ (1994): CLUSTAL W.: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.

Tuite J (1969): *Plant pathological methods: fungi and bacteria*. Minneapolis: Burgess, pp. 239.

ИДЕНТИФИКАЦИЈА И ФИЛОГЕНЕТСКА АНАЛИЗА *Fusarium* sp. FIESC 3 ПРОУЗРОКОВАЧА ТРУЛЕЖИ СЕМЕНА ЦРНОГ ЛУКА (*Allium cepa* L.)

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РЕЗИМЕ: Црни лук (*Allium cepa* L.) спада у најзначајније повртарске биљне врсте у Србији. Гаји се на површини од око 20.000 хектара. Током рутинске контроле квалитета семена црног лука у 2014. години, примећена је појава *Fusarium* sp. у високом проценту – од 28%. Циљ рада био је изолација и идентификација *Fusarium* sp. на основу морфолошких и молекуларних карактеристика патогена. Узорци семена лука сакупљени су с различитих локалитета и из великог броја складишта у Војводини. Након изолације патогена одабрано је и морфолошки окарактерисано 25 изолата *Fusarium* sp. Изолација ДНК извршена је директно из мицелија гљиве (~ 100 mg), коришћењем Dneasy Plant Mini Kit (Qiagen, Hilden, Germany). Амплификација ДНК циљаног гена (translation elongation factor EF-1α gene) обављена је помоћу PCR коришћењем пара прајмера EF1 и EF2. У свим проучаваним изолатима формирани су ампликони величине 700 bp. Идентификација једног одабраног изолата извршена је секвенцирањем транслационог фактора EF-1α гена, који је депонован у NCBI базу података под бројем KP658211 (*Fusarium* sp. FIESC 3).

КЉУЧНЕ РЕЧИ: FIESC3, *Fusarium* sp., црни лук, *EF-1α* ген, семе, секвенцирање

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ANTIOXIDATIVE POTENTIAL OF *Daedaleopsis tricolor* BASIDIOCARPS AND MYCELIUM

ABSTRACT: Although some members of the genus *Daedaleopsis* have been prized for their medicinal and spiritual powers since Neolithic times, modern science has not yet sufficiently dealt with their bioactivities. This study aims at defining the antioxidative activities of extracts of *Daedaleopsis tricolor* wild and cultivated basidiocarps and mycelium and assessing their dependance on substrate type. Ethanol extracts (at a concentrations from 0.25 mg/mL to 16.00 mg/mL) of mycelium and wild fruiting bodies showed a considerable antioxidative potential (88.65% and 81.57%, respectively), which was almost the same as the commercial antioxidant BHA (88.91%). These radical scavenging abilities were reflected in EC_{50} values, which were 12.45 mg/mL for the extract of cultivated basidiocarps, 8.29 mg/mL for the extract of wild basidiocarps, 7.93 mg/mL for mycelium one, and 0.10 mg/mL for commercial antioxidant. Despite the fact that phenol proportion in the extracts was no negligible (between 20.41 μg GAE/mg of the extract of dry wild basidiocarps and 146.37 μg GAE/mg of the extract of dry cultivated basidiocarps), its correlation with antioxidative activity was moderate. Flavonoids, in significant concentration, were detected only in the extract of cultivated fruiting bodies (28.64 μg QE/mg of dry extract), but no correlation with radical scavenging capacity was noted. A remarkable antioxidant potential, especially of the submerged cultivated mycelium, put *D. tricolor* high on the list of promising new natural antioxidants.

KEYWORDS: antioxidative capacity, cultivated basidiocarp, *Daedaleopsis tricolor*, mycelium, wild basidiocarp

INTRODUCTION

The genus *Daedaleopsis*, which was firstly described by Schröter (1888), includes six widespread saprobes and pathogens commonly found on trees and stumps of the *Alnus*, *Betula*, *Salix*, *Corylu*s, *Fagus*, *Quercus*, and *Prunus* species, in the forests of Europe, North America, and Asia (Gorlenko, 1980; Karadžić,

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2011; Marković, 2012). Some members of this genus have been prized for their medicinal and spiritual powers since Neolithic times, which is confirmed by 7,000 years old *D. tricolor* fragments found in cottages in an archaeological site near Rome. These fungal fragments were also the oldest material which DNA was isolated and sequenced (Bernicchia *et al.,* 2006). Despite this ancient knowledge, modern science has not yet sufficiently dealt with the medicinal potential of *Daedaleopsis* spp. so there are only a few reports on their bioactivities. According to Bernicchia *et al.* (2006) and Vidović *et al.* (2011), *D. confragosa* is a source of many bioactive compounds, primarily peptides with anti-hypertensive and analgesic effects, polysaccharides with cytostatic activity, and substances with antioxidant capacity.

The normal cellular metabolism and oxygen consumption are coupled with the generation of free radicals like reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive sulphur species (RSS), superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH). Oxidative stress, which occurs as a result of the disbalance between the concentration of reactive radical species and the potential of defense system of an organism, is the trigger for the most serious diseases of the modern times, such as cancers, cardiovascular diseases, neurodegenerative disorders, etc. (Ćilerdžić *et al.* 2013). Despite the existence of numerous commercial, synthetic antioxidants, which are currently used, there is a growing interest in finding novel natural antioxidative agents that could be continuously used without any side effects on the human organism.

It has already been determined that many mushroom species possess antioxidative, antimicrobial, cytotoxic, immunomodulatory, and many other effects (Ćilerdžić *et al*., 2013; Milovanović *et al*., 2015). Owing to these characteristics, some species, such as *Ganoderma lucidum* and *Lentinus edodes*, have become highly valued and demanded in the health food market. Since the amount of fruiting bodies found in nature is insufficient to meet the needs of a growing market, the current trends deal with finding the optimal substrate for their large scale production (Berovič *et al*., 2003; Ćilerdžić *et al*., 2014). The preference is given to the usage of alternative substrates, which is economically justified and environmentally friendly, contrary to the usage of the traditional ones. Wheat straw is lignocellulosic residue, which is produced in enormous amounts in Europe, and remains as ballast in nature though it has a huge potential as a substrate for production of high-quality fruiting bodies (Stajić *et al*., 2009). In addition to the optimization of fruiting bodies cultivation, great efforts are made to improve the production of mycelium biomass, as a promising alternative for fruiting bodies, which cultivation is carried out under easily controlled conditions for a considerably shorter time.

Considering that there are still no reports about *D. tricolor* antioxidative potential, the aim of this study was to define the activity of basidiocarps and mycelium extracts and the assesment of their dependance on the substrate type.

MATERIALS AND METHODS

Organism and cultivation conditions

The fruiting body collected in Veliko Tarnovo (Bulgaria) was identified as *Daedaleopsis tricolor*, and the isolated culture, designated as BEOFB 720, is maintained on malt agar medium in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (BEOFB).

Inoculum was prepared by inoculation of 100.0 mL of synthetic medium (glucose, 10.0 g/L; NH_4NO_3, 2.0 g/L; K_2HPO_4, 1.0 g/L; NaH_2PO_4 x H_2O, 0.4 g/L; $MgSO_4$ x $7H_2O$, 0.5 g/L; yeast extract, 2.0 g/L; pH 6.5) with mycelial discs (Ø 0.5 cm, from 7-day-old culture from malt agar), incubation at room temperature (22 ± 2 ºC), on rotary shaker (Heidolph Instruments, Germany), for 7 days, washing of obtained biomass with distilled water, and its homogenization in laboratory blender.

Mycelial biomass was obtained by submerged cultivation in 1000-mL Erlenmeyer flasks containing 600.0 mL of synthetic medium inoculated with 30.0 mL of homogenized inoculum, at room temperature, on a rotary shaker, for 21 days. The biomass was separated from the cultivation broth, washed with destilled water, dried at 50 °C for 2 days and stored at -20 °C until usage.

The cultivation of basidiocarps was carried out in 100-mL flasks containing 2.0 g of wheat straw as the carbon source and 10.0 mL of the modified synthetic medium (without glucose) inoculated with 3.0 mL of the inoculum, at 25 ºC for 21 days. The obtained basidiocarps were dried at 40 °C for 2 days and stored at -20 °C until usage.

Preparation of the fungal extracts

Finely powdered mycelium as well as wild and cultivated basidiocarps of *D. tricolor* (10.0 g) were extracted with 300.0 mL of 96% ethanol by stirring on the magnetic stirrer (150 rpm) for 72 h. The obtained extracts were centrifuged (20 ºC, 3,000 rpm, 10 min) (Hettich Universal 32R, Germany) and supernatants were filtered through Whatman No.4 filter paper, concentrated under reduced pressure in a rotary evaporator (Büchi, Rotavapor R-210, Germany) at 40 °C to dryness, and redissolved in 96% ethanol to the initial concentration of 16.0 mg/mL.

Antioxidative activity assay

The free radical scavenging activity of the extracts was determined spectrophotometrically (CECIL CE 2501) by measuring the reduction of 4% methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH•) (Blois, 1958). The mixture of 200.0 μL of extract (series of double dilutions from 16.0 mg/mL to 0.25 mg/mL) and 1,800.0 μL of DPPH• was shaken vigorously, incubated for 30 min in the dark, and absorbance was measured at 517 nm. The scavenging effect was calculated using the equation:

$$\text{DPPH• scavenging effect (\%)} = [(A_0 - A_{sample})/A_0] \times 100$$

A_0 – absorbance of the negative control (all reagents except the extract);
A_{sample} – absorbance of the reaction mixture.

The mixture without extract was used as a negative control and that with commercial antioxidant (butylated hydroxyanisole – BHA) instead of extract as a positive control. Extract concentration (mg/mL) providing 50% of DPPH• reduction (EC_{50}) was obtained by interpolation from linear regression analysis.

Determination of total phenol content

The amount of total phenols in the extracts was estimated by a colorimetric assay based on the procedure described by Singleton and Rossi (1965), using gallic acid as standard. 200.0 µL of extract (1.0 mg/mL) and 1,000.0 µL of 10% Folin-Ciocalteu reagent were reacted in the dark for 6 min before addition of 800.0 µL of 7.5% aqueous solution of Na_2CO_3. The reaction mixture was vortexed vigorously and incubated on a rotary shaker in the dark at room temperature, for 2 h. The absorbance of the reaction mixture was measured spectrophotometrically at 740 nm. The extract was substituted by sterile distilled water in the blank. The total phenol content was determined as µg of gallic acid equivalent (GAE) per mg of dried extract using an equation that was obtained from a standard gallic acid graph:

$$\text{Absorbance} = 0.013 \times \text{total phenols (µg of gallic acid)} + 0.165 \ (R^2 = 0.996).$$

Determination of total flavonoid content

Total flavonoid content was determined by the method described by Park *et al.* (1997) using quercetin as the standard. 1.0 mL of extract (1.0 mg/mL) was diluted with 4.3 mL mixture containing 4.1 mL of 80% ethanol, 0.1 mL of 10% aluminium nitrate ($Al(NO)_3 \times 9H_2O$), and 0.1 mL of 1.0 M aqueous solution of CH_3CO_2K. The reaction mixture was incubated at room temperature for 40 min and absorbance was measured spectrophotometrically at 415 nm. The mixture with ethanol instead extract was used as the blank. Total flavonoid content was expressed as µg of quercetin equivalent (QE) per mg of dry extract using an equation obtained from standard quercetin hydrate graph:

$$\text{Absorbance} = 0.006 \times \text{total flavonoid (µg quercetin hydrate)} - 0.017 \ (R^2 = 0.995).$$

Statistical analysis

The assays were carried out in triplicate and the results are expressed as a mean ± standard error. One-way analysis of variance (ANOVA) and Tukey's

test were performed using STATISTICA, version 6.0 (StatSoft, Inc., Tulsa, USA) in order to test any significant differences among means. Statistical significance was declared at $p<0.05$.

RESULTS AND DISCUSSION

Our results have clearly shown a considerable antioxidative potential of the studied *Daedaleopsis tricolor* extracts, as well as a significant difference ($p<0.05$) among capacities of mycelium extract on the one hand and extracts of cultivated and wild basidiocarps on the other hand (Figure 1). The mycelial and wild basidiocarps' extracts showed a significantly higher DPPH• scavenging activity than the extract of alternatively cultivated basidiocarps. The maximum level of DPPH• reduction (88.65%) was obtained with mycelial extract (at a concentration of 16.00 mg/mL), which was almost the same as with BHA at the same concentration (88.91%). The effectivness of wild basidiocarps' extract at higher concentrations was similar to that of mycelial extract (81.57% at a concentration of 16.00 mg/mL), but at the lowest concentration it was significantly higher (8.73% at a concentration of 0.25 mg/mL). A considerable lower antioxidative potential was noted for the extract of cultivated basidiocarps, namely the maximum DPPH• reduction of 60.72% was noted at a concentration of 16.0 mg/mL (Figure 1). The observed differences between studied *D. tricolor* extracts were also reflected in EC_{50} values, which were 12.45 mg/mL for the

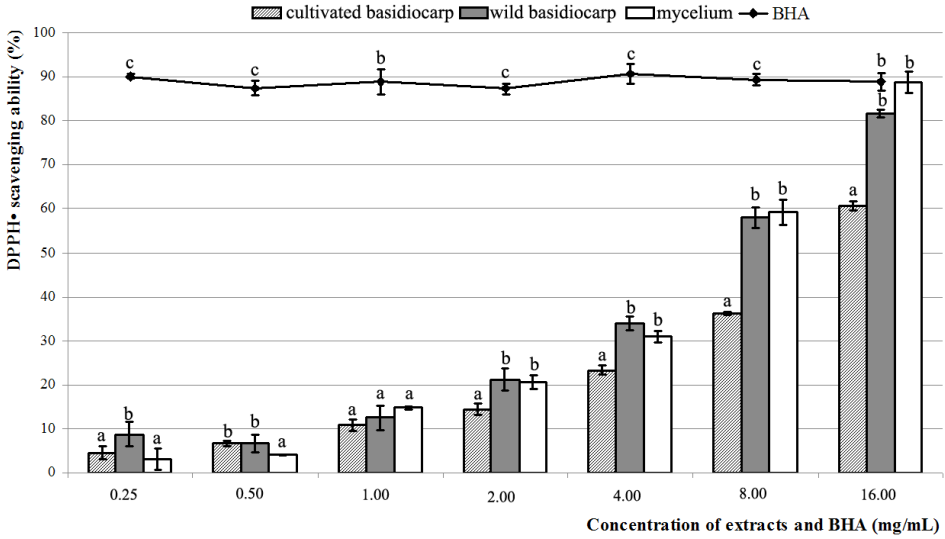

Figure 1. DPPH• scavenging ability of *Daedaleopsis tricolor* extracts and commercial antioxidant (Data represent mean value of activities of three replicates. Variations are given as standard errors. Values within each concentration group with different superscripts differ significantly ($p<0.05$) according to Tukey's test).

cultivated basidiocarps' extract, 8.29 mg/mL for wild basidiocarps' extract, and 7.93 mg/mL for mycelium one. Contrary to the studied *D. tricolor* extracts, which antioxidative ability increased gradually with the rising concentration, BHA showed an enormously high DPPH• scavenging capacity even at the lowest concentrations (0.25 mg/mL) and its EC_{50} value was 0.10 mg/mL.

The amounts of phenols and flavonoids varied significantly ($p<0.05$) between studied *D. tricolor* extracts (Table 1). The lowest content of phenol compounds (20.41 μg GAE/mg of dry extract) was measured in the extract of wild basidiocarps, over two-fold higher content was detected in the mycelial extract, while the extract of cultivated basidiocarps was the richest (146.37 μg GAE/mg of dry extract). The extract of cultivated fruiting bodies was also the only containing flavonoids (28.64 μg QE/mg of dry extract) (Table 1). The moderate degree of correlation ($R^2 = 0.47$) was observed between DPPH• scavenging activity and total phenol content, while there was not noted any correlation between the radical reduction ability and flavonoid amount.

Table 1. Total phenol and flavonoid contents in *Daedaleopsis tricolor* ethanol extracts.

Extracts	Phenol content (μg GAE/mg of dry extract)	Flavonoid content (μg QE/mg of dry extract)
Mycelium	47.86 ± 2.33^{b}*	0.00 ± 0.00^{a}
Wild basidiocarps	20.41 ± 0.80^{a}	0.00 ± 0.00^{a}
Cultivated basidiocarps	146.37 ± 4.69^{c}	28.64 ± 0.32^{b}

* Means within a column with different superscripts differ significantly ($p<0.05$) according to Tukey's test.

Although many previous studies have shown different antioxidative potential of numerous mushroom extracts and its dependance on species, fungal part (fruiting body, mycelium, spores), as well as solvent kind (polar or non-polar) and concentration (Mau *et al*., 2005a, 2005b; Elmastas *et al*., 2007; Saltarelli *et al*., 2009; Ramkumar *et al*., 2010; Sarikurkcu *et al*., 2015; Boonsong *et al*., 2016), our results are the first to show the radical neutralization ability of *D. tricolor*. The capacity was similar to those of *Agaricus bisporus*, *Clitocybe odora* and *Lactarius deliciosus* basidiocarp methanol and ethanol extracts (EC_{50} values were 9.61 mg/mL, 6.77 mg/mL and 8.52 mg/mL, respectively) (Khatua *et al*., 2013). However, it was several-fold higher than methanol extracts of *Polyporus sulphureus* and *Macrolepiota procera* basidiocarps (5.36% and 3.26%, respectively) (Sarikurkcu *et al*., 2015), but significantly lower comparing with 50% ethanol extracts of *Auricularia auricula*, *Lentinus edodes* and *Daedaleopsis confragosa*, which reduced 20%, 65% and 67% of DPPH•, respectively, at considerable lower concentrations (Vidović *et al*., 2011; Boonsong *et al*., 2016).

Although the data reported for methanol and aqueous extracts of *Ganoderma tsugae* basidiocarps and mycelia (Mau *et al*., 2005a, 2005b) and ethanol extract of alternatively cultivated *G. lucidum* basidiocarps (Ćilerdžić *et al*., 2013,

2014) showed that significant phenol concentration was highly correlated with degree of DPPH• reduction, it was not the case with the tested *D. tricolor* extracts. Namely, *D. tricolor* extracts were richer in phenols than extracts of mushrooms tested by Khatua *et al*. (2013) and Kaewnarin *et al*. (2016), but correlation coeficient (R^2) was very low. Likewise, correlation absence can be demonstrated by the lowest antioxidative activity of cultivated basidiocarp extract which contained the highest phenol amount, and the highest activity of mycelial extract where these compounds were represented with 3.5-fold lower amount, which was not in accordance with data of Mau *et al*. (2005a). Wild basidiocarp extract was one more confirmation of independance of scavenging ability and phenol content, i.e. it was poorer in phenols than cultivated basidiocarp extract but more active in radical neutralisation. It seems that antioxidant activity of wild basidiocarps is based on different extract constituents than phenols. Therefore, it is obvious that antioxidative potential of fungal extracts is not obligatory dependent on phenol proportion, but it can be result of complex synergistic or antagonistic interactions among phenols and other extract compounds (Kosanić *et al.,* 2012).

The presented results have clearly showed a remarkable antioxidant potential of the mycelium extract comparing to those of cultivated and wild basidiocarps of *D. tricolor*, which gives them a special importance, bearing in mind that mycelium cultivation is easily controlled, cheaper and faster process than the obtaining of basidiocarps. Further research should go in the direction of the optimization of submerged cultivation conditions and identification of the carriers of the activity in *D. tricolor.*

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REFERENCES

Bernicchia A, Fugazzola MA, Gemelli V, Mantovani B, Lucchetti A, Cesari M, Speroni E. (2006): DNA recovered and sequenced from an almost 7000 y-old Neolithic polypore, *Daedaleopsis tricolor. Mycol. Res.* 110: 1417.

Berovič M, Habijanič J, Zore I, Wraber B, Hodžar D, Boh B, Pohleven F (2003): Submerged cultivation of *Ganoderma lucidum* biomass and immunostimulatory effects of fungal polysaccharides. *J. Biotechnol.* 103: 77–86.

Blois MS (1958): Antioxidant determination by the use of stable free radical. *Nature* 181: 1199–1200.

Boonsong S, Klaypradit W, Wilaipun P (2016): Antioxidant activities of extracts from five edible mushrooms using different extractants. *Agr. Nat. Res*. 50: 89–97.

Ćilerdžić J, Stajić M, Vukojević J, Duletić-Laušević S. (2013): Oxidative stress and species of genus *Ganoderma* (Higher basidiomycetes). *Int. J. Med. Mushrooms.* 15: 21–28.

Ćilerdžić J, Vukojević J, Stajić M, Stanojković T, Glamočlija J (2014): Biological activity of *Ganoderma lucidum* basidiocarps cultivated on alternative and commercial substrate. *J. Ethnopharmacol.* 155: 312–319.

Elmastas M, Isildak O, Turkekul I, Temur N (2007): Determination of antioxidant activity and antioxidant compounds in wild edible mushrooms. *J. Food Compos. Anal*. 20: 337–345.

Gorlenko MB, Bondarceva MA, Garibova LB, Sidorova II, Sizova TP (1980): *Грибы СССР.* Справочники – определители географа и путешественника, Москва.

Kaewnarin K, Suwannarach N, Kumla J, Lumyong S (2016): Phenolic profile of various wild edible mushroom extracts from Thailand and their antioxidant properties, anti-tyrosinase and hyperglycaemic inhibitory activities. *J. Func. Foods* 27: 352–364.

Karadžić D (2011). Najčešće parazitske i saprofitske gljive na grabu (*Carpinus betulus* L.) u Srbiji i njihova uloga u propadanju stabla. *Šumarstvo* 138: 1–11.

Khatua S, Paul S, Acharya K (2013): Mushroom as the potential source of new generation of antioxidant: a review. *Res. J. Pharm. Tech*. 6: 496–505.

Kosanić M, Ranković B, Stanojković T (2012): Antioxidant, antimicrobial, and anticancer activity of 3 *Umbilicaria* species. *J. Food Sci.* 77: T20–T25.

Marković MP (2012): „Proučavanje parazitskih gljiva na divljoj trešnji (*Prunus avium* L.) sa posebnim osvrtom na bioekologiju *Daedaleopsis confragosa*". Doktorska disertacija, Univerzitet u Beogradu, Šumarski fakultet, Beograd.

Mau JL, Tsai SY, Tseng YH, Huang SJ (2005a): Antioxidant properties of hot water extracts from *Ganoderma tsugae* Murrill. *LWT Food Sci.Technol.* 38: 589–597.

Mau JL, Tsai SY, Tseng YH, Huang SJ (2005b): Antioxidant properties of hot water extracts from *Ganoderma tsugae*. *Food Chem*. 93: 641–649.

Milovanović I, Stanojković T, Stajić M, Brčeski I, Knežević A, Ćilerdžić J, Vukojević J (2015): Effect of selenium enrichment of *Lenzites betulinus* and *Trametes hirsuta* mycelia on antioxidant, antifungal and cytostatic potential. *Cur. Pharm. Biotechnol*. 16: 920–926.

Park YK, Koo MH, Ikegaki M, Contado JL (1997): Comparison of the flavonoid aglycone contents of *Apis melifera* propolis from various regions of Brazil. *Arq. Biol. Technol*. 40: 97–106.

Ramkumar L, Ramanathan T, Thirunavukkarasu P, Arivuselvan N (2010): Antioxidant and radical scavenging activity of nine edible mushrooms extract. *Int. J. Pharm*. 6: 950–953.

Saltarelli R, Ceccaroli P, Iotti M, Zambonelli A, Buffalini M, Casadei L, Vallorani L, Stocchi V (2009): Biochemical characterization and antioxidant activity of mycelium of *Ganoderma lucidum* from Central Italy. *Food Chem*. 116: 143–151.

Sarikurkcu C, Tepe B, Kocak MS, Uren MC (2015): Metal concantration and antioxidant activity of edible mushrooms from Turkey. *Food Chem*. 175: 549–555.

Singleton VL, Rossi JA (1965): Colometric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticult*. 16: 144–158.

Stajić M, Vukojević J, Duletić-Laušević S (2009): Biology of *Pleurotus eryngii* and role in biotechnological processes: a review. *Crit. Rev. Biotechnol*. 29: 55–66.

Vidović S, Zeković Z, Mujić I, Lepojević Ž, Radojković M, Živković J (2011): The antioxidant properties of polypore mushroom *Daedaleopsis confragosa*. *Cent. Eur. J. Biol.* 6: 575–582.

АНТИОКСИДАТИВНИ ПОТЕНЦИЈАЛ БАЗИДИОКАРПА И МИЦЕЛИЈЕ *Daedaleopsis tricolor*

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РЕЗИМЕ: Иако су неке врсте рода *Daedaleopsis* биле цењене због својих лековитих својстава још од доба неолита, савремена наука се није довољно бавила њиховом биоактивношћу. Дефинисање антиоксидативне активности екстраката базидиокарпа, сакупљених из природе и гајених, и мицелије *Daedaleopsis tricolor* као и утврђивање њене зависности од састава и типа супстрата циљеви су ове студије. Етанолни екстракти мицелије и самониклих базидиокарпа показали су значајан антиоксидативни потенцијал (88,65% односно 81,57%), готово исти као и код комерцијалног антиоксиданса (88,91%). Способности редукције DPPH радикала огледају се и у EC_{50} вредностима које су биле 12,45 mg/mL за екстракт гајеног базидиокарпа, 8,29 mg/mL за екстракт самониклог базидиокарпа, 7,93 mg/mL за екстракт мицелије и 0,10 mg/mL за комерцијални антиоксиданс. Упркос чињеници да садржај фенолних једињења није био занемарљив (између 20,41 μg GAE/mg сувог екстракта самониклог базидиокарпа и 146,37 μg GAE/mg сувог екстракта култивисаног базидиокарпа), његова корелација с антиоксидативном активношћу била је умерена. Флавоноиди су детектовани само у екстракту гајеног базидиокарпа (28,64 μg QE/mg сувог екстракта) што није било у корелацији с његовим антиоксидативним потенцијалом. Изузетан антиоксидативни потенцијал, посебно за мицелијски екстракт, позиционира *Daedaleopsis tricolor* на високо место на листи нових обећавајућих природних антиоксиданаса.

КЉУЧНЕ РЕЧИ: антиоксидативни капацитет, гајени базидиокарп, *Daedaleopsis tricolor*, мицелија, самоникли базидиокарп

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INHIBITORY EFFECTS OF MEDICAL PLANTS ON THE *Candida albicans* AND BACTERIAL GROWTH IN THE ORAL CAVITY

ABSTRACT: In this mini-review, the authors discuss the effects of ethanol extracts, essential oils and cytotoxicity of some medicinal plants and their compounds used in ethnomedicine in different geographic regions worldwide, including Serbia, on the growth, multiplication and pathogenicity of *Candida albicans* and bacteria that play the main role in the balance of the oral ecosystem. Various medicinal plants, such as *Rosmarinus officinalis* (Fam. Lamiaceae), *Artemisia dracunculus*, *Artemisia absinthium* (Fam. Asteraceae), exist in different geographic regions and continents, as well as in the Balkan region, and among them there are some indigenous species like *Hypericum perforatum* L. (Fam. Hypericaceae), *Urtica dioica* L. (*U. dioica*) (Fam. Urticaceae), *Achillea millefolium* L. (Fam. Asteraceae), *Matricaria chamomilla* L. (Fam. Asteraceae), *Sambucus nigra* L. (Fam. Caprifoliaceae), and *Thymus serpyllum* L. (Fam. Lamiaceae) with impressive antimicrobial activity against microorganisms originating from the oral cavity.

KEYWORDS: medicinal plants, *Candida albicans*, oral cavity microbiota, bacteria

INTRODUCTION

Medicinal plants had been used in traditional medicine as a part of national culture and traditional heritage long before the knowledge of chemical properties of bioactive substances and procedures of classical medicine were

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widely distributed and accepted. In Russia, the first public pharmacy was opened in 1672 in Moscow, and in the same year, a herbalist manuscript entitled "Cool vineyard" was published (Shikov *et al*., 2014). The illustrative examples can be found in Russian phytotherapy, which has accumulated and adopted approaches that originated from European and Asian traditional medicine, due to its specific geographic location (Shikov *et al*., 2014). Besides geographic location, age, gender, farming, and stockbreeding are associated with the knowledge of plant resources. Historical, cultural and socioeconomic traits are essential to understand the local knowledge of useful plant diversity in a particular study area (Monroy-Ortiz *et al*., 2004). In many cases, the cultural factor means that the use of a species is more widespread than its ecological distribution. This may also explain the presence of synonyms and polysemies, which are useful for discussing ethnopharmacological data (Akerreta *et al*., 2007). The correlation between medicinal flora and floristic environment means that adaptation to the local area, even at small spatial scale, is in contrast to the effects of shared ancestry (Saslis-Lagoudakis *et al*., 2015).

Unfortunately, there are some mainly negative influences on the growth of medicinal plants: wild plant collection, residential and commercial development, livestock farming, ecosystem modifications, annual and perennial non-timber crops, wood, and pulp plantations, problematic native species/diseases, etc. Further, climate change and severe weather: droughts, fire and fire suppression, energy production and mining, logging and wood harvesting, dams and water abstraction, invasive non-native/alien species diseases, pollution, transport and infrastructure also should not be neglected (Allen *et al*., 2014).

Medicinal use of plants is common to all cultures, and it is usually part of the national heritage. There are ancient manuscripts like the Egyptian Ebers Papyrus (1500 BC) and Wu Shi Er Bing Fang ("Recipes for Fifty-Two Ailments"), dating from the 2nd century BC, discovered in 1973 in a tomb at Ma Huang Dui in Hunan Province, China. The Atharva Veda (India) dated back to 1200 BC. Egyptian priest doctors influenced the Greek medicine e.g. Hippocrates. Pedanius Dioscorides, a Greek physician, compiled ancient and contemporary herbal knowledge in his book *De Materia Medica* (*Peri hules iatrikēs*), that for more than thirteen centuries remained one of the principle medical textbooks throughout the civilized world. Claudius Galen, also known as Galen of Pergamon, was among the first who systematized the knowledge in the field of medicinal plants. Another mixing of cultures between European Crusaders and their Arab adversaries brought to Europe the knowledge of ancient Greek and Persian medicine (European Herbal & Traditional Medicine Practitioners Association. Herbal and Traditional Medicine).

In Serbia, the *Hilandar Medical Codex* is the most significant and best-preserved medieval Serbian manuscript containing documents on European medical science from the 12th to 15th century. Out of 167 recorded substances, 135 are of plant origin, 13 of animal origin, and 19 are of inorganic origin. The recorded plant species are categorized into 63 families, of which the most frequent are: Apiaceae, Lamiaceae, Asteraceae, Rosaceae and Fabaceae (Jarić *et al*., 2011).

European medicinal plants have been well described and its antibacterial action is very popular. *Dianthus superbus* (Fam. Caryophyllaceae) has long been used for the treatment of bacterial infections. The balsamic sage, *Salvia tomentosa* (LC) (Fam. Lamiaceae), is found to be an antibactericidal agent. Sore throat, cough, and colds have been treated by sage (*Salvia officinalis*). *Gypsophila perfoliata* (Fam. Caryophyllaceae) powder, derived from the root is used for wound healing. Bactericidal effect has formulations from the stems, flowers and fruits (Allen *et al*., 2014). Echinacea (*Echinacea* spp., Fam. *Asteracea*) is useful in relieving colds and upper respiratory infections (approved in Europe for these uses). Cranberry (*Vaccinium macrocarpon*, Fam. *Ericaceae*) is applied for reducing the risk of bladder infection. Calendula (*Calendula officinalis*, Fam. Asteraceae) has long been used to relieve inflammation of the mouth, throat, and stomach; it is popular as a topical cream or ointment to relieve rashes and irritation and to help heal wounds. Elderberry (*Sambucus nigra*, *S. canadensis*, Fam. Capryofoliaceae) flowers have been valued as a remedy for colds and fever for centuries. Garlic (*Allium sativum*, Fam. Amaryllidaceae) is a potent antimicrobial; it is often used to combat colds, ease sinus congestion, and stave off traveler's diarrhea. Hibiscus (Fam. Malvaceae, *Hibiscus sabdariffa*) is used traditionally to ease sore throats and colds. Lemon balm (*Melissa officinalis*, Fam. Lamiaceaae) is applied in topical creams used for fever blisters. Licorice (*Glycyrrhiza glabra*, Fam. Fabaceae,) is excellent for anti-inflammatory remedies, for smoothing mucous membranes, and for healing sore throats and coughs. Mullein (*Verbascum thapsus*, Fam. Scrophulariaceae scrophulariaceae) are commonly used to relieve cough, sore throat, and chest congestion. Thyme (*Thymus vulgaris*), Fam. Lamiaceae) has application in relieving coughs, colds, and congestion. (Pizzorno and Murray, 2013). Its effect is so highly valuated in general population that its Serbian name is Mother's Sweetheart (in Serbian: majčina dušica).

In Serbia, in the region of Mt. Kopaonik, according to Jarić *et al*. (2007) the village people do not visit doctors except for surgical problems, and they use plants as their parents and grandparents did. Among wild plants most commonly used for medicinal purposes are: *Hypericum perforatum* L. (Fam. Hypericaceae), *Urtica dioica* L. (Fam. Urticaceae), *Achillea millefolium* L. (*A. millefolium*) (Fam. Asteraceae), *Matricaria chamomilla* L. (Fam. Asteraceae), *Sambucus nigra* L., and *Thymus serpyllum* L. (Fam. Lamiaceae). These plants were particularly highly recommended by the majority of the interviewed. The most frequently reported medicinal uses were for treating gastrointestinal ailments (50%), skin injuries and problems (25.6%), followed by respiratory, urinary-genital and cardiovascular problems (20.5%, 20.5%, 19.2%, respectively) (Jarić *et al*., 2007). A slightly different situation is in Mt. Rtanj, in Eastern Serbia, where, because of depopulation, mainly elderly people were interviewed. The most common in traditional usage were the species of Labiatae (22%), Rosaceae (20%), and Compositae (13%). *H. perforatum* was the most popular medicinal plant. The most frequently reported medicinal uses of herbal drugs included remedies for the immune system (22.97%), respiratory system (15.77%), and digestive system disorders (15.32%) (Zlatković *et al*., 2014).

Nowadays, in particular, there is a growing interest in herbs and their compounds applicable in contemporary remedies of conventional medicine based on scientific information and data (Clark, 1996; Wurglics *et al*., 2001; Rates, 2001; Salem and Werbovetz, 2006; Veeresham, 2012).

Candida albicans, MICROBIOTA OF ORAL CAVITY

Bacteria, mainly aerobic and microaerophilic streptococci and some yeasts such as *Candida albicans* (*C. albicans*) populate the healthy human oral cavity. Their overgrowth can cause diseases known as candidiasis, stomatitis, gingivitis, or parodontopathy (Lamont *et al*., 2014). Furthermore, changes of pH, a balance between microorganisms, and the presence of specific substrate could enable and enhance the adherence ability of microorganisms involved in dental diseases (Glee *et al*., 1995). There is an opinion that *Candida* overgrowth and adherence to structures in the oral cavity is of paramount importance for the development of an infection because its presence may change the stability of the sensitive ecosystem of the oral microflora (Glee *et al*., 1995).

Candida albicans belongs to the Kingdom of Fungi, Division of Ascomycota, Class Saccharomycetes, Order Saccharomycetales, Family *Saccharomycetaceae* and Genus: *Candida* (Lachance *et al*., 2011). Both yeast and hyphal forms (dimorphic) are present in infected tissue. Virulence properties of *C. albicans* are the consequence of the dimorphic transition. Various sources of carbon and nitrogen possibly accelerate the growth of *C. albicans* over other microbiota. The absence of glucose contributes to the very poor growth conditions for *C. albicans* in the oral cavity (Cannon and Chaffin, 2001; Jenkinson and Douglas, 2002).

Infections caused by *Candida* may be superficial or systemic. Superficial infections of the mucocutaneous tissues may include oropharyngeal candidiasis, involving the palate, tongue, and buccal mucosa (Jenkinson and Douglas, 2002; Alt-Epping *et al*., 2012; Stoopler and Sollecito, 2014). Generally, the risk of *Candida* infection or colonization may be the consequence of the numerous predisposing factors. These factors are poor oral hygiene, cellular immune deficiencies, immunosuppression, inefficiency of the thymus, infection with human immunodeficiency virus (HIV), nutritional deficiencies, long-term use of antibiotic/radiation therapy, dental prostheses, *diabetes mellitus*, high carbohydrate diet, or heavy cigarette smoking (Jenkinson and Douglas, 2002; Wu *et al*., 2013). Regardless of aging, denture wearing constitutes a stable factor that has a positive influence on *Candida* growth (Mizugai *et al*., 2007).

Successful survival of *C. albicans* requires good adaptation, which includes adherence to the oral cavity structures, multiplication and invasiveness provided by degradative enzyme synthesis and secretion (Cannon and Chaffin, 2001). *C. albicans* expresses adhesions rather in the mycelial form than in the form of the yeast (Fukazawa and Kagaya, 1997). In the ability of *C. albicans* to adhere to the surfaces, cell superficies hydrophobicity has paramount importance, since hydrophobic cells adhere better to host tissue compared to hydrophilic cells

(Glee *et al*., 1995). The abundance of phosphodiester-linked, acid-labile mannosyl groups may be associated with hydrophobicity (Masuoka and Hazen, 1997). During *Candida* life cycle, the presence of hydrophobic proteins on surface remains constant, however, hydrophilic proteins vary during growth (Hazen and Hazen, 1993).

C. albicans secretes a range of degradative enzymes such as aspartyl proteinases, lipases and hexosaminidase that may damage host tissues and contribute to *C. albicans* pathogenicity (Cannon and Chaffin, 1999). Adhesion, invasion and damage of the oral cavity by *C. albicans* depend not only on fungal morphology and activity, but also on the epithelial cell type and the differentiation stage of the epithelial cells. Data indicate that *C. albicans* can invade oral epithelial cells by inducing endocytosis and/or by active penetration (Dalle *et al*., 2010). Hyphal formation of *C. albicans* facilitates epithelial invasion via two routes: active penetration and induced endocytosis. While induced endocytosis is predominantly mediated by adhesin and invasin (als adhesin Als3), active penetration seems to be supported by hydrolase activity and mechanical pressure. Besides adhesion and morphogenesis, biofilm formation and morphological changes of multicellular structures are also the virulence factors of *C. albicans*. *Candida* utilizes the Ras/cyclic AMP (cAMP)/protein kinase A (PKA) signal transduction pathway to regulate many of adaptation mechanisms (Inglis and Sherloc, 2013).

ORAL HYGIENE

Oral hygiene, among other factors, depends on ingredients used in modern toothpaste formulations, which include abrasive agents, surfactants, humectants, thickening agents, flavoring, coloring agents, and antimicrobial agents. These antimicrobial agents include metal salts, phenols, herbal extracts, enzymes, essential oils, and bisbiguanides (Stamm, 2007; Yiğit *et al*., 2008). Toothpaste has been formulated to contain chemotherapeutic agents to improve the oral health, to produce inhibitory action on plaque formation as well as bacteria and *Candida* colonization (Gardiner *et al*., 2008; Yiğit *et al*., 2008; Sadeghi and Assar, 2009; Adwan *et al*., 2012). However, there is a different approach to the oral hygiene. Medicinal plants are the basic ingredients of toothpaste on the one hand, and on the other, the presence of herbal components is avoided.

Many investigations support some aspects of the use of traditional approach to the oral health. Traditional medicine can treat various infectious and chronic conditions. A research has shown that all kinds of chewing sticks described in ancient Ayurveda texts have medicinal and anti-cariogenic properties. Its oil pulling practice is claimed to cure about 30 systemic diseases. Amla (*Emblic myrobalan*, Fam. Phyllanthaceae) is a general rebuilder of the oral health. Bilberry fruit (*Vaccinium myrtillus*) and hawthorn berry (*Crateagus oxycanthus*, Fam. Rosaeae) stabilize collagen, strengthening the gum tissue. Liquorice root (*Glycyrrhiza glabra*) promotes anti-cavity action, reduces plaque, and has an antibacterial effect (Singh and Purohit, 2011). In Africa, several medicinal plants

are used for maintaining the oral hygiene: *Antidesma venosum*, Tul. (Fam. Phyllanthaceae), *Casearia barteri*, Mast (Fam. Flacourtiaceae), *Citrus aurantifolia* Swing (Fam. Rutaceae), *Diospirus elliott* F. Whit, (Fam. Ebenaceae), *Garcinia kola* Heckel (Fam. Clusiaceae), *Jatropa curcas* Linn. (Fam. Euphorbiaceae), *Lecaniodiscus cupaniodes* (Fam. Sapindaceae), *Ocimum gratissimun* Linn. (Fam. Lamiaceae), *Vernonia amygdalina* Del. (Fam. Asteraceae), *Zanthoxylum zanthoxiloides* Waterm (Fam. Rutaceae), *Massularia acuminata* (G. Don.) Bull Ex Hoyle (Fam. Rubiaceae), and *Terminalia glaucescens* Planch. (Fam. Combretaceae) (Elujoba *et al.,* 2005).

ANTIFUNGAL ACTIVITY OF PLANTS AND ALGAE AGAINST *C. albicans*

Numerous contemporary investigations support the common knowledge that some plants are used as traditional remedies against microorganisms including *C. albicans*. Since there has been a limited range of available antifungal drugs, especially those for resistant *C. albicans* strains, the prospect of preventing its colonization is becoming increasingly attractive. There are several approaches for preventing colonization by inhibiting *C. albicans* adherence: immunization, physical interference, salivary IgA, mucosal immune response, and application of soluble ligands (Cannon and Chaffin, 2001). However, there are not promising and cost-effective results. Perhaps, the use of traditional medicinal plants may take part in efficient treatment of oral candidiasis or as ingredients in toothpaste for prevention. Yet, there are not many reports related to Serbian autochtonous medicinal plants used in traditional medicine against *C. albicans* in the oral cavity (Tadić *et al.,* 2008; Savikinet *et al*., 2009). Hawthorn [*Crataegus monogyna* Jacq. and *Crataegus oxyacantha* L.; sin. *Crataegus laevigata* (Poiret) DC., Fam. Rosaceae] berries ethanol extract had no effect on *C. albicans* (Tadić *et al*., 2008). Ličina *et al*. (2013) reported that ethanol, acetone, ethil acetate and diethil ether extract of *Origanum vulgare* L. (Fam. Lamiaceae) inhibits the growth of *C. albicans* at concentrations between 2.5 mg/mL and 10 mg/mL.

In one study conducted in Malaysia, *Brucea javanica* (Fam. Simaroubaceae) and *Piper betie* (Fam. Piperaceae) aqueous extracts had effects on the non-specific and specific adherence mechanisms of oral *C. albicans* by modifying the surface hydrophobicity of a cell wall and the characteristics of the experimental pellicle. Namely, exposing to pellicle treated with *P. betle* drastically reduced the adherence of *Candida tropicalis*, *C. albicans*, and *Candida krusei* by 86.01%, 61.41%, and 56.34%, respectively. *B. javanic*a exhibited similar effects on *C. tropicalis* (89.86%), *Candida lusitaniae* (88.95%), *C. albicans* (79.74%), *Candida glabrata* (76.85%), and *C. krusei* (67.61%) (Nordin *et al*., 2013). Also, tincture from *Schinus terebinthifolius* (Fam. Anacardiacae) (Brazilian pepper tree) showed anti-fungal activity against *C. albicans*, probably by inhibition of the fungal cell wall formation (Alves *et al*., 2013). In Nairobi, Kenia, herbal extracts of *Leonotis nepetifolia* (Fam. Lamiaceae), *Biden pilosa* (Fam. Asteraceae), *Senna didymobotrya* (Fam. Fabaceae), *Toddalia asiatica*

(Fam. Rutaceae), and *Physalis peruviana* (Fam. Solanaceae) have a potential to control the multiplication of *C. albicans* (Maobe *et al*., 2013). One study inspired by traditional Indian medicine or Ayurveda showed that the ethanol extract of ginger powder has pronounced inhibitory activities against *C. albicans*. Ethanol itself has antifungal activity; ethanol extract of ginger had a synergistic activity (Supreetha *et al*., 2011). Further, there is an observation coming from India (Rajah Muthiah Medical College and Hospital, Annamalai University) that an expressed antifungal activity is observed for some plants: *Syzygium jambolanum* (Fam. Myrtaceae), *Cassia siamea* (Fam. Fabaceae), *Caulerpa scalpelliformis* (Fam. Caulerpaceae), and algae: green alga consisting of one cell and many nuclei, and *Sargassum wightii* (Fam. Sargassaceae), brown macroalga (Prabhakar *et al*., 2008).

ESSENTIAL OILS

Essential oils of thyme (*T. vulgaris* L.) and tea tree oil (*Melaleuca alternifolia* L.) (Fam. Myrtaceae) both caused changes in cell and colony morphology, but also in the metabolism of *C. albicans* (Rajkowska *et al*., 2014; Yiğit *et al*., 2009). In addition, some data indicate that the essential oil of *Rosmarinus officinalis* (Fam. Lamiaceae) modulates *C. albicans* pathogenicity through its primary virulence factor, which is the suppression of germ tube formation (Gauch *et al*., 2014).

Investigation of the chemical composition and the activity against *C. albicans* of volatile oils obtained from herbs of the family *Asteraceae,* consisting of about 500 species, points out that many of its members possess the antifungal activity. Traditionally, the subgeneric taxonomy species *Artemisia* (Fam. *Asteraceae*) follows a system established by Besser (1829), who separated sections based on various combinations of disc and ray flower occurrences and fertility. Subsequent works of Rydberg (1916), who elevated the sections to the level of subgenera and created subordinate sections including section *Tridentatae* for the North American members of subgenus *Seriphidium,* modified Besser's four sections (*Abrotanum, Absinthium, Dracunculus,* and *Seriphidium*). Current consensus is the recognition of three subgenera: *Artemisia* L. (5 Bessers's *Abrotanum* 1 *Absinthium*), *Dracunculus* (Besser) Rydb., and *Seriphidium* (Besser) Rouy. However, based on karyotypic, chemotaxonomic, and distributional criteria, some authors elevated *Tridentatae* to subgeneric status as *Tridentatae* (McArthur and Sanderson, 1999). Nevertheless, this classification does not accurately represent the natural groups. Molecular phylogenetic studies, based on the analysis of chloroplast (cpDNA) and nuclear ribosomal (nrDNA) sequences have helped elucidating the systematic relationships within *Artemisia*, although important questions remain unresolved. Despite some differences, there is a close phylogenetic relationship between *Artemisia* and *Absinthium*, which is consistent with the available molecular phylogenies presenting species of the subgenera *Artemisia* and *Absinthium* intermixed (Pellicer *et al*., 2008).

Artemisia dracunculus (Subgenus: *Dracunculus*) investigation, *A. abrotanum* (Subgenus: *Artemisia* L. (5 Bessers's Subgenus: *Artemisia* L.)), *A. absinthium* (Subgenus: *Artemisia* L. (Bessers's *Absinthium*)) (McArthur and Sanderson, 1999), and *A. vulgaris* (Subgenus: *Artemisia)* (Pellicer *et al*., 2008) exploration revealed that volatile oils from *A. abrotanum* containing davanone or silphiperfolane derivatives showed the highest antifungal activity, which emphasized that *Artemisia* oils are promising in the development of novel anti-*Candida* drugs (Obistioiu *et al*., 2014). *T. vulgaris* oils are active against virulence factors and biofilms, proteinase and haemolysin producing drug-resistant strains of *Candida* spp. These activities are supposed to be the main contribution due to their major active compound thymol (Khan *et al*., 2014). *S. officinalis* L. essential oil exhibited anticandidal activities and had inhibitory effects on the adhesion of the cells to resin surface of polymethyl methacrylate. There is a possibility to use *S. officinalis* essential oil as an antifungal denture cleanser to prevent candidal adhesion and to reduce the risk of *Candida*-associated denture stomatitis (Sookto *et al*., 2013). *S. officinalis* L. essential oil exhibited anticandidal activities against *C. albicans* and had inhibitory effects on the adhesion of the cells to the surface of polymethyl methacrylate resin.

In Brazil, essential oils from the leaves and/or roots of 35 medicinal plants commonly used in that country were screened for anti-*C. albicans* activity. Essential oils from 13 plants showed anticandidal activity. There was a strong activity against *C. albicans* of oils of *Achillea millefolium*, *Mikania glomerata* (Fam. Asteraceae), and *Stachys byzantina* (Fam. Lamiaceae), (MIC = 0.25 μg mL^{-1}). *Aloysia triphylla* (Fam. Verbenaceae), *Anthemis nobilis* (Fam. Asteraceae), *Cymbopogon martinii* (Fam. Poaceae), *Cyperus articulatus, Cyperus rotundus* (both belong to the Fam. Cyperaceae)*, Lippia alba* (Fam. Verbenaceae), *Mentha arvensis* and *Mentha piperita* (Fam. Lamiaceae) presented a moderate activity (MIC between 0.6 and 1.5 μg mL^{-1}). Weak activity (weak inhibitors – MIC above 1.6 μg mL^{-1}) was shown by *Baccharis dracunculifolia* (Fam. Asteraceae), *O. vulgare*, *Piper regnellii,* and *T. vulgaris*. All the remaining plants presented a MIC above 2.0 μg mL^{-1} (Duarte *et al*., 2005).

One Serbian study included investigation of the essential oils obtained from *R. officinalis, S. officinalis,* and *Satureja kitaibelii* (*S. kitabeli*) (Fam. Lamiaceae) to test *C. albicans* growth *in vitro.* The activity of herb essential oils was expressed as MIC values against *C. albicans* ATCC strain 1023, MIC values for *R. officinalis, S. officinalis*, and *S. kitabeli* were: 50 μg mL^{-1}, 25 μg mL^{-1} and 12.5 μg mL^{-1}, respectively. The essential oil of *S. kitabeli* had the strongest inhibitory activity and the lowest MIC value of 12.5 μg mL^{-1}, and probably can be the best candidate for fighting *C. albicans* oral infection (Tambur *et al*., unpublished).

In addition, testing of the antimicrobial activity of *S. kitaibelii* essential oil against the battery of 30 pathogenic microorganisms showed a significant activity against dermatophyte strains (Mihajilov-Krstev *et al*., 2011). Another investigation showed that essential oil of *Teucrium montanum* (Fam. Lamiaceae) had antifungal effect against three mold species: *Fusarium, Aspergillus* and *Penicillium* (Vukovic *et al*., 2007).

Tanideh and co-workers concluded that experimental oral *C. albicans* mucositis can be treated with *H. perforatum* extract administered orally or topically (Tanideh *et al*., 2014). It may be of interest to notice that *H. perforatum* endophyte *Seimatosporium* sp. (fungus) (Fam. anamorph coelomycete) exhibited a significant antifungal activity against *C. albicans* by (–)-avenaciolide as the only bioactive constituent of the extract (Clark *et al*., 2014).

Investigation of activity of ethanol extracts obtained from other plants such as *Acacia nilotica*, (Fam. Fabaceae), *Syzygium aromaticum*, *Cinnamon zeylanicum* (Fam. Lauraceae), *Eucalyptus globulus* (Fam. Myrtaceae), and *Terminalia arjuna* on *C. albicans* ATCC 10231 growth exhibits strong inhibitory effects of *A. nilotica* and *S. aromaticum* (Khan *et al*., 2014).

Several investigations revealed the antifungal activity of herbs growing in Serbia against *Candida*. Experimental oral mucositis can be treated with *H. perforatum* extract administered orally or topically (Tanideh *et al*., 2014). Methanol extracts of *M. piperita, Mentha longifolia*, *Plantago lanceolata* (Fam. Plantaginaceae), and *Artemisia austriaca* displayed some activities against *C. albicans* (Yiğit *et al*., 2009).

Autochthonous species have been described in a substantial number of studies. Antifungal activity of ethanol extracts of plants in the study undertaken by Rajah Muthiah Medical College and Hospital, Annamalai University, India, was confirmed in investigation on *Candida* strains (*C. albicans* was isolated in 76.08% of the oral lesions). Plants: *Syzygium jambolanum, Cassia siamea, Odina wodier* (Fam. Anacardiaceae)*, Momordica charantia* (Fam. Cucurbitaceae), and *Melia azedarach* (Fam. Melaiceae) and two algal species, *Sargassum wightii* and *Caulerpa scalpelliformis* were tested against 25 strains by disc diffusion method. Antifungal activity was observed for medicinal plants *Syzygium jambolanum* and *Cassia siamea*, and for seaweed *Caulerpa scalpelliformis* at 100 μg mL^{-1} and *Sargassum wightii* at 10 μg mL^{-1} (Prabhakar *et al*., 2008).

Eethanol extracts from the leaves and/or roots of 35 medicinal plants commonly used in Brazil were also screened for anti-*C. albicans* activity. Ethanol extracts of *Achillea millefolium*, *Mikania glomerata*, and *Stachys byzantina* (MIC = 0.25 μg mL^{-1}). *Aloysia triphylla*, *Anthemis nobilis*, *Cymbopogon martinii*, *Cyperus articulatus, Cyperus rotundus, Lippia alba*, *Mentha arvensis* and *M. piperita, Baccharis dracunculifolia*, *O. vulgare*, *Piper regnellii* and *T. vulgaris*, and other investigated plants were not effective against *C. albicans* at any of the concentrations tested (Duarte *et al*., 2005).

In the study conducted in Kisii region, southwest Kenya, on the various leaf extracts of the *Carissa spinarum* (Fam. Apocynaceae)*, Urtica dioica, Warburgia ugandensis* (Fam. Canellaceae)*, Senna didymobotrya, Physalis peruviana, Bidens pilosa* (Fam. Asteraceae)*, Leonotis nepetifolia* and *Toddalia asiatica,* the highest antifungal activity against *C. albicans* was noted in ethanol leaf extracts of *Leonotis nepetifolia* and *Toddalia asiatica* (Maobe *et al*., 2013).

Ethanol extracts of 58 tradional Chinese plants belonging to 45 families were examined for their activity against *A. Fumigatu* and *C. albicans.* The

anti-fungal activities of the selected plant extracts were tested against two kinds of fungal species, namely, *C. albicans* represented the yeast model and *A. fumigatus* represented the filamentous fungus. The activities of these extracts were evaluated in a dose-response curve with 1.00, 0.10 and 0.01 μg mL^{-1} concentrations. These results revealed that 13 plant extracts, *Solanum nigrum* (Fam. Solanaceae), *Poria cocos* (Fam. Polyporaceae), *Eucommia ulmoides* (Fam. Eucommiaceae), *Atractylodes macrocephala* (Fam. Asteraceae), *Polygonum cuspidatum* (Fam. Polygonaceae), *Ligustrum lucidum* (Fam. Oleaceae), *Polygala tenuifolia* (Fam. Polygalaceae), *Saposhnikovia divaricata* (Fam. Apiaceae), *Mahonia fortunei* (Fam. Berberidaceae), *Cynanchum paniculatum* (Fam. Apocynaceae), *Lobelia chinensis* (Fam. Campanulaceae), *Aster tataricus* (Fam. Asteraceae), and *Uncaria rhynchophylla* (Fam. Rubiaceae), showed a high inhibitory activity against *A. fumigatus*, representative of filamentous fungi. However, only two plant extracts, *Codonopsis pilosula* (Fam. Campanulaceae) and *Tussilago farfara* (Fam. Asteraceae), showed a high inhibitory effect against *C. albicans.* These antifungal activities suggest that different plant extracts may contain very selective target compounds that have specific selectivity towards *A. fumigatus* and *C. albicans.* The results are very significant as most of the plants showed an inhibitory effect not only at the concentration of 1.0 μg mL^{-1} (Zhang *et al*., 2013).

There are plenty of herbs used in traditional medicine in South Africa. In the investigation of leaf extracts of *Cussonia zuluensis* (Fam. Araliaceae), *Vepris reflexa* (Fam. Rutaceae), *Curtisia dentata* (*C. dentata*) (Fam. Curtisiaceae), *Trichilia emetica* (Fam. Meliaceae), *Terminalia phanerophlebia*, *Terminalia sambesiaca*, and *Kigelia africana* (Fam. Bignoniaceae), extracts of *C. dentata*, *T. sambesiaca,* and *T. phanerophlebia* had the highest activities against fungal test organisms. The most efficient activity was observed in the acetone extracts of *C. dentata* against *A. fumigatus*, *Micrococcus canis*, *C. albicans*, *Sporothrix schenckii*, and *Cryptococcus neoformans*. The authors concluded that *C. dentata* is a candidate for further work on isolation of compounds active against *C. albicans* (Shai *et al*., 2008).

In a recent study, the effects of some herbs traditionally used in phytotherapy for a wide spectrum of diseases in Serbia have been evaluated (Tambur *et al*., in press). Ethanol extracts activity measured as MIC values for *Sinapis alba* (Fam. Brassicaceae), *T. vulgaris*, *H. perforatum*, *Teucrium montanum*, *Artemisia absinthium*, *Plantago lanceolata*, *S. officinalis*, *C. officinalis*, *Acorus calamus* (Fam. Acoraceae), *Malva mauritanica* (Fam. Malvaceae), *Tilia cordata* (Fam. Malvaceae), *Aesculus hippocastanum* (Fam. Sapindaceae), *Capsella bursa-pastoris* (Fam. Brassicaceae), *Origanum majorana*, and *A. millefolium* were investigated against *Candida albicans* ATCC strain 1023. It has been shown that extracts of *Sinapis alba*, *Teucrium montanum*, *A.absintum*, *Plantago lanceolata*, *C. officinalis*, *Acorus calamus*, *Malva mauritanica*, *Tilia cordata*, *Capsella bursa-pastoris*, *Origanum majorana*, and *Achillea A. millefolium* had MIC = 300 μg mL^{-1}, however, for *S. officinalis* and *H. perforatum*, MIC was > 300 μg mL^{-1} without any effect to *Candida* growth. The extract of *T. vulgaris* had MIC of 150 μg mL^{-1}, while *Aesculus hippocastanum* had a stronger antimicrobial activity (MIC =37.5 μg mL^{-1}) (Tambur *et al*., unpublished).

ANTIFUNGAL ACTIVITY OF HONEY

Since honey is also of plant origin, it is not surprising that it could have the role in antifungal activity (Fallico *et al*., 2004; Djouahri *et al*., 2013). Several factors may influence the antifungal activity of honey. For example, DeMera and Angert (2004) reported that honey from different phytogeographic regions varied in their ability to inhibit the growth of yeasts, suggesting the botanical origin influencing the antifungal activity. However, there are other antibacterial factors such as high sugar or hydrogen peroxide concentration, as well as low pH, which are well known. More recently, identification of methylglyoxal and the antimicrobial peptide bee defensin-1 in honey confirmed that these substances are important antibacterial compounds, but also with strong antifungal activity against many *Candida* species such as *C. albicans*, *C. parapsilosis*, *C. tropicalis, C. kefyr*, *C. glabrata*, and *C. dubliniensis* (Khosravi *et al*., 2008; Moussa *et al*., 2012).

ANTIBACTERIAL HERB EFFECTS

Herbs actively suppress the growth of some bacteria, too. In Serbian traditional medicine, substantial number of plants that belong to several different families such as *Lamiacea*, *Asteracea, Brassicaceae, Hypericaceae*, *Plantaginaceae*, *Acoraceae*, *Malvaceae*, *Tiliaceae*, *Sapindaceae*, and others, have a paramount position. Scientific evidences have confirmed that some extracts of these plants act efficiently against many bacteria (Mihajilov-Krstev *et al*., 2011; Vukovic *et al*., 2007; Samoilova *et al*., 2014).

The essential oil of *Teucrium montanum* had a strong antibacterial effect against 13 bacterial species (Vukovic *et al*., 2007). *Ruscus aculeatus* L. and *Ruscus hypoglossum* L. showed an antimicrobial activity on pathogenic Gram-positive and Gram-negative microflora: *S. aureus* (ATCC 6538), *B. cereus* (human isolate), *Micrococcus flavus* (ATCC 10240), *L. monocytogenes* (NCTC 7973), *P. aeruginosa* (ATCC 27853), *Enterobacter cloacae* (human isolate), *S. typhimurium* (ATCC 13311), and *E. coli* (ATCC 35210). Substances isolated from these plants responsible for this effect are the same as for antifungal activities: phenolic acids such as p-coumaric and caffeic acid and flavonoid rutin pointed to the *R. aculeatus* herb as a potentially new promising herbal material (Hadžifejzović *et al*., 2013).

Investigation of autochthonous medicinal plants from the faraway Himalayas in Ladakhi region, near Siachin glacier, showed that some extracts expressed significant antibacterial effects, especially leaf samples of *Podophyllum hexandrum* (Fam. Berberidaceae), stem of *Verbascum thapsus* against *Bacillus subtilis,* and flower of *Salvia sclarea* against *Pseudomonas aeruginosa* (*P. aeruginosa*) (Kumar *et al*., 2010). Ethanol extracts of 58 traditional Chinese plants belonging to 45 families were examined for their activity against *Acinetobacter baumannii*, *P. aeruginosa*, and *S. aureus*. A total of 30 plant extracts showed significant antimicrobial activities against these test microbial strains (Zhang *et al*., 2013).

Some plants (*Allium sativum* L.) may ameliorate effects on gentamicin nephrotoxicity in the kidney (Nasri *et al*., 2013). In Pakistan, investigation of the crude extracts and fractions of six medicinal plants revealed that methanol fraction of *Pistacia integerrima* (Fam. Anacardiaceae), chloroform fractions of *Debregeasia salicifolia* (Fam. Urticaceae) and *Toona ciliata* (Fam. Meliacea), and aqueous fraction of *Aesculus indica* are suitable candidates for the development of novel antibacterial compounds (Bibi *et al*., 2011). In a study where twenty-one herbal extracts were screened for antiadhesive activity against *Campylobacter jejuni*, the highest anti-adhesion effects were obtained for *Zingiber officinale* (ginger) (Fam. Zingiberaceae), *Capsicum annuum* (cayenne) (Fam. Solanaceae), and *Glycyrrhiza glabra* (licorice) (Bensch *et al*., 2011).

Essential oil of *R. officinalis* expressed *in vitro* antibacterial effects against meticillin-resistant (MR), and meticillin-sensitive *Staphylococcus aureus* (MSSA), *Escherichia coli*, *P. aeruginosa*, *Salmonella* Typhimurium, and *Salmonella* Enteritidis (Barbosa *et al*., 2015). The extract from *Urtica dioica* reduced biofilm formation in *E. coli* BW 25113 (Samoilova *et al*., 2014). The ethanol extracts of 15 traditionally used medicinal plants against ESBL-producing drug-resistant enteric isolates demonstrated a broad-spectrum activity against all test isolates (Ahmad and Aqi, 2007).

C. officinalis and *Camellia sinensis* L. (Fam. Theaceae) demonstrate an antimicrobial activity inhibiting the adherence of microorganisms to sutures after extraction of unerupted third molars (Faria *et al*., 2011). During the evaluation of the antibacterial effects of some medicinal plants, *Thymus caramanicus* and *Zataria multiflora* (Fam. Lamiaceae) were the most effective ones against several pathogens, including *S. aureus*, *Shigella dysenteriae*, *S. typhimurium*, *E. coli*, *Staphylococcus epidermidis*, *Bacillus subtilis*, MRSA, and *P. aeruginosa* (Mahboubi *et al*., 2014). Investigation on *A. absintum* revealed the effects of 49,59-O-dicaffeoylquinic acid (49,59-ODCQA) as a pump inhibitor with a potential of targeting efflux systems in a wide panel of Gram-positive human pathogenic bacteria (Fiamegos *et al*., 2014).

Medicinal plants may be a valuable alternative to the control of bacteria in raw beef. *R. officinalis* and especially *T. vulgaris* essential oils can reduce the presence of *Listeria monocytogenes* (Oliveira *et al*., 2013).

It is worth mentioning that extracts of one of the species of lichen *Usnea* (lichen is a symbiosis between a fungus and an alga or cyanobacterium Hale, ME.) (Fam. Parmeliaceae), where the fungus belongs to the division Ascomycota, while the alga is a member of the division Chlorophyta), *Usnea barbata,* showed a strong antibacterial activity against the most of the tested strains of *Streptococcus*, *Enterococcus,* and *Staphylococcus* of different origin (Zizovic *et al*., 2012).

The acetone extracts of *C. dentata* expressed the most efficient activity. *C. dentata* extracts also had five compounds active against other tested bacterial species (*S. aureus*, *E. coli*, *Enterococcus faecalis* and *P. aeruginosa*). An opposing, microbial growth inhibitory effect of *C. dentata* extracts was non-selective (Shai *et al*., 2008).

CYTOTOXICITY

In addition, it may be of interest to note that evaluation of the antimicrobial activity and analysis of the cytotoxicity of Brazilian plant extracts of *Equisetum arvense* L (Fam. Equisetaceae), *Glycyrrhiza glabra* L., *Punica granatum* L. (Fam. Lythraceae), and *Stryphnodendron barbatimam* Mart. (Fam. Fabaceae) against *S. aureus*, *S. epidermidis*, *Streptococcus mutans*, *C. albicans*, *C. tropicalis*, and *C. glabrata* revealed that all plant extracts were effective against the microorganisms tested. The *G. glabra* L. extract exhibited least cytotoxicity and the *E. arvense* L. extract was the most cytotoxic (Oliveira *et al*., 2005). When investigation was extended to other virulence mechanisms of *C. albicans*, it was noticed that the concentration-dependent activity of *Carum copticum* (Fam. Apiaceae) and *T. vulgaris* oils exist against virulence factors and biofilms, proteinase and haemolysin producing drug-resistant strains of *Candida* spp. Thymol is also major active compound which paly a significant role against biofilms (Khan *et al*., 2014).

One species of a genus of evergreen coniferous tree, *Tetraclinis articulata* (Vahl) Masters, also known as *Thuja articulata* (Vahl) or *Callitris quadrivalvis* (Vent) (Fam. Cupressaceae) (Barrero *et al*., 2005) is native to northwestern Africa, to Morocco, Algeria, and Tunisia. It grows also in small populations in Malta and near Cartagena (southeast Spain) (Bourkhiss *et al*., 2010). This tree is used in traditional and popular medicine to cure numerous infections both in childhood and adulthood: respiratory and intestinal infections, gastric pains, diabetes, hypertension and rheumatism (Bourkhiss *et al*., 2007; Bourkhiss *et al*., 2010). It was observed that this plant not only has antibacterial and antifungal effects (Bourkhiss *et al*., 2007; Bourkhiss *et al*., 2010; Djouahri *et al*., 2014), but it also possesses cytotoxic, antioxidative, and anti-inflammatory characteristics (Djouahri and Boudarene, 2012). Having in mind a broad spectrum of treated illnesses, the chemical composition of *Thuja articulata* (Vahl) has been successfully evaluated (Djouahri *et al*., 2013).

CONCLUSION

Medicinal plants are a substantial reservoir of positive effects on human health. There are records about their use in the early history, but we can assume that herbs were used for healing before the historical documents existed. Some herbs are studied in details and some of their compounds including useful ones are well-known. However, as humans, we have to be very careful with the flora generally, and with medicinal plants especially, because they are very sensitive to different influences including human behavior. In addition, we have to be very careful about our relationship with wild areas in order to preserve the natural floral ecosystems.

Medicinal plants remain a reservoir of undiscovered drugs, or substances that have antimicrobial effects. In Serbia, many healing species grow. Our

autochthonous medicinal plants are investigated to some extent, but perhaps the application of the isolated substances is not as wide as we could expect. In addition, their investigation could help the accumulation of knowledge and changing of attitude towards their use: they can be used instead of conventional drugs or together with remedies of conventional medicine. Interest in medicinal plants could influence the evolving relationship between pharmaceutical industry and pharmacognosy. Investigation of medicinal plant compounds and their effect on the target microorganisms can develop chemical synthesis or plantation of medicinal plants.

Many authors and numerous studies have shown that extracts of some plant species autochthonous in Serbia, as well as the exotic ones, can have strong antimicrobial effects. Studies coming from almost all geographic regions in the world revealed results on the application of their autochthonous medicinal plants to some pathogenic fungi such as *C. albicans*.

Toothpaste ingredients can involve only chemical compounds, but some individuals prefer toothpastes with addition of herbal extracts, or only with herbal extracts in vehiculum. For that reasons, the effects of some medicinal plants to *C. albicans* growth should be taken into consideration. It seems that toothpaste that contains both herbal extracts and sodium fluoride is more effective in the control of *C. albicans* than toothpaste containing monofluorophosphate only. In addition, some herbal toothpastes appear to be equally effective as fluoride dental formulations. They can be an acceptable alternative in the future to the individuals who prefer natural approach in the prevention of oral cavity diseases.

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REFERENCES

Adwan, G, Salameh Y, Adwan K, Barkat A (2012) Assessment of antifungal activity of herbal and conventional toothpastes against clinical isolates of *Candida albicans*. *Asian Pac. J. Trop. Biomed*. 2: 375–379.

Ahmad I, Aqi F (2007): In vitro efficacy of bioactive extracts of 15 medicinal plants against ESBL-producing multidrug-resistant enteric bacteria. *Microbiol. Res*. 162: 264–267.

Akerreta S, Cavero RY, López V, María Isabel Calvo MI (2007): Analyzing factors that influence the folk use and phytonomy of 18 medicinal plants in Navarra. *J. Ethnobiol. Ethnomed*. 3: 16.

Allen D, Bilz M, Leaman DJ, Miller RM, Timoshyna A, Window J (2014): *European Red List of Medicinal Plants*, Luxembourg: Publications Office of the European Union.

Alt-Epping, B, Nejad RK, Jung K, Gross U, Nauck F (2012): Symptoms of the oral cavity and their association with local microbiological and clinical findings- a prospective survey in palliative care. *Support Care Cancer*. 20: 531–537.

Alves LA, Freires IA, Pereira TM, Dias de castro R (2013): Effect of Schinus terebinthifolius on Candida albicans growth kinetics, cell wall formation and micromorphology. *Acta Odontol Scand*. 2013; 71: 965–971.

Barbosa LN, Probst Ida S, Andrade BF, Alves FC, Albano M, da Cunha Mde L, Doyama JT, Rall VL, Fernandes Júnior A (2015): In vitro antibacterial and chemical properties of essential oils including native plants from Brazil against pathogenic and resistant bacteria. *J. Oleo. Sci*. 64: 289–298.

Barrero AF, Herrador MM, Arteaga P, Quilez J, Akssira M, Mellouki F (2005): Chemical composition of the essential oil of leaves and wood of *Tetraclinis articulata* (Vahl) Masters. *J. Essent. Oil Res*. 17: 166–168.

Bensch K, Tiralongo J, Schmidt K, Matthias A, Bone KM, Lehmann R, Tiralongo E (2011): Investigations into the antiadhesive activity of herbal extracts against *Campylobacter jejuni*. *Phytother Res*. 25: 1125–1132.

Bibi Y, Nisa S, Chaudhary FM, Zia M (2011): Antibacterial activity of some selected medicinal plants of Pakistan. *BMC Complem Altern M*. 11: 52.

Bourkhiss B, Ouhssine, M, Hnach M, Bourkhiss M, Satrani B, Farah A (2007): Chemical composition and bioactivity of branches essential oil of *Tetraclinis articulata*. *Bull. Soc. Pharm. Bordeaux*. 146: 75–84.

Bourkhiss M, Hnach M, Lakhlifi T, Bourkhiss B, Ouhssine M, Satrani B (2010): Production and characterization of the sawdust of wood essential oil of *Tetraclinis articulata* (Vahl) Masters. *Bull. Soc. R. Sci. Liège*. 79: 4–11.

Cannon RD, Chaffin WL (2001): Colonization is a crucial factor in oral candidiasis. *J. Dent. Educ*. 65: 785–787.

Cannon RD, Chaffin WL (1999): Oral colonization by *Candida albicans*. *Crit. Re.v Oral. Biol. Med*. 10: 359–383.

Clark AM (1996): Natural products as a resource for new drugs. *Pharm. Res.* 13: 1133–1144.

Clark TN, Bishop AI, McLaughlin M, Calhoun LA, Johnson JA, Gray CA (2014): Isolation of (-)-avenaciolide as the antifungal and antimycobacterial constituent of a *Seimatosporium* sp. endophyte from the medicinal plant *Hypericum perforatum*. *Nat. Prod. Commun*. 9: 1495–1496.

Dalle F, Hebecker B, L'Ollivier C, Hube B (2010): Cellular interactions of *Candida albicans* with human oral epithelial cells and enterocytes. *Cell. Microbiol*. 12: 248–271.

De Oliveira MM, Brugnera DF, Piccoli RH (2013): Essential oils of thyme and Rosemary in the control of *Listeria monocytogenes* in raw beef. *Braz. J. Microbiol*. 44: 1181–1188.

DeMera JH, Angert ER (2004): Comparison of the antimicrobial activity of honey produced by *Tetragonisca angustula* (Meliponinae) and *Apis mellifera* from different phytogeographic regions of Costa Rica, *Apidologie*, 35: 411–417.

Djouahri A, Boudarene L and Meklati BY (2013): Effect of extraction method on chemical composition, antioxidant and anti-inflammatory activities of essential oil from the leaves of Algerian *Tetraclinis articulata* (Vahl) Masters. *Ind. Crop. Prod*. 44: 32–36.

Djouahri A, Boudarene L (2012): Antioxidant and anti-inflammatory activity of methanol, chloroform and ethyl acetate extracts of leaves *Tetraclinis articulata* (Vahl) Masters Algerian. *Int. J. Res. Phytochem. Pharmacol*. 2: 7–11.

Djouahri A, Saka B, Boudarene L, Baaliouamer A (2014): In vitro synergistic/antagonistic antibacterial and anti-inflammatory effect of various extracts/esential oil from cones of

Tetraclinis articulata (Vahl) Masters with antibiotic and anti-inflammatory agents. *Ind. Crop. Prod*. 56: 60–66.

Duarte MC, Figueira GM, Sartoratto A, Rehder VL, Delarmelina C (2005): Anti-Candida activity of Brazilian medicinal plants. *J. Ethnopharmacol*. 97: 305–311.

Elujoba AA, Odeleye OM, Ogunyemi CM (2005): Traditional medicine development for medical and dental primary health care delivery system in Africa. *Afr. J. Trad. CAM* 2: 46–61.

European Herbal & Traditional Medicine Practitioners Association. Herbal and Traditonal Medicine. http://ehtpa.eu/herbal_traditional_medicine/index.html. Accessed on 20. 3. 2017.

Fallico B, Zappalã M, Arena E (2004): Effects of heating process on chemical composition and HMF levels in Sicilian monofloral honeys. *Food Chem*. 85: 305–313.

Faria RL, Cardoso LM, Akisue G, Jungueira JC, Jorge AO, Santos Júnior PV (2011): Antimicrobial activity of *Calendula officinalis*, *Camellia sinensis* and chlorhexidine against the adherence of microorganisms to sutures after extraction of unerupted third molars. *J. Appl. Oral. Sci.* 19: 476–482.

Fiamegos YC, Kastritis PL, Exarchou V (2011): Antimicrobial and efflux pump inhibitory activity of caffeoylquinic acids from *Artemisia absinthium* against gram-positive pathogenic bacteria. *Plos One* 2011; 6: e18127.

Fukazawa Y, Kagaya K (1997): Molecular bases of adhesion of *Candida albicans*. *J. Med. Vet. Mycol*. 35: 87–99.

Gardiner J, Freeman S, Leach M, Green A, Alcock J, D'Emanuele A (2008): PAMAM dendrimers for the delivery of the antibacterial triclosan. *J. Enzym. Inhib. Med. Ch*. 23: 623–628.

Gauch LM, Silveira-Gomes F, Esteves RA, Pedrosa SS, Gurgel S, Arruda AC, Margues-da-Silva SH (2014): Effects of *Rosmarinus officinalis* essential oil on germ tube formation by *Candida albicans* isolated from denture wearers. *Rev. Soc. Bras. Med. Tro*. 47: 389–391.

Glee PM, Sundstrom P, Hazen KC (1995): Expression of surface hydrophobic proteins by *Candida albicans* in vivo. *Infect. Immun*. 63: 1373–1379.

Hadžifejzović N, Kukić-Marković J, Petrović S, Nahrstedt A (2013): Bioactivity of the extracts and compounds of *Ruscus aculeatus* L. and *Ruscus hypoglossum* L. *Ind. Crop. Prod*. 49: 407–411.

Hale ME (1967): *The biology of lichens*. London: Edward Arnold.

Hazen KC, Hazen BW (1993): Surface hydrophobic and hydrophilic protein alterations in *Candida albicans*. *FEMS Microbiol. Lett*. 107: 83–88.

Inglis DO, Sherlock G (2013): Ras signaling gets fine-tuned: Regulation of multiple pathogenic traits of *Candida albicans*. *Eukaryot. Cell*. 12: 1316–1325.

Jarić S, Mitrović M, Djurdjević L, Kostić O, Gajić G, Pavlović D, Pavlović P (2011): Phytotherapy in medieval Serbian medicine according to the pharmacological manuscripts of the Chilandar Medical Codex (15–16th centuries). *J. Ethnopharmacol*. 137: 601–619.

Jarić S, Popović Z, Macukanovic-Jocic M, Djurdjevic L, Mijatovic M, Karadzic B, Mitrovic M, Pavlovic P (2007): An ethnobotanical study on the usage of wild medicinal herbs from Kopaonik Mountain (Central Serbia). *J. Ethnopharmacol.* 111: 160–175.

Jenkinson HF, Douglas LJ (2002): *Candida* interactions with bacterial biofilms, In: *Polymicrobial Infections and Disease*. Eds.: KA Brogden, JM Guthmiller. ASM Press, Washington (DC), 357–373.

Khan MS, Ahmad I, Cameotra SS, Botha F (2014): Sub-MICs of *Carum copticum* and *Thymus vulgaris* influence virulence factors and biofilm formation in *Candida* spp. *BMC Complem. Altern. M*. 14: 337.

Khosravi AR, Shokri H, Katiraee F, Ziglari T, Forsi M (2008): Fungicidal potential of different Iranian honeys against some pathogenic *Candida* species. *J. Apicult. Res*. 47: 256–260.

Kumar PG, Kumar R, Badere R, Singh SB (2010): Antibacterial and antioxidant activities of ethanol extracts from trans Himalayan medicinal plants. *Phcog. J.* 17: 66–69.

Lachance MA, Boekhout T, Scorzetti G, Fell JW, Kurtzman CP (2011): *Candida* Berkhout, In: *The Yeasts: a taxonomic study*, 5. Eds.: CP Kurtzman, JW Fell, T Boekhout, Elsevier, Amsterdam, 987–1279.

Lamont RJ, Hajishengallis GN, Jenkinson HF (2014): *Oral microbiology and immunology*. No. Ed.2 pp.xxvi + 504 pp. ASM Press, Washington, USA.

Ličina BZ, Stefanović OD, Vasić SM, Radojević ID, Dekić MS, Čomić LjR (2013): Biological activities of the extracts from wild growing *Origanum vulgare* L. *Food Control.* 33: 498–504.

Mahboubi A, Kamalinejad M, Ayatollahi AM, Babaeian M (2014): Total phenolic content and antibacterial activity of five plants of labiatae against four foodborne and some other bacteria. *Iran J. Pharm. Res*. 13: 559–566.

Maobe MAG, Gitu L, Gatebe E, Muingai C (2013): Antifungal activity of eight selected medicinal herbs used for the treatment of diabetes, malaria and pneumonia in Kisii Region, Southwest Kenya. *World J. Med. Sci*. 8: 74–78.

Marsh P, Martin M(2009): *Oral Microbiology.* 5th Edition, Churchill Livingstone, Elsevier.

Masuoka J, Hazen KC (1997): Cell wall protein mannosylation determines *Candida albicans* cell surface hydrophobicity. *Microbiology* 143: 3015–3021.

Mihajilov-Krstev T, Kitić D, Radnović D, Ristić M, Mihajlović-Ukropina M, Zlatković B (2011): Chemical composition and antimicrobial activity of *Satureja kitaibelii* essential oil against pathogenic microbial strains. *Nat. Prod. Commun*. 6: 1167–1172.

Mizugai H, Isogai E, Hirose K, Chiba I (2007): Effect of denture wearing on occurrence of *Candida* species in the oral cavity. *J. Appl. Res*. 7: 250–254.

Monroy-Ortiz C, Monroy R (2004): Análisis preliminar de la dominancia cultural de las plantas útiles en el estado de Morelos. *Bol. So.c Bot. México.* 74: 77–95.

Moussa A, Djebli N, Saad A, Abdelmalek M (2012): Influence of temperature on the inhibitory potency of *Eucalyptus* honey against *Candida albicans*. *Asian Pac. J. Trop. Dis*. S567–S570.

Nasri H, Nematbakhsh M, Rafieian-Kopaei M (2013): Ethanol extract of garlic for attenuation of gentamicin induced nephrotoxicity in wistar rats. *Iran. J. Kidney Dis*. 7: 376–382.

Nordin MA, Wan Harun WH, Abdul Razak F (2013): An in vitro study on the anti-adherence effect of *Brucea javanica* and *Piper betle* extracts towards oral *Candida*. *Arch. Oral. Biol*. 58: 1335–1342.

Obistioiu D, Cristina RT, Schmerold I, Shizzola R, Stolze K, Nichita I, Chiurciu V (2014): Chemical characterization by GC-MS and in vitro activity against *Candida albicans* of volatile fractions prepared from *Artemisia dracunculus*, *Artemisia abrotanum*, *Artemisia absinthium* and *Artemisia vulgaris*. *Chem. Cent. J.* 8: 6.

Oliveira JR, Castro VC, Graças Figueiredo Vilela P, Camargo SE, Talge CA, Jorge AO, Oliveira LD (2013): Cytotoxicity of Brazilian plant extracts against oral microorganisms of interest to dentistry. *BMC Complem. Altern. M.* 13: 208.

Pellicer J, Garcia S, Garnatje T, Hidalgo O, Siljak-Yakovlev S, Vallès J (2008): Molecular cytogenetic characterization of some representatives of the subgenera *Artemisia* and *Absinthium* (genus *Artemisia*, Asteraceae). *Collectanea Botanica (Barcelona).* 27: 19–27.

Pizzorno JE, Murray MT (2013): *Textbook of Natural Medicine*. 4th Edition. Churchill Livingstone, Elsevier.

Prabhakar K, Kumar LS, Rajendran S, Chandrasekaran M, Bhaskar K, Sajit Khan AK (2008): Antifungal activity of plant extracts against *Candida* species from oral lesions. *Indian J. Pharm. Sci*. 70: 801–803.

Rajkowska K, Kunicka-Styczyńska A, Maroszyńska M, Dąbrowska M (2014): The effect of thyme and tea tree oils on morphology and metabolism of *Candida albicans*. *Acta Biochim. Pol*. 61: 305–310.

Rates SM (2001): Plants as source of drugs. *Toxicon*. 39: 603–613.

Sadeghi M, Assar S (2009): An in vitro antimicrobial activity of ten Iranian-made toothpastes. *Dent. Res. J*. 6: 87–92.

Salem MM, Werbovetz KA (2006): Natural products from plants as drug candidates and lead compounds against leishmaniasis and trypanosomiasis, *Curr. Med. Chem*. 13: 2571–2598.

Samoilova Z, Muzyka N, Lepekhina E, Oktyabrsky O, Smirnova G (2014): Medicinal plant extracts can variously modify biofilm formation in *Escherichia coli*. *Ant. Leeuw. J. Microb.* 105: 709–722.

Saslis-Lagoudakis CH, Hawkins JA, Greenhill SJ, Pendry CA, Watson MF, Tuladhar-Douglas W, Baral SR, Savolainen V (2014): The evolution of traditional knowledge: environment shapes medicinal plant use in Nepal. *Proc. Royal Soc. B: Biol. Sci.* DOI: 10.1098/rspb.2013.2768

Savikin K, Menković N, Zdunić G, Stević T, Radanović D, Janković T (2009): Antimicrobial activity of *Gentiana lutea* L. extracts. *Z. Naturforsch C*. 64: 339–342.

Shai LJ, McGaw LJ, Masoko P, Eloff JN (2008): Antifungal and antibacterial activity of seven traditionally used South African plant species active against *Candida albicans*. *S. Afr. J. Bot*. 74: 677–684.

Shikov AN, Pozharitskaya ON, Makarov VG, Wagner H, Verpoorte R, Heinrich M (2014): Medicinal plants of the Russian Pharmacopoeia; their history and applications. *J. Ethnopharmacol*. 154: 481–536.

Singh A, Purohit B. Tooth brushing, oil pulling and tissue regeneration: A review of holistic approaches to oral health, *J. Ayurveda Integr. Med*. 2: 65–68.

Sookto T, Srithavaj T, Thaweboon S, Thaweboon B, Shrestha B (2013): In vitro effects of *Salvia officinalis* L. essential oil on *Candida albicans*. *Asian Pac. J. Trop. Biomed*. 3: 376–380.

Stamm JW (2007): Multi-function toothpastes for better oral health: a behavioural perspective. *Int. Dent. J*. 57: 351–363.

Stoopler ET, Sollecito TP (2014): Oral mucosal diseases: evaluation and management. *Med. Clin. N. Am*. 98: 1323–1352.

Supreetha S, Sharadadevi M, Simon SP, Jain J, Tikare S, Amit M (2011): Antifungal activity of ginger extract on *Candida albicans*: an *in-vitro* study. *J. Dent. Sci. Res*. 2: 1–5.

Tadić VM, Dobrić S, Marković GM, Đorđević SM, Arsić IA, Menković NR, Stević T (2008): Anti-inflammatory, gastroprotective, free-radical-scavenging, and antimicrobial activities of hawthorn berries ethanol extract. *J. Agric. Food Chem*. 56: 7700–7709.

Tanideh N, Namazi F, Tadbir A (2014): Comparative assessment of the therapeutic effects of the topical and systemic forms of *Hypericum perforatum* extract on induced oral mucositis in golden hamsters. *Int. J. Oral Maxilofac. Surg*. 43: 1286–1292.

Veeresham C (2012). Natural products derived from plants as a source of drugs. *J. Adv. Pharm. Technol. Res*. 3: 200–201.

Vukovic N, Milosevic T, Sukdolak S, Solujic S (2007): Antimicrobial activities of essential oil and methanol extract of *Teucrium montanum*. *Evid Based Complement Alternat Med*. 4: (Suppl 1), 17–20.

Wu L, Feng J, Shi L, Shen X, Liu W, Zhou Z (2013): Candidal infection in oral leukoplakia: a clinicopathologic study of 396 patients from eastern China. *Ann Diagn Pathol*. 17: 37–40.

Wurglics M, Westerhoff K, Kaunzinger A, Schubert-Zsilavecz M (2001): Comparison of German St. John's Wort products according to hyperforin and total hypericin content. *J. Am. Pharm. Assoc. (Wash).* 41: 560–566.

Yiğit D, Yiğit N, Ozgen U (2009): An investigation on the anticandidal activity of some traditional medicinal plants in Turkey. *Mycoses.* 52: 135–140.

Yiğit N, Aktas E, Ayyildiz A (2008): Antifungal activity of toothpastes against oral *Candida* isolates. *J. Mycol. Med*. 18: 141–146.

Zhang L, Ravipati AS, Koyyalamudi SR, Jeong SC, Reddy N, Bartlett J, Smith PT, De la Cruz M, Monteiro MC, Melguizo A, Jimenéz E, Vicente F (2013): Anti-fungal and anti-bacterial activities of ethanol extracts of selected traditional Chinese medical herbs. *Asian Pac. J. Trop. Med*. 6: 673–661.

Zizovic I, Ivanovic J, Misic D, Stamenic M, Djordjevic S, Kukic-Markovic J, Petrovic SD (2012): SFE as a superior technique for isolation of extracts with strong antibacterial activities from lichen *Usnea barbata* L. *J. Supercrit. Fluids*. 72: 7–14.

Zlatković BK, Bogosavljević SS, Radivojević AR, Pavlović MA (2014): Traditional use of the native medicinal plant resource of Mt. Rtanj (EasternSerbia): Ethnobotanical evaluation and comparison. *J. Ethnopharmacol*. 151: 704–13.

ИНХИБИТОРНИ ЕФЕКАТ МЕДИЦИНСКИХ БИЉАКА НА РАСТ *Candida albicans* И БАКТЕРИЈА У УСНОЈ ДУПЉИ

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РЕЗИМЕ: У овом прегледном раду разматра се деловање етанолских екстраката, есенцијалних уља и цитотоксичности појединих медицинских биљака које се користе у традиционалној медицини у различитим географским подручјима широм света, укључујући и Србију, на раст, умножавање и патогеност кваснице *Candida albicans,* и бактерија које имају водећу улогу у одржавању оралног екосистема. Разноврсне медицинске биљке, као што су *Rosmarinus officinalis* (Fam.

Lamiaceae), *Artemisia dracunculus* и *Artemisia absinthium* (Fam. Asteraceae), настањују различите географске регије, континенте као и Балканско полуострво, међу којима су неке и аутохтоне *Hypericum perforatum* L. (Fam. Hypericaceae), *Urtica dioca* L. (Fam. Urticaceae), *Achillea millefolium* L. (Fam. Asteraceae), *Matricaria chamomilla* L. (Fam. Asteraceae), *Sambucus nigra* L. (Fam. Caprifoliaceae) и *Thymus serpyllum* L. (Fam. Lamiaceae) с импресивном антимикробном активношћу у усној дупљи.

КЉУЧНЕ РЕЧИ: медицинске биљке, *Candida albicans*, микрофлора усне дупље, бактерије

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MOLECULAR IDENTIFICATION OF *Bradyrhizobium japonicum* STRAINS ISOLATED FROM ROOT NODULES OF SOYBEAN (*Glycine max* L.)

ABSTRACT: The aim of this study was to isolate and identify *Bradyrhizobium japonicum* strains on the basis of molecular characteristics. From root nodules of different soybean cultivars were obtained 56 isolates, characterized according to morphological, cultural, and biochemical properties. Among these isolates, 33 isolates showing resemblance with *Bradyrhizobium* sp. were further subjected to molecular identification. Following DNA extraction, a partial 16S rDNA gene sequence from the isolates was amplified by PCR using universal primers fD1 (27F) and rP3 (1492R). Purification and sequencing of the amplified fragments were done in the biotechnology company Macrogen, Seoul, South Korea. Sequences were analyzed using the program FinchTV and BLAST (Basic Local Alignment Search Tool) and compared to sequences in GenBank and the *Bradyrhizobium* ID-database for identification. Comparison of the sequences with the *Bradyrhizobium* ID-database showed that all tested isolates were identified as *Bradyrhizobium japonicum*. Each isolate was deposited in the NCBI GenBank database under a unique accession number. Identification of *Bradyrhizobium* species from root nodules of soybean is of great importance because the symbiosis between rhizobia and legumes are a cheaper and usually more effective agronomic practice for ensuring an adequate supply of nitrogen for legumes, while preserving and improving fertility and productivity of soils.

KEYWORDS: *Bradyrhizobium japonicum*, biological nitrogen fixation, identification, soybean

INTRODUCTION

Great agricultural, ecological and economic importance of legumes, besides quality and chemical composition of the grain, is reflected in the ability of these plants to fix atmospheric nitrogen in the community with the root nodulating bacteria (Sengupta and Reddy, 2011). Atmospheric nitrogen is converted into plant-available forms through symbiotic nitrogen fixation of legumes and

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bacteria from the family *Rhizobiaceae* (Dixon and Kahn, 2004). Annual return of nitrogen to the soil ranges from 20 to 400 kg per hectare depending on the plant species, bacterial strains and numerous biotic and abiotic factors (Zahran, 1999).

As an important source of proteins and oils in human and animal nutrition, soybean (*Glycine max* L. Merr.) is one of the most cultivated legumes in the world (Nouri *et al.*, 2011). With area exceeding 100,000 ha, soybean is an important factor in the crop production in Serbia (Hrustić and Miladinović, 2008). Nitrogen--fixing bacteria provide "free" nitrogen for soybean plants, increases the yield by 20–50%, and improve the quality of grain without disturbing the natural soil microflora (Milošević and Jarak, 2005).

The most common microsymbionts of soybean are *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* (Zhang *et al*., 2011). The number of *Bradyrhizobium* sp. in our agricultural soils is very small, therefore it is necessary to inoculate legume seeds with nitrogen-fixing bacteria using microbiological inocula (Marinković *et al.,* 2010). Application of microbiological fertilizers containing selected and effective strains of *Bradyrhizobium japonicum* was introduced as a regular measure in the cultivation of soybean (Milošević and Marinković, 2009).

Bacteria that form nodules on the roots of legumes have long been placed in a common genus *Rhizobium*. Nitrogen-fixing bacteria were divided into fast--growing and slow-growing on the basis of culture growth, until Jordan (1982) proposed the separation of slow growing species in a separate genus *Bradyrhizobium*. However, the development and application of molecular techniques in microbiology enabled a simple, fast and reliable genotypic characterization of rhizobia and pointed to their great genetic diversity and divergence. The search for effective strains capable of eliciting and invading root or stem nodules on leguminous plants require isolation and identification of a large number of desirable *Bradyrhizobium* species. Effective strains of *Bradyrhizobium japonicum*, besides the capacity for nitrogen fixation, must also have the competitive ability in relation to the natural population which is most often inefficient in fixing nitrogen (Marinković, 2012).

Therefore, the aim of this study was to perform identification of *Bradyrhizobium* sp. isolated from root nodules of different soybean cultivars on the basis of molecular characteristics.

MATERIALS AND METHODS

Root Nodules Collection

Nodules were randomly collected from field grown soybean during the four-year period (2010–2013). Four soybean cultivars of medium late and late maturity were selected for the root nodules collection: Balkan (maturity group I), Novosađanka (maturity group I), Venera (maturity group II), and Rubin (maturity group II). Cultivars were obtained from different locations of the Province of Vojvodina, from agricultural fields where soybeans were not previously grown (last five years). All nodules from four plants per each cultivar were separately collected at the full bloom stage of soybean, placed in sterilized polythene bags, transported to the laboratory.

Isolation of Bradyrhizobium sp.

Root nodules were surface sterilized and crushed to obtain the bacteria on yeast extract mannitol agar media (YEMA) (Somasegaran and Hoben 1994). Followed by several successive isolations and recultivations of individual pure colonies on the same medium, the isolates were further characterized according to morphological, cultural and biochemical properties (Vincent 1970). Isolates were cultured in yeast extract mannitol broth (YEMB) for 5 days at optimal temperature of 28±2 °C and stocked at 4 °C.

DNA isolation and PCR analysis

Isolates showing resemblance with *Bradyrhizobium* sp. were grown on YEMA plates for 72 hrs. DNA was isolated from single bacterial colonies by using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany), following the manufacturer's instructions. For the amplification of 16S rDNA gene fragments, primers fD1 (27F) (AGAGTTTGATCMTGGCTCAG) and rP3 (1492R) (TACGGYTACCTTGTTACGACTT) were used (Weisburg *et al*., 1991). The polymerase chain reaction (PCR) was done in 25-µl aliquots using S-thermal cycler (Eppendorf, Germany) (Table 1).

Table 1. PCR protocol

Components	**Final concentration**	**25 µl reaction**
2x MMix (Eppendorf)	1x	12.5 µl
10 µM Forward Primer	0.2 µM	0.5 µl
10 µM Reverse Primer	0.2 µM	0.5 µl
Template DNA	~1,000 ng	1 µl
Nuclease-free water		10.5 µl

The PCR reactions were performed with an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, at 55 °C primer annealing for 1 min and at 72 °C extension for 2 min, followed by a final extension step at 72 °C for 3 min (Laguerre *et al*., 1994). Amplicons were electrophoresed in 1.5% agarose gel (Invitrogen) with ethidium bromide. Purification and sequencing of the PCR-amplified DNA fragments were done in the biotechnology company MACROGEN, Seoul, South Korea (http://dna.macrogen.com). FinchTV Version 1.4.0. was used for sequence analysis, and nucleotide sequences were filed in the GenBank Database at the National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

When searching for efficient microsymbiotic nitrogen-fixing bacteria, among 56 isolates obtained from different soybean cultivars grown in the Province of Vojvodina, 33 isolates belonged to the genus *Bradyrhizobium*. Based on the morphological characteristics of isolates, species of *Bradyrhizobium* are characterized as rod-shaped, aerobic, non-spore forming and motile by one polar or subpolar flagellum. Colonies are circular, opaque, rarely translucent, white and convex, with entire margins. Strains are usually slow growing, not exceeding 1 mm in diameter within 5–7 days incubation on YEMA, while faster growing strains are uncommon.

Isolates showed negative chemical reaction for indole, methyl red, Voges--Proskauer, hydrogen sulphide production, utilization of carbohydrates and gelatin hydrolysis, and positive reaction for citrate utilization, catalase and ammonia production from peptone and urea (Gachande and Khansole, 2011). Strains are characteristically able to invade the root hairs of leguminous plants and incite the production of root nodules, wherein the bacteria occur as intracellular symbionts with host "specificity" (Gage, 2004). The bacteria are present in root nodules as swollen forms which are normally involved in fixing atmospheric nitrogen into combined forms utilizable by the host plant, while some strains fix nitrogen in the free living state under special conditions (Holt *et al*., 1994).

Characterization of rhizobia based on genetic characteristics is more precise and more informative compared to the morphological and physiological classification. Until 1992, only one species was known within the genus *Bradyrhizobium* – *Bradyrhizobium japonicum* (Jordan, 1982), while the application of molecular methods in the past 20 years enabled the separation of several new species (Ramirez-Bahena *et al.,* 2009).

It has been reported that *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium liaoningense*, *Bradyrhizobium yuanmingense* and *Sinorhizobium fredii* could nodulate soybean. Recently, *Bradyrhizobium huanghuaihaiense*, *Bradyrhizobium daqingense*, *Sinorhizobium sojae*, and several unnamed species were also found to be effective microsymbionts of soybeans (Zhang *et al*., 2011).

In this study, identification of *Bradyrhizobium* isolates based on 16S rDNA homology was performed using PCR with the universal primers 27F and 1492R, probably the most widely used primer pair for amplification of a taxonomically diverse eubacterial 16S rDNA gene fragments by PCR (Weisburg *et al*., 1991). Comparison of the sequences with the *Bradyrhizobium* ID-database showed that all isolates were identified as *Bradyrhizobium japonicum*. BLASTn queries of GenBank and the *Bradyrhizobium* ID-database, showed 100% identity to *B. japonicum* to accessions EU010398.1, KF995085.1, KP219176.1, KC736659.1, JN392462.1, KR092322.1, KX242473.1, CP010313.1, AB680665.1, FJ390915.1, AP012206.1, DQ133343.1, respectively. Isolates were deposited in the NCBI GenBank database under a unique accession number (Table 2).

Table 2. Isolates of *Bradyrhizobium japonicum* from root nodules of soybean

Isolate Code	Soybean Cultivar	Region of Origin	Year of Isolation	Acc. No
Bj1	Balkan	Rimski Šančevi	2010	KY000628
Bj2	Balkan	Rimski Šančevi	2010	KY000629
Bj3	Balkan	Bačka Topola	2010	KY000630
Bj4	Balkan	Srbobran	2010	KY000631
Bj5	Balkan	Rimski Šančevi	2011	KY000632
Bj6	Balkan	Srbobran	2011	KY000633
Bj7	Balkan	Bačka Topola	2011	KY000634
Bj8	Balkan	Sombor	2011	KY000635
Bj9	Novosađanka	Sombor	2010	KY000636
Bj10	Novosađanka	Karavukovo	2010	KY000637
Bj11	Novosađanka	Pančevo	2010	KY000638
Bj12	Novosađanka	Rimski Šančevi	2010	KY000639
Bj13	Novosađanka	Pančevo	2011	KY000640
Bj14	Novosađanka	Hajdučica	2011	KY000641
Bj15	Novosađanka	Srbobran	2011	KY000642
Bj16	Novosađanka	Karavukovo	2011	KY000643
Bj17	Novosađanka	Sremska Mitrovica	2011	KY000644
Bj18	Venera	Sremska Mitrovica	2012	KY000645
Bj19	Venera	Bačka Topola	2012	KY072854
Bj20	Venera	Ruma	2012	KY072855
Bj21	Venera	Sombor	2012	KY072856
Bj22	Venera	Vršac	2013	KY072857
Bj23	Venera	Plavna	2013	KY072858
Bj24	Venera	Plavna	2013	KY072859
BJ25	Venera	Rimski Šančevi	2013	KY072860
Bj26	Rubin	Sombor	2012	KY072861
Bj27	Rubin	Zrenjanin	2012	KY072862
Bj28	Rubin	Kikinda	2012	KY072863
Bj29	Rubin	Rimski Šančevi	2012	KY072864
Bj30	Rubin	Subotica	2013	KY072865
Bj31	Rubin	Zrenjanin	2013	KY072866
Bj32	Rubin	Hajdučica	2013	KY072867
Bj33	Rubin	Rimski Šančevi	2013	KY072868

Partial and complete sequencing of 16S rRNA made a significant step in the phylogeny and classification of rhizobia, and allowed description of several new genera and species (Germano *et al*., 2006). However, the conservative nature of 16S rRNA gene allows the characterization to the species level, while the differences between the strains of the same species cannot be determined.

More molecular procedures enable the identification and classification of bacteria at a high level of taxonomic resolution, such as using rep-PCR genomic fingerprinting to achieve genetic differences at subspecies and strain levels (Melchiorre *at al*., 2011). Unlike the 16S rRNA gene region, intergenic region 16S-23S rRNA (ITS) shows a high degree of variation among different strains. Variability in the sequences and length of ITS region proved to be very informative in taxonomic evaluation and characterization of indigenous *Bradyrhizobium* populations (Tan *et al*., 2001).

CONCLUSION

The research confirmed the presence of indigenous *Bradyrhizobium japonicum* in root nodules collected from different soybean cultivars. Further identification using rep-PCR genomic fingerprinting will be necessary to establish genetic differences at the strain level. Also, the selection of strains through inoculation assays in greenhouse and field conditions is needed in order to determine their efficiency in soybean production.

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REFERENCES

Dixon R, Kahn D (2004): Genetic regulation of biological nitrogen fixation. *Nat. Rev. Microbiol.* 2: 621–631.

Gachande BD, Khansole GS (2011): Morphological, cultural and biochemical characteristics of *Rhizobium japonicum* syn. and *Bradyrhizobium japonicum* of soybean. *Biosci. Discov. J.* 2: 1–4.

Gage D (2004): Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol. Biol. Rev.* 68: 280–300.

Germano MG, Menna P, Mostasso FL, Hungria M (2006): RFLP analysis of the rRNA operon of a Brazilian collection of bradyrhizobial strains from 33 legume species. *Int. J. Syst. Evol. Microbiol.* 56: 217–229.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994): *Bergey's Manual of determinative bacteriology*, ninth ed. Williams and Wilkins Pub., MD, USA.

Hrustić M, Miladinović J (2008): Značaj, poreklo i širenje soje. In: Miladinović J, Hrustić M, Vidić M (eds.), *Soja*, Institut za ratarstvo i povrtarstvo, Novi Sad, Sojaprotein, Bečej, 13–43.

Jordan DC (1982): Transfer of *Rhizobium japonicum* Buchanan 1980 to Bradyrhizobium gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. *Int. J. Syst. Bacteriol.* 32: 136–139.

Laguerre G, Allard MR, Revoy F, Amarger N (1994): Rapid Identification of Rhizobia by Restriction Fragment Length Polymorphism Analysis of PCR-Amplified 16S rRNA Genes. *Appl. Environ. Microbiol.* 60: 56–63.

Marinković J (2012): „Biohemijska i molekularna karakterizacija simbioze soje i različitih sojeva *Bradyrhizobium japonicum* Kirchner (Jordan) u uslovima suše". Doktorska disertacija, Univerzitet u Novom Sadu, Novi Sad.

Marinković J, Mrkovački N, Aćimović R, Đorđević V (2010): Effect of NS-Nitragin application on soybean yield and yield components agriculture. *Field Veg. Crop Res.* 47: 545–548.

Melchiorre M, de Luca MJ, Anta GG, Suarez P, Lopez C, Lascano R, Racca RW (2011): Evaluation of bradyrhizobia strains isolated from field-grown soybean plants in Argentina as improved inoculants. *Biol. Fertil. Soils* 47: 81–89.

Milošević N, Jarak M (2005): Značaj azotofiksacije u snabdevanju biljaka azotom. In: Kastori R (ed.), *Azot: agrohemijski, agrotehnički, fiziološki i ekološki aspekti*, Naučni institut za ratarstvo i povrtarstvo, Novi Sad, 307–352.

Milošević N, Marinković J (2009): Rizobiumi – biođubriva u proizvodnji leguminoza. *Zbornik radova*, Institut za ratarstvo i povrtarstvo, Novi Sad, 46: 45–54.

Nouri MZ, Toorchi M, Komatsu S (2011): Proteomics approach for identifying abiotic stress responsive proteins in soybean. In: Sudaric A (ed.): *Soybean – molecular aspects of breeding.* InTech, 187–214.

Ramirez-Bahena MH, Peix A, Rivas R, Camacho M, Rodriguez-Navarro DN, Mateos PF, Martinez-Molina E, Willems A, Velazquez E (2009): *Bradyrhizobium pachyrhizi* sp. nov. and *Bradyrhizobium jicamae* sp. nov., isolated from effective nodules of *Pachyrhizus erosus*. *Int. J. Syst. Evol. Microbiol.* 59: 1929–1934.

Sengupta D, Reddy AR (2011): Water deficit as a regulatory switch for legume root responses. *Plant Signal. Behav.* 6: 914–917.

Somasegeran P, Hoben HJ (1994): Handbook for Rhizobia: methods in legume-*Rhizobium* technology, Springer-Verlang, New York.

Tan Z, Hurek T, Vinuesa P, Müller P, Ladha JK, Reinhold-Hurek B (2001): Specific detection of *Bradyrhizobium* and *Rhizobium* strains colonizing rice (*Oryza sativa*) roots by 16S-23S ribosomal DNA intergenic spacer-targeted PCR. *Appl. Environ. Microbiol.* 67: 3655–3664.

Vincent JM (1970): *A manual for the practical study of the root nodule bacteria*. Int. Biol. Prog. Handbook, vol. 15. Blackwell Scientific Publications, Oxford, USA.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991): 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173: 697–703.

Zahran HH (1999): Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev.* 63: 968–989.

Zhang YM, Li Y Jr, Chen WF, Wang ET, Tian CF, Li QQ, Zhang YZ, Sui XH, Chen WX (2011): Biodiversity and biogeography of rhizobia associated with soybean plants grown in the North China Plain. *Appl. Environ. Microbiol.* 77: 6331–6342.

МОЛЕКУЛАРНА ИДЕНТИФИКАЦИЈА *Bradyrhizobium japonicum* СОЈЕВА ИЗОЛОВАНИХ ИЗ КОРЕНСКИХ КВРЖИЦА СОЈЕ (*Glycine max* L.)

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РЕЗИМЕ: Циљ овог рада је изолација и молекуларна идентификација сојева *Bradyrhizobium japonicum*. На основу морфолошке и биохемијске карактеризације, од 56 изолата из коренских квржица различитих сорти соје, 33 изолата за које је утврђена сличност с *Bradyrhizobium* sp. били су предмет даље идентификације. Након екстракције ДНК, парцијална 16S rDNA генска секвенца из изолата је умножена PCR методом употребом универзалних прајмера fD1 (27F) и rP3 (1492P). Пречишћавање и секвенционирање умножених фрагмената урађено је у компанији Macrogen Ltd. (Сеул, Јужна Кореја). Помоћу програма FinchTV и BLAST (Basic Local Alignment Search Tool) анализе, извршено је вишеструко поређење добијених секвенци с GenBank базом података. Поређењем добијених секвенци с *Bradyrhizobium* ID-базом података сви испитивани изолати идентификовани су као *Bradyrhizobium japonicum*. Секвенце су депоноване у светску NCBI базу уз добијање приступног броја (NCBI Acc. number). Идентификација врста *Bradyrhizobium*-а пореклом из коренских квржица соје од великог је значаја јер је симбиоза између ризобиума и легуминоза исплативији и обично ефикаснији начин снабдевања биљака азотом, а важно је и због очувања и унапређења плодности и продуктивности земљишта.

КЉУЧНЕ РЕЧИ: *Bradyrhizobium japonicum*, биолошка фиксација азота, идентификација, соја

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BIOFILM FORMING MICROORGANISMS ON VARIOUS SUBSTRATA FROM GREENHOUSE OF BOTANICAL GARDEN "JEVREMOVAC"

ABSTRACT: Diversity of subaerial biofilm forming cyanobacteria, algae and fungi was investigated on 10 different substrata from greenhouse of Botanical Garden "Jevremovac". Out of 37 documented taxa, 16 cyanobacterial and 10 algal taxa were identified. Remaining 11 taxa belong to the Kingdom of Fungi. The highest diversity of biofilm forming microorganisms, a total of 24 taxa, was detected on the corroded metal surface, while significantly lower number of taxa was recorded on other examined substrata. Cyanobacterium *Porphyrosiphon* sp., diatom *Achnanthes* sp. and green algae *Chlorella* sp. and *Chlorococcum minutum* were the most frequently encountered photosynthetic components of biofilms. In all analyzed samples, *Trichoderma* sp., followed by *Cladosporium* sp. and *Rhizopus stolonifer*, were the most frequently identified fungi.

KEYWORDS: algae, biofilm, cyanobacteria, fungi, greenhouse

INTRODUCTION

In natural conditions, phototrophic and heterotrophic microorganisms are able to colonize and subsequently form ubiquitous, self-sufficient, miniature microbial ecosystems on all substrata where direct contact with the atmosphere and solar radiation occurs (Gorbushina, 2007). Process of establishing these complex microbial communities, known as subaerial biofilms (SABs), depends on

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substratum bioreceptivity, biology of colonizing microorganisms and wide range of environmental factors such as temperature, humidity, pH, solar radiation, water and nutrient availability (Gu and Mitchell, 2006; Macedo *et al*., 2009). In early stages of SAB formation, cyanobacteria and algae use CO_2 from the atmosphere and sunlight as their carbon and energy source and produce metabolites which serve as nutrient source for incoming heterotrophic bacteria and fungi (Ljaljević Grbić *et al.,* 2010). In addition, various air-borne organic and inorganic deposits and animal remains help fill nutrient requirements for heterotrophic SAB forming microorganisms (Suihko *et al*., 2007). Although most biofilms only contain complex consortia of algae, cyanobacteria, heterotrophic bacteria, fungi and protozoa, in advance stages of the colonisation, more complex organisms such as lichens, mosses and vascular plants can occur (Ljaljević Grbić *et al*., 2009; Stupar *et al*., 2014).

Changes in the structure and appearance of the substratum occur as a result of biofilm development. Discoloration, due to pigment excretion, depends on specific physiology of the SAB involved species and is influenced by changes in physiological state of the cells and the environmental conditions (Cappitelli *et al*., 2008; Warscheid and Braams, 2000). Additionaly, SAB forming microorganisms secrete various extracellular polymeric substances (EPS) to maintain moisture levels, enable mutual binding of microbial cells and adhesion to the substratum (Warscheid and Braams, 2000; Macedo *et al*., 2009). However, EPS may potentially cause alteration of physico-chemical properties of the substrata due to retained water (Brehm *et al*., 2005; Keshari and Adhikary, 2013). Fungi produced organic acids and enzymes interact with released CO_2 resulting in pH change of the substratum, which further facilitates the mechanical degradation (Gorbushina *et al*., 2007). Moreover, chemical reactions between organic acids and minerals ensue bio-weathering and formation of secondary minerals on the attacked substrata.

The aim of this research was to study the diversity of SAB forming microorganisms on different substrata from greenhouse of Botanical Garden "Jevremovac".

MATERIALS AND METHODS

Sampling was done in 2010 from different substrata within the greenhouse of Botanical Garden "Jevremovac", University of Belgrade, Faculty of Biology, Institute of Botany.

Sampling site

Sampling of SAB forming cyanobacteria, algae and fungi was conducted on various substrata from the greenhouse of Botanical Garden "Jevremovac". The Botanical Garden was founded in 1874 by the decree of the Ministry of Education of the Kingdom of Serbia, at the suggestion of famous Serbian botanist Josif Pančić. The greenhouse, from which samples were taken, was built in 1892 and covers the area of 500 m^2. Since the time of its construction, numerous tropical, sub-tropical

and desert plants have been grown in two wings connected by a central dome. From 1892 to 2010 no work was done on the reconstruction of the greenhouse. Today, due to its exceptional architectural value, it is protected by the law.

Sampling

Samples for algological and mycological analyses were collected from the surfaces of 10 different substrata with visible SAB formation: wood (Wd), stone (St), sand (Sd), clay (Cl), mortar (Mr), concrete (Co), metal (Mt), nylon (Ny), putty (Pt), and glass (Gl) (Figure 1).

Figure 1. Examined substrata, with visible alterations, from the greenhouse of Botanical Garden "Jevremovac": a. wood; b. stone; c. mortar; d. clay; e. sand; f. concrete; g. putty; h. metal; i. glass; j. nylon.

Algological analyses

Algological analyses were conducted on samples acquired using two methods: scraping and non-aggressive adhesive tape sampling (Gaylarde and Gaylarde, 1998). After rehydration in modified Knöps medium, samples were analyzed using stereomicroscope (Zeiss Stemi DV4) and a light microscope (Zeiss Axio-Imager M1, with software AxioVision Release 4.6). The observed cyanobacteria and algae were identified to species or genus level, on the base of cellular morphology, using appropriate literature (Starmach, 1972; Krammer and Lange-Bertalot, 1988; Komarek and Anagnostidis, 1998; Komarek and Anagnostidis, 2005).

Mycological analyses

Sampling for the mycological analyses was done using sterile cotton swabs and adhesive tape method. Sterile swab samples were diluted in 10 mL sterile distilled water and shaken mechanically for 10 min, after which 1 mL of the resulting suspensions was inoculated on malt extract agar (MEA) medium with 500 mg streptomycin per liter (Booth, 1971). The inoculated plates were incubated in a thermostat at 25 ± 2 °C. After incubation period of 7 days, pure fungal cultures were obtained by re-isolation of primary isolates onto the selective nutrient media: MEA, potato dextrose agar (PDA), and Czapek Dox agar (CzA). Re-isolated cultures were incubated 7 days at 25 ± 2 °C. Isolated fungi were identified to species or genus level, based on the macroscopic features of colonies and the micro-morphology of the reproductive structures, using the appropriate identification keys (Ainsworth *et al*., 1973; Von Arx, 1974; Ellis, 1971; Ellis and Ellis, 1997; Samson *et al*., 2004).

To confirm the existence of fungal growth and identify the type of fungi present at the sampling points, the non-aggressive adhesive tape sampling method was used (Urzì and de Leo, 2001). Samples were collected by pulling the adhesive tape off the surface of substrata with a slow and steady force, after which they were stained with Lactophenol Cotton Blue and put on slides for light microscopy.

RESULTS AND DISCUSSION

A total of 37 biofilm forming cyanobacteria, algae and fungi was identified on the surfaces of 10 substrata examined from greenhouse of Botanical Garden "Jevremovac". All identified taxa are presented in Table 1. The highest microbial diversity, a total of 24 taxa, was detected on the corroded metal surface. In contrast, significantly lower number of taxa was identified as SAB forming microorganisms on other examined substrata.

Table 1. Identified taxa on various substrata from greenhouse

	Identified taxa	Substrata									
		Wd	St	Sd	Cl	Mr	Co	Mt	Ny	Pt	Gl
Cyanobacteria	*Aphanothece pallida* (Kützing) Rabenhorst							+			
	Chondrocystis dermochroa (Nägeli) Komárek & Anagnostidis							+			
	Chroococcus lithophilus Ercegovic							+	+		
	Chroococcus varius A. Braun				+			+			
	Chroococcus Nägeli sp.		+								
	Gloeocapsa atrata Kützing							+			
	Gloeocapsa novacekii Komárek & Anagnostidis							+			
	Gloeocapsa Kützing spp.							+	+		
	Gloeocapsopsis crepidinium (Thuret) Geitler ex Komárek						+				+
	Gloeocapsopsis Geitler ex Komárek sp.							+			
	Lyngbya truncicola Ghose			+							
	Nostoc Vaucher ex Bornet & Flahault sp.						+		+		
	Phormidium Kützing ex Gomont sp.		+					+			
	Porphyrosiphon Kützing ex Gomont sp.		+		+			+			
	Pseudocapsa dubia Ercegovic							+			
	Synechococcus elongatus (Nägeli) Nägeli							+			
Algae	*Achnanthes* Bory de Saint-Vincent sp.		+		+			+			+
	Amphora Ehrenberg ex Kützing sp.				+						
	Chlorella M.Beijerinck sp.	+	+					+	+		
	Chlorococcum minutum R.C.Starr					+		+	+	+	
	Dynobrion Ehrenberg sp.							+			
	Hantzschia amphioxys (Ehrenberg) Grunow				+			+			
	Oedogonium Link ex Hirn sp.				+						
	Pediastrum duplex Meyen							+			
	Stichococcus Nägeli sp.							+			
	Trentepohlia umbrina (Kützing) Bornet	+					+				+

Fungi	*Acrogenospora sphaerocephala* (Berk. & Broome) M.B. Ellis									+	
	Alternaria Nees ex Wallroth sp.		+					+			
	Aspergillus niger Tiegh	+									
	Aspergillus ochraceus Micheli	+	+								
	Cladosporium Link ex Gray sp.	+						+	+		+
	Curvularia lunata (Wakker) Boedijn								+		
	Fusarium Link ex Gray sp.	+						+			
	Penicillium Link spp.	+									+
	Rhizopus stolonifer (Ehrenb.) Vuill			+	+		+		+		
	Trichoderma Persoon ex Gray sp.			+		+	+	+	+	+	+
	Ulocladium Preuss sp.							+			
	Total	7	7	3	7	2	5	24	9	3	6

Identified photosynthetic organisms

Algological analysis showed that photosynthetic component of the examined biofilms samples were composed of 16 cyanobacterial and 10 algal taxa in total. The highest diversity of SAB forming cyanobacteria, a total of 12 taxa, was documented on corroded metal surface, while no cyanobacteria were detected on mortar, putty and wooden substrata. *Porphyrosiphon* sp. was the most frequently encountered cyanobacterium, detected as photosynthetic component of 3 different biofilms. On the other hand, the highest diversity of algae was noted on corroded metal, while no algal taxa was recorded on porous sand. Out of 10 identified algae, most frequently encountered were diatom *Achnanthes* sp. and green algae *Chlorella* sp. and *Chlorococcum minutum*, each found on 4 different substrata (Figure 2).

Identified fungi

From all the samples analyzed, 11 fungal taxa were identified. In contrast to photosynthetic microorganisms, fungi were detected in all examined biofilm samples. These fungi belonged to the genera *Acrogenospora*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Penicillium*, *Rhizopus*, *Trichoderma*, and *Ulocladium* (Figure 2). The most frequently encountered micromycete was *Trichoderma* sp. found on 7 substrata, followed by *Cladosporium* sp. and *Rhizopus stolonifer*, detected as heterotrophic biofilm components on 4 different substrata. Other identified fungi were detected sparingly.

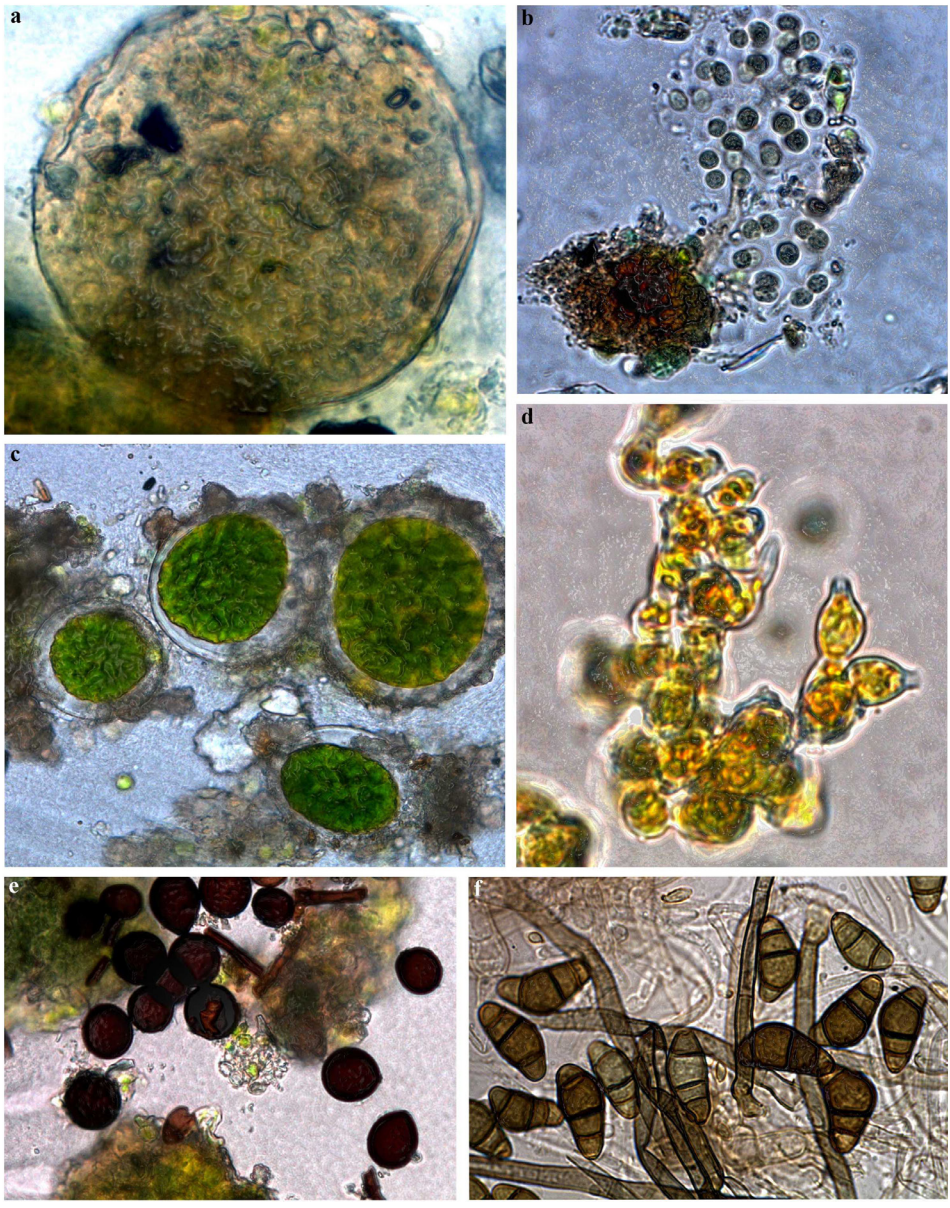

Figure 2. SAB forming microorganisms: a. *Nostoc* sp.-colony; b. *Aphanothece pallida*--colonies (cyanobacteria); c. *Chlorococcum minutum*; d. *Trentepohlia umbrina* (algae); e. *Acrogenospora sphaerocephala*-conidia; f. *Curvularia lunata*-conidia (fungi)

Investigation of microbial communities present in dense layers of highly developed sub-aerial biofilms, documented on all examined substrata within greenhouse, showed high diversity of SAB forming cyanobacteria, algae and

fungi. This result was expected since favourable conditions for development of microorganisms, such as adequate illumination, high humidity levels and constant temperature are present in greenhouse environment. However, in regard to the examined substrata, substantial differences in taxa diversity were detected. The highest diversity of biofilm forming microorganisms, a total of 24 taxa, was documented on the corroded metal surface. Constant moistening of metal substratum, due to frequent watering of plants, is the main cause of a high diversity of phototropic microorganisms and the presence of typically aquatic algae *Dynobrion* sp. and *Pediastrum duplex*. Such conditions facilitate metal corrosion since favorable environment, for colonization by heterotrophic bacteria and fungi, was established. Presence of these aerobic microorganisms results in decrease of oxygen levels beneath biofilm and allows development of anaerobic microbiota. The difference in oxygen concentrations beneath and around microbial biofilm generates an electrochemical potential and electron flow resulting in metal biodeterioration (Gu and Mitchell, 2006). In addition, fungi *Alternaria* sp., *Cladosporium* sp., *Trichoderma* sp., and *Ulocladium* sp. produced dark pigments that bound to the substratum particles and formed aesthetically detrimental discoloration of metal surface.

In regard to taxa diversity, microbial community present on nylon substratum is second to the corroded metal. Highly dense green biofilm was documented on surface of nylon sheets covering windows of greenhouse. Although synthetic in nature, this substratum proved suitable for development of biofilm. This is to a great extent due to high humidity, present in greenhouse interior, and adequate lighting, which comes through windows. However, adverse environmental conditions, such as high temperature and UV radiation, are also present. Nonetheless, SAB forming microorganisms are present in high diversity due to many adaptations for surviving UV exposure and high temperatures. All identified cyanobacterial taxa have gelatinous sheaths, composed of polysaccharides, which act as a water reservoir and play a role in adhesion to the substratum (Macedo *et al*., 2009; Keshari and Adhikary, 2013). Additionally, scytonemin, UV absorbing yellow-brown pigment, accumulates in the extracellular sheaths of cyanobacteria upon exposure to solar radiation (Balskus and Walsh, 2008). On the other hand, identified dematiaceous fungi produce fungal melanin which protects them from UV light (LJaljević Grbić *et al*., 2010). These microorganisms may be responsible for biodeterioration of nylon substrata. However, little is known about microbial degradation of synthetic polymers, due to their relatively recent discovery and very slow rate of degradation in natural habitats. Degradation generally depends on chemical structure, molecular weight, crystallinity and physical form of polymer, but environmental conditions may determine the dominant groups of microorganisms that play a role in polymer degradation (Gu and Mitchell, 2006). In this sense, it is important to note the presence of dematiaceous hyphomycete *Curvularia lunata* only on the surface of nylon substratum. Species of this genera were earlier reported to secrete extracellular enzyme-like factor, with esterase properties, which degrade ester-based polyurethane in a polyurethane-agar clearing assay (Crabbe *et al*., 1994).

Samples of biofilm from clay substratum were characterised by presence of 3 typically aquatic diatoms: *Achnanthes* sp., *Amphora* sp., and *Hantzschia amphioxys*. In general, diatoms are among the most successful contemporary groups of photosynthetic microorganisms that occur in virtually every environment containing water (Vanormelingen *et al*., 2008). This holds true not only for freshwater and marine habitats, but also for temporary aquatic and moist soil habitats. Constant moistening of clay and other substrata, due to frequent watering of plants in greenhouse, created favorable conditions for colonization by diatoms.

Highly developed biofilms were documented on surfaces of all examined substrata. However, in regard to documented taxa, mortar and putty are considered much less diverse, with only 2 and 3 identified taxa, respectively. Nonetheless, algological and mycological analyses of all biofilm samples showed a large number of cyanobacteria (*Aphanothece pallida*, *Gloeocapsa* spp., *Nostoc* sp.), algae (*Chlorococcum minutum*, *Trentepohlia umbrina*), and fungi (*Alternaria* sp., *Cladosporium* sp., *Curvularia lunata*, *Trichoderma* sp. and *Ulocladium* sp.), causing discoloration and biodeterioration of the substrata (Ljaljević Grbić *et al*., 2009, 2010). This finding is consistent with documented symptoms on the surfaces of examined substrata.

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REFERENCES

Ainsworth GC, Sparrow FK, Sussman SA (1973): *The Fungi. Volume IVA, Taxonomic Review with Keys: Ascomycetes and Fungi Imperfecti*. Acad. Press, New York and London.

Balskus EP, Walsh CT (2008): Investigating the initial steps in the biosynthesis of cyanobacterial sunscreen scytonemin. *J. Am. Chem. Soc.* 130: 15260–15261.

Booth C (1971): *The fungal culture media. In: Booth C (ed.), Methods in Microbiology.* Acad. Press, London. Volume IV, pp. 49–94.

Brehm U, Gorbushina AA, Mottershead D (2005): The role of microorganisms and biofilms in breakdown and dissolution of glass and quartz. *Paleogeogr. Paleoclimatol. Paleoecol*. 219: 117–129.

Cappitelli F, Abbruscato P, Foladori P, Zanardini E, Ranalli G, Principi P, Villa F, Polo A, Sorlini C (2008): Detection and elimination of cianobacteria from frescoes: The case of the St. Brizio Chapel (Orvieto Cathedral, Italy). *Microb. Ecol.* 57: 633–639.

Crabbe J, Campbella J, Thompson L, Walza S, Schultza W (1994): Biodeterioration of colloidal ester-based polyurethane by soil fungi. *Int. Biodeter. Biodegr.* 33: 103–113.

Ellis MB (1971): *Dematiaceous hyphomycetes*. Commonwelth Mycological Institute Kew, Surrey, England.

Ellis MB, Ellis PJ (1997): *Microfungi on Land Plants*. An Identification Handbook. The Rich. Publ. Co. Ltd.

Gaylarde PM, Gaylarde CC (1998): A rapid method for the detection of algae and cyanobacteria on the external surfaces of buildings. In: Gaylarde CC, Barbosa TCP, Gabilan NH (eds.), *Biodegradation and Biodeterioration in Latin America* – Third Latin American Biodegradation & Biodeterioration Symposium, 1998. The British Phycological Society, UK, Paper No 37.

Gorbushina AA (2007): Life on the rocks. *Environ. Microbiol.* 9: 1613–1631.

Gu JD, Mitchell R (2006): Biodeterioration. In: Rosenberg E, De Long EF, Lory S, Stackebrandt E, Thompson F (eds.), *The Prokaryotes. Applied Bacteriology and Biotechnology 1*, Springer, New York, pp. 864–903.

Keshari N, Adhikary SP (2013): Characterization of cyanobacteria isolated from biofilms on stone monuments at Santiniketan, India. *Biofouling* 29: 525–536.

Komarek J, Anagnostidis K (1998): Cyanoprokaryota. 1. Teil: Chroococcales. In: Ettl H., Gärtner G, Heynig H & Mollenhauer D (eds.), *Süßwasserflora von Mitteleuropa*. Spektrum Akademischer Verlag, Berlin. Band 19/1, pp. 1–548.

Komarek J, Anagnostidis K (2005): Cyanoprokaryota. 2. Teil: Oscillatoriales. In: Büdel B., Gärtner G, Krienitz L & Schagerl M (eds.), *Süßwasserflora von Mitteleuropa*. Spektrum Akademischer Verlag, Berlin, Band 19/2, pp. 1–759.

Krammer K, Lange-Bertalot H (1988): *Bacillariophyceae*. 1. Teil. Gustav Fischer Verlag, Stuttgart.

Krammer K, Lange-Bertalot H (1988): *Bacillariophyceae*. 2. Teil. Gustav Fischer Verlag, Stuttgart.

LJaljević Grbić M, Subakov Simić G, Krizmanić J, Lađić V (2009): Cyanobacterial, algal and fungal biofilm on sandstone substrata of Eiffel's Lock in Bečej (Serbia). *Botanica Serbica* 33: 101–105.

LJaljević Grbić M, Vukojević J, Subakov Simić G, Krizmanić J, Stupar M (2010): Biofilm forming cyanobacteria, algae and fungi on two historic monuments in Belgrade, Serbia. *Arch. Biol. Sci.* 62: 625–631.

Macedo MF, Miller AZ, Dionisio A, Saiz-Jimenez C (2009): Biodiversity of cyanobacteria and green algae on monuments in the Mediterranean Basin: an overview. *Microbiology* 155: 3476–3490.

Samson RA, Hoekstra ES, Frisvad JC (2004): *Introduction to food- and airborne fungi*. The Netherlands: Ponsen & Looyen, Wageningen.

Starmach K (1972): Chlorophyta III. In: Starmach K, Sieminska J (eds.), *Flora Slodkowodna Polski* 10, Panstwowe Wydawnictwo Naukowe. Warszawa, pp. 1–750.

Stupar M, LJaljević Grbić M, Subakov Simić G, Jelikić A, Vukojević J, Sabovljević M (2014): A sub-aerial biofilms investigation and new approach in biocide application in cultural heritage conservation: Holy Virgin Church (Gradac Monastery, Serbia). *Indoor. Built. Environ.* 23: 584–593.

Suihko LM, Alakomi LH, Gorbushina AA, Fortune I, Marquardt J, Saarela M (2007): Characterization of aerobic bacterial and fungal microbiota on surfaces of historic Scottish monuments. *Syst. Appl. Microbiol.* 30: 494–508.

Urzì C, de Leo F (2001): Sampling with adhesive tape strips: an easy and rapid method to monitor microbial colonization on monument surfaces. *J. Microbiol. Meth.* 44: 1–11.

Vanormelingen P, Verleyen E, Vyverman W (2008): The diversity and distribution of diatoms: from cosmopolitanism to narrow endemism. *Biodivers. Conserv.* 17: 393–405.
Von Arx JA (1974): *The Genera of Fungi: Sporulating In Pure Culture*. Vaduz: J. Cramer.
Warscheid T, Braams J (2000): Biodeterioration of stone: a review. *Int. Biodeter. Biodegr.* 46: 343–368.

МИКРООРГАНИЗМИ У САСТАВУ БИОФИЛМА С РАЗЛИЧИТИХ СУПСТРАТА СТАКЛЕНИКА БОТАНИЧКЕ БАШТЕ „ЈЕВРЕМОВАЦ”

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РЕЗИМЕ: Испитиван је диверзитет цијанобактерија, алги и гљива у биофилму с 10 различитих супстрата из стакленика Ботаничке баште „Јевремовац“. Од 37 документованих таксона, идентификовано је 16 цијанобактерија и 10 алги. Преосталих 11 таксона припадају „Петом царству“. Највећа разноврсност микроорганизама, укупно 24 таксона, забележена је у биофилму на кородираној металној површини, док је значајно нижи број таксона регистрован на осталим испитиваним супстратима. Цијанобактерија *Porphyrosiphon* sp., дијатома *Achnanthes* sp. и зелене алге *Chlorella* sp. и *Chlorococcum minutum* су најчешће фотосинтетичке компоненте биофилма. У свим испитиваним узорцима *Trichoderma* sp., заједно са *Cladosporium* sp. и *Rhizopus stolonifer* су најчешће идентификоване гљиве.

КЉУЧНЕ РЕЧИ: алге, биофилм, гљиве, стакленик, цијанобактерије

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BLOOD PARASITES IN DOGS FROM THE BELGRADE AREA IN THE PERIOD 2014–2015

ABSTRACT: Prevalence of blood parasites in dogs in the Belgrade area has been investigated continuously during the last 20 years, especially in clinically suspicious dogs. In the period from 2014 to 2015, 249 blood samples of pets (182) and shelter dogs (67) were examined. Using Giemsa-stained blood smears, the presence of *Babesia spp.* was examined in erythrocytes and the presence of morulae of *Ehrlichia spp.* and *Anaplasma spp.* in circulating monocytes and granulocytes. To confirm positive findings of ehrlichiosis and anaplasmosis in blood smears, CaniV-4 Test Kit or IDEXX SNAP 4DX test was used. Infection with two pathogens was found in 78/249 (31.32%) cases; in all cases, the infection with one of the protozoa or bacteria was in combination with heartworms. In blood-smears, babesiosis was found in 39.75% of pet dogs and in 71.64% of shelter dogs, ehrlichiosis in 15.93% and 28.35%, and anaplasmosis in 6.04% and 19.40%, respectivelly. From colected ticks, relative abundance analysis revealed that the species *Ixodes ricinus* was absolutely dominant and found in 50.53% (47/93), followed by *Rhipicephalus sanguineus* – 38.70% (36/93), *Dermacentor marginatus* – 9.67% (9/93), *D. reticulatus,* and *Ixodes persulcatus* found in 3.22% (3/93), which for the first time occurred in dogs in the Belgrade area and in Serbia.

KEYWORDS: dogs, babesiosis, ehrlichiosis, anaplasmosis, ticks

INTRODUCTION

Blood parasites represent an actual health problem in dogs in the Belgrade area. Of the tick-borne diseases of protozoan and bacterial etiology – babesiosis, ehrlichiosis and anaplasmosis are the most important (Ristic and Holland, 1993)

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and have a permanent increase (Pavlović *et al*., 2014). The principal vector of babesiosis and ehrlichiosis are ticks: i.e. *Rhipicephalus sanguineus,* which occurs throughout the world; species of the genus *Dermacentor – D. marginatus* and *D. reticulatus*, which have been incriminated in Europe, including Russia (Bowman, 2008). *Ixodes ricinus* is a principal vector of anaplasmosis and the most abundant tick species in Europe and occurs throughout the world (Milutinović *et al*., 2012).

These diseases have a seasonal character primarily because the population density of ticks varies throughout the year. Ticks tend to be more active during warmer months, though this varies by geographic region and climate. Areas with woods, bushes, high grass, or leaf litter are likely to have more ticks (Bowman, 2008; Pavlović *et al*., 2012a).

During the examination performed in the Belgrade green areas, in the central city parks, picnic areas and walkways along the rivers I. ricinus, R. sanguineus, D. marginatus and D. reticulatus tick species have been reported (Milutinović *et al*., 1997a,b; Dimitrić, 1999; Pavlović *et al*., 1999, 2011; Pavlović, 2016). The presence of I. persulcatus in Serbia for the first time was reported in 2014 and 2015, first in foxes and later in dogs in the Belgrade area (Stojanov *et al*., 2014).

At the same time, an increased number of cases of tick-borne diseases in dogs has been registered. Babesiosis in dogs was for the first time recorded in the Belgrade area during early 1990s (Pavlović *et al*., 1999); ehrlichiosis was for the first time recorded in 2002 (Pavlović *et al*., 2002b) and anaplasmosis in 2012 (Pavlović *et al*., 2012c).

MATERIALS AND METHODS

In the period 2014–2015, 249 blood samples of dogs have been examined. Examinations were performed in 182 pet dogs and in 67 dogs with no owner in shelters. Positive results were confirmed by identification of species of blood parasites in the laboratory of parasitology at the Scientific Veterinary Institute of Serbia.

Of the examined animals 37 originated from old housing district (central Belgrade municipalities where there is the largest number of parks). In the western part of Belgrade, near the Sava and the Danube Rivers (New Belgrade and Zemun), there are large green areas and walkways along the rivers where the owners are walking dogs. From these areas originated 61 of the examined dogs. In the northern part of Belgrade near the Danube River (Ovča and Borča) there is a space with rural character, with a large arable area, forests, partially swampy areas and shrub. There is also a hunting ground for small wild game, roe deer and wild boar, with lots of ticks and mosquitoes. Blood samples of 84 dogs from these places were examined. At same time, blood samples of 67 non-owner dogs from dog shelters were examined.

Tested dogs were exposed to tick bite and ticks were collected from some of them during the observation. The ticks were removed and then species

identification was performed. The tick species were determined using the keys given by Pomerancev (1950) and Kapustin (1955).

Using Giemsa-stained blood smears, the presence of babesia in erythrocytes and morulae of erlichia and anaplasma in circulating monocytes and granulocytes was examined. The affirmative tests (CaniV-4 TEST KIT and SNAP (IDEXX 4Dx)) were used for *in vitro* diagnostics for the detection of *Dirofilaria immitis* antigen, and antibody to *Anaplasma phagocytophilum*, antibody to *Anaplasma platys*, antibody to *Borrelia burgdoferi*, antibody to *Ehrilichia canis* and antibody to *Ehrlichia ewingii* in canine serum, plasma or whole blood. In laboratory of parasitology at the Scientific Veterinary Institute, blood samples were tested to *Ehrilichia spp.* by ELISA test using Ingezim Ehrilchia 1.5.EHR.K1 plate kit. In the same laboratory was performed the identification of *Babesia, Anaplasma* and *Ehrilichia* species in all positive samples using morphometric methods.

RESULTS AND DISCUSSIONS

Ticks findings

During the study, ticks were found in 93/249 (37.34%) of the examined dogs. All ticks were collected from owner dogs. Relative abundance analysis revealed that the species *I. ricinus* was dominant, found in 50.53% (47/93), and followed by *R. sanguineus* 38.70% (36/93), *D. marginatus* 9.67% (9/93), D*. reticulatus* and *I. persulcatus* found in 3.22% (3/93). Usually 2–3 ticks occurred per animal and in several cases were found two tick species at the same animal. In total, 271 ticks were collected. Overall male-female ratio in the course of the study was 61.02% : 38.98% in favor of females for the two most commonly found species *Ixodes ricinus* and *Rhipicephalus sanguineus*. This ratio was 69.50% : 30.50% and 63.42% : 36.58% in favor of females.

The population dynamics of *I. ricinus* and *I. persulcatus* shows two phases of season fluctuation: spring and autumn, because two generations mature every year. *R. sanguineus* reached their maximum in June and *D. marginatus* and *D. reticulatus* had a spring peak (Pavlović *et al*., 2015b). Occurrence of infection was directly correlated with the seasonal dynamics of ticks. Seasonal variations and changes in the microclimate caused the permanent presence of ticks throughout the year but the degree of infection was higher in the periods when the tick population was the densest (Pavlović *et al*., 2011).

Results of blood examination

In blood-smears of pet dogs, babesiosis was found in 39.75% (99/182), ehrlichiosis in 15.93% (29/182), and anaplasmosis in 6.04% (11/182) of the samples (Table 1). In non-owner dogs from shelters, dog babesia was found in 71.64% (48/67), ehrlichiosis in 28.35% (21/67), and anaplasmosis in 19.40% (13/67) of the samples (Table 2). During blood examination with CaniV-4 TEST KIT or

SNAP (IDEXX 4Dx) tests infection with the two pathogens was found in the 78/249 (31.32%) cases. In all cases, infection with one of protozoa/bacteria was in combination with heartworms. By ELISA test using Ingezim Ehrilchia 1.5.EHR.K1 plate kit only the presence of ehrlichiosis was confirmed.

Table 1. Prevalence of blood parasites infections in pet dogs in the Belgrade area

Location in Belgrade	No. exam.	No. infected					
		babesisos		ehrlichiosis		anaplasmosis	
	total	Positive	%	Positive	%	Positive	%
Old housing districts of Belgrade	37	17	6.82	6	3.29	1	0.54
New Belgrade and Zemun	61	39	15.66	4	2.19	3	1.64
Ovča and Borča	84	43	17.26	19	10.43	7	3.84
Total	182	99	39.75	29	15.993	11	6.04

Table 2. Prevalence of blood parasites infections in non-owner dogs from shelters

Shelters	No. examined	No. infected					
		babesiosis		ehrlichiosis		anaplasmosis	
	total	Positive	%	Positive	%	Positive	%
Shelter dogs	67	48	71.64	21	28.35	13	19.40

Babesiosis

In shelter dogs, prevalence was 71.64% (48/67) and in pet animals prevalence was 39.75% (99/182). *B. canis* was found in 117 and *B. gibsoni* in 30 animals (distinguished in the smear). In the Belgrade area, *B. canis* for the first time occurred in dogs during 1993 and *B. gibsoni* in 2009 (Pavlović *et al*., 2002a, 2012). Pathogenicity increased in young dogs, heavily parasitized and immunosuppressed dogs and when there was exposure to a virulent strain or concurrent infection with other tick-borne pathogens.

Commonly encountered was the acute form accompanied with weakness, fever, lethargy, haemolytic anaemia, pale of mucous membranes, a yellow coloring of the eyes (and skin), and red or orange urine color (haemoglobinuria). These symptoms, in various combinations, were found in 79 pet dogs. In only five dogs were found ascites, peripheral edema, and gastroenteritis (Pavlović *et al*., 2012a). If parasites infest the central nervous system, a dog with babesiosis can display neurological problems, as well as local inflammation, which rarely occurred in seven cases. Acute infections of virulent strains of *Babesia canis* have been associated with induction of the systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) secondary to massive immunostimulation and cytokine release. Signs of MODS can include

coagulopathies (DIC), adult respiratory distress syndrome (ARDS), cerebral dysfunction, and acute renal failure (Riek, 1968). These symptoms were observed in one dog, which was infected also with heartworm. In shelter dogs, an acute form of babesiosis occurred with moderate level of mortality (29.85%).

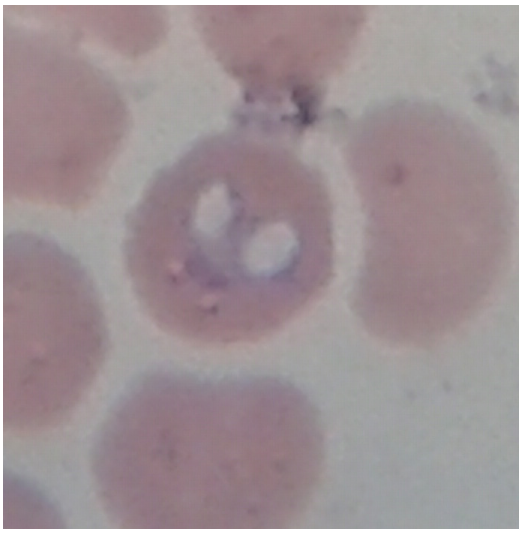

Figure 1. Babesia canis

Ehrlichiosis

Average determined prevalence for ehrlichiosis in pet dogs was 15.93% (29/182) but in shelter dogs it was 28.35% (21/67). In the Belgrade area, *E. canis* was for the first time identified in dogs in 2002 and *E. ewingii* in 2006 (Pavlović *et al*., 2002b, 2006, 2012a). Dog ehrlichiosis caused by *E. canis* was more common than *E. ewingii.* Determination was performed by morphometric characteristic and location in adequate blood element. However, *E. canis* parasitizes monocytes and *E. ewingii* is smaller than *E.canis* and produces morulae in granulocytes rather than in monocytes. It is easy to determinate but requires qualification and adequate experience.

During the examination, *E. ewingii* was identified in four cases in pet dogs, which travelled previously to Croatia and Montenegro. Clinically, the infection is characterized by acute, subclinical, and chronic stage of infection. In this study, there were nine cases of acute stage of the disease. Ehrlichiosis was determined during blood smear examination (at first, symptoms look like babesiosis). Dogs may resolve the disease, but develop subclinical persistent infections, and thus, become asymptomatic carriers of the infection.

The acute stage of the disease is due largely to vasculitis. This was found in 12 pet dogs and all shelter dogs. The organism replicates in circulating monocytes, and subsequently in mononuclear phagocytic cells throughout the body.

The infected monocytes bind to vascular endothelial cells and initiate vasculitis and subendothelial cell infection. Acute phase of the disease is characterized with fever (found in 37 shelter dogs), anorexia (4 cases), and lethargy (12 cases). In all infected pet dogs we confirmed lymphadenopathy and thrombocytopenia by blood analyses performed at "Vetlab" in Belgrade.

These symptoms were similar to those given by Shipov *et al.* (2008). The thrombocytopenia in ehrlichiosis may be due to consumption of platelets, sequestration of platelets in the spleen, immune-mediated destruction of platelets, decreased bone marrow production of platelets, or some combination of these mechanisms. Overall, however, the basis for ehrlichia thrombocytopenia remains unclear (Waner *et al*., 1997).

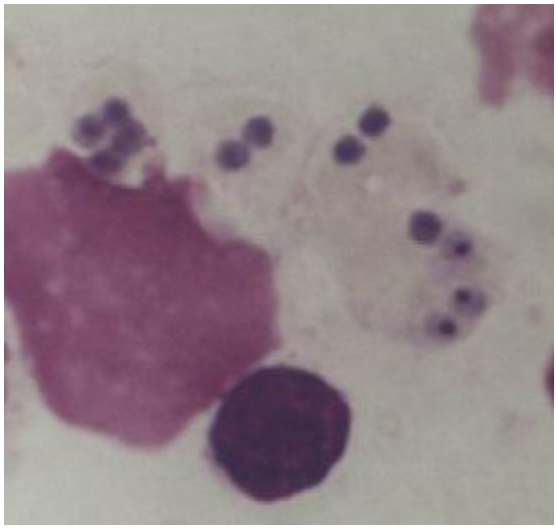

Figure 2. Ehrilichia canis

Anaplasmosis

Average determined prevalence for anaplasmosis in pet dogs was 6.04% (11/182) and in shelter dogs 19.40% (13/67). Anaplasmosis is a tick-borne disease caused by bacteria *Anaplasma phagocytophilum*. In the Belgrade area, anaplasmosis was for the first time reported in dogs in 2012 (Pavlović *et al*., 2012c).

Infection often causes lameness, joint pain, fever, lethargy, and loss of appetite (Carrade *et al*. 2009). During the examination, ten infected dogs had these symptoms for 1 to 7 days; however, four dogs had only minor symptoms or no symptoms (Pavlović *et al*., 2015b). Other less commonly observed clinical signs include gastrointestinal problems such as vomiting (found in one dog), diarrhea, or both (found in seven dogs). Respiratory signs described by Kohn *et al.* (2008) were not determined. There were no data for non-owner dogs about clinical signs of anaplasmosis.

Because animals had clinical signs of polyarthritis and possibly a history of tick exposure, clinical signs of canine anaplasmosis may be indistinguishable from those seen in Lyme disease. In addition, the same tick transmits both diseases. During this research, there were 19 suspect cases, of which 15 were positive to anaplasma and in the other four cases Lyme boreliosis was determined by SNAP test.

Prevention and control

The best way to prevent these diseases is by preventing the exposure to the ticks that carry all tick-borne diseases. This is especially important during the peak tick season or if a dog spends time in the woods or tall grass (these areas should be avoided in tick season).

Owners should inspect their dogs daily for ticks. Prompt removal of ticks within 24 hours should prevent disease transmission, because it has been reported that a tick must be attached for two to three days to transmit the organism. In kennels where puppies are being lost to disease, aggressive tick-control measures should be instituted including spraying the environment as well as treating animals.

One of the best measures for protection against ticks is the use of spot on drugs, which is applied to the skin, like fipronile or other drugs. Drop on method is used when a small amount of the solution is squeezed from a tiny tube and gently rubbed into the pet's coat at the base of the neck, right where it connects to the shoulders at the back of the head. Through a process called translocation, the ointment works its way through the pet's coat. The oil in the coat slowly dispenses through the hair over 30 days. The initial translocation normally completes in about 24 hours.

CONCLUSIONS

In the period 2014–2015 in the Belgrade area, 249 blood samples of dogs were examined for the presence of babesiosis, ehrlichiosis and anaplasmosis.

Rate of established infection with babesia, ehrilichia and anaplasma was significantly higher in shelter (non-owner) dogs, compared to pet dogs.

During the study, ticks were found in 93/249 (37.34%) of examined dogs. *Ixodes persulcatus* occurred for the first time in dogs from the Belgrade area and in Serbia.

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REFERENCES

Bowman AS, Nuttall PA (2008): *Ticks Biology, Disease and Control*, Cambridge University Press, 21–98.

Carrade DD, Foley JE, Borjesson DL (2009): Canine granulocytic anaplasmosis: a review. *J. Vet. Intern. Med*. 23: 1129–1141.

Dimitrić A (1999): „Fauna i ekologija krpelja (Acari: Ixodidae) kao prenosioca metazoonoza", MSc thesis, Faculty of Veterinary Medicine Belgrade

Kapustin FU (1955): *Atlas parazitov krovi životnih i klešćei iksodid*, Ed. Gasudarstvenoei zdetejlstvo seljskohazjajstvenoi literaturi, Moskva, 29–33, 104–109, 150–164.

Kohn B, Galke D, Beelitz P (2008): Clinical features of canine granulocytic anaplasmosis in 18 naturally infected dogs. *J. Vet. Intern. Med*. 22: 1289–1295

Milutinović M, Aleksić-Bakrač N, Pavlović I (1997a): Faunistic and ecological notes on ticks (Acari: Ixodidae, Argasidae) in the extended area of Belgrade, *Magy. Állatorvosok Lapja* 120: 434–436.

Milutinović M, Aleksić-Bakrač N, Pavlović I (1997b): Ticks (Acari: Ixodidae, Argasidae) in the Belgrade area, *Acta Ent. Serb.* 2: 77–85.

Milutinović M, Radulović Ž, Tomanović S, Petrović Z (2012): *Krpelji (Acari: Ixodidae, Argasidae) Srbije*. SANU, Beograd, 207–222, 229–232.

Pavlović I, Milutinović M, Kulišić Z, Dimitrić A, Pavlović V (1999): Prisustvo artropoda od biomedicinskog značaja na zelenim površinama grada Beograda, *Zbornik radova II Gradske konferencije o suzbijanju štetnih artropoda i glodara sa međunarodnim značajem*, Beograd, 81–87.

Pavlović I, Milutinović M, Terzin V, Petković D, Terzin D (2002a): Tick fauna of dogs from Belgrade area and its role in epizootiology of dog babesiosis, *12th Federation of Asian Veterinary Association Congress – Globalization – Challenges to the Veterinary Profession*, Kuala Lumpur, Malaysia, 26.

Pavlović I, Pavlović I, Terzin V, Petković D, Terzin D (2002b): The first occurence of *Erlichia canis* at dogs in Belgrade area, *12th Federation of Asian Veterinary Association Congress – Globalization – Challenges to the Veterinary Profession*, Kuala Lumpur, Malaysia, 28.

Pavlović I, Milutinović M, Radulović Ž, Petković D, Terzin V, Terzin D (2003): Epizootiological research of canine babesiosis in the Belgrade district, *Lucrari Stiintifice Medicina Veterinara* 46: 365–370.

Pavlović I, Terzin V, Petković D, Ćurčin Lj, Stanković B, Terzin D, Ćurčin K (2006): Erlihioza pasa na području Beograda, *Zbornik radova i kratkih sadržaja 18. savetovanja veterinara Srbije*, Zlatibor, 97–98.

Pavlović I, Jovičić D, Terzin V, Savatić I, Marković S (2011): Influence of ecological factors to spread of dog babesiosis at Belgrade area. *Book of Abstracts of International Scientific Conference on Sustainable Development in the Function of Environment Protection*, Belgrade, Serbia, 221.

Pavlović I, Petković D, Kukovska V, Stamenković V, Jovčevski S, Pavlović M, Jovčevski St, Elezović M (2012a): Most important food-borne disease of dogs caused by ticks and its control, *Proceedings 3rd International Scientific Meeting Days of Veterinary Medicine*, Ohrid, Macedonia, 34–37.

Pavlovic I, Terzin V, Pavlovic M., Petkovic D, Terzin D, Stankovic B (2012b): Babesiosis of dogs in Belgrade area between 2009–2011. *Abstracts of XI European Multicolloquium of Parasitology, Parasites in the Changing World*, Cluj-Napoca, Romania, 329.

Pavlovic I, Milojkovic N, Curcin Lj, Kovacevic M, Novak N, Ivanovic O (2012c): Prevalence of erlichiosis, anaplasmosis and boreliosis in dogs in Serbia. *Abstracts of XI European Multicolloquium of Parasitology, Parasites in the Changing World*, Cluj-Napoca, Romania, 330.

Pavlović I, Rogožarski D, Jovčevski Sr, Csordás F, Mitrović N, Mijatović I, Ćirković D, Jovčevski St, Ristić M (2015): Biodiversity of ticks and fleas of dogs in the Western Balkans – results of preliminary examinations. *Book of abstracts of the 14th International Symposium "Prospects for the 3rd Millennium Agriculture"*, Cluj-Napoca, Romania, 619.

Pavlović I, Terzin V, Petković D, Terzin D, Savić B, Cvetojević Đ (2015b): Nalaz *Anaplasma phagocytophilum* kod pasa u Beogradu. *Zbornik kratkih sadržaja XVII Simpozijum epizootiologa i epidemiologa (XVII Epizootiološki dani)*, Niš, 119–120.

Pavlović I (2016): Biodiversity and seasonal distribution of ticks on green areas of Belgrade. *Proceeding of Second International Symposium of Veterinary Medicine* (ISVM 2016), Beograd, 24–29.

Pomerancev BL (1950): *Fauna SSSR. Paukobraznie. Iksodovie kleščei (Ixodidae)*. Ed. Akademem Nauk SSSR, Moskva-Leningrad, 31–52.

Riek PF (1968): Babesiosis: In: *Infectious Blood Diseases of Man and Animals*, vol. 2, Waineman D. & Ristić M. (ed), Academic Press, New York-London, 220–268.

Ristic M, Holland CJ (1993): Canine ehrlichiosis. In: Z. Woldehiwet, M. Ristic (Eds.): *Rickettsial and chlamydial diseases of domestic animals*. Pergamon Press, Oxford; 169–186.

Shipov A, Klement E, Reuveni-Tagar L, Harrus S (2008): Prognostic indicators for canine monocytic ehrlichiosis, *Vet. Paras.* 153: 131–138.

Stojanov I, Pušić I, Pavlović I, Prodanov Radulović J, Kapetanov M, Ratajac R (2014): Findings of ticks in some species of wild carnivores. *Proceeding 3rd International Symposium on Hunting, Modern aspects of sustainable management of game populations*, Zemun – Belgrade, Serbia, 154–158.

Waner T, Harrus S, Bark H (1997): Characterization of the subclinical phase of canine ehrlichiosis in experimentally infected beagle dogs. *Vet. Paras.*, 69: 307–317.

КРВНИ ПАРАЗИТИ ПАСА НА ПОДРУЧЈУ ГРАДА БЕОГРАДА У ПЕРИОДУ 2014–2015. ГОДИНЕ

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РЕЗИМЕ: Испитивање преваленце крвних паразита код паса с подручја Београда континуирано се врши у последњих 20 година, поготову код клинички сумњивих животиња. У периоду 2014–2015. испитано је 249 узорака крви од којих су 182 потицала од животиња љубимаца а 67 од невласничких паса. Вршен је преглед нативних крвних размаза бојених по Гимси при чему смо присуство *Babesia spp.* утврђивали у еритроцитима а морула *Ehrlichia spp.* и *Anaplasma spp* у моноцитима и неутрофилима. Позитивне налазе на ерлихиозу и анаплазмозу потврђивали смо применом CaniV-4 TEST KIT или SNAP (IDEXX 4Dx) теста. Током прегледа у 31,32% (78/249) узорка установљено је присуство два патогена – уобичајени налаз био је протозоа или рикеција у комбинацији с дирофиларијама. Током прегледа присуство бабезиозе је установљено код 39,75% власничких и 71,64% невласничких паса, ерлихиоза код 15,93% и 28,35% и анаплазмозе код 6,04% и 19,40% паса. Истовремено, установљено је и присуство крпеља који су вектори ових патогена при чему је *Ixodes ricinus* био доминанта врста, нађена у 50,53% (47/93), следе *Rhipicephalus sanguineus* 38,70%, (36/93), *Dermacentor marginatus* 9,67% (9/93), *D. reticulatus* док је *Ixodes persulcatus* нађен у 3,22% (3/93) по први пут установљен код паса с подручја Београда и у Србији.

КЉУЧНЕ РЕЧИ: пси, бабезиоза, ерлихиоза, анаплазмоза, крпељи

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MULTIPLICATIVE VERSION OF *PROMETHEE* METHOD IN ASSESMENT OF PARKS IN NOVI SAD

ABSTRACT: Decision support methods have an important role regarding the environmental and landscape planning problems. In this research, one of the decision support methods – multiplicative version of Promethee – has been applied for assessment of five main parks in Novi Sad. The procedure required defining a set of criteria that were as follows: aesthetic, ecological and social values of analyzed parks. For each criterion an appropriate Promethee preference function was adopted with corresponding threshold values. The final result of the process was the ranking of parks by their aesthetic, ecological and social quality and importance for the City of Novi Sad. The result can help urban planners and responsible city bodies in their future actions aimed at improving development and management of analyzed parks. Two main directions of a future research were identified: (a) testing applicability of other decision support methods, along with Promethee, on the same problem and comparison of their results; and (b) analysis of the criteria set more closely by expanding it and/or including a set of indicators.

KEYWORDS: assessment of parks, Multiplicative *Promethee*, Novi Sad

INTRODUCTION

Urban parks represent oases of nature within city structures. They have an important role in maintaining environmental qualities and are linked with numerous aspects of wellbeing and health in urban zones (Larson *et al*., 2016). Planning of urban parks has to be approached and analyzed from different perspectives and by taking into account numerous criteria. Regarding the multiple criteria, decision support methods can provide a valuable aid and they have been recognized as suitable for diverse types of environmental projects (Huang *et al*., 2011). Among decision support methods Analytic Hierarchy Process (AHP)

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(Saaty, 1980) tends to be the most intensively applied (e.g. Jiangping and Yichuan, 2014; Srdjevic *et al*., 2013), but the application of other well-known methods can be find in pertinent literature (e.g. Lakicevic *et al*., 2014).

The Preference Ranking Organization Method for Enrichment of Evaluations, known mainly in its two standard versions as Promethee I and II (Brans *et al*., 1986), also has a sizeable number of applications in the subject area (Behzadian *et al*., 2010). Besides standard versions, Promethee has its multiplicative version used in this research. Both standard and multiplicative Promethee are efficient in processing qualitative and quantitative criteria. The main prerequisite for their application is having a competent decision maker able to estimate if an alternative is preferred over the other for a given criterion (Kangas *et al*., 2015).

This research aims to promote the application of Multiplicative Promethee (MP) method in landscape planning tasks, demonstrated on the example of assessment of parks in Novi Sad. In this research there was a group of decision makers who assessed the values of parks, and a moderator who suited and processed all data in the MP framework. At the end of the process, ranking of parks was provided along with the subsequent research agenda.

METHOD OF RESEARCH

Multiplicative Promethee (Parreiras and Vasconcelos, 2007) is a similar method with Promethee II (Brans *et al*., 1986). It starts with choosing a preference function for each criterion being considered. The preference of alternative *a* over alternative *b* for a criterion *i*, labeled as $F_i(a, b)$, belongs to the range [0, 1], where 0 means no preference (indifference) and 1 means strict preference.

In order to calculate an exact value of the preference one must choose among six preference functions (for details consult Brans *et al*., 1986). In this research we used: linear function (type III) and linear function with an indifference area (type V), both presented in Figure 1. Even though these functions are commonly referred to as separate ones, it is clear that linear function can be derived from linear function with an indifference area when $q = 0$.

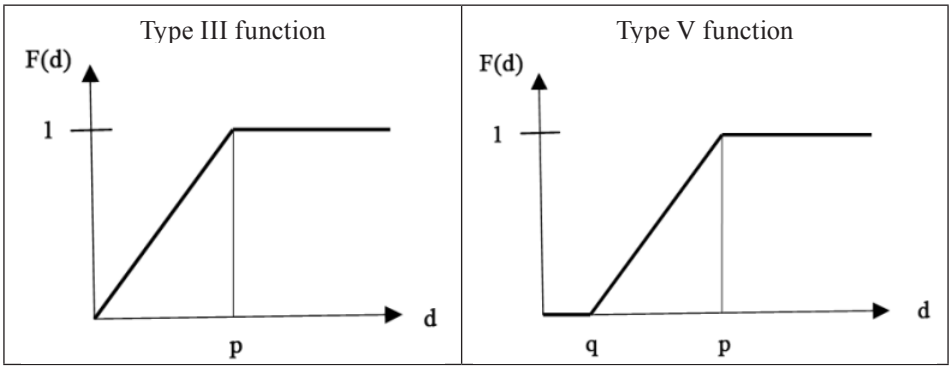

Figure 1. Type III and V preference functions in Promethee

When considering a maximizing criterion, d_i is calculated as $d_i = g_i(a) - g_i(b)$ and the intensity of preference as:

$$F_i = \begin{cases} 0 & \text{if } d_i \le q_i \\ \dfrac{d_i - q_i}{d_i - q_i} & \text{if } q_i < d_i \le p_i \\ 1 & \text{if } d_i \le p_i \end{cases} \quad (1)$$

where q_i refers to a indifference threshold and p_i refers to a preference threshold, for a given criterion i.

Statements given above are the same for both Promethee II and Multiplicative Promethee, and these versions of the method differ from this point on. In the Multiplicative Promethee net flow ϕ for criterion i is defined as:

$$\phi_i(a) = \sum_{\text{œ}b \text{O} A} F_i(a, b) - F_i(b, a). \quad (2)$$

For criterion i, it takes into account, at the same time, the intensity of preference of alternative a over b and the intensity of preference of alternative b over a. The graphical representation of the multiplicative net flow for any three alternatives being considered is presented in Figure 2.

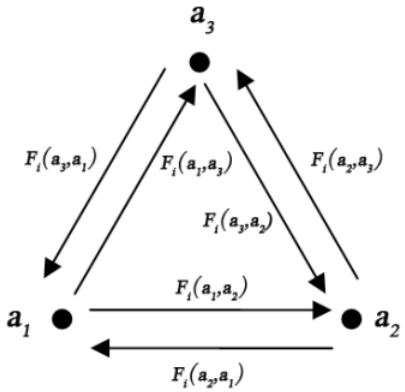

Figure 2. Preference relations between a_1, a_2 and a_3

Before arriving at the alternatives' ranking, it is necessary to calculate the Outranking Index as:

$$O(a) = \prod_{i=1}^{m} \phi_i(a)^{wi} \quad (3)$$

where ϕ(a) is defined as ϕ_i(a) + $|\phi \min_i|$, and $\phi \min_i$ is the minimum value of ϕ_i for the alternatives included, and w_i is the weight of criterion i. The following rule applies: $0 < w_i < 1$ and $\Sigma_{i=1}^{m} w_i = 1$.

Based on the values of Outranking Index, alternatives can be ranked accordingly:

1. if $0(a) = 0(b)$, then a is indifferent over b,
2. if $0(a) > 0(b)$, then a outranks b.

CASE STUDY DESCRIPTION

The research included five major and largest parks in the City of Novi Sad. Their basic description is provided in Table 1.

Table 1. Basic description of parks in Novi Sad

Park	Area [ha]	Year of establishment	City center distance [km]	Main tree species
Dunavski Park	3.9	1895	0.7	Bald-cypress, English oak, Horse-chestnut, European nettle tree, London plane, Cherry plum
Limanski Park	12.9	1950s	2.6	Poplars, Willows, Autumn olive, European nettle tree, Norway maple, Black pine, Black locust
Futoški Park	12.0	1910	1.8	Sweet-gum, Red oak, White poplar, Black walnut, Pagoda tree, Serbian spruce, Tulip tree
Železnički Park	4.2	1970s	2.3	Poplars, Turkish hazel, Horse-chestnut, Silver birch
Kamenički Park	42.0	1834	3.6	English oak, Austrian Oak, Silver lime, Norway maple, White mulberry, Kentucky coffeetree

For the assessment of the parks it was necessary to come up with the set of criteria. In this research, three criteria were selected: aesthetic, ecological, and social values. Aesthetic values included all the visual qualities of a park – including the visual features of plant material, monuments, fountains, and architectonic objects, as well as the design concept of the entire park zone. Ecological values corresponded to biodiversity, share of green areas versus concrete and other artificial covers, physiological condition and maturity (age) of tree species, and so on. Social values were related to the capacity of a park in providing a place for diverse social and gathering activities including sports and recreation, places for sitting/resting, location and accessibility of the park, etc.

Each criterion can be described by a comprehensive set of indicators, but in this research the analysis was based on decision makers' evaluations, having in mind that description of each criterion is previously explained in detail. There were 10 decision makers included in the research and they were landscape architects – master's students at the Faculty of Agriculture, University of Novi Sad. They evaluated the aesthetic, ecological and social values of each park

using a scale from 1 to 5, where value of 1 corresponded to a poor and 5 to an extraordinary value of a park for a given criterion. Before assessing the parks, they were asked to visit all parks again and to focus on the features they were supposed to evaluate. In addition, students attended an audio-visual presentation of parks and had a detailed explanation about the criteria set. The whole process was moderated by the first author of this paper, who adapted the results of assessment for the Promethee analysis and determined the weights of criteria and threshold values.

RESULTS AND DISSCUSSION

Table 2 presents the results of assessment of parks in Novi Sad with respect to analyzed criteria (aesthetic, ecological, and social values). The results provided in the table are the arithmetic means of scores (belonging to a scale 1–5) estimated by the group of ten decision makers included in the research.

Table 2. Assessment of values of parks in Novi Sad

Park (alternative)	Criteria		
	C_1	C_2	C_3
Dunavski Park	4.85	4.30	4.70
Limanski Park	3.70	3.50	4.00
Futoški Park	4.30	4.40	3.90
Železnički Park	2.00	2.83	2.33
Kamenički Park	4.45	4.90	4.15

These values were input data for the Promethee analysis, but before applying this method, it was necessary to define the weights of criteria and threshold values for each criterion; and that was done by the moderator of the decision-making process, according to her knowledge and experience in the subject area. The Promethee treshold values for each criterion are presented in Table 3. It is worth mentioning that Promethee method does not deal with defining the weights of criteria, therefore these values can be estimated directly (like in this research), or by applying AHP method as suggested by (Macharis *et al*., 2004), or by giving scores belonging to the range 1–7 (Hokkanen and Salminen, 1997), etc. Here the weights of criteria were estimated directly as: w_1=0.20; w_2=0.45 and w_3=0.35.

Table 3. Promethee threshold values (*q, p*)

Criteria	q	p
C_1 (aesthetic value)	0.50	2.0
C_2 (ecological value)	0.00	1.0
C_3 (social value)	0.75	2.0

Table 4 presents the values of the multiplicative net flow for each alternative. In the original Promethee method, values of the net flow for a set of alternatives are both positive and negative, belonging to the range [-1, 1] and their sum is equal to zero. In the multiplicative version, those features disappear and the value of the multiplicative net flow starts from 0 for the lowest ranked alternative with respect to given a criterion, and the other values of a multiplicative net flow show how 'far' is each given alternative from the one with the lowest rank.

Table 4. Multiplicative net flows of alternatives (ϕ)

Park (alternative)	Multiplicative net flow (ϕ)		
	C_1	C_2	C_3
Dunavski Park	5.27	4.77	4.28
Limanski Park	3.93	1.64	3.97
Futoški Park	4.82	5.17	3.85
Železnički Park	0.00	0.00	0.00
Kamenički Park	4.97	6.77	4.09

Table 5 presents the values of outranking index which enabled the final ranking of the alternatives. The obtained results show that Kamenički park is the first ranked according to adopted criteria set and applied group decision-making procedure based on the Multiplicative Promethee.

Table 5. Outranking indices and ranks of alternatives

Park (alternative)	Outranking Index (*0*)	Rank
Dunavski Park	4.685	2
Limanski Park	2.662	4
Futoški Park	4.599	3
Železnički Park	0.000	5
Kamenički Park	5.334	1

CONCLUSION

This paper presents the results of a group multi-criteria decision-making procedure aimed at assessment of five major parks in Novi Sad. The multiplicative version of the Promethee method is used and the complete exercise proved that it is an efficient and trustful for this type of the analysis. Based on 'performance' of parks on selected three criteria understood as principal values of the parks (aesthetic, ecological and social), the one known as 'Kamenički Park' turned out to be the highest ranked, and the one known as 'Železnički Park' as the lowest ranked. Being aware that another decision support methods can also

be applied for a broad range of tasks in landscape analysis and planning, future research might focus on various aspects of their concurrent application and possible differences in results, which is a well-known effect when several methods are applied to solve the same landscape related decision-making problem. Also, it is anticipated that criteria set used in presented research can be additionally inspected by including sets of sub criteria (indicators). This is an emerging issue when it comes to estimation of biodiversity/ecological values. Improving the quality of input values would probably make the decision-making process more complete and objective, and expectedly more reliable.

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REFERENCES

Behzadian M, Kazemzadeh RB, Albadvi A, Aghdasi M (2010): PROMETHEE: A comprehensive literature review on methodologies and applications. *Eur. J. Oper. Res.* 200: 198–215.

Brans JP, Vincke P, Mareschal B (1986): How to select and how to rank projects: The Promethee method. *Eur. J. Oper. Res.* 24: 228–238.

Hokkanen J, Salminen P (1997): Locating a Waste Treatment Facility by Multicriteria Analysis. *J. Multi-Crit. Decis. Anal.* 6: 175–184.

Huang IB, Keisler J, Linkov I (2011): Multi-criteria decision analysis in environmental sciences: Ten years of applications and trends. *Sci. Total. Environ.* 409: 3578–3594.

Jiangping W, Yichuan Z (2014): Service quality evaluation of urban parks based on AHP method and SD software. *J. Appl. Sci.* 14: 291–295.

Kangas A, Kurttila M, Hujala T, Eyvindson K, Kangas J (2015): *Decision Support for Forest Management, Managing Forest Ecosystems*. Springer International Publishing, Cham.

Lakicevic M, Srdjevic Z, Srdjevic B, Zlatic M (2014): Decision making in urban forestry by using approval voting and multicriteria approval method (case study: Zvezdarska forest, Belgrade, Serbia). *UFUG* 13: 114–120.

Larson LR, Jennings V, Cloutier SA (2016): Public Parks and Wellbeing in Urban Areas of the United States. *PLoS One* 11: e0153211.

Macharis C, Springael J, De Brucker K, Verbeke A (2004): PROMETHEE and AHP: The design of operational synergies in multicriteria analysis.: Strengthening PROMETHEE with ideas of AHP. *Eur. J. Oper. Res.* 153: 307–317.

Parreiras RO, Vasconcelos JA (2007) A multiplicative version of Promethee II applied to multi-objective optimization problems. *Eur. J. Oper. Res.* 183: 729–740.

Saaty TL (1980): *The Analytical Hierarchy Process*. McGraw Hill, New York.

Srdjevic Z, Lakicevic M, Srdjevic B (2013): Approach of decision making based on the analytic hierarchy process for urban landscape management. *Environ. Manage.* 51: 777–785.

МУЛТИПЛИКАТИВНА ВЕРЗИЈА МЕТОДА *PROMETHEE* ЗА ВРЕДНОВАЊЕ НОВОСАДСКИХ ПАРКОВА

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РЕЗИМЕ: Методи за подршку одлучивању имају значајну улогу у решавању проблема везаних за животну средину и планирање предела. У раду је за вредновање пет главних паркова у Новом Саду примењен један од метода за подршку процеса одлучивања – мултипликативна верзија метода *Promethee*. Поступак је подразумевао дефинисање скупа критеријума и то су биле: естетске, еколошке и социјалне вредности истраживаних паркова. За сваки критеријум је одабрана одговарајућа *Promethee* функција преференције и дефинисани су одговарајући прагови (индиференције и преферентности). На крају примене *Promethee* поступка добијено је рангирање паркова у складу с њиховим естетским, еколошким и социјалним функцијама и значајем за Град Нови Сад. Добијени резултати могу користити урбанистима и одговорним градским органима у њиховим будућим задацима који имају за циљ унапређење развоја и управљања анализираним парковима. Главни правци наредних истраживања могу бити: а) тестирање примене других метода, упоредо с методом за *Promethee*, за решавање истог проблема и поређење добијених резултата, и б) детаљнија анализа скупа критеријума, његовим проширивањем и/или укључивањем одговарајућих индикатора.

КЉУЧНЕ РЕЧИ: вредовање паркова, мултипликативна верзија метода *Promethee*, Нови Сад

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THE CONCENTRATION RATIO OF ALKALINE EARTH ELEMENTS CALCIUM, BARIUM AND STRONTIUM IN GRAINS OF DIPLOID, TETRAPLOID AND HEXAPLOID WHEAT

ABSTRACT: Even though calcium (Ca), strontium (Sr) and barium (Ba) belong to the same group of the periodic table of elements, and thus have similar chemical features, their importance for plants differs greatly. Since plants do not have the ability to completely discriminate between essential (e.g. Ca) and non-essential elements (e.g. Sr and Ba), they readily take all of them up from soil solution, which is reflected in the ratios of concentrations of those elements in plant tissues, and it influences their nutritive characteristics. The ability of plant species and genotypes to take up and accumulate chemical elements in their different tissues is related to their genetic background. However, differences in chemical composition are the least reflected in their reproductive parts. Hence, the aim of this study was to evaluate ratios of concentrations of Ca, Sr and Ba in the whole grain of diploid and tetraploid wheat – ancestors of common wheat, as well as in hexaploid commercial cultivars, grown in the field, at the same location, over a period of three years.

The investigated genotypes accumulated Ca, Sr and Ba at different levels, which is reflected in the ratio of their concentrations in the grain. The lowest ratio was established between Ba and Sr, followed by Ca and Ba, while the highest ratio was between Ca and Sr. Moreover, the results have shown that the year of study, genotype and the combination highly significantly affected the ratio of the concentration Ca:Sr, Ca:Ba, and Ba:Sr.

KEYWORDS: barium, calcium, concentration ratio, grain, ploidy levels, strontium, wheat

INTRODUCTION

The alkaline earth metals, belonging to the group II A of the periodic table of elements, include beryllium (Be), magnesium (Mg), calcium (Ca),

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strontium (Sr), barium (Ba), and radium (Ra). They are widely distributed, in varying concentrations, in all parts of the biosphere (Kabata-Pendias, 2000). A common feature of alkaline earth metals is that their degree of oxidation is +2. Two electrons in the highest quantum state are characteristic of the electronic configuration of the alkaline earth metals. Moreover, compounds of these elements, except for Ba, have mainly ionic character. The similarity in physical and chemical properties of Ca, Ba and Sr is important in some physiological processes of plants.

Ca, Ba and Sr are not equally distributed in plants. Whereas Sr and Ba are regarded as trace elements, Ca belongs to macronutrients. Their ecological importance is also different. Contamination of the environment with Ba (Suwa *et al*., 2008) and Sr (^{90}Sr) (ATSDR, 2014) jeopardizes the living world, which is not the case with Ca. Nevertheless, Ba and Sr can take over the role of Ca in some physiological processes in plants (Vanselow, 1966 a,b), but most probably only non-specific ones. They can precipitate in the form of oxalates in some plant species (Fink, 1991; Wyttenbach *et al*., 1995), their mobility in plants is poor (Marschner, 1995; Kastori *et al*., 2007), and they have similar geochemical behavior.

Plant species differ in their ability to take up, accumulate, translocate and use mineral elements (Mengel, 1982). Differences exist also between genotypes, lines and individual parts of the whole plant within a species or variety (Sarić, 1981; Clark, 1983). The ratio between concentrations of alkaline earth metals differs not only between different plant species, but also between different genotypes of the same species (Young and Rasmusson, 1966), and also between different organs and tissues within each individual plant (Watmough, 2014). The ratio between Ca and Sr in edible plant parts is particularly important having in mind the potential risk of Sr (and its ^{90}Sr isotope) entering the food chain, and the importance of Ca as essential macronutrient. In this context, and considering also the importance of wheat as food and feed, the aim of this research was to assess ratios between concentrations of alkaline earth elements Ca, Ba and Sr in the whole grain of wheat genotypes of different levels of ploidy (diploid, tetraploid and hexaploid), grown under the same agro-ecological conditions. The results of this research may contribute to the creation of genotypes with more favorable ratio between these elements in wheat grain.

MATERIALS AND METHODS

Six diploid genotypes of wheat with different genome formula (BB, AA or DD), five tetraploids (BBAA) and nine hexaploids (BBAADD) were used in the experiment. Among the diploid wheat, four were wild and one (*Triticum monococcum* subsp. *monococcum*) was primitive cultivated wheat. Among the tetraploid wheat included in the experiment, three genotypes were wild einkorn while two were cultivated. All hexaploids were cultivated genotypes. The names of the cultivars are given in Table 1.

The wheat genotypes were planted in the experimental field of the Institute of Field and Vegetable Crops, Novi Sad, in 2011, 2012 and 2013, on Calcic, Gleyic Chernozem, well-provided with total nitrogen and rich in available phosphorus and potassium. Wheat genotypes were sown in a randomized complete block design, in three replications. Surface of field plots was 2.5 m^2. The soil of the experimental area was fertilized with 50 kg N ha^{-1}, and 50 kg P_2O_5 ha^{-1}. Top-dressing was conducted once, with 50 kg N ha^{-1}.

Table 1. Genotypes of *Aegilops* and *Triticum* species (classified according to van Slageren, 1994) included in the experiments.

No	Species and subspecies	Genome(s)	Name	Source/Origin
1	*Aegilops speltoides* subsp. *speltoids* 1	BB		IPK*
2	*Aegilops speltoides* subsp. *speltoids* 2	BB		IPK
3	Triticum urartu	AA		IPK
4	*Triticum monococcum* subsp. *Aegilopoides*	AA	Wild einkorn	IFVC, SRB**
5	*Triticum monococcum* subsp. *Monococcum*	AA	Cultivated einkorn	IFVC, SRB
6	*Aegilops tauschii* subsp. *Tauschii*	DD	Goat grass	IPK
7	*Triticum turgidum* subsp. *Dicoccoides* (IPK)	BBAA	Wild emmer	IPK
8	*Triticum turgidum* subsp. *Dicoccoides* (IFVC)	BBAA	Wild emmer	IFVC, SRB
9	*Triticum turgidum* subsp. *dicoccon*	BBAA	Cultivated emmer	IFVC, SRB
10	*Triticum turgidum* subsp. *turgidum*	BBAA	Ri vet wheat	IPK
11	*Triticum turgidum* subsp. *Durum* (cv. *Durumko*)	BBAA	Durum wheat	IFVC, SRB
12	*Triticum aestivum* subsp. *spelta* (cv. *Nirvana*)	BBAADD	Spelt wheat	IFVC, SRB
13	*Triticum aestivum* (cv. *Panonnia*)	BBAADD	Common wheat	IFVC, SRB
14	*Triticum aestivum (*cv. *Bankut* 1205)	BBAADD	Common wheat	HUN
15	*Triticum aestivum* (cv. *Bezostaja 1*)	BBAADD	Common wheat	RUS
16	*Triticum aestivum* (cv. *Siete Cerros*)	BBAADD	Common wheat	MEX
17	*Triticum aestivum* (cv. *Florida*)	BBAADD	Common wheat	USA
18	*Triticum aestivum* (cv. *Renan*)	BBAADD	Common wheat	FRA
19	*Triticum aestivum* (cv. *Condor*)	BBAADD	Common wheat	AUT
20	*Triticum aestivum* (cv. *Bolal*)	BBAADD	Common wheat	TUR

* IPK – Genebank Gatersleben of the Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany; ** IFVC, SRB – Institute of Field and Vegetable Crops, Novi Sad, Serbia; HUN – Hungary; RUS – Russia; MEX – Mexico; USA – United State of America; FRA – France; AUT – Australia; TUR – Turkey.

Genotypes included in the experiment were harvested at crop maturity and all hulled genotypes were manually de-hulled. After digestion of grain whole meal in a mixture of 10 mL HNO_3 (65%) and 2 mL of H_2O_2 (30%), the

concentration of Ca, Ba and Sr were determined by inductively coupled plasma emission spectroscopy.

The data were subjected to a combined analysis of variance, treating environment as the main plot and the species as the sub-plot, and the differences among genotypes and environments were determined using the Tukey test. Standard deviations, analysis of variance, Pearson's linear correlation coefficients among all the traits were obtained by Infostat (Di Rienzo *et al*., 2016). Principal coordinates analysis (PCO) was used to find the eigenvalues and eigenvectors of a matrix containing the distances between all data points (Davis, 1986) applying Euclidean correlation.

RESULTS AND DISCUSSION

Concentration ratios Ca:Sr, Ca:Ba and Ba:Sr in the whole grain of *Aegilops* and *Triticum* species varied significantly between different genomes but also between different years (Table 2). However, the differences were more pronounced between genomes than within them. Significant differences were recorded with respect to all three ratios between *Triticum* species, but not within *Aegilops* (Table 2). The ratio Ba:Sr was affected the most by the year (Table 2). The ratio between concentrations of alkaline earth metals and the other elements in particular plant species, genotypes, organs, tissues, and cell organelles depends, besides genetics, on many ecological factors, as well as on physical and chemical features of particular ions. The most important ecological factor is the concentration of elements in the root zone, although in this respect opinions vary. According to White (2001), the accumulation of Ca, Sr, and Ba in shoots is often linear in relation to their concentration in the nutrient solution, and the ratio Ca:Ba:Sr in the shoot is the same as in the nutrient solution in which the root is embedded. During the transport of Ca, Sr, and Ba towards the shoot there is no competition or interaction between these cations, in spite of the fact that there is competition between them during their uptake into root cells. Russell and Squire (1958) found that the absorption of Sr in the presence of Ca declined and translocation of Sr in plants insufficiently supplied with Ca was lower probably due to binding of Sr on the root surface or close to it.

Combined analyses of variance showed that the year of study, genotype, and the combination highly significantly affected the three examined ratios (Table 3). When the analysis was done with respect to the genomes, the result was the same (highly significant differences) with the exception of Ca:Sr which was significant (Table 4). Standard deviation and coefficient of variation were quite variable within and between genotypes and years (Table 5). The ratio Ca:Sr was the smallest in genotype 13 (ranging from 121 to 263) and the highest in genotype 7 (423–612); the ratio Ca:Ba was the smallest in genotype 2 (74–124) and the highest in genotype 10 (174–535); the ratio Ba:Sr was the lowest in genotype 15 (0.64–2.23) and the highest in genotype 2 (1.78–3.41). Coefficient of variation was also pretty variable and ranged from 1.9 for ratio Ca:Sr in genotype 19 to over 52 for ratio Ca:Ba in genotypes 6, 10 and 18 (Table 5). With respect

Table 2. Concentration ratios: Ca:Sr, Ca:Ba, and Ba:Sr in the whole grain of *Aegilops* and *Triticum* species over the period of 3 years.

	Ca:Sr	Ca:Ba	Ba:Sr
Genotype no.*			
1	262.6^{i}	97.2^{i}	2.9^{a}
2	258.4^{i}	97.5^{i}	2.8^{ab}
3	374.3^{e}	273.3^{c}	1.4^{j}
4	439.3^{cd}	211.8^{de}	2.2^{ef}
5	440.1b^{cd}	167.1^{fg}	2.7^{abc}
6	469.3^{b}	239.9^{d}	2.4^{cde}
7	524.1^{a}	223.4^{de}	2.4d^{ef}
8	309.8^{g}	194.2^{ef}	1.6^{j}
9	502.7^{a}	349.0^{d}	1.5^{j}
10	459.4^{bc}	308.3^{b}	1.9^{hi}
11	458.4^{bc}	222.0^{de}	2.1^{fgh}
12	340.3^{f}	149.7^{gh}	2.3^{efg}
13	206.7^{j}	91.2^{i}	2.3^{efg}
14	279.4^{hi}	122.1^{hi}	2.3^{defg}
15	332.1^{fg}	223.4^{de}	1.6^{j}
16	303.8^{gh}	122.8^{hi}	2.6^{bgd}
17	258.3^{i}	138.0^{gh}	2.1^{gh}
18	268.3^{i}	210.1^{de}	1.6^{ij}
19	416.9^{d}	311.1^{b}	1.4^{j}
20	303.8^{dh}	144.5^{gh}	2.2^{efgh}
Genome			
BB	260.5^{c}	217.4^{a}	2.8^{a}
AA	469.3^{a}	97.3^{c}	2.1^{bc}
DD	417.9^{b}	239.9^{a}	2.4^{b}
BBAA	450.9^{ab}	259.4^{a}	1.9^{c}
BBAADD	301.1^{c}	168.1^{b}	2.1^{bc}
Year			
2011	345.2^{b}	183.1^{b}	2.1^{b}
2012	373.8^{a}	235.9^{a}	1.8^{c}
2013	362.3^{ab}	165.5^{b}	2.4^{a}

*Genotypes of *Aegilops* and *Triticum* species examined in the experiments are given in Table 1.
Average ratios were obtained from three independent measurements and significance of differences between the average ratios was assessed by Tukey's test; different letters indicate a significant difference at 5% probability level.

to genomes and experimental years, in each ratio of concentrations, all examined ranges had overlaps (Table 5).

Table 3. Combined analysis of variance (ANOVA) of concentration ratios: Ca:Sr, Ca:Ba, and Ba:Sr in the whole grain of *Aegilops* and *Triticum* species over the period of 3 years.

	DF	Ca:Sr		Ca:Ba		Ba:Sr	
		SS	(%)[a]	SS	(%)[a]	SS	(%)[a]
Year (Y)	2	24810[b]	1	161043[b]	9	10.41[b]	11
Genotype (G)	19	1561592[b]	73	990100[b]	54	34.25[b]	36
Y x G	38	505355[b]	24	654806[b]	35	46.31[b]	49
Error	120	35145	2	42541	2	3.39	4
Total	179	2126902		18448191		94.36	

[a] % explained and calculated from ANOVA as factor SS (sum of squares)/total SS.
[b] Significant at P <0.01.

Table 4. Combined analysis of variance (ANOVA) of concentration ratios: Ca:Sr, Ca:Ba, and Ba:Sr in the whole grain of 20 genotypes and 5 genomes over the period of 3 years.

	DF	Ca:Sr		Ca:Ba		Ba:Sr	
		MS	F	MS	F	SS	F
Year (Y)	2	12405**	4.3	80522**	25.7	5.21**	24.4
Genome (Gen)	4	257316*	88.8	112091**	35.7	3.15**	14.7
Y x Gen	8	13214**	4.6	28261**	9.0	2.21**	10.3
Gen/Gen>G	15	35489**	12.2	36116**	11.5	1.44**	6.7
Error	150	2899		3140		0.21	

* Significant at P < 0.05; ** Significant at P < 0.01

Pearson's correlation coefficients for ratios of concentrations of examined elements were significant between all genomes for Sr/Ca:Sr, Sr/Ca:Ba, Ba/Ba:Sr, and Ca:Sr/Ca:Ba (Table 6). Antagonism between Ca, Sr, and Ba was investigated in the middle of the last century by Epstein and Leggett (1954) and later by Kabata-Pendias (2000). It is generally accepted that the Ca:Sr ratio does not change significantly during their uptake (Blum *et al*., 2002). In eight woody species Watmough (2014) found that the ratios Ca:Sr and Ca:Ba were different in shoots with respect to the soil. In ten pasture species, the ratio Sr:Ca depended on plant species but also on soil features (Veresoglou *et al*., 1996). In wheat, the ratio Sr:Ca to a small and Ba:Ca to a higher extent depended on their ratios in the soil extract (Smith, 1971a). On the bases of these results, it can be concluded that ratios of concentrations of Ca, Sr, and Ba in the nutrient medium under certain conditions may affect their ratios in shoots. Therefore, studies of genotypic

Table 5. Range, standard deviation and coefficient of variation of concentration ratios: Ca:Sr, Ca:Ba, and Ba:Sr in the whole grain of *Aegilops* and *Triticum* species over the period of 3 years.

		Ca:Sr			Ca:Ba			Ba:Sr		
	N	range	SD	CV	range	SD	CV	range	SD	CV
Genotype no.*										
1	3	251–268	8.0	3.1	77–132	24.6	25.3	1.91–3.48	0.69	24.0
2	3	221–321	44.6	17.3	74–124	20.4	22.0	1.78–3.41	0.71	25.7
3	3	292–417	58.2	15.6	202–322	51.0	18.7	1.29–1.45	0.07	4.7
4	3	321–568	101.2	23.0	188–240	21.49	10.2	1.59–3.09	0.67	30.9
5	3	403–502	44.3	10.1	142–192	20.7	12.4	2.41–2.96	0.23	8.5
6	3	425–525	41.5	8.9	124–415	101.2	52.5	1.27–3.69	0.99	40.4
7	3	423–612	77.5	14.8	215–236	9.5	4.2	2.01–2.84	0.35	14.7
8	3	224–405	74.0	23.9	159–238	33.0	17.0	1.41–1.71	0.12	7.73
9	3	428–600	72.3	14.4	259–442	74.9	21.5	0.97–1.89	0.41	26.6
10	3	429–500	29.9	6.5	174–535	152.1	52.2	0.84–2.87	0.83	43.8
11	3	407–510	41.9	9.1	190–278	39.9	18.0	1.84–2.34	0.21	9.7
12	3	213–438	94.1	27.7	136–163	11.2	7.5	1.56–2.94	0.56	24.9
13	3	121–263	61.4	29.7	81–108	11.8	12.9	1.43–3.23	0.74	32.3
14	3	269–292	9.5	3.4	113–128	6.9	5.6	2.09–2.61	0.22	9.4
15	3	318–346	11.5	3.5	152–305	62.9	28.1	1.09–2.09	0.41	25.71
16	3	281–318	16.4	5.4	98–164	29.3	23.9	1.94–2.94	0.45	17.6
17	3	251–273	10.2	3.9	106–200	44.2	32.0	1.25–2.57	0.57	27.9
18	3	230–334	46.9	17.5	108–363	110.2	52.4	0.64–2.23	0.72	43.6
19	3	409–428	7.7	1.9	197–490	128	41.1	0.87–2.08	0.50	32.5
20	3	211–375	68.5	22.5	125–168	17.6	12.2	1.26–2.67	0.64	29.7
Genome										
BB	6	221–321	32.1	12.3	74–132	22.6	23.3	1.78–3.48	0.71	24.9
AA	9	292–569	78.3	18.8	142–322	55.3	25.4	1.29–3.09	0.66	32.1
DD	3	425–525	41.5	8.9	124–415	101.2	52.5	1.27–3.69	0.99	40.4
BBAA	15	224–612	97.4	21.6	159–535	101.6	39.2	0.84–2.87	0.55	29.2
BBAADD	27	121–438	73.3	24.3	81–490	90.1	53.6	0.64–3.24	0.66	32.0
Year										
2011	20	121–525	101.6	29.5	83–443	95.3	52.0	0.97–3.41	0.67	31.4
2012	20	211–612	126.8	33.9	108–535	117.4	49.8	0.64–3.09	0.69	38.0
2013	20	221–536	89.7	24.8	74–322	67.6	40.8	1.29–3.69	0.65	27.1

* Genotypes of *Aegilops* and *Triticum* species examined in the experiments are given in Table 1

differences with respect to ratios between concentrations of these elements have to be conducted under the identical soil and other agro-ecological conditions, and the experiment described here was done in line with this recommendation.

Table 6. The Pearson's correlation coefficient (*r*) for concentration of Ca, Sr, and Ba and their ratios in the whole grain of *Aegilops* and *Triticum* genomes over the period of 3 years. Genotypes of *Aegilops* and *Triticum* species examined in the experiments are given in Table 1.

Elements	Genome				
	BB	AA	DD	BBAA	BBDD
Ca/Sr	0.853**	0.521**	0.955**	0.158	0.290**
Ca/Ba	-0.446	0.513**	-0.707*	0.286	0.280*
Ca/Ca:Sr	-0.353	-0.047	-0.819**	0.296*	0.369**
Ca/Ca:Ba	0.398	0.014	0.923**	-0.379*	-0.285**
Ca/Ba:Sr	0.870**	0.539**	0.923**	0.284	0.253*
Sr/Ba	-0.254	0.410*	-0.608	0.526**	0.527**
Sr/Ca:Sr	-0.781**	-0.832**	-0.946**	-0.838**	-0.692**
Sr/Ca:Ba	0.816**	0.857**	0.996**	0.837**	0.826**
Sr/Ba:Sr	0.677**	0.133	0.839**	-0.306*	-0.224*
Ba/Ca:Sr	-0.103	-0.182	0.517	-0.372*	-0.358**
Ba/Ca:Ba	0.077	0.181	-0.574	0.367*	0.321**
Ba/Ba:Sr	-0.825**	-0.439*	-0.919**	-0.792**	-0.751**
Ca:Sr/Ca:Ba	-0.990**	-0.926**	-0.970**	-0.887**	-0.897**
Ca:Sr/Ba:Sr	-0.179	0.152	-0.713*	0.445**	0.422**
Ca:Ba/Ba:Sr	0.218	-0.169	0.801**	-0.436**	-0.326**

* significance level alpha=0.05; ** significance level alpha=0.01

The intensity of transport from the root and redistribution of elements from vegetative parts to the fruit may significantly determine their ratios in generative parts. Indexes of translocation of Sr and Br were found to be the lowest out of examined microelements (Ni, Cu, Zn, Mo, Sr, and Ba) in five plant species, including wheat, and it was not significantly influenced by the increase in their concentration available to plants (Kastori *et al*., 2007). According to Smith (1971a) Sr, Ba, and Ra are less mobile in wheat in comparison to Ca, and a significant part of Ba and Ra is bound in stems. Russell and Squire (1958) found that redistribution of Sr in barley is very low. Also, low concentration of Ca, Sr, and Ba in generative parts and grain of wheat may be explained by their low mobility (translocation) in plants.

Distribution of examined alkaline earth metals in plant organs is variable, and this is reflected in the ratios of their concentrations in the other plant species as well. For example, concentration of Sr in rapeseed was the highest in chaff, of Ba in stems, and the lowest concentration of both elements was in seed. The ratio of concentrations Sr:Ba in stems was 9.7, in chaff 14.4, and in seeds 8.9 (Kastori *et al*., 2003). According to Watmough (2014) in eight woody species the highest molar ratios Ca:Sr, Ca:Ba, and Sr:Ba were found in leaves, and the lowest in stems. Sr and Ba accumulate in the endosperm of Brazil nut and their ratio to Ca was found to be 20 times higher than in stem tissues (Smith, 1971b).

Regression analysis of concentration ratios Ca:Sr against Ca:Ba was in 2011 and 2013 highly significant, whereas concentration ration of Ca:Ba against Ba:Sr was significant in all experimental years. On the contrary, concentration ratio of Ca:Sr against Ba:Sr was not significant (Figure 1). It is clear that concentrations of individual elements are reflected in the analyses of their ratios. Significant differences with respect to the concentrations of particular alkaline earth elements were recorded between the genotypes of the same species. Young and Rasmusson (1966) found significant differences in the accumulation of Ca and Sr between genotypes of barley: those genotypes which accumulated higher amounts of Sr accumulated Ca as well. Barley genotypes differed significantly with respect to Sr:Ca ratio in the shoots. Significant differences were recorded with respect to the concentration of Ba (Denčić *et al*., 2015), Sr (Kastori *et al*., 2017) and Ca in the whole wheat grain. All three elements accumulated most in the grain of *Aegilops speltoides*.

Principal coordinate analysis of ratios of concentrations Ca:Ba, Ba:Sr, and Ca:Sr during three years allowed grouping of genotypes according to their genome into distinct groups (Figure 2) and these results are further supported by discriminant analysis (Figure 3). Therefore, the results described here underline the significance of the genetic background with respect to the ability to accumulate Ca, Ba, and Sr in the wheat and *Aegilops* grain. These findings are important since they can be used in the breeding programs to obtain wheat grains of better nutritional quality.

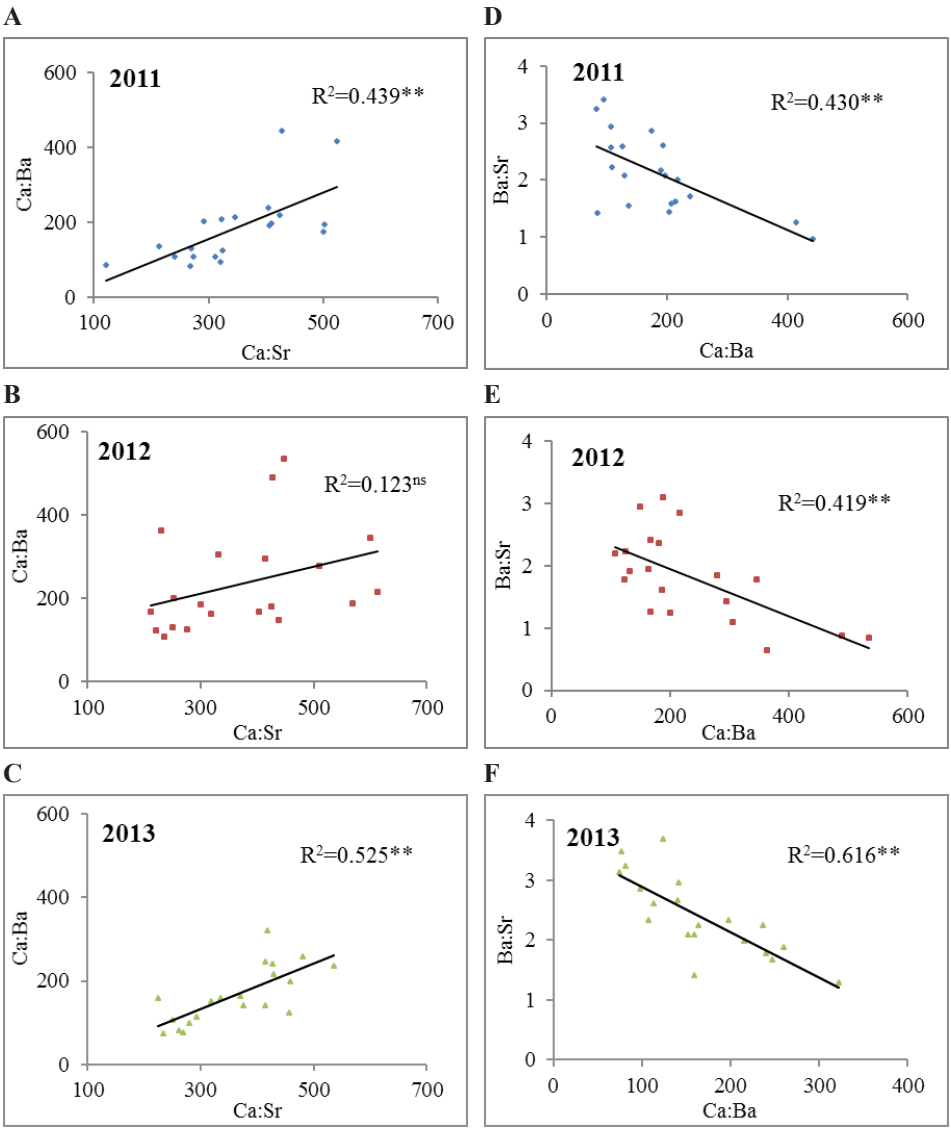

Figure 1. Pearson's liner correlation coefficients among ratios of concentrations of Ca:Ba and Ca:Sr (**A, B, C**), and Ba:Sr and Ca:Ba (**D**, **E**, **F**) in the whole grain of *Aeglops* and *Triticum* species in three experimental years. Ratios between Ba:Sr and Ca:Sr were not statistically significant and therefore are not shown. Genotypes of *Aegilops* and *Triticum* species examined in the experiments are given in Table 1

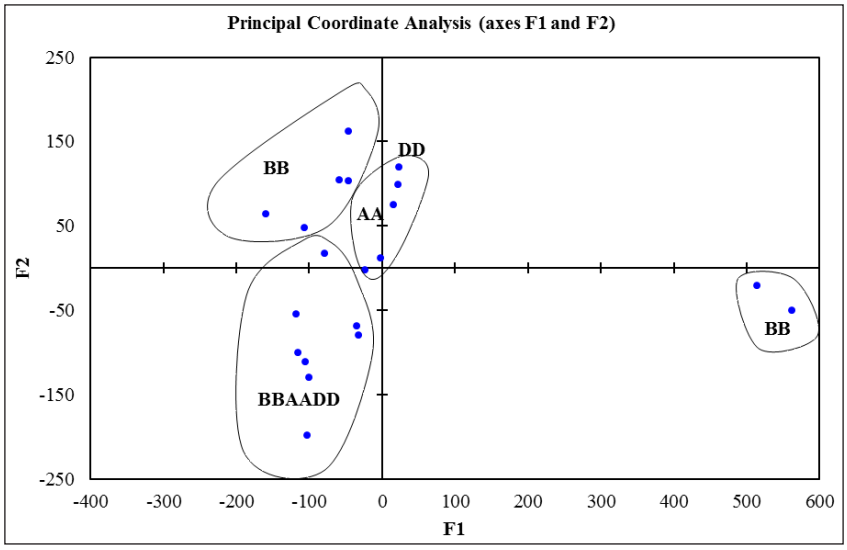

Figure 2. Principal coordinate analysis of concentration ratios: Ca:Sr, Ca:Ba and Ba:Sr in the whole grain of *Aegilops* and *Triticum* species over a 3-year period shows segregation groups with respect oto their respective genetic backgrounds. Genotypes of *Aegilops* and *Triticum* species examined in the experiments are given in Table 1

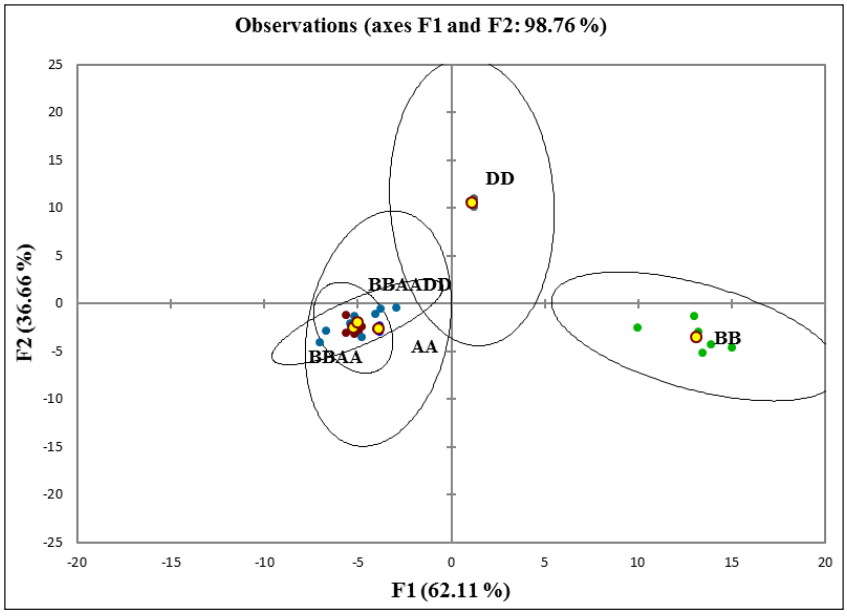

Figure 3. Discriminant analysis of concentration ratios: Ca:Sr, Ca:Ba, and Ba:Sr in the whole grain of *Aegilops* and *Triticum* species over a 3-year period shows segregation groups with respect to their respective genetic backgrounds. Genotypes of *Aegilops* and *Triticum* species examined in the experiments are given in Table 1

CONCLUSION

Examined alkaline earth metals have different physiological and ecological significance. Calcium is a necessary element for all living things, while strontium, under certain circumstances, is considered as an ecological hazard. Therefore, the knowledge of their concentrations and ratios of their concentrations in wheat grain, beside scientific, has practical significance as well. Diploid, tetraploid, and hexaploid wheats accumulated Ca, Sr, and Ba at different levels. Overall ratios of their concentrations in the grain were the lowest for Ba:Sr, than for Ca:Ba, and the highest for Ca:Sr. Besides genotype, the year of study, and also the combination of genotype and the year of study highly significantly affected the ratios of concentration Ca:Sr, Ca:Ba, and Ba:Sr.

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REFERENCES

ATSDR (2014): Agency for toxic substances and disease registry, public health statement, strontium CAS#: 7440-24-6, Available from: <www.atsdr.ede.Gov/>

Blum JD, Klaue A, Nezat CA, Driscott CT, Johnson CE, Sicama TG, Eagar C, Fahey TJ, Likens GE (2002): Mycorrhizal weathering of apatite as an important calcium source in base poor forest ecosystems. *Nature* 417: 729–731.

Clark RB (1983): Plant genotype differences in the uptake, translocation and use of mineral elements required for plant growth. *Plant Soil* 72: 175–196.

Davis JC (1986): *Statistics and data analysis in geology.* New York: John Wiley & Sons,.

Denčić S, Kastori R, Kádár I, Maksimović I, Putnik-Delić M, Momčilović V (2015): Barium concentration in grain of *Aegilops* and *Trtiticum* species. Novi Sad: *Matica Srpska J. Nat. Sci.* 129: 27–34.

Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW (2016): InfoStat version. InfoStat Group, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina, Available from: <http://www.infostat.com.ar>.

Epstein E, Leggett J (1954): The absorption of alkaline earth cations by barley roots: kinetics and mechanism. *Am. J. Bot.* 41: 785–791.

Fink S (1991): Comparative microscopical studies on the patterns of calcium oxalate distribution in the needles of various conifer species. *Bot. Acta* 104: 306–315.

Kabata-Pendias A (2000): *Trace elements in soil and plants*. 3rd edition CRS Press, Boca Raton London New York Washington D.C.

Kastori R, Kádár I, Sekulić PĐ, Zeremski-Škorić TM (2003): Effect of Mo, Zn, Sr and Ba loads on these elements' uptake and oil content and fatty acid composition of rapeseed. Novi Sad: *Matica Srpska Proc. Nat. Sci.* 105: 5–14.

Kastori, R, Kádár, I, Maksimović, I (2007): Translocation of heavy metals and Sr and Ba from vegetative parts to seeds of some crops. *Proceedings of the 14th Symposium on Analytical and Environmental Problems*. SAB Szakbizottsága Analitikai és Környezetvédelmi Munkabizottsága, Szeged, Hungary, 121–125.

Kastori R, Maksimović I, Denčić S, Kádár I, Putnik-Delić M, Momčilović V (2017): Strontium accumulation in whole grain of *Aegilops* and *Triticum* species. *J. Plant. Nutr. Soil Sci*. 180: 212–219.

Marschner H (1995): *Mineral nutrition of higher plants*. 2nd Edition, Academic Press, London.

Mengel K (1982): Response of various crop species and cultivars to mineral nutrition and fertilizer application. In: *Genetic specificity of Mineral Nutrition of Plants*. Belgrade: Serb. Acad. Sci. Arts, 22: 233–245.

Russell RS, Squire HM (1958): The absorption and distribution of strontium in plants I. Preliminary studies in water culture. *J. Exp. Bot*. 9: 262–276.

Sarić M (1981): Genetic specificity in relation to plant mineral nutrition. *J. Plant Nutr*. 3: 743–766.

Smith KA (1971a): The comparative uptake and translocation by plants of calcium, strontium, barium and radium. *Plant Soil* 34: 369.

Smith KA (1971b): The comparative uptake and translocation by plants of calcium, strontium, barium and radium II. *Triticum vulgare* (wheat). *Plant Soil* 34: 643–651.

Suwa R, Jayachandran K, Nguyen NT, Boulenouar A, Fujita K, Saneoka H (2008): Barium toxicity effects in soybean plants. *Arch. Environ. Contam. Toxicol*. 55: 539–403.

Van Slageren MWSJ (1994): *Wild wheats: A monograph of Aegilops L. and Amblyopyrum (Jaub. et Spach) Eig (Poaceae): a revision of all taxa closely related to wheat, excluding wild Triticum species, with notes on other genera in the tribe Triticeae, especially Triticum*. Wageningen: Wageningen Agricultural University Papers, The Netherlands, 94–97.

Vanselow AP (1966a): Barium. In: Chapman, HD (eds.), *Diagnostic criteria for plants and Soils. Division of agricultural sciences*, Berkeley: University of California, 24–32.

Vanselow AP (1966b): Strontium. In: Chapman HD (eds.), *Diagnostic criteria for plants and soils. Division of agricultural sciences*, Berkeley: University of California, 433–443.

Veresoglou DS, Barhayiannis N, Matsi T, Anagostopoulos C, Zalidis GC (1996): Shoot Sr concentrations in relation to shoot Ca concentrations and to soil properties. *Plant Soil* 178: 95–100.

Watmough SA (2014): Calcium, strontium and barium biogeochemistry in a forested catchment and insight into elemental discrimination. *Biogeochemistry* 118: 357–369.

White PJ (2001): The pathways of calcium movement to the xylem. *J. Exp. Bot*. 52: 891–899.

Wyttenbach A, Bajo S, Bucher J, Furrer V, Schleppi P, Tubler L (1995): The concentration of Ca, Sr, Ba and Mg in successive needle age classes of Norway spruce (*Picea abies* (L.) Karst.), *Trees* 10: 31–39.

Young WI, Rasmusson DC (1966): Variety differences in strontium and calcium accumulation in seedlings of barley. *Agron. J*. 58: 181–483.

ОДНОС КОНЦЕНТРАЦИЈЕ ЗЕМНОАЛКАЛНИХ МЕТАЛА КАЛЦИЈУМА, БАРИЈУМА И СТРОНЦИЈУМА У ЗРНУ ДИПЛОИДНЕ, ТЕТРАПЛОИДНЕ И ХЕКСАПЛОИДНЕ ПШЕНИЦЕ

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РЕЗИМЕ: Калцијум (Ca), стронцијум (Sr) и баријум (Ba) припадају истој групи елемената Периодног система и имају сличне хемијске особине. Међутим, њихове улоге у биљном организму веома се разликују. Обзиром да биљке немају способност да у потпуности разликују есенцијалне (Ca) од неесенцијалних елемената (Sr и Ba), оне их све усвајају из земљишног раствора, а то се одражава у различитом односу концентрација ових елемената у биљном ткиву и утиче на њихове нутритивне особине. Способност врста и генотипова да усвајају и акумулирају хемијске елементе у различитим ткивима, зависи од њихове генетике. И поред тога, разлике у хемијском саставу огледају се у њиховој репродуктивној улози. Циљ истраживања био је да се одреде односи концентрација Ca, Sr и Ba у целом зрну диплоидне и тетраплоидне пшенице – претка данашње пшенице, као и хексаплоидних комерцијалних врста, гајених на истом пољу и локалитету током три године.

Испитивани генотипови су акумулирали Ca, Sr и Ba у различитим количинама, што се одразило на однос њихових концентрација у зрну. Најнижи однос установљен је између Ba и Sr, затим Ca и Ba, а највећи између Ca и Sr. Такође, резултати су показали да година испитивања, генотип и комбинација значајно утичу на однос концентрације Ca:Sr, Ca:Ba и Ba:Sr.

КЉУЧНЕ РЕЧИ: баријум, калцијум, однос концентрације, нивои плоидности, пшеница, стронцијум

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IVANA MAKSIMOVIĆ

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