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*Hans P. van Egmond**

MYCOTOXINS: RISKS, REGULATIONS AND EUROPEAN CO-OPERATION

ABSTRACT: Mycotoxins and mycotoxicises have been problems of the past and the present, but scientific attention for mycotoxins did not start until the early 1960's. Nowadays, many mycotoxins are known, and their occurrence in food and animal feed may cause various adverse effects on human and animal health, including carcinogenic, hepatotoxic, immunotoxic, nephrotoxic, neurotoxic, oestrogenic and teratogenic effects. Some important mycotoxins include the aflatoxins, ochratoxin A, the fumonisins and the trichothecenes, and their significance is briefly described. To protect human and animal health, many countries have enacted specific regulations for mycotoxins in food and animal feed. Risk assessment is a major factor for scientific underpinning of regulations, but other factors such as availability of adequate sampling and analysis procedures also play an important a role in the establishment of mycotoxin regulations. In addition, socio-economic factors such as cost-benefit considerations, trade issues and sufficiency of food supply are equally important in the decision-taking process to come to meaningful regulations. Nowadays, more than 100 countries have formal mycotoxin regulations for food and feed. The mycotoxin regulations are the most stringent in the EU, where various organizations and pan-European networks contribute to combat the mycotoxin problem. It is to be expected that mycotoxins will stay with us in the future and climate change might have a negative influence in this respect. Several possibilities exist to mitigate the problems caused by mycotoxins. In particular prevention of mould growth and mycotoxin formation is key to the control of mycotoxins.

KEYWORDS: aflatoxin, climate, Europe, fumonisin, mycotoxin, ochratoxin, prevention, regulation, risk, trichothecene

INTRODUCTION

Mycotoxins are secondary metabolites of fungi which are capable of producing acute toxic, carcinogenic, mutagenic, teratogenic and estrogenic effects on animals at the levels of exposure. Mycotoxins occur worldwide and they are considered significant food and feed safety issues. Mycotoxins form an important class of the natural toxins – toxic substances of natural origin. The natural toxins also include the bacterial toxins, the phycotoxins, the plant toxins and the animal toxins. Toxic symptoms resulting from the intake of mycotoxins by man and animals are known as "mycotoxicoses".

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Mycotoxicoses have been known for a long time, and they have been described in history many times. The first recognized mycotoxicosis was probably ergotism (Tulasne, 1853), better known as "Saint Anthony's fire" or "Holy fire". The disease, caused by ergots alkaloids formed in the sclerotia of the fungus *Claviceps purpurea* was already described more than 1000 years ago. This disease, leading to gangrene, loss of limbs and hallucinations, often occurred in Europe in the Middle Ages. In 1890 cardiac beriberi (yellow rice disease) was described in Japan, caused by citreoviridin, a neurotoxin produced by *Penicillium citreo-viride* (Kinosita and Shikata, 1965). The ingestion of "yellow rice" by men caused vomiting, convulsions and ascending paralysis. Death could also occur within 1–3 days after the appearance of the first signs of the disease. In 1913 and again in 1944 Alimentary Toxic Aleukia broke out in Russia, due to the consumption of overwintered wheat which contained high levels of trichothecenes, produced by *Fusarium sporotrichioides* (Mayer, 1953). These toxins caused hemorrhages and malfunctioning of the human immune system, leading to many fatalities in Russian villages (Joffe, 1965). In 1952 Balkan Endemic Nephropathy was described for the first time. This disease is characterized by severe shrinkage of the kidneys, leading to human death. Ochratoxin A, produced by e.g. *Penicillium verrucosum* is, among other components, considered responsible for this serious disease.

Despite many examples of mycotoxin-caused diseases in men, mycotoxicoses remained the "neglected diseases" until the early 1960's, when Turkey X Disease in Great Britain broke out, and within a few months more than 100,000 turkeys and other poultry suddenly died, mainly in East Anglia and southern England (Stevens et al. 1960). The problem of Turkey X Disease led to a multidisciplinary approach to investigate the cause of the disease. These efforts were fruitful and the cause of the disease was found to be due to aflatoxins, highly toxic and carcinogenic compounds, found in the Brazilian groundnut meal fed to the poultry. The aflatoxins were mainly formed by two fungi, *Aspergillus flavus* and *Aspergillus parasiticus*, and hence the name 'aflatoxin' – an acronym derived from the name of the first fungus. Further elucidation of the toxic factor demonstrated that the material could be separated chromatographically into four distinct spots (Nesbitt et al., 1962). Distinction of the four substances (aflatoxins B_1, B_2, G_1 and G_2) was made on the basis of their fluorescent colour with subscripts relating to the relative chromatographic mobility.

In the period following the outbreak of Turkey X disease, a wealth of information about the aflatoxins has been produced, and many other mycotoxins have also been isolated and characterized. Since the discovery of the aflatoxins, tens of thousands of publications on mycotoxins have been published, dozens of mycotoxin conferences have been held, several international organisations got involved with mycotoxins, thematic interlaboratory research and networking projects have been initiated, professional networks have been founded and mycotoxin-dedicated scientific journals were created. Moreover, the growing knowledge about the real and potential hazards of mycotoxins to human and animal health led many countries to establish legal measures to control mycotoxin contamination of foodstuffs and animal feedstuffs.

SOME IMPORTANT MYCOTOXINS AND THEIR CHARACTERISTICS

Currently hundreds of mycotoxins are known, many or these are produced by *Aspergillus*, *Penicillium* and *Fusarium* species. Some important mycotoxins formed by these genera include the aflatoxins, the ochratoxins, the fumonisins and the trichothecenes. They probably play a role in serious human and animal diseases. Without intentionally ignoring the potential significance of the many other mycotoxins that exist, only these mentioned mycotoxins will be briefly reviewed hereafter.

Aflatoxins

Aflatoxin B_1 (Figure 1) is the most notorious of the aflatoxins, feared for its high toxicity and carcinogenicity to humans and animals. Aflatoxins are found worldwide, especially in warm and humid climate zones. Aflatoxins have also recently caused fatal human intoxications. For instance, human aflatoxicosis has occurred several times in Kenya in the last decade, due to human consumption of aflatoxin-contaminated maize, leading to more than 100 fatalities in 2004, including many children. Whereas the aflatoxins were not found in Europe until the turn of the millennium, they were detected in maize in Northern Italy a decade ago, and subsequently in milk from dairy cattle fed with this maize. Since then their occurrence gradually spread over south-eastern Europe, a phenomenon which is possibly due to climate change. Also in the Balkan, where a lot of maize is produced, the occurrence of aflatoxins have become an issue of concern, including problems with exports to the EU in 2013. An important feature in dairying is the fact that aflatoxin B_1 shows a significant carry-over (up to 6%; Veldman et al., 1992) into the milk in its hydroxylated form aflatoxin M_1 (figure 1). Aflatoxin M_1 is a suspected human carcinogen and its occurrence in milk and milk products should therefore be limited as much as possible.

Ochratoxin A

Ochratoxin A, formed from isocoumarin and phenylalanine (Figure 2) is another carcinogenic mycotoxin, produced by *Aspergillus* and *Penicillium* species, *A. ochraceus* and *P. verrucosum* in particular. *Penicillium* tends to be more prevalent in cooler climates and *Aspergillus* in warmer climates. Ochratoxin A has been found in many countries. The toxin is a renal carcinogen in mice and rats and it is nephrotoxic in all animal species tested, while pigs are particularly sensitive. Ochratoxin A is also highly immunotoxic in mice. In addition, it has been associated with Balkan Endemic Nephropathy (BEN), a human kidney disease leading to shrinkage of the kidneys. It has also been related with the occurrence of urogenital tract tumors in animals and possibly in humans. Nowadays, it is believed that also other compounds may play a

role in the etiology of BEN, e.g. aristolochic acid, a toxic plant constituent (Schmeiser et al., 2012). The main route of human exposure to ochratoxin A is through consumption of vegetable products, contaminated with ochratoxin A (e.g. cereals, coffee, cocoa, beans, wine, beer, spices, dried vine fruit, liquorice), but it can also occur by transfer via animals such as pigs. Pig blood and plasma are used in the preparation of various sausages, thus meat products can become contaminated with this toxin.

Fumonisin B_1

Natural occurrence of fumonisins is specifically associated with maize, where they are produced specifically by *Fusarium verticillioides* and *F. proliferatum*. Fumonisin B_1 (Figure 3) is the most significant of the fumonisins in terms of occurrence and toxicity. Contrary to most of the other mycotoxins, the fumonisins do not have cyclic structures, but possess long aliphatic chains with two ester-linked hydrophilic side chains and a primary amine moiety. Fumonisin B_1 can induce hepatic cancer in rats and epidemiological studies suggest a possible role in human esophageal cancer in Africa and Asia. In husbandry animals, dramatic effects caused by fumonisin B_1 in the feed have been observed in horses and pigs. In horses leukoencephalomalacia (ELEM or hole-in-the-head syndrome) has been described, a neurological disorder in the brains, which may lead to death within 2-3 days. In pigs fumonisin B_1 may cause pulmonary edema, a fatal disease in which the animals' lungs are filled with fluid. Residues of fumonisins are not known to occur in animal products such as meat, milk and eggs and this is not an issue of potential concern.

Trichothecenes

Trichothecenes constitute a family of more than 100 structurally related compounds, primarily produced by *Fusarium* species. Deoxynivalenol (Figure 4) is the most commonly occurring trichothecene, detected frequently in maize, wheat and barley, in virtually all regions of the world. The toxin is particularly produced by *F. graminearum* and *F. culmorum*. Deoxynivalenol exhibits toxic effects in all animal species investigated and like most of the mycotoxins it has an adverse effect on the immune system. The susceptibility to deoxynivalenol may vary among species, and pigs are particularly sensitive, where the intake of the toxin may lead to nausea, vomiting (deoxynivalenol = vomitoxin), feed refusal, reduced feed intake and weight reduction. These phenomena may lead to significant economic damage in the feed industry. The emetic effect also occurs in humans, and vomiting is one of the earliest symptoms of trichothecene poisoning. The exposure of children to deoxynivalenol may lead to growth retardation. Carry-over of deoxynivalenol from animal feed into animal products hardly occurs. Deoxynivalenol survives some food processing, such as milling, baking and boiling.

MYCOTOXIN REGULATIONS

The first food regulation was probably promulgated approx. 3500 years ago by a king of the Hittites in what is now Turkey. That law already focused on the two goals of modern food laws: the protection of health and the prevention of fraud. In the early days of food legislation the protection of health was mostly a local affair, and municipal ordinances were promulgated for the purpose. But in the beginning of the 20th century this situation changed and national statutory food legislation was enacted, thanks to the development of auxiliary sciences such as chemistry, bacteriology and microscopy. Specific mycotoxin regulations did not appear until the late 1960s, approx. 10 years after the discovery of the aflatoxins. Around the turn of the millennium regional harmonization of mycotoxin regulations in food and feed started to take place in the EU (European Union), MERCOSUR (Mercado Comun del Sur), Australia/New Zealand, GCC (Gulf Cooperation Council), ASEAN (Association of South East Asian Nations) and COMESA (Common Market of Eastern and Southern Africa).

A most important factor for drafting meaningful mycotoxin regulations is risk assessment, which is the scientific underpinning of regulations and limits. Formal health risk assessments have been carried out by authoritative international organizations, such as JECFA (FAO/WHO Joint Expert Committee on Food Additives and Contaminants) and EFSA (European Food Safety Authority). Risk assessment involves a number of steps: hazard identification, hazard characterization, exposure assessment and risk characterization. Risk assessment is one of the pillars of the Risk Analysis Framework, the other pillars are risk management and risk communication. Whereas risk assessment is primarily the responsibility of scientific committees, risk management is primarily the responsibility of regulators and policy makers. Risk communication is the communication between risk assessors and managers, and with the public.

In addition to hazard assessment and exposure assessment other factors are also important when it comes to the establishment of mycotoxin regulations in food and feed. These include the availability of methods of sampling and analysis. The distribution of the concentration of mycotoxins in products is an important factor to be considered in establishing regulatory sampling criteria. The distribution can be very heterogeneous, as is the case with aflatoxins in peanuts and figs. The number of contaminated peanut kernels in a lot is usually very low, but the contamination level within a kernel can be very high. If insufficient care is taken for representative sampling, the mycotoxin concentration in an inspected lot may therefore be wrongly estimated. Reliable analytical methods will have to be available to allow enforcement of the regulations in daily practice. In addition to reliability, simplicity is desired, as it will influence the amount of data that will be generated and the practicality of the ultimate measures taken. Socio-economic factors play an important role, such as cost-benefit considerations and trade issues. Finally, there must be sufficiency of food supply, which can be a problem in the developing coun-

tries that may be faced with shortage of food and severe mycotoxin problems in their food and feed supply at the same time. Weighing the various factors in the decision-taking process to come to regulations is not a trivial task, and common sense is a major factor in reaching a decision. Despite the dilemmas, many countries have established formal mycotoxin regulations and limits.

Over the years there have been several international inquiries on mycotoxin regulations, from 1981 to 2012. These yielded details about tolerances, legal bases, responsible authorities and official protocols of sampling and analysis, and have resulted in various publications, e.g. by FAO (2004) and by Van Egmond et al. (2007). In 2013 there were more than 100 countries worldwide that have enacted regulations or detailed guidelines for the control of mycotoxins in food and animal feed. These regulations are related to aflatoxins (B_1, B_2, G_1, G_2), aflatoxin M_1, trichothecenes (deoxynivalenol, diacetoxyscirpenol, T-2 toxin and HT-2 toxin), fumonisins (B_1, B_2, B_3), agaric acid, ergot alkaloids, ochratoxin A, patulin, phomopsins, sterigmatocystin and zearalenone.

When reviewing the existing legislation on mycotoxins in certain parts of the world, it can be observed that mycotoxin regulations develop continuously. In Europe for instance, and particularly in the European Union, the regulatory and scientific interest in mycotoxins has undergone a development in the last 15 years from autonomous national activity towards more EU-driven activity with a structural and network character. In 2013 harmonized EU limits exist for 63 mycotoxin-food/feed combinations. In addition, harmonized guidance limits are in place for 18 mycotoxin-feed combinations, while indicative levels for 15 mycotoxin-feed combinations have been set. The direct or indirect influence or involvement of European organisations and programs in the EU mycotoxin regulatory developments is significant. They include the Rapid Alert System for Food and Feed (RASFF), the European Food Safety Authority (EFSA), the European Standardization Committee (CEN) and the EU and National Reference Laboratories for Mycotoxins (EU-RL and NRLs); see also next section "EUROPEAN CO-OPERATION".

Regulatory limits on mycotoxins in food and feed are under constant debate among trading countries. Their enactment has increased awareness of the issues caused by mycotoxins and has led research, industry and policy makers to work on solutions to combat the problems.

EUROPEAN CO-OPERATION

Since the discovery of the aflatoxins, several international organizations got involved with mycotoxins, thematic interlaboratory research and networking projects have been initiated and professional networks have been founded. Moreover the growing knowledge about the real and potential hazard of mycotoxins to human and animal health led many countries to establish legal measures to control mycotoxins in foodstuffs and animal feedstuffs. Especially

in Europe various activities take place to combat the mycotoxin problem. Examples of European collaboration on mycotoxin issues can be found in the Rapid Alert System for Food and Feed, the European Food Safety Authority, the European Committee for Standardization and the EU- and National Reference Laboratories for Mycotoxins.

Rapid Alert System for Food and Feed

Already for several decades the European Union has a rapid alert system for the risk of contaminants in food and feed (RASFF) that are harmful to human health. The system involves a quick information exchange among the competent authorities of the Member States (including EFTA/EEA countries), the European Commission itself and the EFSA. In the case of problems in the food chain of direct risk to human health, the RASFF facilitates that necessary measures can be taken to recover the consumer safety. In 2011, the RASFF received over 600 border rejection notifications connected to risks for human health by mycotoxins. Figure 5 shows that the problems with mycotoxins were much greater than those of other food and feed menaces. Almost 90% of these mycotoxin notifications were related to aflatoxins in the product group of nuts (pistachio nuts, peanuts, hazelnuts, almonds) and nut products (peanut butter) which were imported in 2011 in the EU. The RASFF notifications provide useful data for the development of new EU regulations and safeguard measures, and thus contribute to the improvement and maintenance of European food safety.

European Food Safety Authority

The European Food Safety Authority (EFSA) is an independent body of the European Commission, established in 2002 and, among other tasks, charged with the development of risk assessments on issues of concern in the food and feed supply. EFSA publishes its advices in the form of scientific opinions, which form the main scientific basis for the preparation of EU regulations (Anonymus, 2006). Opinions about risks of mycotoxins in food and feed are adopted in EFSA's Panel on Contaminants in the Food Chain, and are based on comprehensive documents produced in dedicated working groups of European experts. Over the years EFSA has published various opinions on its website and in the EFSA Journal about mycotoxins in food and animal feed, and several more are in preparation. Published EFSA mycotoxin risk assessments include those about aflatoxins, *Alternaria* toxins, citrinin, ergot alkaloids, fumonisins, ochratoxin A, phomopsins, several trichothecenes and zearalenone. EFSA opinions are quite influential in the decision-making process by European risk managers to come to meaningful EU-harmonized mycotoxin regulations and limits.

European Committee for Standardization

The European Committee for Standardization (CEN) is the European equivalent of ISO. Among other tasks, CEN standardizes mycotoxin methods, which are selected according to a performance criteria approach. In Europe, CEN methods are getting increasingly important. Currently, 22 mycotoxin methods have been standardized by CEN, and their number will further grow in the coming years. CEN mycotoxin methods are not mandatory for official food control in the EU. However, all CEN mycotoxin methods can be used in the EU for official food control purposes because their performance characteristics fulfil the criteria, laid down in the EU directives for sampling and analysis (Commission of the European Communities, 2006a). Regulation EC no. 882/2004 (European Commission, 2006b) provides that sampling and analysis methods used in the context of official controls shall comply with relevant Community rules or if no such rules exist, with internationally recognized rules or protocols, for example those that CEN has accepted. The view of the European Commission on CEN standards is clear: "*The establishment of standardized methods of analysis is of utmost importance to guarantee a uniform application and control of the European legislation in all Member States. Standardized methods of analysis are an indispensable element in guaranteeing a high level of food safety*".

EU- and National Reference Laboratories for Mycotoxins

The European Commission's Joint Research Centre/Institute for Reference Materials and Measurements (Geel, Belgium) fulfills the role of EU Reference Laboratory (EU-RL) for Mycotoxins. These tasks are related to mycotoxins in vegetable products, and human exposure through this route is in fact, much more significant than the indirect exposure through carry-over of mycotoxins from feed into animal products. However, the latter route is also considered relevant (e.g. in relation to aflatoxin M_1 in dairy products) and the RIKILT Institute of Food Safety (Wageningen, the Netherlands) is formally charged with the task of EU-RL for mycotoxins in products of animal origin. The tasks, duties and requirements for EU-RLs for food and feed and for animal health have been published (Commission of the European Communities, 2006b). The EU-RL for mycotoxins has been created in order to take, among other duties, initiatives and to co-ordinate activities related to the development, improvement and application of sample preparation and methods of analysis for the official control of maximum levels for mycotoxins in food and feed. Among the tasks mentioned is the function "*to provide technical assistance to the Commission and, upon its request, to participate in international forums relating to the area of competence, concerning in particular the standardization of analytical methods and their implementation*". So, it is evident that the link with the European Standardization Committee (CEN) is important.

In addition to these structural Europe-coordinated activities, several EU projects in the 7th Framework Programme had a strong focus on mycotoxins, such as CON*ff*IDENCE and MycoRed. CON*ff*IDENCE (www.conffidence.eu) was a Large Collaborative Project in the Food, Agriculture and Fisheries, and Biotechnology Program, focusing on the development and validation of simple, fast, multi-analyte, multi-class detection methods. The project, which ended in late 2012, contained a Work Package Biotoxins, in which (among many other tasks) a multiplex dipstick method was successfully developed and validated, able to simultaneously detect the *Fusarium* mycotoxins deoxynivalenol, zearalenone, T-2 and HT-2 toxins, and fumonisins. MycoRed (www.mycored.eu) is another Large Collaborative Project in the same program, dedicated to the "Reduction of mycotoxins in food and feed chains". In MycoRed the focus is on aflatoxins, ochratoxin A, trichothecenes, zearalenone and fumonisins, and a lot of research was devoted to prevention and reduction of mycotoxin formation. Within the successful project, which finishes in late 2013, various international mycotoxin workshops and conferences were also organized in Europe, Asia, Africa and Latin America. These and other EU-sponsored research and networking projects, in their own right and collectively, are very valuable to increase our knowledge, to advance the analytical state-of-the-art and to create strong networks of collaboration on mycotoxins in Europe and beyond.

CLIMATE CHANGE, PREVENTION AND CONTROL

A contemporary issue that also has a potential influence on mycotoxins is climate change. Due to this phenomenon changes, may occur in the latitudes where certain fungi are able to compete. An example is the increasing occurrence of *Fusarium graminearum*, a nivalenol producer, in certain areas in Europe. Climate change may also lead to drought and flooding, which may result in the occurrence of more mycotoxins and changed toxin profiles. For example, since about 10 years ago aflatoxins have been found in south-eastern Europa in particular in maize, and the affected areas are becoming larger. Increases of plant diseases and insect manifestations are also expected as a result of climate change, and these may have a significant effect on mycotoxins as well.

Potentially successful measures to combat and control mycotoxins include (but are not limited to) the following:

- Prevention; possibilities exist with programs to develop plants resistant to certain fungi, to improve the proper application of fungicides, and to control insects, which may act as vectors of fungal spores and may damage crops.
- Improved agricultural practices; e.g. application of irrigation of maize may assist in preventing damage from drought stress, which may lead to significantly increased aflatoxin formation.

- Biocontrol; biocompetetive exclusion is a newer technique, where non-toxigenic fungal strains are inoculated in the field to outcompete toxigenic strains. Successful application has been achieved in Nigeria with non-aflatoxigenic strains in maize and peanuts producing areas.
- Development of predictive models; theoretical models have been developed, e.g. at RIKILT, that forecast the occurrence of emerging mycotoxins in food and feed chains at an early stage so that timely management measures can be taken to minimize their impact on food and feed safety.
- Post-harvest; it is essential that agricultural harvest is rapidly dried and stored under cool and dry conditions to avoid fungal growth and mycotoxin production.
- Hygienic conditions at storage; not only control of water activity and temperature are important, but possibilities of (re-) infection by moulds must be excluded as good as possible.
- HACCP in production chains, novel food processing; the rigorous application of Hazard Analysis Critical Control Point procedures at diverse stages of the food and feed production and processing chain is recommended to achieve final products that fulfill to desired criteria with respect to Mycotoxin contamination. The development of novel food processing techniques that exclude the possibility of fungal re-infection can be also effective.
- Cleaning, sorting, application of decontamination systems; various physical, chemical and biological procedures have been developed to reduce or degrade mycotoxin contamination of food and animal feed. Not all of them are (economically) feasible in practice, and they should be considered with care.
- Improved methods to detect fungi; their application by phytopathologists may help others to be alarmed at an early stage that undesired fungi are developing or have developed, able to produce mycotoxins.
- Improved sampling and analytical methods for mycotoxins; errors made in sampling and in analytical methodology can be quite substantial, and provide little comfort for those who must pay for analytical measurements, or who base potentially important decisions on the data generated.
- Drafting of regulations; whilst mycotoxin regulations themselves will not directly lead to reduction of mycotoxins, they may force food and feed business operators to apply good agricultural, good manufacturing, good storage and good hygienic practices with regard to mycotoxins, eventually leading to reduced chances of undesired human exposure.
- Training, education and networking; awareness, experience, capacity-building and international collaboration are all essential elements in the combat of mycotoxins, both at local and at global level.

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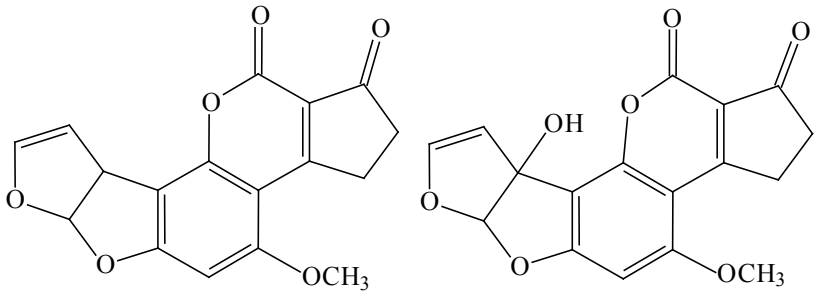

Figure 1: Chemical structures of aflatoxin B_1 (left) and aflatoxin M_1 (right)

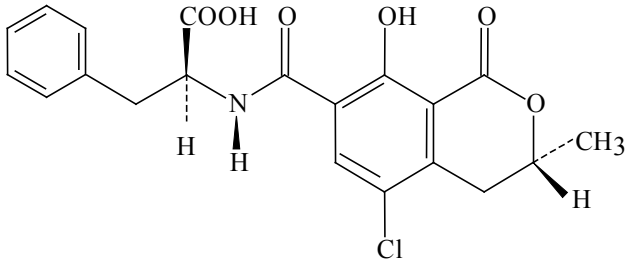

Figure 2: Chemical structure of ochratoxin A

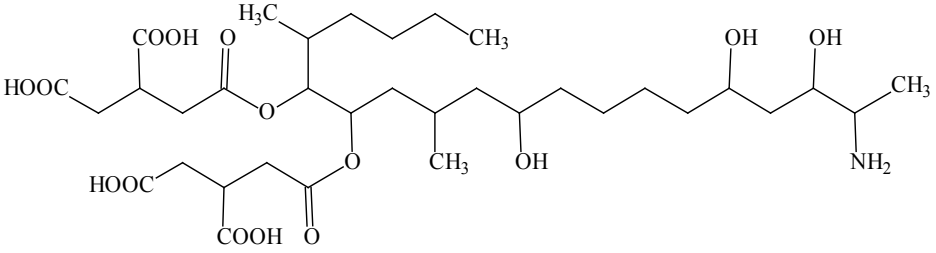

Figure 3: Chemical structure of fumonisin B_1

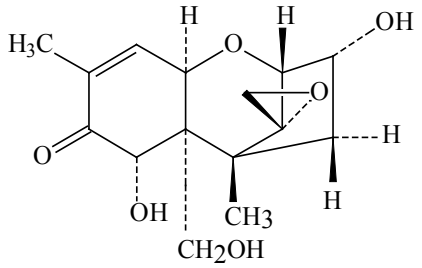

Figure 4: Chemical structure of deoxynivalenol

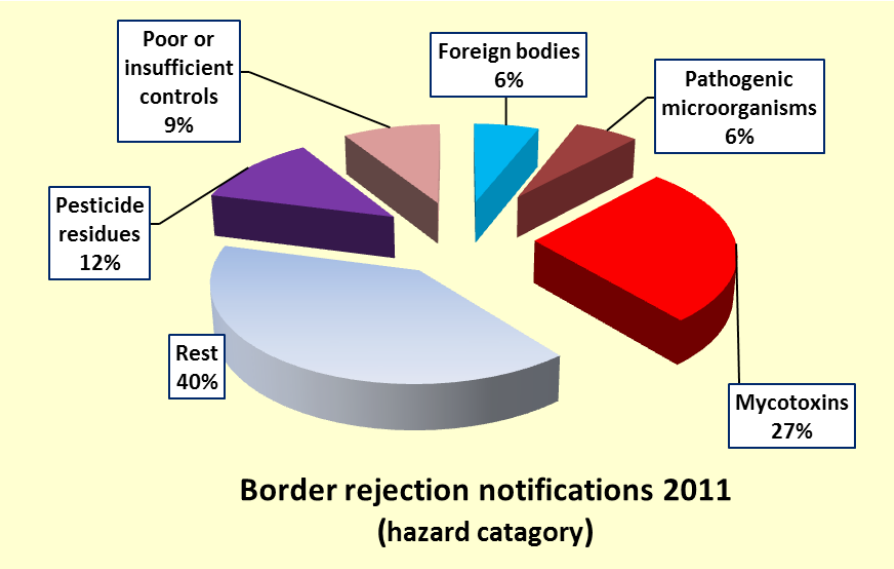

Figure 5: Rapid Alert System for Food and Feed: Border rejection notifications 2011

МИКОТОКСИНИ: РИЗИЦИ, ПРОПИСИ И ЕВРОПСКА САРАДЊА

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РЕЗИМЕ: Микотоксини и микотоксикозе као и данас, и у прошлости су представљали проблем, али научног интересовања за њих није постојало све до почетка шездесетих година прошлог века. Многи микотоксини данас су познати, а њихова појава у храни за људе као и у храни за животиње може да изазове различите негативне ефекте на здравље људи и животиња, укључујући канцерогене, хепатотоксичне, имунотоксичне, нефротоксичне, неуротоксичне, естрогене и тератогене ефекте. Неки од важних микотоксина су афлатоксин, охратоксин А, фумонизин и трихотецен, а њихов значај је укратко описан. Да би заштитиле здравље људи и животиња, многе земље донеле су посебне прописе за микотоксине у храни за људе и у храни за животиње. Процена ризика главни је фактор за научну потврду прописима, али и други фактори као што су доступност адекватног узорковања и анализа процедура који такође играју важну улогу у формирању прописа у вези са микотоксинима. Поред тога, социо-економски фактори, као што су трошкови, трговина и довољна снабдевеност храном, подједнако су важни у процесу доношења одлука да би се донели важни прописи. Данас више од 100 земаља има формалне прописе у вези са микотоксинима у храни за људе и храни за животиње. Прописи у вези са микотоксинима најстрожији су у ЕУ, где различите организације и пан-европске мреже доприносе борби против проблема

микотоксина. Треба очекивати да ће микотоксина бити и у будућности, а климатске промене могу да имају утицај у том погледу. Постоји неколико могућности за ублажавање проблема изазваних микотоксинима. Спречавање појаве плесни и формирања микотоксина кључно је за контролу микотоксина.

КЉУЧНЕ РЕЧИ: афлатоксин, клима, Европа, фумонизин, микотоксин, охратоксин, превенција, пропис, ризик, трихотецен

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THE INCIDENCE OF *PYRENOCHAETA TERRESTRIS* IN ROOT OF DIFFERENT PLANT SPECIES IN SERBIA

ABSTRACT: Root samples of cereals (oats, wheat, barley, maize and sorghum), vegetables (garlic, onion, pepper, cucumber, pumpkin, carrot and tomato), industrial plant (soya bean) and weeds (Johnson grass, barnyard grass and green bristle-grass) collected in different agroecological conditions in Serbia were analysed for the presence of *Pyrenochaeta terrestris*. The fungus was found in 42 out of 51 samples (82.4%), while the incidence varied from 2.5 to 72.5%. The highest incidence was detected in cereals (average 30.3%), and then in weeds of the *Poaceae* family (average 14.2%). Considering single species, maize (up to 72.5% in root) and Johnson grass (up to 37.5%) were mostly attacked by this fungus. The lowest incidence of the fungus was determined in vegetable crops (average 6.7%). Red to reddish discoloration of root was correlated with the incidence of the fungus. Obtained data indicate that *P. terrestris* is widespread in Serbia and conditions for its development are favourable.

KEYWORDS: *Pyrenochaeta terrestris*, root, incidence, cereals, soya bean, vegetables, weeds

INTRODUCTION

Pyrenochaeta terrestris (Hansen) Gorenz, Walker & Larson (syn. *Phoma terrestris* Hansen), a common soil inhabitant, affects root of many plants, particularly monocots, including maize, cereals, grasses, sorghum and sugar cane (Walker, 1952). It has also been reported in cucumber, carrot, tomato, pepper and other plant species (Walker, 1952). This pathogen has been identified most frequently in onion (Yassin et al., 1982) and in maize (Mao et al., 1998). Rotting results in total plant collapse. It appears in the initial growing stages, but most commonly occurs in root of nearly mature plants.

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Since the 1950s there have been a few published papers on the appearance of the fungus in other plants than onion or maize.

The first identification of *P. terrestris* in Serbia was in root of three types of *Allium* species (onion, garlic and leek) (Aleksić et al., 1989), and then in root of maize and other hosts (Petrović and Lević, 1999). Recent studies indicate that this fungus is common in root of maize in Serbia (Lević et al., 2011, 2012a, 2012b), while there is no data on its incidence in root of other hosts. Therefore, we have collected root samples different plant species belonging to the group of cereals, vegetables, soya bean and weeds in maize crops. The incidence of *P. terrestris* in Serbia was determined on the basis of the mycological analysis of collected samples.

MATERIAL AND METHODS

Sample collection. Root samples were collected from 16 plant species: cereals (oats, wheat, barley, maize and sorghum), vegetables (garlic, onion, pepper, cucumber, pumpkin, carrot and tomato), weeds (Johnson grass, barnyard millet and green bristle-grass) and soya bean. These samples have been collected from 27 localities in Serbia, mostly from the Province of Vojvodina (Fig. 1). Samples of maize leaf sheaths have also been collected from these localities. Symptoms of root rot in the collected samples have been described and the fungus has been isolated on potato dextrose agar (PDA).

Isolation and incidence of the fungus. Roots of individual plants were washed gently in running tap water to remove soil, and then they were excised. A small section (3-4 mm) was usually cut from the margin of lesions developed in root. Then, the sections were washed for 2 h in running tap water, sterilised in 1% sodium hypochlorite (NaOCl) for 3 min, rinsed three times in sterile distilled water, and blotted dry between two layers of soft paper. Forty root sections of each plant were arranged in five Petri dishes on PDA and incubated for seven days under laboratory conditions. This procedure was also applied to the samples of maize leaf sheaths. After incubation, root and leaf sheath sections were examined for a fungal growth under a stereomicroscope (x15-25).

Identification and maintenance of isolates. In order to reliably identify the fungus, the fragments of colonies developed on root and leaf sheath sections were transferred to PDA and carnation leaf agar (CLA). The fungus was incubated on PDA at 25° C in the dark and on CLA at 25° C with alternating 12h periods of darkness and combined fluorescent and near ultraviolet light. The composition and the preparation of these media were described by Burgess et al. (1994).

The identification of *P. terrestris* was performed according to the description by Punithalingam and Holliday (1973), Zitter et al. (1996), Gorenz et al. (1948) and Ferreira et al. (1991), and on the basis of our experience gained during studying this fungus (Petrović and Lević, 1999; Lević et al., 2011, 2012a, 2012b).

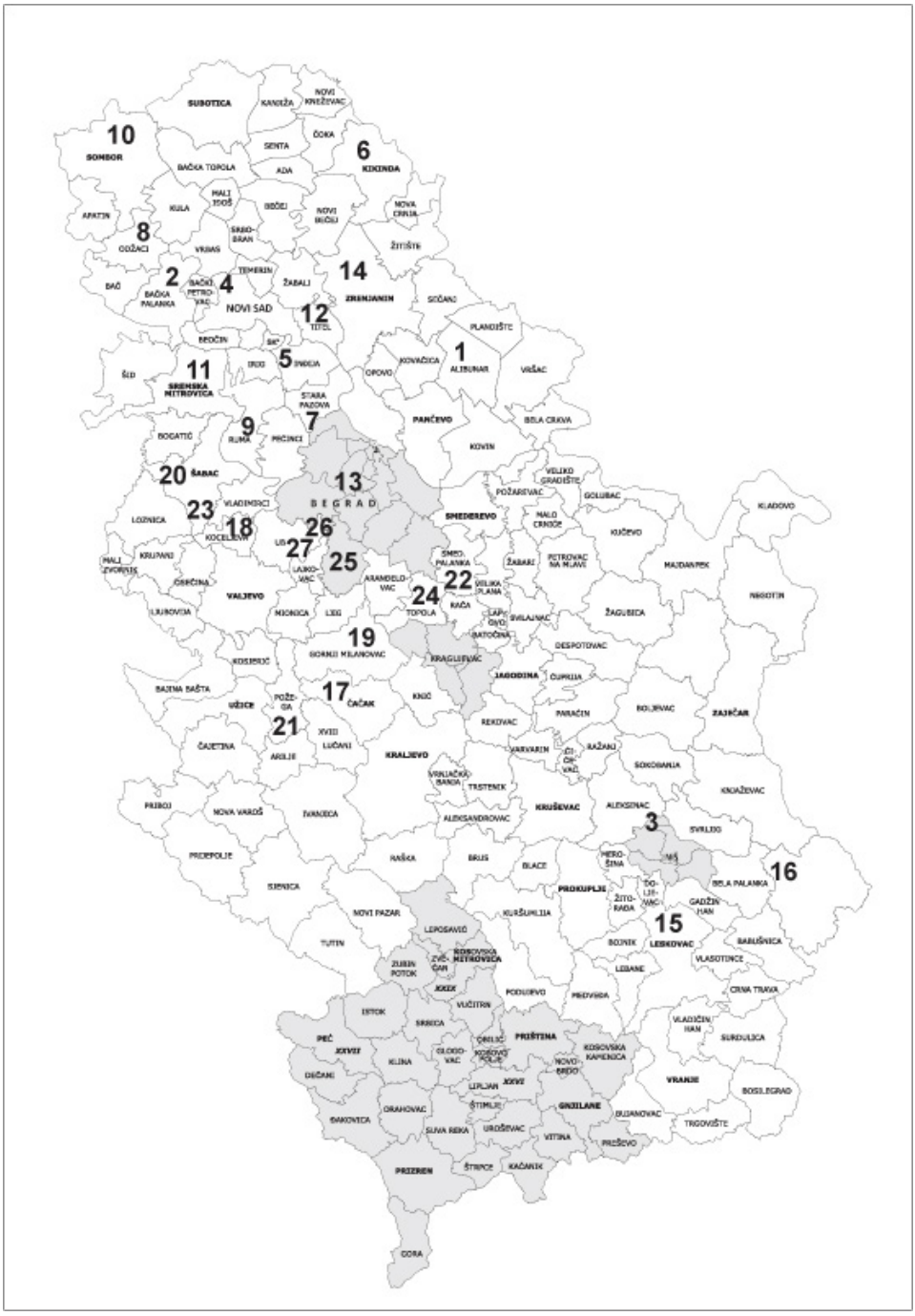

Figure 1. Map of Serbia and marked localites in which plant samples were collected.

P. terrestris cultures, isolated in the course of this study and designated as MRIZP, were stored on agar (PDA and CLA) slants in 5 ml vials within the collection of the Maize Research Institute, Zemun Polje.

Statistical analysis. The incidence (I) was calculated from the number of sections in a surface sterilised root sample colonised by *P. terrestris* as follows (Lević et al., 2011): I = [Number of root sections in which *P. terrestris* was detected/Total number of root sections] x 100.

RESULTS

The prevalent symptom in root samples on the majority of studied plant species was a combination of red (pinkish, reddish, red, purple) and brown discoloration (Table 1). Moreover, brown discoloration without redness was determined in root samples of pepper (*Capsicum annum* L), cucumber (*Cucumis sativus* L.) and squash (*Cucurbita pepo* L). Pycnidia formed below the root epidermis were observed only in the case of tomato (*Lycopersicon esculentum* Mill.). Fusiform lesions were formed in root of Johnson grass (*Sorghum halepense* L.) and maize (*Zea mays* L.).

Table 1. Description of symptoms on root samples of tested plant species

No.	Plant species	Symptoms on root samples
	Allium cepa L.	Pinkish, reddish brown & pale brown discoloration
	Allium sativum L.	Pinkish, reddish brown & brown discoloration
	Avena sativa L.	Reddish brown & brown discoloration
	Capsicum annuum L.	Brown discoloration
	Cucumis sativus L.	Brown discoloration
	Cucurbita pepo L.	Brown discoloration
	Daucus carota L.	Brown & reddish grey discoloration
	Echinochloa crus-galli (L.) P. Beauv.	Purple red & brown discoloration
	Glycine max (L.) Merr.	Reddish brown & brown discoloration
	Hordeum vulgare L.	Reddish brown & brown discoloration
	Lycopersicon esculentum Mill.	Brown reddish root & formed pycnidia under the epidermis
	Setaria viridis (L.) P.B.	Brown & reddish brown discoloration
	Sorghum bicolor (L.) Moench.	Pinkish, reddish brown, brown & scab root
	Sorghum halepense Pers.	Red, reddish brown & scab root, formed fusiform spots on root
	Triticum aestivum L.	Reddish brown & brown discoloration
	Zea mays L.	Reddish, purple red & brown root, formed fusiform spots on root

P. terrestris was determined in 42 out of 51 root samples or in 12 out of the 16 plant species. The incidence of this fungus varied depending on plant species and agroecological conditions under which these species were culti-

vated or grew in the wild (Table 2). On the average, the highest incidence of *P. terrestris* was noticed in cereals (30.3%), then in weeds (14.2%) and less in root of vegetables (6.7%). The incidence of *P. terrestris* varied from 2.5% to 75% in cereals, from 0% to 22% in vegetables and from 2.5% to 37% iin root of weeds.

Table 2. Incidence of *P. terrestris* on roots of different plant species

No.	Location (No. on map)	Plant species	*P. terrestris* incidence (%)
		Cereals	
	Zemun Polje (13)	*Avena sativa* L.	50.0
	Krnješevci (7)	*Hordeum vulgare* L.	5.0
	Zemun Polje (13)	*Sorghum bicolor* (L.) Moench.	14.2
	Inđija (5)	*Triticum aestivum* L.	2.5
	Ruma (9)	*Triticum aestivum* L.	2.5
	Zemun Polje (13)	*Triticum aestivum* L.	25.0
	Badince (15)	*Zea mays* L.	22.5
	Blato (16)	*Zea mays* L.	47.5
	Družetić (18)	*Zea mays* L.	37.5
	Gornji Milanovac (19)	*Zea mays* L.	72.5
	Inđija (5)	*Zea mays* L.	10.0
	Krnješevci (7)	*Zea mays* L.	37.5
	Krnješevci (7)	*Zea mays* L.	22.5
	Požega (21)	*Zea mays* L.	42.5
	Smederevska Palanka (22)	*Zea mays* L.	45.0
	Titel (12)	*Zea mays* L.	22.5
	Zemun Polje (13)	*Zea mays* L.	55.5
	Average		30.3
		Vegetables	
	Alibunar (1)	*Allium cepa* L.	5.0
	Bajevac (27)	*Allium cepa* L.	5.0
	Šabac (23)	*Allium cepa* L.	15.0
	Veliki Crljeni (25)	*Allium cepa* L.	0.0
	Zemun Polje (13)	*Allium cepa* L.	20.0
	Alibunar (1)	*Allium sativum* L.	22.5
	Smederevska Palanka (22)	*Allium sativum* L.	12.5
	Topola (24)	*Allium sativum* L.	2.5
	Veliki Crljeni (25)	*Allium sativum* L.	0.0
	Badince (15)	*Capsicum annuum* L.	0.0
	Veliki Crljeni (25)	*Capsicum annuum* L.	0.0
	Veliki Crljeni (25)	*Cucumis sativus* L.	0.0
	Veliki Crljeni (25)	*Cucurbita pepo* L.	0.0
	Badince (15)	*Daucus carota* L.	0.0
	Veliki Crljeni (25)	*Daucus carota* L.	0.0

Badince (15)	*Lycopersicon esculentum* Mill.	20.8
Sremska Mitrovica (11)	*Lycopersicon esculentum* Mill.	17.5
Veliki Crljeni (25)	*Lycopersicon esculentum* Mill.	0.0
Average		6.7
	Industrial plants	
Zemun Polje (13)	*Glycine max* (L.) Merr.	27.5
	Weeds	
Družetić (18)	*Echinochloa crus-galli* (L.) P.Beauv.	20.0
Zemun Polje (13)	*Echinochloa crus-galli* (L.) P.Beauv.	30.0
Zemun Polje (13)	*Setaria viridis* (L.) P.B.	15.0
Bačka Palanka (29	*Sorghum halepense* Pers.	7.5
Čačak (17)	*Sorghum halepense* Pers.	7.5
Donja Toponica (3)	*Sorghum halepense* Pers.	20.0
Futog (4)	*Sorghum halepense* Pers.	7.5
Inđija (5)	*Sorghum halepense* Pers.	5.0
Jelenča (20)	*Sorghum halepense* Pers.	15.0
Kikinda (6)	*Sorghum halepense* Pers.	2.5
Odžaci (8)	*Sorghum halepense* Pers.	20.0
Sombor (10)	*Sorghum halepense* Pers.	5.0
Zemun Polje (13)	*Sorghum halepense* Pers.	37.5
Zrenjanin (14)	*Sorghum halepense* Pers.	15.0
Zvečka (26)	*Sorghum halepense* Pers.	5.0
Average		14.2

The incidence of *P. terrestris* in small grain cereals was 50% in root of oats (*Avena sativa* L.), 25% in wheat (*Triticum aestivum* L.) and 5% in barley (*Hordeum vulgare* L.) at Zemun Polje, and 2.5% in wheat root in two localities (Inđija and Ruma).

The fungal incidence in maize root varied, depending in the locations, from 10% (Inđija) to 72.5% (Gornji Milanovac). High incidence of *P. terrestris* in maize root was determined at the locations of Zemun Polje (55.5%), Blato (47.5%), Smederevska Palanka (45%) and Požega (42.5%).

In some *P. terestris* was isolated from maize leaf sheaths (Titel and Zemun Polje) localilies. From the leaf sheath with purple red symptoms the fungus was isolated in 16.7% samples, and from the bluish tissue it was 27.5% (data not presented in table).

P. terrestris was found in only half of the collected samples of vegetables, and with the incidence ranging from 2.5% to 22%. The incidence of the fungus in positive samples varied from 5% (Alibunar and Bajevac) to 20% (Zemun Polje) in onion root (*Allium cepa* L.), from 2.5% (Topola) to 22.5% (Alibunar) in garlic (*Allium sativus* L.) and from 17.5% (Sremska Mitrovica) to 20.8% (Badince) in tomato (*Lycopersicon esculentum* Mill.). The fungus was not found in root of pepper, cucumber, squash and carrot.

As far as industrial plants are concerned, only soya bean root was analysed and 27.5% of analysed sample showed the presence of the fungus.

The root samples of all weed species were infected with *P. terrestris*. The fungal incidence in Johnson grass varied from 2.5% (Kikinda) to 37.5% (Zemun Polje). The moderate incidence (≤20%) was determined in two localities (Donja Toponica and Odžaci), while low incidence (5-15%) was observed in eight localities. Root of barnyard grass (*Echinochloa crus-galli* L. P. Beauv.) was infected with the fungus in the amount of 20% (Družetić) and 30% (Zemun Polje), while 15% of green bristle-grass root (*Setaria viridis* L. P. B.) was infected.

DISCUSSION

The obtained results indicate that *P. terrestris* is a widespread pathogen in Serbia. This pathogen was determined in 25 out of 27 localities or in 12 of the 16 plant species. It is known that *P. terrestris* attacks root of numerous crop plants, such as onion, soya bean, oats, barley, wheat, maize, cucumber, tomato, pepper, carrot and other plant species (Kreutzer, 1941). In the present research, positive results were obtained for these species as well, with the exception of cucumber and carrot.

The analysis of symptoms and the detection of the fungus in root of certain plant species showed their interrelationship. In fact, *P. terrestris* was determined in all plant species with a symptom of reddish discoloration of root. On the other hand, the fungus was not present in plant species only with brown root discoloration, such as in cases of pepper, cucumber, squash and carrot. *P. terrestris* differs from other species of *Pyrenochaeta*, not only in morphological characteristics, but also as it causes red root rot (Watanabe and Imamura, 1995; Zitter et al., 1996; Lević et al., 2011, 2012a, 2012b), while other species cause black (Ramsey, 1990) or brown root rot (Ball, 1979).

The highest fungal incidence was determined in maize root (up to 72.5%), then in oats (50.0%) and Johnson grass (37.5%). In previous studies we have found that this fungus occurred early in the season and intensively developed in root of maize (Lević et al., 2012a, 2012b), which is in accordance with results obtained in this study. The relatively high incidence of the fungus in root of Johnson grass can be explained by the fact that this plant species belongs to the family *Poaceae* in which the fungus often appears (Sprague, 1944). Furthermore, perennial species of the *Poaceae* family, including Johnson grass, are widespread weeds in maize crops in Serbia (Stefanovic and Simic, 2006; Simić and Stefanović, 2006).

The incidence of *P. terrestris* in root of different plant species was affected by agroecological conditions in some localities. The effects of local agroecological conditions on the fungal incidence was most obvious in Veliki Crljeni because all the samples collected in this locality were negative for the presence of this fungus. On the other hand, the fungus was found in all samples collected in the locality of Zemun Polje and its incidence varied from 14.2% to 55.5% (average, 28.1%). The highest incidence was recorded in maize sample from Gornji Milanovac (72.5%).

In conclusion, the data show that *P. terrestris* is a very important root pathogen of maize, oats and weeds (Johnson grass and barnyard grass), which are often associated with maize crops in Serbia. These data are in accordance with the literature data indicating that this fungus usually occurs on root of plants belonging to the family of *Poaeceae*. Furthermore, the fungus was rarely and with a lower incidence determined in root of vegetable crops, and even in root of *Allium* species that are worldwide known as common hosts of *P. terrestris*.

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УЧЕСТАЛОСТ ПОЈАВЕ *PYRENOCHAETA TERRESTRIS* НА КОРЕНУ РАЗЛИЧИТИХ БИЉНИХ ВРСТА У СРБИЈИ

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РЕЗИМЕ: Узорци корена жита (овас, пшеница, јечам, кукуруз и питоми сирак), поврћа (бели лук, црни лук, паприка, краставац, бундева, шаргарепа и парадајз), индустријског биља (соја) и корова (дивљи сирак, коровски просо и зелени мухар), који су прикупљени у различитим агроеколошким условима у Србији, анализирани су на присуство *Pyrenochaeta terrestris*. Гљива је утврђена у 42 од 51 узорка (82,4%), а степен напада је варирао од 2,5% до 72,5%. Генерално, највећи степен напада гљиве утврђен је на корену жита (просек 30,3%), а затим на корену корова (просек 14,2%) из породице *Poaceae*. Међу појединачним врстама, гљива је у највећем степену утврђена на корену кукуруза (до 72,5%) и дивљег сирка (до 37,5%). Насупрот томе, гљива је ређе утврђена на корену повртарских култура (просек 6,7%). Црвенило (ружичаста, црвенкаста, црвена и љубичаста

боја) корена било је у корелацији са учесталошћу појаве гљиве. Добијени подаци указују на то да је *P. terrestris* широко распрострањена у Србији и да су повољни услови за њен развој.

КЉУЧНЕ РЕЧИ: *Pyrenochaeta terrestris*, корен, учесталост, жита, соја, поврће, коров

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DETECTION OF PHOSPHATASE ACTIVITY IN AQUATIC AND TERRESTRIAL CYANOBACTERIAL STRAINS

ABSTRACT: Cyanobacteria, as highly adaptable microorganisms, are characterized by an ability to survive in different environmental conditions, in which a significant role belongs to their enzymes. Phosphatases are enzymes produced by algae in relatively large quantities in response to a low orthophosphate concentration and their activity is significantly correlated with their primary production. The activity of these enzymes was investigated in 11 cyanobacterial strains in order to determine enzyme synthesis depending on taxonomic and ecological group of cyanobacteria. The study was conducted with 4 terrestrial cyanobacterial strains, which belong to *Nostoc* and *Anabaena* genera, and 7 filamentous water cyanobacteria of *Nostoc*, *Oscillatoria*, *Phormidium* and *Microcystis* genera. The obtained results showed that the activity of acid and alkaline phosphatases strongly depended on cyanobacterial strain and the environment from which the strain originated. Higher activity of alkaline phosphatases, ranging from 3.64 to 85.14 μmolpNP/s/dm^3, was recorded in terrestrial strains compared to the studied water strains (1.11-5.96 μmolpNP/s/dm^3). The activity of acid phosphatases was higher in most tested water strains (1.67-6.28 μmolpNP/s/dm^3) compared to the activity of alkaline phosphatases (1.11-5.96 μmolpNP/s/dm^3). Comparing enzyme activity of nitrogen fixing and non-nitrogen fixing cyanobacteria, it was found that most nitrogen fixing strains had a higher activity of alkaline phosphatases. The data obtained in this work indicate that activity of phosphatases is a strain specific property. The results further suggest that synthesis and activity of phosphatases depended on eco-physiological characteristics of the examined cyanobacterial strains. This can be of great importance for the further study of enzymes and mechanisms of their activity as a part of cyanobacterial survival strategy in environments with extreme conditions.

KEYWORDS: acid phosphatases, alkaline phosphatases, cyanobacteria, enzyme activity

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INTRODUCTION

Cyanobacteria, the oxygen-evolving photosynthetic prokaryotes originating about 3.5 billion years ago, occupy a credential position between pro- and eukaryotes (Atzenhofer et al., 2002). Cyanobacteria successfully colonise almost all kinds of terrestrial and aquatic ecosystems, due to their high ecological adaptability to different environmental conditions (Oren, 2000). They also play an important role in the global cycling of elements, such as carbon, nitrogen and phosphorus (Sanudo-Wilhelmy et al., 2001). Cyanobacteria represent productive and efficient biological system due to the fact that many cyanobacteria have ability to perform both photosynthesis and nitrogen fixation together with their efficient nutrient uptake mechanisms (Parveen and Pandey, 2011).

In the environment with widely fluctuating nutrient availability, cyanobacteria synthesize new proteins which contribute to survival of the organisms and become a part of their unique survival strategy. In order to deal with phosphate deprivation, cyanobacteria have devised a number of different measures (Pandey, 2006). Since inorganic phosphate is the only form of phosphorus that is directly used by cells in most ecosystems, there is a deficiency of phosphorus (Thingstad et al., 2005). Three main components involved in phosphorous metabolism in cyanobacteria include: inorganic phosphate (Pi) uptake, dissolved organic phosphorus (DOP) hydrolysis, and polyphosphate (polyp) biosynthesis and catabolism (Duncan, 2010). During short periods of phosphorous starvation, cyanobacteria use accumulated phosphate stored in the form of polyphosphate reserves for cellular metabolism (Bhaya et al., 2000). This enables cyanobacteria to propagate 3-4 cell divisions even when the dissolved phosphate is entirely depleted (Chorus and Mur, 1999). During long periods of phosphorus starvation, cyanobacteria produce extracellular phosphatases, extracting phosphate from a wide spectrum of organic compounds and converting it into biologically available inorganic phosphate and organic moiety (Stihl et al., 2001).

Phosphatases (phosphomonoester hydrolases – PME) represent the group of phosphohydrolases which play an important role in phosphate release in aquatic environments (Matavulj and Flint, 1987; Chróst and Suida, 2002). Phosphatases represent inducible catabolic ectoenzymes and their expression is generally regulated by the external concentration of inorganic phosphate, but the internal N:P ratio may also play a role in this process (Hoppe, 2003). After the early phase of enzyme synthesis, phosphatases accumulate in the periplasmic space and at the later stage they are released outside the cell (Pandey, 2006). Their activity is modulated by different physicochemical factors like temperature, light, pH, micro and macronutrients, salinity and heavy metals (Singh et al., 2006).

Alkaline phosphatases (APA) include a group of inducible isoenzymes (Luo et al., 2010) which optimally react in pH ranging from 7.6 to 9.6 (Chróst and Suida, 2002). Their role is to catalyze the hydrolysis of a variety of phosphate esters and to liberate inorganic phosphate (Chróst and Suida, 2002). The active center of enzyme is conserved well, although the protein features of

alkaline phosphatase are strongly divergent. Regulation of the APA synthesis is carried out through a repression-derepression mechanism and by competitive inhibition (Chróst and Suida, 2002). Phosphate causes repression of the enzyme activity in a concentration dependent manner while lower amounts of phosphate lead to derepression of the PMEase enzyme (Pandey, 2006).

Acid phosphatases represent a group of isoenzymes that optimally react in pH ranging from 4.0 to 5.5. Regulation of synthesis often takes place without any form of repression with inorganic phosphorus present in the environment (Chróst and Suida, 2002).

The importance of increased phosphorus loading in the process of eutrophication of water ecosystems was recognized at the end of the sixties (Matavulj *et al*., 1990; Pandey and Tiwari, 2003). The low TN:TP ratio, together with thermal stratification, reduces transparency and increases water temperature and pH, and frequently enhances the occurrence of cyanobacterial blooms (Mischke, 2003). Since phosphorus is an important factor in the growth of cyanobacteria and phosphorus concentration greater than 0,1 mg/L is sufficient to cause cyanobacterial blooms in aquatic ecosystems (Bartram et al., 1999), it is of great significance to test phosphatase enzyme activity in cyanobacterial strains.

The aim of the present study was to investigate the changes in PMEase activity of 11 cyanobacterial strains during the stationary phase of growth. The other objectives include comparison of PMEase activity in terrestrial and freshwater cyanobacterial strains as well as determining whether there is a difference in enzyme activity between nitrogen-fixing and non nitrogen-fixing strains.

MATERIALS AND METHODS

Cyanobacteria and culture conditions

Detection of phosphatase activity was performed in the cultures of 11 different cyanobacterial strains. Seven water cyanobacterial strains were isolated from surface waters in the region of Vojvodina (Simeunović, 2010) and four strains were isolated from different soil types in Vojvodina (Simeunović, 2005). All cyanobacterial strains that were examined belong to the Novi Sad Cyanobacterial Culture Collection-NSCCC. The studied terrestrial strains belong to the *Nostoc* and *Anabaena* genera, whereas the studied aquatic cyanobacterial strains belong to the *Microcystis, Nostoc, Phormidium* and *Oscillatoria* genera. Strain *Microcystis* PCC 7806 was purchased from Pasteur Culture Collection (http://www.pasteur.fr/bio/PCC). The cyanobacterial strains were grown in the laboratory conditions in liquid synthetic mineral medium BG-11 (Rippka et al., 1979), with or without nitrogen, depending on the ability of the strains to fix atmospheric nitrogen. The cultures were incubated photo-autrophically at 22–24ºC under illumination of cool white fluorescent light. Phosphatase activity was determined on the 21st day of incubation, during the stationary phase of growth.

Enzyme assay

Phosphatase activity (PA) in the cyanobacterial cultures was measured using the spectrophotometric method and enzyme activity was measured as the rate of hydrolysis of the phosphatase substrate p-nitrophenylphosphate (p-NPP, Sigma Aldrich), by detecting the released product, p-nitrophenol (Matavulj, 1986). Activities of alkaline and acid phosphatases of tested strains were determined at pH values of the appropriate sterile buffer (pH5 and pH9) as their potential activities at a temperature of 30°C. Phosphatase activities were determined by adding 0.3 ml of 5% p-nitrophenylphosphate into 2.4 ml of sample. After one hour of incubation, the reaction was interrupted directly by adding 10 M NaOH and the result of enzymatic reaction was a yellow product, para-nitrophenol (pNP). The intensity of the yellow color was proportional to the level of phosphatase activity of the sample and therefore the sample absorbance was measured at 420 nm using a spectrophotometer (Beckman 25). The calculation of enzyme activity was performed according to Matavulj (1986). Sterile distilled water was used as a control. All enzyme assays were done in triplicate and the results are expressed as mean values.

RESULTS

In order to determine the possible relationship between phosphatase activity and taxonomic and ecological background of the studied cyanobacteria, comparison of the enzyme activity was made during the stationary phase of the growth between water and terrestrial strains, as well as between nitrogen-fixing and non-nitrogen-fixing strains.

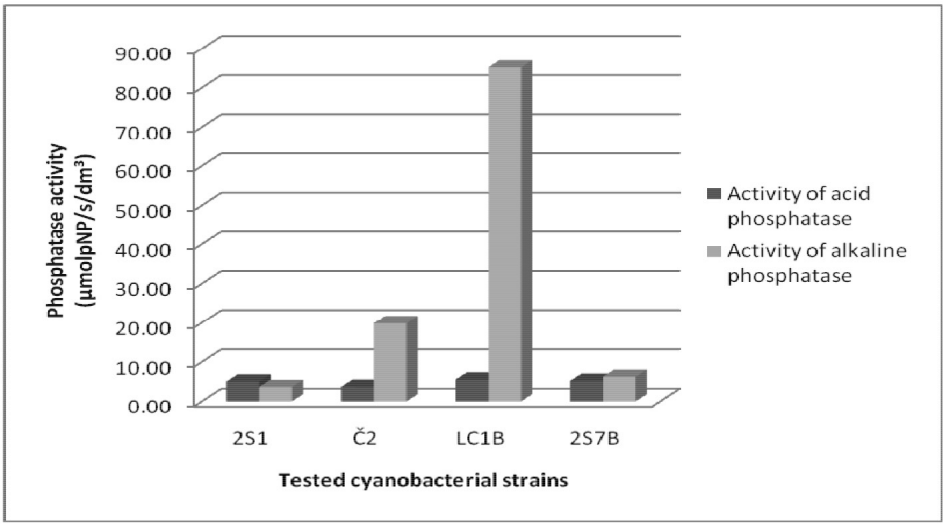

Fig. 1

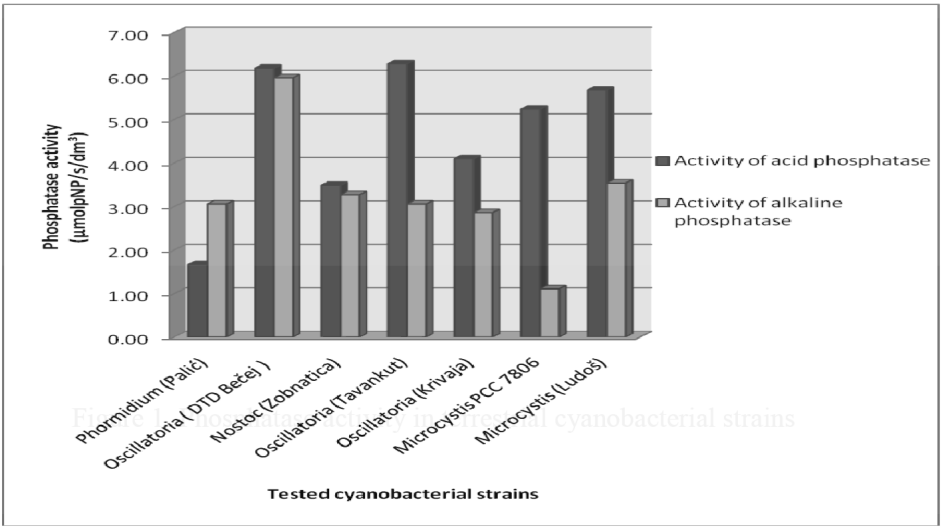

Fig. 2

When analyzing the phosphatase activity in terrestrial strains, it was observed that the activity of alkaline phosphatase ranged from 3.64 to 85.14 µmolpNP/s/dm^3, while the activity of acid phosphatase ranged from 3.66 to 5.48 µmolpNP/s/dm^3, indicating a higher activity of the enzyme alkaline phosphatase (Figure 1). The highest AP activity was detected only in *Anabaena* strain LC_1B (85.14 µmolpNP/s/dm^3) compared to the other terrestrial strains, and the lowest activity was recorded in *Nostoc* strain $2S_1$ (3.64 µmolpNP/s/dm^3). Acid phosphatase was less active in the studied terrestrial strains, ranging from 3.66 to 5.48 µmolpNP/s/dm^3. The exception was the strain $2S_1$ and in case of this strain an increased activity of acid phosphatase was observed (5.01 µmolpNP/s/dm^3) in comparison with the alkaline phosphatase (3.64 µmolpNP/s/dm^3).

Unlike terrestrial strains, most of the water strains (86%) were characterized by a higher activity of the acid phosphatase in comparison to alkaline phosphatases (Figure 2). An exception was the strain *Phormidium* (Palić) in which acid phosphatase showed a lower activity than the alkaline phosphatase. The activity of alkaline phosphatase ranged from 1.11 to 5.96 µmolpNP/s/dm^3, while the acid phosphatase activity ranged from 1.67 to 6.28 µmolpNP/s/dm^3. Acid phosphatase in the examined strains was almost equally active in strains *Oscillatoria* (DTD Bečej) and *Oscillatoria* (Tavankut) with values of 6.18 µmolpNP/s/dm^3 and 6.28 µmolpNP/s/dm^3, respectively (which also represent the highest recorded value of acid phosphatase activity in the examined aquatic strains). The strain with the lowest activity of acid phosphatase was strain *Phormidium* (Palić) (1.67 µmolpNP/s/dm^3).

In case of nitrogen fixing cyanobacteria, the activity of alkaline phosphatase ranged from 3.27 to 85.14 µmolpNP/s/dm^3, while the range of acid phosphatase enzyme activity was lower, with values between 3.49 and 5.48 µmolpNP/s/dm^3 (Figure 3). Thus, in most nitrogen-fixing strains (60%) a higher

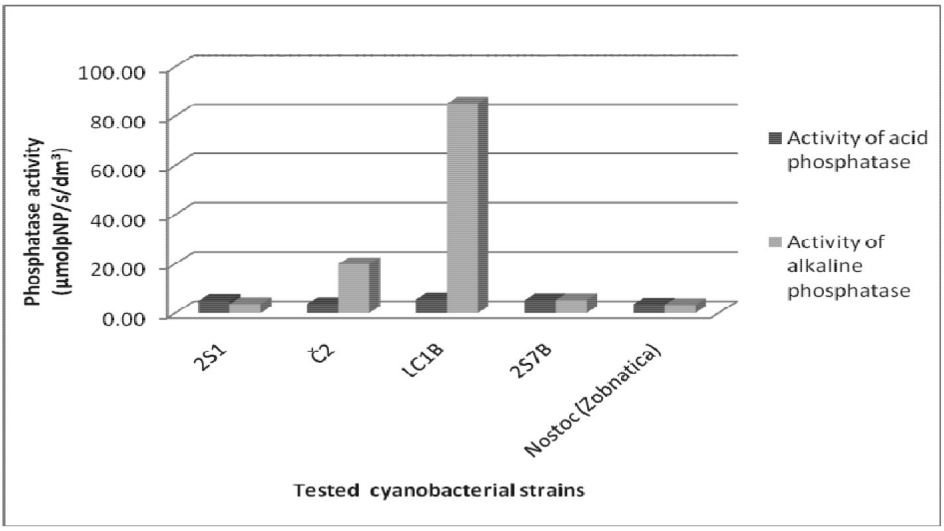

Fig. 3

activity of alkaline phosphatases was observed. The exceptions were strains $2S_1$ and *Nostoc* (Zobnatica) in which a higher activity of acid phosphatase was detected. The highest AP activity, reaching the value of 85.14 µmolpNP/s/dm^3, was detected in cyanobacterial strain *Anabaena* LC_1B.

The results obtained for non-nitrogen-fixing strains showed dominant activity of acid phosphatase, and the values ranged from 1.67 to 6.28 µmolpNP/s/dm^3 (Figure 4). Alkaline phosphatase in these strains showed a lower activity than the acid phosphatase, with values between 1.11 and 5.98

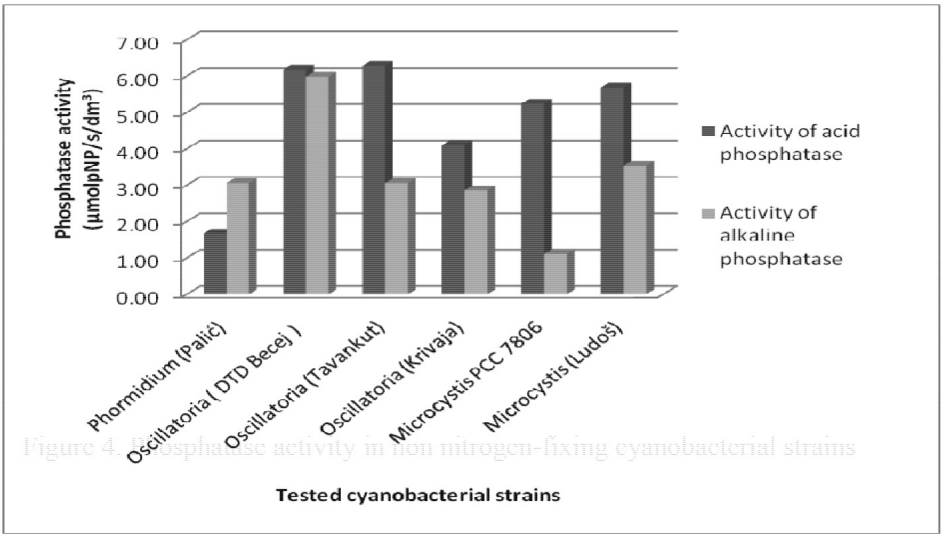

Figure 4. Phosphatase activity in non nitrogen-fixing cyanobacterial strains

Fig. 4

μmolpNP/s/dm^3. The exception was the strain *Phormidium* (Palić) which was characterized by increased activity of alkaline phosphatase (3.06 μmolpNP/s/dm^3) compared to acid phosphatase (1.67 μmolpNP/s/dm^3).

DISCUSION

Based on the results obtained by examining cyanobacterial strains, it can be noticed that the phosphatase activity is a strain specific property. The results suggest that during growth under laboratory conditions each strain reacts differently to the environmental conditions; some strains are characterized by the dominance of alkaline phosphatase, while in the others there was a higher activity of acid phosphatase. This leads to a conclusion that an organism of a given genotype is a very much product of its environment (Tempest and Neigssel, 1978). Singh et al. (2007) have collected similar data indicating that the phosphatase activity is a species-specific property. The results of Tetu et al. (2009) indicate that different species and even different strains of the same species are likely to react quite differently to phosphate deficiency. The physiological manifestation of P stress, nutrient requirements and uptake capacity are complex and variable among cyanobacterial species and strains (Schreiter et al., 2001).

In this study the enzymatic activity was compared between aquatic and terrestrial strains, as well as between nitrogen-fixing and non-nitrogen-fixing strains. It was observed that the phosphatase activity was clearly during the stationary phase of growth differs between terrestrial and water strains, as well as between nitrogen-fixing and non-nitrogen-fixing strains. In 3 out of 4 terrestrial strains alkaline phosphatase had greater activity than acid phosphatases. Unlike terrestrial strains, 6 of 7 water strains were characterized by high activity of the enzyme acid phosphatase during the stationary phase of growth. The results indicate that there is a connection between enzymatic activity and the processes of cell differentiation (sporulation, formation of heterocyst) in cyanobacteria. This may explain the higher activity of alkaline phosphatase in the majority of nitrogen-fixing cyanobacterial strains examined in this study, because during their life-cycle they form heterocysts and often permanent spores. Pandey et al. (1991) had similar results and they observed a higher activity of alkaline phosphatase in a wild type *Anabaena dolium* during sporulation which suggests that the enzyme activity is related to sporulation rather than the phosphate starvation. The assumption that the induction of the APase activity during P-stress may be considered as an early biochemical event preceding sporulation is strengthened by the observation that excess phosphate inhibits both sporulation and alkaline phosphatase activity (Pandey, 2006). Banerjee and John (2005) showed that the phase of rapidly increasing phosphatase activities correlates with the gradual loss of ability to form hormogonia in some examined *Rivularia* strains.

The results of this study show different activity of acid and alkaline phosphatases depending on their origin and eco-physiological characteristics. On the basis of these results, we may assume that there could be a connection between enzymatic activity and the taxonomic and ecological groups of cyanobacteria.

Whitton et al. (1998) indicate that the taxonomic affiliation and origin of cyanobacterial strains play an important role in the phosphatase activity in cyanobacteria, and their results suggest that representatives of family *Rivulariacae* have a greater activity in comparison with the strains which do not belong to this family.

Enzymatic activity is modulated by macro- and microelements present in the medium and in the cell (Pandey, 2006). The examined cyanobacterial strains were grown in mineral medium containing EDTA complex and Mg^{2+} and Zn^{2+} ions. Therefore, there is a possibility that the mineral medium could influence the enzyme activity in some strains. In this study enzymatic activity was measured during the stationary phase of growth, when concentration of nutrients in the medium is considerably reduced, which could also affect the activation of certain types of phosphatase enzymes. Pandey (2006) reported the requirement for Mg^{2+} in the APase activity in four diazotrophic cyanobacterial strains. There are several reports of enhanced phosphatase activity in cyanobacterial strains in response to elevated calcium (Whitton et al., 2005). Relatively low level (1 μM) of all micronutrients (Mn^{2+}, Cu^{2+}, Zn^{2+} and Fe^{3+}) enhanced the activity of PMEase or kept it stable (Pandey, 2006). Ions such as Na^{+}, K^{+}, Fe^{3+} and Zn^{2+} at moderate concentrations had a stimulating effect on phosphatase activity in *Lyngbya majuscula* (Al-Shehri, 2006). Singh et al. (2007) show that salinity (NaCl) significantly stimulated phosphate uptake which is followed by a greater P-accumulation in the cells. Therefore, the availability and concentration of certain nutrients may play an important role in regulation of phosphatase synthesis in cyanobacteria. Liu et al. (2011) showed that the lower concentrations of inorganic phosphate led to inhibition of the cell growth rather than cell death. Pandey (2006) registered a decrease in phosphatase activity during incubation, which correlates with a gradual increase in internal phosphorus content. In cyanobacterial cells internal phosphate pool regulates the synthesis of repressible phosphatases (Fitzgerald and Nelson, 1996). Thus, the concentration of phosphate initially supplied in the medium and the cellular phosphate level significantly affect the time required for the expression of phosphatase (Kumar et al., 1992). Banerjee (2007) found the presence of significant phosphatase activity in cyanobacteria strain *Calothrix anomala* 182 even when the concentration of P in the medium was high. Besides this, physical factors like temperature and light significantly affect the enzymatic activity. In case of cyanobacteria *Anabaena oryzae* enzymatic activity was greatly reduced in cells that were incubated in the dark, compared with cells incubated in light conditions, which indicates that photo energy is required for the synthesis of APases (Singh and Tiwari, 2000). From this point of view, further study of the influence of different factors on enzyme phosphatases in the examined cyanobacterial strains would be of great importance.

CONCLUSION

Analysis of phosphatase activity in water and terrestrial cyanobacterial strains provided the evidence that there is a strong connection between enzy-

matic activity and the taxonomic and ecological groups of cyanobacteria. Activity of alkaline phosphatases was dominant in most of the examined terrestrial and nitrogen-fixing cyanobacteria during the stationary phase of growth. On the other hand, acid phosphatases showed a higher activity in the largest number of water and non nitrogen-fixing cyanobacteria. The results suggest that synthesis and activity of these enzymes are the specific property of every cyanobacterial strain. The obtained results are significant for the study of cyanobacterial metabolism and their responses to environment conditions. In that respect it is very important to understand how different factors affect phosphatase activity of cyanobacteria, which requires further investigations.

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ДЕТЕКЦИЈА ФОСФАТАЗНЕ АКТИВНОСТИ КОД ВОДЕНИХ И ЗЕМЉИШНИХ СОЈЕВА ЦИЈАНОБАКТЕРИЈА

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РЕЗИМЕ: Цијанобактерије се као врло адаптибилни микроорганизми одликују способношћу преживљавања у различитим неповољним условима спољашње средине у чему значајна улога припада њиховим ензимима. Фосфатазе (фосфомоноестеразе) представљају ензиме које микроорганизми, укључујући и микроалге, продукују у релативно великим количинама као одговор на ниску концентрацију неорганског фосфора. Активност две групе фосфатаза (киселих и алкалних) испитивана је код 11 филаментозних сојева цијанобактерија у циљу одређивања синтезе и активности ензима у зависности од таксономске и еколошке групе цијанобактерија. Испитивања су вршена са 4 азотофиксирајућа земљишна соја цијанобактерија који припадају родовима *Nostoc* и *Anabaena*, као и са 7 водених сојева који су представници родова *Nostoc, Oscillatoria, Phormidium* и *Microcystis*. Резултати испитивања указали су на доминантну активност алкалних фосфатаза код већине испитиваних земљишних сојева цијанобактерија (75%) при чему се активност кретала од 3,64 до 85,14 μmolpNP/s/dm^3. Нижа активност алкалних фосфатаза (1,11 до 5,96 μmolpNP/s/dm^3) констатована је код већине водених сојева у поређењу са земљишним сојевима. Киселе фосфатазе су показале значајно већу активност код већине водених сојева (86%), при чему су се детектоване вредности кретале од 1,67 до 6,28 μmolpNP/s/dm^3. Резултати испитивања су указали на то да је активност ензима фосфатаза својство специфично за сваки цијанобактеријски сој (сој-специфично својство) и да значајно зависи од њиховог порекла. Поредећи активност ензима фосфатаза између азотофиксирајућих и неазотофиксирајућих сојева, констатовано је да је већа активност алкалних фосфатаза била карактеристична за већину испитиваних азотофиксирајућих сојева (60%), док је код већине неазотофиксатора забележена доминација активности киселих фосфатаза (83%). Добијени резултати иду у прилог томе да активност ових ензима значајно зависи и од екофизиолошких карактеристика тестираних цијанобактеријских сојева. Свакако би од великог значаја било спровести даља испитивања активности ових ензима у зависности од различитих фактора спољашње средине и механизама њиховог деловања као дела стратегије преживљавања цијанобактерија у неповољним условима спољашње средине.

КЉУЧНЕ РЕЧИ: киселе фосфатазе, цијанобактерија, алкалне фосфатазе, ензимска активност

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LEAF STRUCTURAL ADAPTATIONS OF TWO *LIMONIUM* MILLER (PLUMBAGINALES, PLUMBAGINACEAE) TAXA

ABSTRACT: *Limonium gmelinii* (Willd.) O. Kuntze 1891 subsp. *hungaricum* (Klokov) Soó is Pannonian endemic subspecies that inhabits continental halobiomes, while *Limonium anfractum* (Salmon) Salmon 1924 is one of the indicators of halophyte vegetation of marine rocks and its distribution is restricted to the southern parts of Mediterranean Sea coast. In this work, micromorphological and anatomical characters of leaves of these two *Limonium* taxa were analyzed, in order to examine their adaptations to specific environmental conditions on saline habitats. The results showed that both taxa exhibited strong xeromorphic adaptations that reflected in flat cell walls of epidermal cells, thick cuticle, high palisade/spongy tissue ratio, high index of palisade cells, the presence of sclereid idioblasts in leaf mesophyll and mechanical tissue by phloem and xylem. Both taxa are crynohalophytes and have salt glands on adaxial and abaxial epidermis for excretion of surplus salt. Relatively high dimensions of mesophyll cells, absence of non-glandular hairs and unprotected stomata slightly increased above the level of epidermal cells, are also adaptations to increased salinity.
KEYWORDS: adaptations, anatomy, epidermis, halophytes, leaf, salt glands

INTRODUCTION

Genus *Limonium* includes 87 species grouped in three sections. The typical section contains the highest number of species and some European endemic species (Pignatii, 1972). *Limonium anfractum* (Salmon) Salmon 1924 and *Limonium gmelinii* (Willd.) O. Kuntze 1891 belong to the typical section. Like a number of species from this section and this genus in Europe, they inhabit xerotermic habitats, thus being exposed to physical or, more often, physiological drought.

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L. gmelinii, especially subsp. *hungaricum* (Klokov) Soó spreads in regions with continental climate, with some influences of maritime climate. It is present in the Aralocaspian-Southsiberian-Pontic-Pannonian area, and inhabits marshy terrains of the steppe zone that spreads to the west up to South Slovakia and Austria (Meusel et al., 1978). This species has two subspecies with deffined areas of distribution. Eastern subspecies, *hypanicum* (Klokov) Soó, is distributed in steppe regions of Ukraine and Moldavia (Romania) and inhabits the eastern parts of species area (Soö, 1970; Pignatii, 1972). The Pannonian endemic subspecies *hungaricum* (Klokov) Soó is distributed in Romania, Hungary and Slovakia, where it inhabits continental halobiomes as a member of the *Festucion pseudovinae* alliance (Soö, 1970; Řehorek and Maglocký, 1999). In the marshes in north Serbia it was recorded for the first time near Kovilj (Zorkóczy, 1896). Today, it is present in three forms – *f. hungaricum*, f. *obtusum* (Schur) Soó and f. *acuminatum* (Schur) Soó – in different types of marsh habitats in the regions of Bačka and Banat (northern Serbia), mostly in the soils with lower salt content (Bodrogközy, 1966; Knezevic, 1994; Budak, 1998).

L. anfractum inhabits places with maritime climate. Its distribution is restricted to the southern parts of Mediterranean Sea coast (Albania, Montenegro) (Pignatii, 1972), and southern parts of the Croatian coast, up to Dubrovnik coast, with islands (Ilijanic, 1994). This is Illyrian-Adriatic endemic species which lives on limestone rocks as a characteristic species of halophytic *Limonietum anfracti* association (Ilijanic and Hecimovic, 1982). It is one of the indicators of halophytic vegetation of marine rocks and cliffs, where it is often pounded by the sea waves. Both taxa are exposed to direct sunlight during their vegetation.

Anatomical structures of plant organs, especially leaves, change, thus enabling the plant to adaptate to its environment. Therefore, the histological components of the leaf seem are the most satisfactory parameters for the study of the relations between xeromorphous structures of plants and their habitat, although the anatomy of other plant organs could also give additional information (Colombo and Trapani, 1992). Colombo (2002) examined morpho-anatomical characters of 25 *Limonium* species from Sicily and found that their differences in root structure were of little importance, and the differences the stem and inflorescence axis insignificant, and therefore unsuitable from taxonomic point of view. He proposed foliar architecture as remarkable discriminating character for *Limonium* species. The presence of different types of sclereids, which proved to be morphologically different in different species, is also typical for this genus (Colombo, 2002). They strengthen the leaves and give them mechanical support.

Both examined *Limonium* taxa are crynohalophytes and they have epidermal salt glands for excretion of surplus salt (Fahn and Cutler, 1992; Stevanovic and Jankovic, 2001). In genus *Limonium* those glands consist of four secreting cells arranged in a circle, each with a secreting pore, four internal cup-cells and two circles of four collector cells (Metcalfe and Chalk, 1957; Janjatovic and Merkulov, 1981; Vassilyev and Stepanova, 1990; Co-

lombo and Trapani, 1992). In *Limonium* species investigated by Colombo (2002), glands were 12 to 16 celled, small in number and large on the upper side, and small in size and numerous on the lower side. In 12-celled types, four cells are secretional, four internal and four external. Each internal cell bears a pore, separated by a cross and enclosed in a ring. At the base of the gland appear four storing cells which secrete salt. Glands appear on both leaf sides, but are more numerous abaxially. Salama et al. (1999) described the structure of salt glands of three *Limonium* species. They found that the glands were composed of 16 secretory cells, arranged in four circles, and four subbasal collecting cells. NaCl was the most abundant salt excreted.

The aim of this research was to examine leaf anatomical characteristics and particularly leaf epidermal tissue of two *Limonium* taxa endemic to Serbia, with special attention to their adaptations to specific environmental conditions in saline habitats.

MATERIAL AND METHODS

Specimens of *Limonium gmelinii* subsp. *hungaricum* were collected from salt marsh near Secanj (northern Serbia) and *Limonium anfractum* from Valdanos (the Mediterranean coast, Montenegro), where those species grow as endemic. Voucher specimens were deposited in the Herbarium of the Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad (BUNS). For anatomical investigations leaves of ten plants were used. For light microscopy, cross sections of the fresh leaves were made at the region of the main vein and at 1/4 of leaf width using Leica CM 1850 cryostat, at a temperature of -20° C and at cutting intervals of 25mm. Sections were observed and measurements made using Image Analyzing System Motic 2000. Data were statistically processed using STATISTICA for WINDOWS version 10.0. Significance of differences between the taxa was determined using t-test. For scanning electron microscopy (SEM) small pieces of leaves fixed in FAA were frozen in liquid N_2 and viewed with JEOL JSM-6460LV under the pressure of 50Pa, using BEI, at an acceleration voltage of 10 kV.

RESULTS

The leaves of *Limonium gmelinii* subsp. *hungaricum* and *L. anfractum* have a single layer of epidermis, formed of almost isodiametric, relatively large cells, with flat cell walls (Fig. 1. and Fig. 2). Adaxial and abaxial epidermal cells of *L. gmelinii* subsp. *hungaricum* leaves show no significant differences in size and cuticle thickness (Tab. 1). The cells of abaxial epidermis are thick walled and smaller only in the region of the main vein. The epidermal cells of *L. anfractum* leaves have thick outer cell walls and are significantly larger adaxially. The cuticle is thicker, but not significantly, on the adaxial epidermis, with cuticular ornamentations, which are not very pronounced.

Stomata of anisocytic type occur on both leaf surfaces of these species and are slightly increased above the level of epidermal cells. They are significantly more numerous and narrower on the adaxial epidermis, but of almost the same length on surfaces in *L. gmelinii* subsp. *hungaricum*. In *L. anfractum* they are significantly more numerous and narrower on the abaxial epidermis, while of similar length on both epidermises (Tab. 1).

Secretory glands are present on both leaf surfaces. They are equally numerous on leaf surfaces in *L. gmelinii* subsp. *hungaricum*, while significantly more numerous on abaxial epidermis in *L. anfractum* (Tab. 1). The glands are of almost the same diameter on both surfaces. In *L. anfractum,* they are surrounded by a ring of raised, large epidermal cells.

The *L. gmelinii* subsp. *hungaricum* leaves have dorsiventral structure (Fig. 3). The mesophyll consists of 2–3 layers of palisade and 5–6 layers of spongy tissue cells. The index of palisade tissue cells (length/width ratio) is rather high (7.1); cells are very narrow, which is the characteristic of plants exposed to a strong insolation (Tab. 2). Spongy tissue cells are rounded in shape, but the cells of the first layer under abaxial epidermis are elongated. The thickness of the palisade tissue is 47.2% and spongy tissue 41.9% of leaf thickness, their ratio being 1.1. In the mesophyll, branched sclereids of irregular shape occur. The closed collateral vascular bundles are linearly arranged, with sclerenchyma tissue by phloem and xylem. Lamina rostrum is thin and elongated.

The main vein is prominent abaxially, the ratio of leaf thickness at the main vein/leaf thickness at 1/4 of leaf width being 2.47. It contains 4-6 randomly arranged vascular bundles, completely surrounded by a few layers of sclerenchyma cells and one layer of parenchyma sheath containing starch grains. A layer of collenchyma occurs under abaxial epidermis.

The *L. anfractum* leaves are isolateral (Fig. 4.). Palisade tissue under adaxial epidermis is composed of 2–3 layers of elongated cells (length/width ratio being 5.1), while of 1–3 layers under abaxial epidermis (Tab. 2). Between them, 2–4 layers of rounded spongy tissue cells occur. The palisade tissue is much thicker than spongy tissue (their ratio being 2.9) and it makes 63.7% of the leaf thickness. In mesophyll, branched sclereids in the form of idioblasts are also present, as well as linearly arranged vascular bundles. Rostrum is short, composed of only a few cells.

The main vein is not prominent (the ratio of the leaf thickness at the main vein/leaf thickness at the 1/4 of leaf width being 1.28). Only one vascular bundle, with groups of sclerenchyma by phloem and xylem, occurs in it. Palisade tissue cells under the abaxial epidermis are not present in the region of the main vein.

DISCUSSION

On the basis of leaf anatomical characteristics of the two examined *Limonium* taxa, it could be seen that both of them show a combination of halomorphic and xeromorphic structures. The leaves of both taxa had a relatively

thick cuticle, flat anticlinal walls of epidermal cells, better developed palisade than spongy tissue (high palisade/spongy tissue ratio), elongated, narrow palisade tissue cells (relatively high palisade cell's index), sclereid idioblasts in leaf mesophyll and mechanical tissue by phloem and xylem, which were adaptations to constant direct insolation and physiological drought. Moreover, on *L. anfractum* leaves, cuticular ornamentations, significantly thicker cuticles, thick outer walls of epidermal cells, presence of palisade tissue under abaxial epidermis and significantly higher palisade/spongy tissue ratio were noticed as an additional adaptations to higher insolation on marine cliffs.

According to Colombo (2002) all groups of *Limonium* species are anatomically very heterogeneous. In the species investigated by this author, salt glands were more numerous and smaller on abaxial lamina side. Our results showed that only *L. anfractum* had more salt glands abaxially, whilst their size was similar on both lamina sides. The structure of salt glands corresponded to the one previously described by several authors (Metcalfe and Chalk, 1957; Janjatovic and Merkulov, 1981; Vassilyev and Stepanova, 1990; Colombo and Trapani, 1992; Colombo, 2002). The average lamina thicknesses in species investigated by Colombo (2002) ranged from 260 to 500μm, and the average palisade tissue thickness was about 65 μm. In our examined material these values were significantly higher, especially for palisade tissue.

Compared to the mesophyll structures of *L. lopadusanum, L. intermedium* and *L. albidum*, three species endemic to Pelagic Islands, two examined taxa had thicker palisade tissue and higher ratio of palisade and spongy tissue thickness (Colombo and Trapani, 1992). For *Limonium* species only dorsiventral leaf structure was previously reported (Metcalfe and Chalk, 1957, Colombo and Trapani, 1992, Colombo, 2002). According to de Fraine (1916, in Metcalfe and Chalk, 1957) *L. binervosum*, which is the species that inhabits marine clifs and rocks, has normally isobilateral mesophyll, but showing dorsiventral structure when raised from seed in cultivated ground. It also has vascular bundles surrounded by sclerenchyma, and branched sclereids in mesophyll. In the mesophyll of *L. intermedium* and *L. bellidifolium* sclereids were not recorded.

The two examined taxa also showed halomorphic adaptations to an increased salinity, such as relatively high dimensions of epidermal and mesophyll cells, absence of protective structures on the epidermis, relatively small number of stomata per mm^2, stomata unprotected and slightly increased above the level of epidermal cells, and salt glands on both leaf surfaces. Comparison of halomorphic characteristics of two taxa showed that *L. anfractum* had significantly larger cells of adaxial epidermis and mesophyll and significantly smaller number of stomata on the adaxial epidermis, which could be explained by the higher salinity of the habitat.

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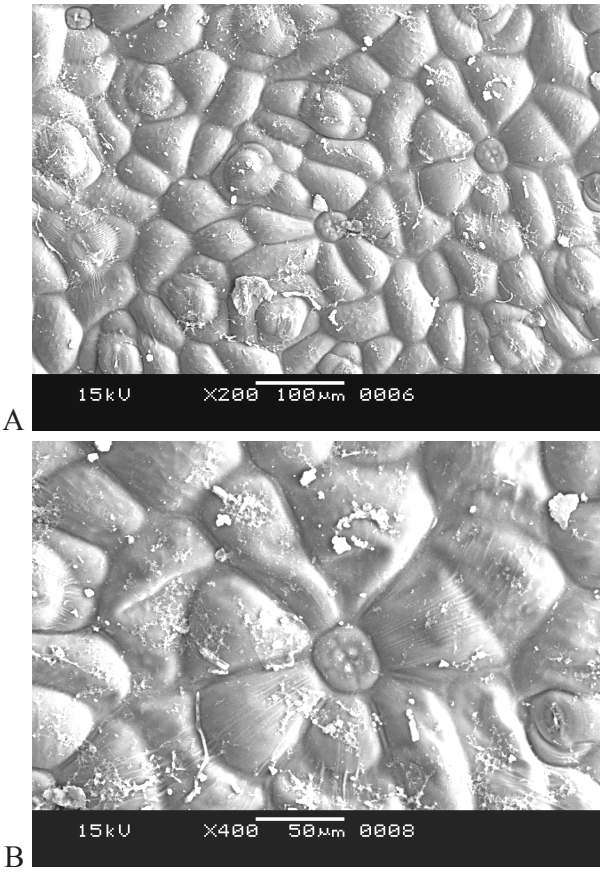
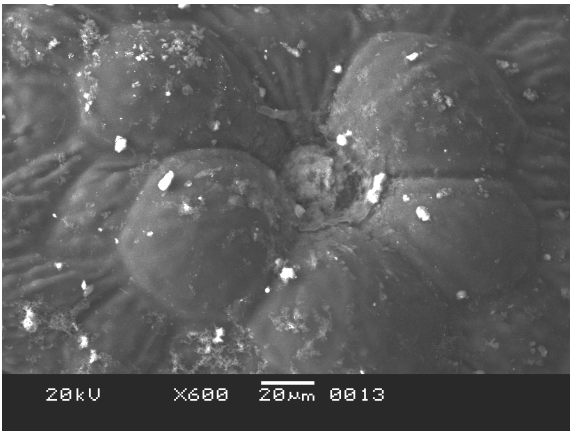


Figure 1. *L. gmelinii* subsp. *hungaricum* adaxial epidermis and salt glands:
SEM micrographs of adaxial surface (A, B) and light micrograph of the cross-section (C)



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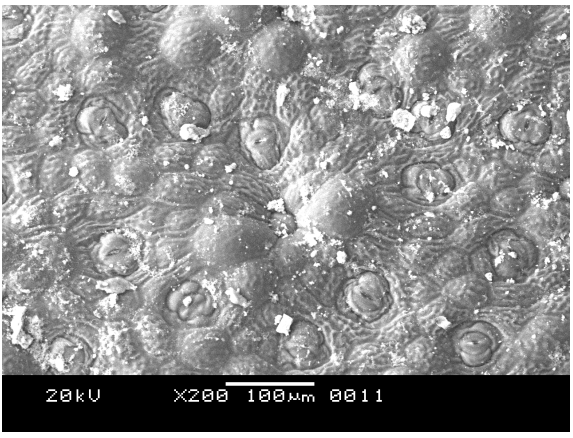

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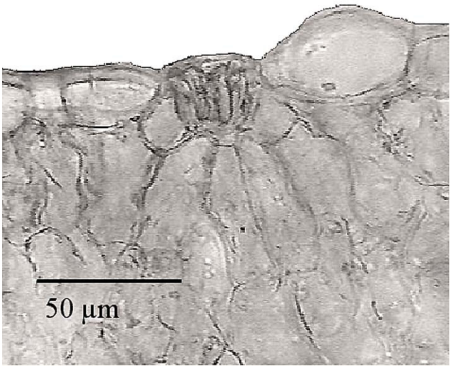

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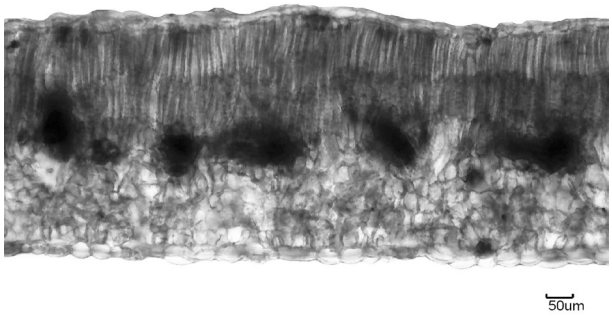
Figure 2. *L. anfractum* adaxial epidermis and salt glands: SEM micrographs of adaxial surface (A, B) and light micrograph of the cross-section (C)



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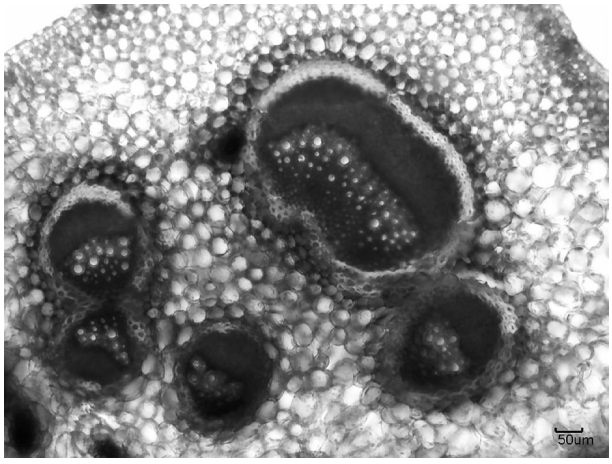

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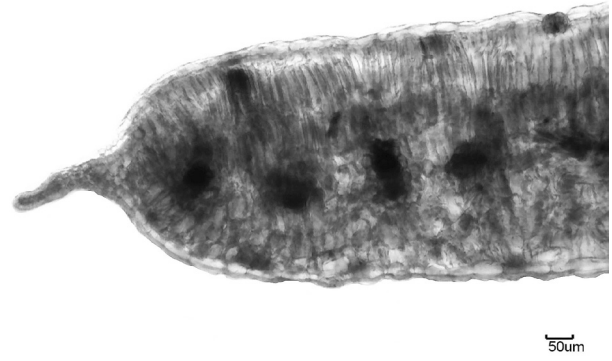

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Figure 3. Light micrographs of *L. gmelinii* subsp. *hungaricum* lamina cross sections: A – lamina at ¼ of the width; B – the main vein; C – leaf margin

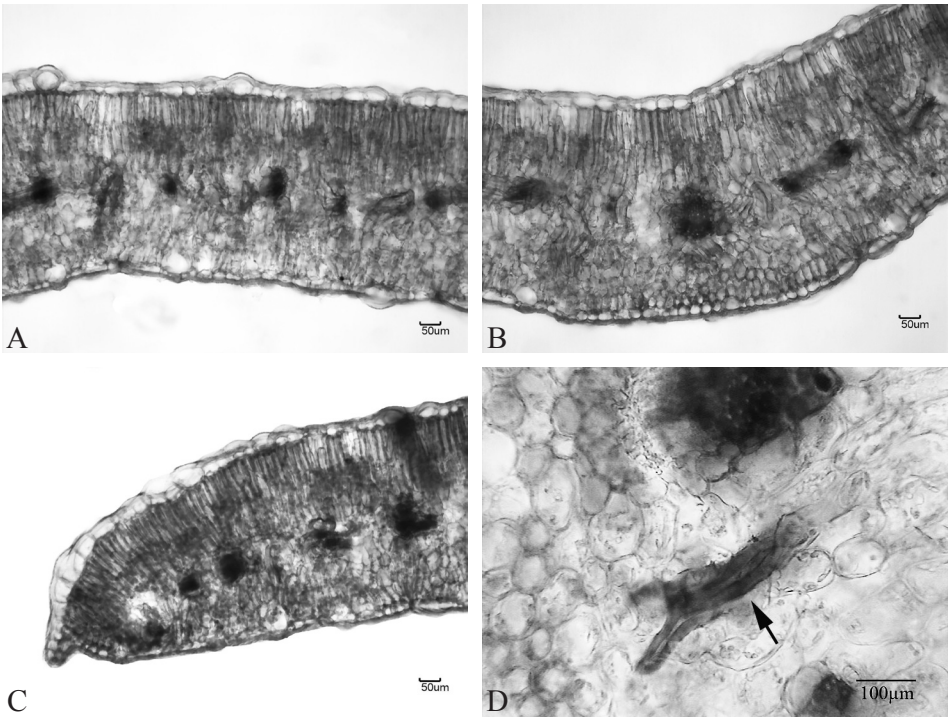

Figure 4. Light micrographs of *L. anfractum* lamina cross sections: A – lamina at ¼ of the width; B – the main vein; C – leaf margin; D – sclereid in mesophyll

Tab. 1. Characteristics of the epidermis (mean values ± standard error).

	Limonium gmelinii subsp. *hungaricum*	Limonium anfractum	T-test
Adaxial epidermis			
Thickness (µm)	28.8 ± 1.2	34.1 ± 1.7	*
Percentage of lamina thickness (%)	5.6% ± 0.3%	7.0% ± 0.5%	*
Area of abe cells (µm^2)	1178 ± 49.5	1096 ± 68.8	ns
No. of stomata /mm^2	94.3 ± 4.7	57.2 ± 2.4	**
Stomata length (µm)	36.7 ± 1.0	38.1 ± 0.7	ns
Stomata width (µm)	22.7 ± 0.6	28.1 ± 0.6	**
No. of salt glands/mm^2	10.9 ± 0.5	6.7 ± 0.2	**
Diameter of salt glands (µm)	33.9 ± 0.6	34.4 ± 0.8	ns
Cuticle thickness (µm)	2.3 ± 0.1	3.2 ± 0.1	**
Abaxial epidermis			
Thickness (µm)	28.9 ± 0.9	30.4 ± 1.5	ns
Percentage of lamina thickness (%)	5.6% ± 0.2%	6.1% ± 0.3%	ns
Area of ade cells (µm^2)	1012 ± 70.1	1594 ± 123.6	**
No. of stomata /mm^2	74.2 ± 2.0	78.0 ± 2.8	ns
Stomata length (µm)	36.3 ± 0.8	36.3 ± 0.7	ns

Stomata width (µm)	26.8 ± 0.8	25.5 ± 0.6	ns
No. of salt glands/mm^2	10.3 ± 0.5	9.3 ± 0.5	ns
Diameter of salt glands (µm)	33.4 ± 0.7	32.9 ± 0.8	ns
Cuticle thickness (µm)	2.5 ± 0.1	2.9 ± 0.1	**

*, **, ns – according to t-test, differences between the taxa significant at p≤0.05, p≤0.01 or not significant, respectively.

Tab. 2. Characteristics of mesophyll leaf (mean values ± standard error).

	Limonium gmelinii subsp. *hungaricum*	Limonium anfractum	T-test
Tissue thickness			
Lamina (µm)	529 ± 17.4	481 ± 30.1	ns
Mesophyll (µm)	470 ± 18.9	413 ± 30.4	ns
% of lamina thickness	89.1% ± 0.8%	87.0% ± 1.1%	ns
Palisade tissue (adaxially) (µm)	249 ± 11.7	206 ± 16.5	*
% of lamina thickness	47.2% ± 1.2%	42.3% ± 1.5%	*
Palisade tissue (abaxially) (µm)	0	106 ± 11.0	**
% of lamina thickness	0	21.4% ± 1.0%	**
Spongy tissue (µm)	221 ± 10.7	111 ± 6.6	**
% of lamina thickness	41.9% ± 1.1%	23.3% ± 1.3%	**
Palisade/spongy tissue ratio	1.1 ± 0.1	2.9 ± 0.2	**
The size of the cells			
Area of palisade cells (µm^2)	1526 ± 72.4	1964 ± 89.8	**
Area of spongy cells (µm^2)	1205 ± 61.9	1295 ± 84.5	ns
Palisade cells height (µm)	102 ± 2.9	102 ± 4.2	ns
Palisade cells width (µm)	14.6 ± 0.5	20.3 ± 0.5	**
Index of palisade cells	7.1 ± 0.2	5.1 ± 0.2	**
No. of palisade cell layers	3	2-3 (ad); 1-2(3) (ab)	
The main vein			
Main vein thickness (µm)	1303 ± 39.5	615 ± 46.6	**
No. of vascular bundles	4-6	1	

*, **, ns – according to t-test, differences between the taxa significant at p≤0.05, p≤0.01 or not significant, respectively.

СТРУКТУРНЕ АДАПТАЦИЈЕ ЛИСТА ДВА ТАКСОНА РОДА *LIMONIUM* MILLER (PLUMBAGINALES, PLUMBAGINACEAE)

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РЕЗИМЕ: *Limonium gmelinii* (Willd.) O. Kuntze 1891 subsp. *hungaricum* (Klokov) Soó је панонски ендем који насељава континенталне халобиоме, док је врста *Limonium anfractum* (Salmon) Salmon 1924 један од индикатора халофитске морске вегетације и њена дистрибуција ограничена је на јужни део медитеранске обале. У раду су анализиране микроморфолошке и анатомске карактеристике листова два таксона рода *Limonium* у циљу испитивања њихове адаптације на специфичне услове заслањеног станишта. Резултати су показали да оба таксона поседују карактеристичне ксероморфне адаптације у виду равних ћелијских зидова епидермалних ћелија, задебљале кутикуле, високе вредности односа палисадног и сунђерастог ткива, високе вредности индекса палисадних ћелија, присуства склереида у мезофилу листа и механичког ткива уз ксилем и флоем. Оба таксона су кринохалофите и на адаксијалном и на абаксијалном епидермису поседују слане жлезде за излучивање вишка соли. Релативно крупне ћелије мезофила, одсуство нежлезданих трихома као и незаштићене стоме које су благо издигнуте изнад површине епидермиса такође су адаптација на повећану концентрацију соли на станишту.

КЉУЧНЕ РЕЧИ: адаптација, анатомија, епидермис, халофите, лист, слане жлезде

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STRUCTURAL ADAPTATION OF *SALSOLA SODA* L. (CHENOPODIACEAE) FROM INLAND AND MARITIME SALINE AREA

ABSTRACT: The microscopic analysis of leaf and stem in two populations of *Salsola soda* was carried out in order to examine mechanism of anatomical adaptations to environmental condition on saline habitats and to determine if there exists a morpho-anatomical differentiation between populations from maritime and inland saline area. Analysis included 26 quantitative characters of leaf and stem. The results showed that both populations exhibited halomorphic and xeromorphic adaptations, which refered to ecological plasticity and adaptations of plants to their habitats. Our research also showed that *S. soda* had quite a stable morpho-anatomical structure, since only quantitative changes were recorded.
KEYWORDS: anatomy, halophytes, leaf, *Salsola soda,* stem

INTRODUCTION

Soil salinization is an increasing problem world-wide. Global estimates indicate that at least 1.5 billion hectares of land are salt-affected (Choukr-Allah, 1996). In many oil production areas, contamination of soils with oilfield brines is a significant environmental problem (Merrill et al., 1990). Brine salts are predominantly chlorides, 90% or more NaCl (McMilion, 1965). Some ions contained in the brine solution, such as N^{a+}, Cl^{-}, Ca^{2+}, Mg^{2+}, K^{+} and SO_4^{2-}, can be phytotoxic if concentrations are above levels that plants can tolerate, even though most of these elements are essential for plant growth (Munn and Stewart, 1989).

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Running title: Milić, D. et al., Structural adaptation of *Salsola soda*

The United Nations Environment Program (UNEP) estimates that 20% of the agricultural land and 50% of the cropland in the world is salt stressed (Flowers and Yeo, 1995). Because of this the latest investigations were focused on amelioration methods (Qadir et al., 2001, 2002, 2005; Li et al., 2004). On the other hand salt-accumulating halophytes could be used to revegetate and improve the quality of saline soils (Flowers et al., 1986; Zhao, 1991). Halophytes have adopted different strategies in order to survive periodic soil saturation. They are usually divided into euhalophytes, which have succulent structure and accumulate salt in their tissues, and crinochalophytes, which are capable of excreting salt, usually through salt glands and bladders (Zhao et al., 2002; Voronkova et al., 2008). Specific conditions of salt soils, primarily high concentration, different quality and quantity of salts and variability of water content in soils, affects specific morpho-anatomical adaptations of halophytes (Knežević et al., 1998; Polić et al., 2009). The leaf histological components are the most satisfactory parameters for the study of the relations between halomorphic structures of plants and their habitat, although anatomy of other plant organs could give additional information (Colombo & Trapani, 1992).

Of the Dicotyledoneae, the Chenopodiaceae has by far the highest proportion of halophytic genera (44%). With 312 halophytic species it is probably the family in which salt-tolerance is most widespread (Flowers et al., 1986). Many genera of Chenopodiaceae, especially *Atriplex, Suaeda* and *Salicornia*, are extremely salt tolerant and have been studied for their potential use as forage and oilseed crops (Watson, 1990; Glenn et al., 1991, Rozema et al., 1995).

Salsola L., a genus of 100 to nearly 200 species, is one of the largest genera within the Chenopodiaceae (Botschantzev, 1969, 1979; Cronquist, 1981).

S. soda is an annual, succulent shrub up to 70 cm tall. It has fleshy green leaves and either green or red stems. The tiny flowers develop from inflorescences that grow out of the base of the leaves near the stem (Slavnić, 1972; Akeroyd, 1993). *S. soda* is native in Eurasia and North Africa. It is also found on the Atlantic coasts of France and Portugal and on the Black Sea coast (Jalas and Suominen, 1989). It has become naturalized along the Pacific coast of North America, and there is concern about its invasiveness in California's salt marshes (Baye, 1998). In Serbia *S. soda* is an endangered species which distribution is limited to saline areas in the northern part of Serbia, while in Montenegro this plant can be found only on the Adriatic coast (Slavnić, 1972).

This study was performed in order to reveal structural features of *S. soda*, particularly anatomical characteristics of leaf and stem, allowing them to survive under specific environmental conditions. Another aim was to determine the structural differences and variability rate between populations from maritime and inland saline areas.

MATERIAL AND METHODS

Morpho-anatomical analyses were done of plant samples from two populations of *S. soda* growing in Ulcinj salina (maritime saline area, Montenegro)

and Okanj (inland saline area, Serbia; Table 1). The soil at the collection site contained very small amount of salts in inland saline area – 0.03% compared with locality from maritime saline area (0.8%). The alkaline reaction was higher at Okanj locality (8.8), than in Ulcinj salina locality (7.5).

Table 1. Voucher data for *Salsola soda* specimens used in the study with climate and soil characteristics of their habitats in the year 2006

Collection site	Date	Voucher Number	Average annual temperature	Annual precipitation	% of salts in soil	Ph soil
Montenegro, Ulcinj salina UTM 34T CM4 54	09.09.2006.	2-1994	15.4° C	1272mm	0.8	7.5
Serbia, Okanj UTM 34T DR2 44	15.09.2006.	2-1995	11.1° C	571mm	0.03	8.8

Plants were determined at the Department of Biology and Ecology, University of Novi Sad. Voucher specimens were deposited in the Herbarium of the Department of Biology and Ecology, University of Novi Sad – BUNS (Table 1). For anatomical investigation ten plants of each population were used. For light microscopy observations leaf epidermal prints were made after Wolf (1954). The leaf surfaces were covered with liquid transparent lac, and epidermal prints removed using transparent adhesive tape. Stomata were counted on five randomly selected areas of the adaxial and abaxial surfaces and calculated per mm^2 of the leaf surface. The segments of leaves and stems from the middle part of the plants were separated and fixed in 50% ethanol. For light microscopy, cross sections were made using Leica CM 1850 cryostat, at a temperature of -18° C to -20° C, at cutting intervals of 25 mm. Sections, epidermal cells and stomata were observed and measurements made using Image Analyzing System Motic 2000. Relative proportions were calculated for leaf and stem tissues, and expressed as a ratio of the whole cross section area of each organ. Data were statistically processed by analysis of variance and means, and standard errors and coefficients of variation were calculated using STATISTICA for Windows version 10.0 (StatSoft, 2011). The significance of differences in measured parameters between the populations was determined using t-test ($p \leq 0.05$ and $p \leq 0.01$). The general structure of sample variability was established by Principal Component Analysis (PCA), based on correlation matrix.

RESULTS

The stem cross sections are rounded to elliptical in shape, with incisions. The stem has a single layer of epidermis (Figure 1).

Stem cortex was differentiated into one layer of collenchyma located subepidermally (Figure 2A), one layer of chlorenchyma beneath it and several layers of thin-walled parenchyma cells that do not contain chloroplasts. Paren-

chyma cells are generally large, except in incisions of peripheral part of cortex where they are much smaller. In central cylinder, numerous collateral vascular bundles are arranged in a circle, with well developed sclerenchyma tissue above them (Figure 2B). Pith parenchyma is compact, composed of relatively large parenchyma cells, with no cavity present.

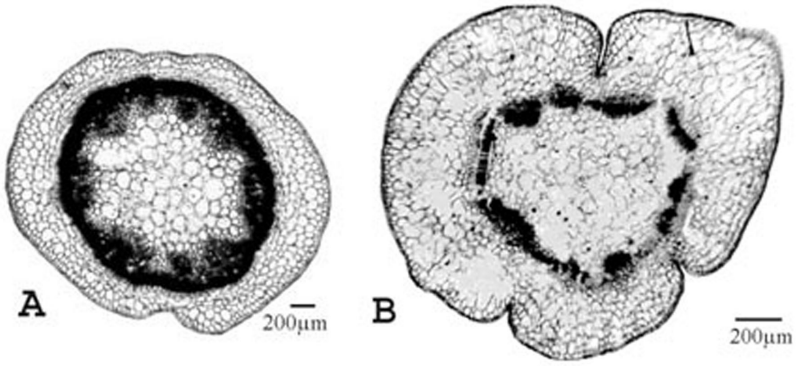

Figure 1. Cross section of the stem: A- Okanj, B- Ulcinj salina

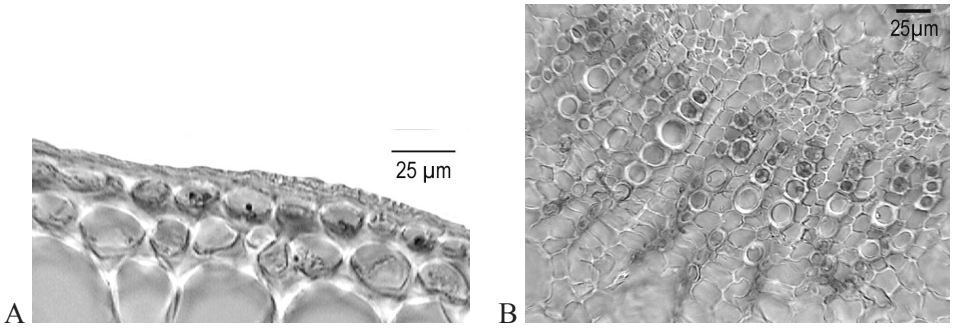

Figure 2. Cross section of the stem: A – collenchyma, B – vascular bundles

The plants of the population from Ulcinj salina had significantly higher stems (22.3 cm) than plants from Okanj (14.6 cm), with larger diameter and cross-section area (Table 2). They also had higher percentage of cortex and pith parenchyma. Significantly higher proportions of epidermis and vascular bundles with sclerenchyma were only recorded in plants from Okanj. The mean cortex thickness: stem radius ratio was 0.352 and 0.376, from population from Ulcinj and Okanj, respectively.

Table 2. Stem anatomical characteristics (mean values ± standard error and coefficient of variation %)

Characters	Ulcinj salina		Okanj		t-test
	$\bar{x}$±se	cv	$\bar{x}$±se	cv	
stem height (cm)	22.2±1.2	17.4	14.6±1.8	38.3	**
stem cross section area of (mm^2)	7.6±0.4	16.9	2.5±0.3	35.4	**
stem diametar (mm)	3.1±0.1	8.6	1.8±0.1	19.4	**
% epidermis	2.0±0.1	10.1	3.6±0.2	18.3	**
% cortex	60.2±1.6	8.6	52.9±2.9	17.5	*
cortex thickness (mm)	0.6±0.02	13.1	0.3±0.03	33.4	**
% cylinder	37.8±1.6	13.8	43.4±3.1	22.6	ns
% v.bundles+ sclerenchyma	15.8±0.8	16.7	25.9±1.9	23.7	**
% pith parenchyma	22.07±1.0	14.4	17.5±1.4	24.5	*

*, ** – differences between the localities significant for 0,05 and 0.01 level of significance respectively; ns – differences between the localities not significant

The leaves are of succulent structure (Figure 3). Their cross sections are round to triangular in shape.

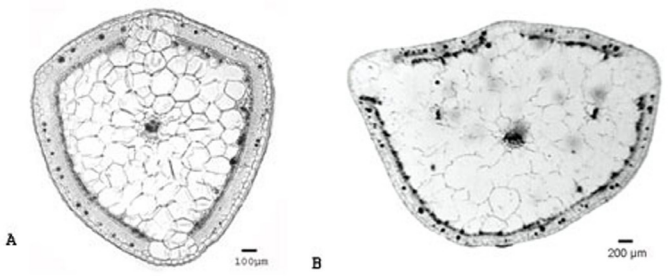

Figure 3. Cross section of the leaf from A-Okanj and B-Ulcinj salina

The leaves have a single layer of epidermis, formed of almost isodiametric, relatively large cells. The epidermis is provided with a thick cuticle. Stomata of paracytic type occur on both leaf surfaces of these species and are slightly under the level of epidermal cells. Underlying the epidermis is a layer of chloroplast containing hypodermal cells, which have calcium oxalate crystals. The mesophyll is differentiated into palisade tissue and atypical spongy tissue which expands to water storage tissue. Palisade tissue is uniseriate, composed of cylindrical cells, placed between hypodermis and chlorenchymatous bundle sheath (Figure 4). Together with bundle sheath layers it forms a discontinuous ring. Water storage cells are large and thin-walled. Those adjacent to bundle sheath often contain cubic crystals.

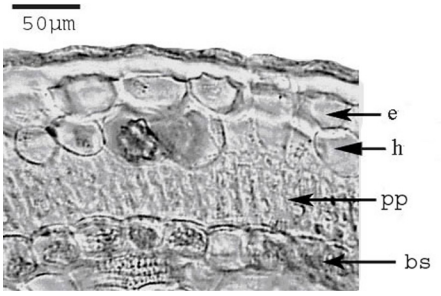

Figure 4. Cross section of the leaf: e – epidermis, h – hypodermis, pp – palisade tissue, bs – bundle sheath

The main vascular bundle is in the center of the leaf, surrounded by water storage parenchyma. Smaller, peripheral vascular bundles are in contact with bundle sheath cells (Figure 5).

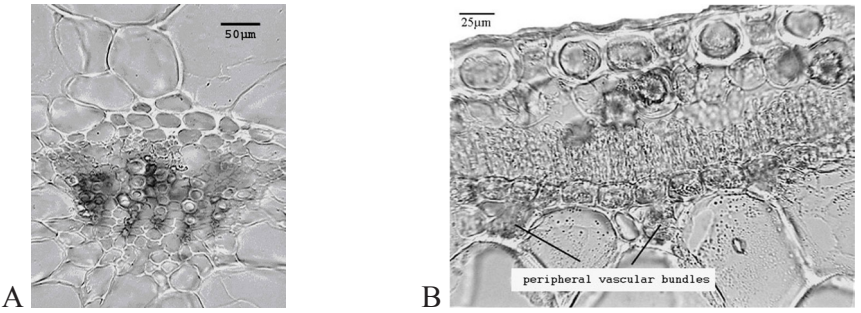

Figure 5. Cross section of the leaf. A – main vascular bundle, B – smaller, peripheral vascular bundles

The leaf cross section area was significantly higher in plants from Ulcinj salina locality, due to better developed water storage tissue (Table 3). Number of stomata /mm^2 on adaxial and abaxial leaf side, proportions of epidermis, hypodermis, vascular bundles and palisade tissue were significantly higher in plants from Okanj locality. No significant differences between plants of these two populations were recorded in stomata size on adaxial and abaxial leaf side, leaf and cuticle thickness, percentage of bundle sheath and the size of palisade cells.

Principal Components Analysis (PCA) showed that examined characters had generally low variability. It indicated three groups of characters, which explained 69.08% of the total variation (Table 4). The first principal component explained 43.51% of the variation. It was defined by the stem cross-section area, proportions of stem epidermis and vascular bundles with sclerenchyma, number of stomata per unit on adaxial leaf side, length of stomata on abaxial leaf side, leaf cross-section area and thickness, as well as proportions of all

leaf tissues, except the bundle sheath. The second principal component explained 16.14% of variation due to the variability of proportions of stem cortex, cylinder, pith parenchyma and stomata width on adaxial leaf side. The third principal component explained 9.43% of variation due to the variability of cuticle thickness only. The most stable parameters, which did not contribute significantly to the total variation, were the most of the stomata parameters, percentages of bundle sheath tissue and the size of palisade cells.

Table 3. Leaf anatomical characteristics (mean values ± standard error and coefficient of variation %)

Characters	Ulcinj salina		Okanj		t-test
	$\bar{x}$±se	cv	$\bar{x}$±se	cv	
no. of stomata /mm^2(adaxially)	89.4±3.9	13.7	119.7±4.4	11.7	**
stomata length (adaxially)	30.8±0.8	8.1	28.5±1.0	11.6	ns
stomata width (adaxially)	19.9±0.4	6.6	20.4±0.6	10.1	ns
no. of stomata /mm^2(abaxially)	76.9±3.8	15.6	89.0±3.5	12.4	*
stomata length (abaxially)	31.4±1.0	10.6	29.4±0.7	7.3	ns
stomata width (abaxially)	20.1±0.3	5.2	20.1±1.3	20.8	ns
leaf cross section area (mm^2)	5.1±0.4	23.8	2.2±0.1	17.1	**
leaf thickness (µm)	2378±9	11.9	1682±6	11.6	ns
cuticle thickness (µm)	8.3±0.1	5.2	8.02±0.3	10.4	ns
% epidermis	4.9±0.2	12.9	6.9±0.2	10.3	**
% hypodermis	3.4±0.2	14.2	4.9±0.2	13.7	**
% vascular bundles	3.8±0.4	31.8	5.8±0.3	16.6	**
% bundle sheat	3.4±0.3	23.9	4.0±0.2	12.5	ns
% pallisade tissue	6.9±0.3	15.8	8.5±0.4	16.2	**
length of palisade cells (µm)	40.2±2.1	16.5	42.8±1.9	14.1	ns
width of palisade cells (µm)	8.3±0.4	15.1	8.7±0.3	11.7	ns
% water storage tissue	77.4±0.5	2.2	69.9±0.8	3.7	**

*, ** – differences between the localities significant for 0,05 and 0.01 level of significance respectively; ns – differences between the localities not significant

According to the type of variability, examined populations were grouped by PCA (Figure 6). The projection of the cases for the first two components showed that the two examined populations could be clearly separated according to the type of variation of the examined parameters along the first axis. Population from Montenegro showed higher level of homogeneity.

Table 4. Principal components analysis (PCA) of measured parameters. Factor coordinates of the variables, based on correlations and cumulative percentages of the vectors

Anatomical characters		Factor 1	Factor 2	Factor 3
stem	cross section area	-0.83891*	0.333944	0.055640
	% epidermis	0.75443*	-0.536530	-0.016999
	% cortex	-0.53440	-0.786692*	0.026596
	% cylinder	0.44617	0.849792*	-0.024612
	% v.bundles+ sclerenchyma	0.78259*	0.489109	-0.045702
	% pith parenchyma	-0.41126	0.825360*	0.026753
leaf	no. of stomata /mm^2 (adaxially)	0.82459*	0.077839	-0.111648
	stomata length (adaxially)	0.50039	-0.318316	-0.055230
	stomata width (adaxially)	-0.02652	-0.770675*	-0.285822
	no. of stomata /mm^2 (abaxially)	0.53883	-0.023148	-0.352040
	stomata length (abaxially)	0.76060*	0.239433	-0.366002
	stomata width (abaxially)	0.68880	0.149738	-0.523641
	cross section area	-0.88233*	0.252356	-0.022143
	thickness	-0.86765*	0.152934	-0.009624
	cuticle thickness	-0.15584	-0.119269	0.750124*
	% epidermis	0.88851*	-0.035401	0.186393
	% hypodermis	0.83737*	-0.112614	0.079522
	% vascular bundles	0.71987*	-0.020031	0.310511
	% bundle sheat	0.54703	-0.085024	0.606772
	% pallisade tissue	0.71701*	0.133486	0.448626
	length of palisade cells	0.28324	0.299464	-0.134504
	width of palisade cells	0.11422	-0.196672	-0.465631
	% water storage tissue	-0.85029*	-0.010936	-0.168549
cumulative percentages of the vectors		43.51	59.65	69.08

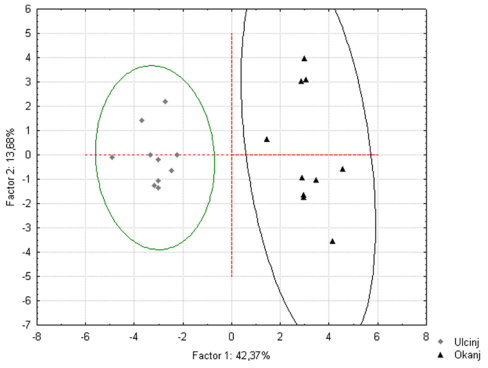

Figure 6. The projection of the cases of the first two components of the Principal Component Analysis

DISCUSION

On the basis of leaf and stem anatomical characteristics of the two examined *S. soda* populations, it could be seen that both of them show a combination of halomorphic and xeromorphic structures.

The ratio of the cortex thickness to stem diameter was 0.352 (Ulcinj salina) and 0.376 (Okanj), which is within the usual values found in xeromorphic stems (Fahn and Cutler, 1992).

The leaf is the organ that reacts the most to external environmental conditions, like those on examined localities (Fahn and Cutler, 1992). This was also proved in our examination. The results of PCA analysis showed that leaf parameters were dominantly present on the first axis and defined the most of the total variability. Therefore, two analyzed populations were clearly separated by PCA analysis along the first axis, mostly based on the variability of leaf anatomical parameters. Different environmental conditions on the two habitats and different types of leaf anatomical responses, induced this high variability.

Compared with the leaf thickness of *Salsola* species from South Africa, as well as *Salsola oreophila* and *Salsola australis*, the two examined populations of *S. soda* have thicker leaves (P'yankov et al., 1997; Klopper and Wyk, 2001).

The presence of a hypodermis is a common feature of many members of the Chenopodiaceae, as well as other *Salsola* species (Solereder, 1908, Carraro et al., 1993, Patrignati et al., 1993). The abundance of calcium oxalate crystals present in the hypodermis, might serve as a protective measure against insects and other small herbivores (Franceschi and Nakata, 2005). They also protected cells from excess calcium, regulate ion balance and help detoxication of the plant. Carolin et al. (1975) stated that presence or absence of a hypodermis appears to have no taxonomic significance in the Chenopodiace, but that statement was not supported by Klopper and Wyk (2001). These authors recorded that presence or absence of a hypodermis has been used to divide *Salsola* species into two main groups.

According to Solereder (1908), Metcalfe and Chalk (1983), Klopper and Wyk (2001), Voznesenkaya et al. (2001), the leaves of *Salsola* species have a thick mesophyll. Our results showed that palisade mesophyll is well developed in both studied populations. The spongy mesophyll is not typical, but is rather characterized by the absence of intercellular spaces. It consists of centrally placed aqueous tissue enclosing the main vascular bundle.

S. soda has so-called "Salsoloid" or Kranz type of photosynthetic cell arrangement (Voznesenkaya and Gamaley, 1986), which can occur in succulent cylindrical leaves. However, some *Salsola* species have different cross-section leaf anatomy. Carolin et al. (1975) described *S. webbii* as lacking Kranz type anatomy and they considered it an example of a reversion to non-Kranz anatomy in *Salsola*. Such features were also recorded in the genus *Sympegma* and were designated as "Sympegmoid" type. These authors defined Sympegmoid as having non-Kranz type anatomy when they observed multiple layers of mesophyll chlorenchyma. P'yankov et al. (1997) gave a hypotheses that reversion process of the Salsoloid Kranz type leaf anatomy from a C_4 to C_3 photosyn-

thesis, in genus *Salsola* was in the first place connected with the reduction of biochemical systems for the C_4 dicarboxylic acid cycle and then with changes in anatomical features of the photosynthetic tissues. Our results showed that *S. soda* from Serbia and Montenegro has Kranz type of photosynthetic cell arrangement.

The inland saline locality in Serbia had significantly lower precipitation and lower percentage of salt in the soil, which induced the formation of certain xeromorphic anatomical adaptations in leaves of the plants from this habitat. These plants had significantly larger proportion of leaf epidermis, hypodermis, palisade and vascular tissue with sclerenchyma, as well as higher number of stomata on both leaf sides. These findings are in accordance with the descriptions of leaf xeromorphic characteristics of plants from dry habitats, given by Fahn and Cutler (1992).

Flowers et al. (1986) gave anatomical features of halophytes which respond to changes in salinity. These authors found that plants that grow on high concentration of salt had relatively small number of stomata per mm^2, thicker leaf and cuticle, larger water storage tissue and lower stelar diameter. Moreover, comparision of halomorphic characteristics of two populations indicated that plants from Montenegro (maritime saline area) had more halomorphic characteristics than plants from Pannonian plane, which could be explained by the higher salinity of the soil in Adriatic coast.

CONCLUSION

In Ulcinj salina (maritime saline area, Montenegro) and Okanj (inland saline area, Serbia) localities, studied populations grow in saline area with longer or shorter intervals of summer drought, which explains xeromorphic and halomorphic characteristics in their anatomical structure. These xeromorphic and halomorphic morpho-anatomical characteristics of two studied populations refer to ecological plasticity and adaptations of plants in their habitats. Beside this, our research has shown that *S. soda* has quite a stable morpho-anatomical structure, since only quantitative changes were recorded.

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СТРУКТУРНЕ АДАПТАЦИЈЕ ВРСТЕ *SALSOLA SODA* L. (CHENOPODIACEAE) СА КОНТИНЕНТАЛНИХ И МАРИТИМНИХ ХАЛОБИОМА

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РЕЗИМЕ: Детаљно упознавање еколошке варијабилности и диференцијације популација унутар врста неопходан услов је за сагледавање биолошког потенцијала врста. У том смислу, одабрана је врста *Salsola soda* фамилије Chenopodiaceae која је присутна и на континенталним (Окањ, Срем) и на маритимним халобиомима (Улцињска солана). Истраживања су обухватила анализу 26 квантитативних карактера листа и стабла. Резултати су показали да биљке и са континенталних и са маритимних халобиома поседују халоморфне и склероморфне карактеристике које им омогућавају да опстану на стаништима са повећаном концентрацијом соли у подлози. Јединке популације са континенталног халобиома имају већи број склероморфних карактеристика, док маритимна популација поседује већи број халоморфних карактеристика. Најважнији анатомски карактери који имају статистички значај у формирању разлика између популација са континенталног и маритимног халобиома су: број стома на адаксијалној страни листа, површина попречног пресека и дебљина листа, процентуални удео површине епидермиса, хиподермиса, ткива за магационирање воде и проводних снопића листа, као и површина попречног пресека и дебљине коре стабла, полупречник стабла, процентуални удео површине епидермиса и проводних снопића стабла. Истраживања су такође показала да врста *S. Soda* има стабилне морфо-анатомске карактере.

КЉУЧНЕ РЕЧИ: анатомија биљака, халофите, лист, Salsola soda, стабло

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PREDICTION OF CURRENT SPECIES DISTRIBUTION OF *CHEILOSIA PROXIMA* GROUP (DIPTERA: SYRPHIDAE) ON THE BALKAN PENINSULA

ABSTRACT: Predicting species distribution in different climates is most often made by climate models ("climate envelope models" – CEM) which are using the current geographical distribution of species and climate characteristics of the area. Hoverflies (Insecta: Diptera: Syrphidae) can act as bioindicators and monitors of climate change and habitat quality. *Cheilosia* Meigen, 1822 is one of the largest hoverflies genera, with about 450 described species. The aim of this study was to model the current potential distribution of six species from *Cheilosia proxima* group on the Balkan Peninsula (*Cheilosia aerea* Dufour, 1848, *C. balkana* Vujić, 1994, *C. gigantea* Zetterstedt, 1838, *C. pascuorum* Becker, 1894, *C. proxima* Zetterstedt, 1843 and *C. rufimana* Becker, 1894) using maximum entropy modeling (Maxent). It is observed that parameters with highest influence on the analyzed species are Altitude and BIO 15 (Precipitation Seasonality) for all species, except *C. rufimana*. Parameter that also substantially influenced for all species, except *C. pascuorum*, is BIO 18 (Precipitation of Warmest Quarter). The models of current distribution have shown that the most important area of the Balkan Peninsula, for species from *Cheilosia proxima* group, is Dinaric mountains. Information obtained in this paper can help in future monitoring of species, as well as for the conservation measures, especially for endemics and rare species.
KEYWORDS: Syrphidae, climate envelope modeling, MAXENT, distribution

INTRODUCTION

Great loss of global species biodiversity is one of the biggest threats in the modern era. A large number of species is affected by a recent climate change,

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which is reflected in narrowing and moving its range to higher latitudes and altitudes (Parmesan and Yohe, 2003; Root et al., 2003). Without the diversity of species, ecosystems are more fragile and Liable to natural disasters and climate change. Idea of protecting species entails the question how to determine protection priorities and which conservation strategies should be applied. The fact is that species cannot be protected if their habitats are not protected. Monitoring of biodiversity, especially diversity of bioindicator species, has great importance for the implementation of active strategies in the management of protected areas.

Lack of detailed knowledge of a species distribution has been serious concern in wildlife management and conservation. Understanding species biology and thus their living requirements has to be the first priority before any decision making and action planning (Nazeri et al., 2012). One of the available tools for mapping the geographical distribution and potential suitable habitats are species distribution models. These models are generally based on various hypotheses how environmental factors control the distribution of species and communities (Guisan and Zimmermann, 2000). Predicting species distribution in different climates is most often made by climate models ("climate envelope models" – CEM) which are using the current geographical distribution of species and climate characteristics of the area (Hijmans and Graham, 2006).

Hoverflies (Insecta: Diptera: Syrphidae) can be used for the recognition and assessment of different types of habitats and they can be effective indicators of level of pollution as well. Basic aspects of hoverfly ecology, such as breeding sites, larval feeding habits, outline life cycles and habitat preferences are well enough understood for hoverflies to be incorporated into qualitative and quantitative methods of assessment. There are hoverflies that have conservation significance because, unfortunately, they are endangered by human activities and require action to ensure their survival. As such, they can act as flag species for whole communities and by conserving them, many other species will also be conserved. Also they can be used for monitoring the effects of climate change. Ball and Henshall (2007) used presumed climate preferences obtained from current distribution and other data (topography and land cover) to predict ranges of two species from genus *Chrysotoxum* Meigen, 1803 in United Kingdom under climate changes. Their results showed that *C. arcuatum* (Linnaeus, 1758) would contract in range and move to higher elevation, whereas *C. cautum* (Harris, 1776) would expand its range to northern England. Stuart Ball also undertook a similar kind of analysis for *Cheilosia sahlbergi* (Becker, 1894) which, according to his results, would become almost extinct in 2080 and *Platycheirus melanopsis* Loew, 1856 with little change in distribution (Rotheray and Gilbert, 2011).

Genus *Cheilosia* Meigen, 1822 is one of the largest genera in the family Syrphidae, with about 450 species, mostly Holarctic, from which 300 are present in the Palearctic (Peck, 1988), more than 80 in Nearctic, about 50 in the Oriental region, and several in the northern part of Neotropic (Ståhls et al., 2004). So far, there is about 175 European species, of which more than 50% is present on the Balkan Peninsula (Vujić, 1996).

There is no key that deals with all European species of genus *Cheilosia*, but Vujić et al. (2013) gave an identification key for the European species of the *proxima* species group, including newly described species *C. barbafacies* Vujić et Radenković, 2013. *Proxima* species group comprises following species: *Cheilosia aerea* Dufour, 1848, *C. balkana* Vujić, 1994, *C. gigantea* Zetterstedt, 1838, *C. ingerae* Nielsen & Claussen, 2001, *C. pascuorum* Becker, 1894, *C. proxima* Zetterstedt, 1843, *C. rufimana* Becker, 1894, *C. velutina* Loew, 1840 and *C. vulpina* (Meigen, 1822). All these species were recorded on the Balkan Peninsula, except for North European species *C. ingerae*.

Species from *Cheilosia proxima* group prefer open habitats in oak and beech forests and mountain and alpine pastures up to 2000 meters on the Balkan Peninsula. Flight period of these species is from April to October for *C. aerea* and *C. proxima*, from April to June for *C. pascuorum* and *C. rufimana*, from May to June for *C. gigantea*, from June to July for *C. balkana* and from July to August for *C. velutina* (Vujić, 1996).

The aim of this study was to model the current potential distribution of species from *Cheilosia proxima* group on Balkan Peninsula.

MATERIALS AND METHODS

All distribution data of *Cheilosia proxima* group of species from the Balkan Peninsula were obtained from following publications (Strobl, 1898, 1902; Drensky, 1934; Glumac, 1955, 1959, 1968; Coe, 1960; Bankowska, 1967; Vujić and Glumac, 1994; Vujić and Šimić, 1994; Vujić, 1995, 1996; Vujić et al., 2000) and hoverflies collections deposited at: National Museum of Bosnia and Herzegovina, Sarajevo, Bosnia and Herzegovina; Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad; Natural History Museum, Belgrade, Serbia; Croatian Natural History Museum, Zagreb, Croatia; Zoological Institute and Museum, Sofia, Bulgaria and private collection of J. Lucas (database:

http://www.dbe.uns.ac.rs/o_departmanu/laboratorije/laboratorija_za_istrazivanje_i_zastitu_biodiverziteta/prilog).

Environmental data was obtained from WORLDCLIM (version 1.3, http://www.worldclim.org) which is explained in detail in Hijmans et al. (2005). WORLDCLIM contains climate data (monthly precipitation and monthly mean, minimum and maximum temperature) and elevation at a spatial resolution of 2.5 arc-minutes (~5x5 km resolution) obtained by interpolation of climate station records from 1950–2000.

Environmental suitability was modeled using maximum entropy modeling (Maxent) (Phillips et al., 2006). Maxent calculates the potential geographic distribution of species by finding the probability distribution of maximum entropy and is an effective method for modeling species distributions from presence-only data. This program can work with a small number of samples and with records that have not been collected as a part of systematic biological

surveys, which is useful for processing the data that are based on museum collections (Elith et al., 2011).

Choosen Maxent default settings for all models were: regularization multiplier = 1, maximum iterations = 500, convergence threshold = 10^{-5}, maximum number of background points = 10.000. Seventy-five percent of occurrence records were randomly selected by Maxent as training data and 25% reserved for model testing. We ran cross-validation replicates for each model scenario. Covariates were tested for muticolinearity with VIF (various inflation factors) analysis in R package. Selected covariates for SDM analysis are shown in Table 1. The area under the curve (AUC) of the receiver operator characteristic was used to test the agreement between observed species presence and projected distribution (Manel et al., 2001). A jack-knife test was used to evaluate the importance of each environmental variable and to explain the native distribution of the species from *C. proxima* group.

Table 1: Bioclimatic variables* used for modeling the potential distribution of species from *C. proxima* group on Balkan Peninsula

C. aerea	*C. balkana*	*C. gigantea*	*C. pascuorum*	*C. proxima*	*C. rufimana*
BIO 4	BIO 2	BIO 8	BIO 8	BIO 2	BIO 2
BIO 9	BIO 15	BIO 9	BIO 15	BIO 9	BIO 18
BIO 15	BIO 18	BIO 15	BIO 18	BIO 15	
BIO 18	BIO19	BIO 18		BIO 18	
BIO 19				BIO19	

*BIO 2 – Mean Diurnal Range (Mean of monthly (max temp – min temp)); BIO 4 – Temperature Seasonality (standard deviation *100); BIO 8 – Mean Temperature of Wettest Quarter; BIO 9 – Mean Temperature of Driest Quarter; BIO 15 – Precipitation Seasonality (Coefficient of Variation); BIO 18 – Precipitation of Warmest Quarter; BIO 19 – Precipitation of Coldest Quarter

RESULTS AND DISCUSION

Prediction of current distribution was analyzed for six species from *Cheilosia proxima* group registered on the Balkan Peninsula (*Cheilosia aerea, C. balkana*, *C. gigantea*, *C. pascuorum*, *C. proxima* and *C. rufimana*). For two species, *C. velutina* and *C. barbafacies* there were not enough data for modeling.

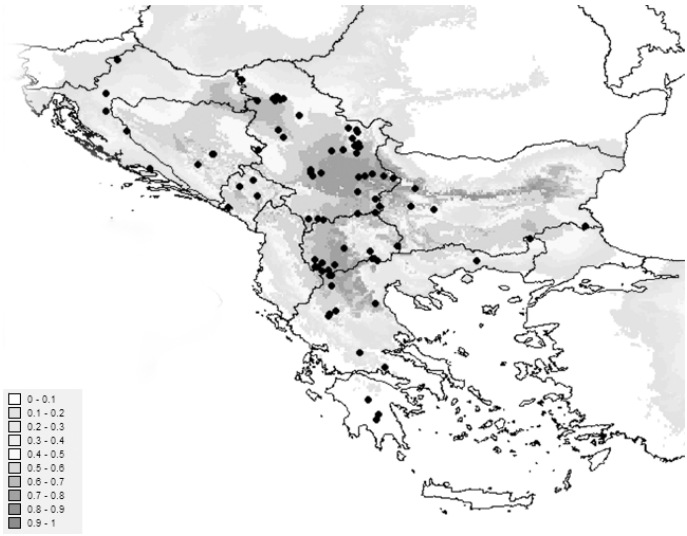
To estimate success rate of climate models for each species, we calculated training and test AUC values. According to training AUC (0,870-0,993) and test AUC (0,500-0,939), this model can be evaluated as good and excellent (Tab. 2). It is observed that parameters with highest influence on the analyzed species are Altitude and BIO 15 (Precipitation Seasonality) for all species, except for *C. rufimana*. Parameter that also showed substantial influence for all species, except for *C. pascuorum*, is BIO 18 (Precipitation of Warmest Quarter).

Table 2: AUC training/test values and variable contributions for analysed species

	AUC training	AUC test	Variable contributions (%)
Cheilosia aerea	0.870	0.761	BIO 15 (40%) BIO 4 (21.1%) BIO 18 (16.9%) BIO 19 (8.5%) BIO 9 (4.12%) Altitude (0.7%)
Cheilosia balkana	0.993	0.939	BIO 15 (51.6%) Altitude (33.1%) BIO 19 (10%) BIO 18 (5.2%) BIO 2 (1.8%)
Cheilosia gigantea	0.981	0.925	BIO 15 (49.2%) Altitude (20.9%) BIO 8 (15.9%) BIO 9 (10.1%) BIO 18 (2.1%)
Cheilosia pascuorum	0.976	0.624	BIO 15 (65.8%) Altitude (34.2%)
Cheilosia proxima	0.923	0.819	Altitude (39.3%) BIO 15 (36.4%) BIO 19 (10.2%) BIO 18 (9.6%) BIO 2 (4.1%) BIO9 (0.4%)
Cheilosia rufimana	0.984	0.500	Altitude (78.9%) BIO 18 (13.8%) BIO 2 (7.3%)

Habitat and elevation can restrict species ranges, and they are important in explaining the distribution of species (Harris and Pimm, 2008; Sekercioglu and Schneider, 2008; Newbold et al., 2009; Virkkala et al., 2010). Species do not respond directly to elevation, but they change in abiotic variables which are regulated by elevation. However, it may be argued that elevation is a surrogate for other non-climate related factors that may restrict species geographically, e.g. food availability (Remonti et al., 2009), or for climatic parameters when spatially explicit estimates of climate are unavailable. Precipitation (BIO 15, 18) indirectly influences larval development of *Cheilosia proxima* group, because immature stages of these phytophagous species live in stems, roots or rhizomes of different plants.

C. aerea and *C. proxima* are widely distributed species. According to current prediction they can be found on the whole territory of the Balkan Peninsula, not only on known localities (Fig. 1). *C. aerea* does not have special habitat preferences and could be expected, not only on mountains, but also on hills at lower altitudes. *C. proxima* has similar distribution pattern, but lower percentage of occurrence on the coast of the Mediterranean Sea and hills (Fig. 2). This could be explained by different ecological demands of these two species: *C. aerea* prefers lower altitudes and more dry habitats while *C. proxima* usually inhabits humid forests (Vujić, 1996; Speight, 2012).



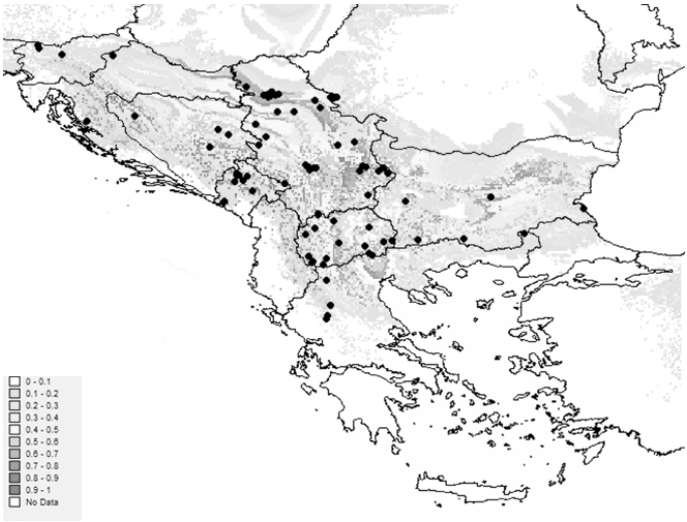


Figure 1: Potential distribution of *C. aerea* Figure 2: Potential distribution of *C. proxima* Legends represent percentage of potential species distribution and spots represent current data of species

C. *gigantea* has wide Palaearctic range, but on the Balkans does not reach extreme south of the Peninsula (Greece). The current prediction corresponds to the actual distribution on high mountains and also confirms the south edge of its range on the Balkan Peninsula (Fig. 3).

Palaearctic species *C. rufimana* is very rare on the Balkan Peninsula. It was found at few localities of the south Dinaric mountains and the Rilo-Rhodopes (Vujić, 1996). According to the current prediction this species could

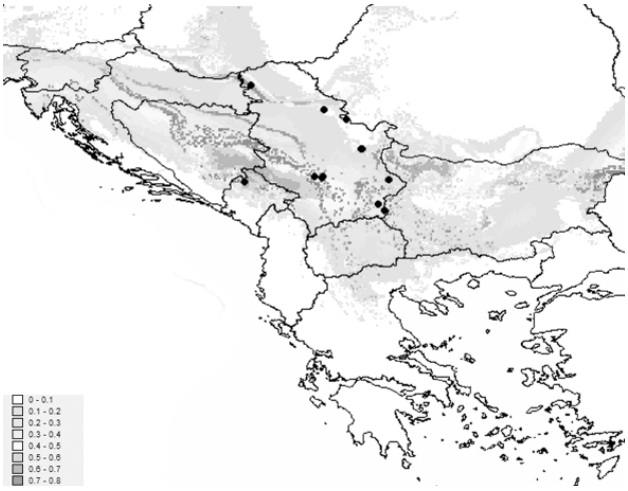
also find appropriate habitats in the entire ranges of the Dinaric mountains and the Carpathians, but also in the Alps, where has already been registered (Speight, 2010) (Fig. 4).

Figure 3: Potential distribution of *C. gigantea* Figure 4: Potential distribution of *C. rufimana* Legends represent percentage of potential species distribution and spots represent current data of species

Range of *C. pascourum* extends from Alps, across the Balkan Peninsula to the European part of Russia (Speight, 2012). Except on the high mountains, this species was also recorded from lowland oak forests in the Pannonian

Plain (Vujić, 1996). The similar pattern of distribution is visible in the model where the area with highest percentage of appearance is, besides the high Dinaric Mountains, also Sub-Pannonian hills, low mountains and coast of the Black Sea in the eastern part of the Peninsula (Fig. 5).

C. balkana is endemic species for Alps and south Dinaric mountains (Vujić, 1996). Data of the current prediction almost completely coincide with the records of its relatively narrow distribution. Model predicts that this species could also be present on north and central Dinaric mountains between Alps and south Dinaric mountains, where already has been registered (Fig. 6).



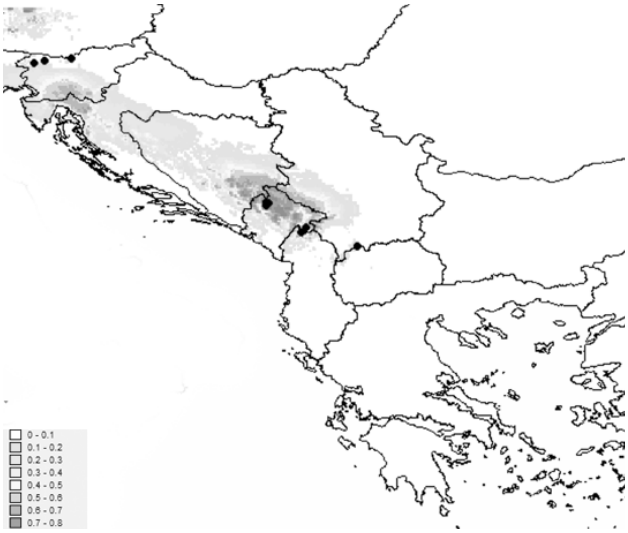


Figure 5: Potential distribution of *C. pascuorum* Figure 6: Potential distribution of *C. balkana* Legends represent percentage of potential species distribution and spots represent current data of species

CONCLUSION

The models of current distribution of species from *Cheilosia proxima* group have shown that the most important area on the Balkan Peninsula is the Dinaric mountains. Current predictions, based on climate and altitude, highly correspond to actual distribution data, but also reveal new localities with suitable habitats and ecological conditions for particular species (for *C. gigantea*, these are mountains in the south-western part of Serbia and mountain Prokletije and for *C. pascuorum* Strandža mountain in Bulgaria). These information can help in future monitoring of species, as well as for the conservation measures, especially for endemic and rare species.

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ПРЕДВИЂАЊЕ САДАШЊЕ ПОТЕНЦИЈАЛНЕ ДИСТРИБУЦИЈЕ ВРСТА ИЗ *CHEILOSIA PROXIMA* ГРУПЕ (DIPTERA: SYRPHIDAE) НА БАЛКАНСКОМ ПОЛУОСТРВУ

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РЕЗИМЕ: Предвиђање дистрибуције врста у различитим климатским условима најчешће се врши помоћу климатских модела (Climate envelope models – CEM) који користе тренутну географску дистрибуцију врста и климатске карактеристике подручја. Осолике муве (Insecta: Diptera: Sirphidae) могу послужити као биоиндикатори климатских промена и квалитета станишта. *Cheilosia* Meigen, 1822 је један од највећих родова осоликих мува, са око 450 врста. Циљ овог истраживања је моделовање тренутне потенцијалне дистрибуције шест врста из *Cheilosia proxima* групе (*Cheilosia aerea* Dufour, 1848, *Cheilosia balkana* Vujić, 1994, *Cheilosia gigantea* Zetterstedt, 1838, *Cheilosia pascuorum* Becker, 1894, *Cheilosia proxima* Zetterstedt, 1843 и *Cheilosia rufimana* Beker, 1894) на Балканском полуострву помоћу модела максималне ентропије (Maxent). Примећено је да су параметри са највећим утицајем надморска висина и сезонске падавине (BIO 15) за све врсте, осим *C. rufimana*. Параметар који је такође показао значајан утицај на све врсте, осим *C. pascuorum* је BIO 18 (падавине током најтоплијег тромесечја). Модели потенцијалне дистрибуције врста из *Cheilosia proxima* групе показали су да је најважније подручје на Балканском полуострву регија Динарских планина. Информације добијене у овом раду могу да помогну у будућим мониторингу врста, као и успостављању конзервационих мера, посебно за ендеме и ретке врсте.

КЉУЧНЕ РЕЧИ: Сифриде, климатски модели, MAXENT, дистрибуција

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FLAVONOID EXTRACTION FROM *FICUS CARICA* LEAVES USING DIFFERENT TECHNIQUES AND SOLVENTS

ABSTRACT: The current study presents the best method for a rapid and efficient extraction of flavonoids from *Ficus carica*. Dried leaves were extracted using distilled water and ethanol 70% by extraction method of maceration, microwave and stirring. Using of TLC and HPLC techniques, the rutin and kaempferol were detected. For flavonoids extraction ethanol 70% was more efficient than water. The relative concentration of rutin and kaempferol was higher by microwave methods ussing ethanol.

KEYWORDS: *Ficus carica,* flavonoids, maceration, microwave

INTRODUCTION

Ficus carica L.(Moraceae) is a deciduous tree, which grows in a tropical and subtropical regions of India and is commonly known as fig tree.

The leaves and the fruits of *Ficus carica* are traditionally used as laxative, stimulant, against throat diseases, antitussive, emollient, emmenagogue and resolvent (Bellakhdar et al., 1991; Guarrera et al., 2003, Trifunschi and Ardelean, 2012).

The fig leaf decoction is used for hemorrhoids, whereas an infusion of its fruit can safely be used as a laxative for children. The fresh leaves are dabbed on warts (Baytop, 1984).

However, the antioxidant activity and citotoxicity against various cancer cell lines reported in fig are potentially promising for its future therapeutic uses. (Wang et al., 1996; Kikuzaki et al, 1993).

Ficus species are rich sourse of polyphenolic compounds, flavonoids which are responsable for strong antioxidant properties that help in prevention and

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therapy of various diseases. *Ficus carica* leaves are known to be reach in flavonoids (Leung et al., 1996). The flavonoids of this species have remarkable medicinal properties. Vaya et al. (2006) isolated and identified the flavonoids, rutin and kaempferol by HPLC-MS, which have a high medicinal value in therapeutic uses.

Recent studies mentioned the microwave and ultrasonic methods as efficient in flavonoid extraction (Pan et al., 2001, 2003). Type of solvent and method of extraction are important factors for optimizing yield extraction (Turkmen et al., 2006).

The purpose of this work was to develop and evaluate efficient and simple procedures for extraction of flavonoids from *Ficus carica* leaves in short time.

MATERIAL AND METHODS

Plant material: Leaves of *Ficus carica* were collected in may 2012 from Timisoara (Romania) and were dried at room temperature in a dark place

Material: Methanol, acetonitrile and phosphoric acid (HPLC grade) were purchased from Merck, rutin and kaempferol were purchased from Sigma Aldrich and other chemicals purchased from "Reactivul" Bucureşti and "Chimopar" Bucureşti.

Preparation of extracts

Three extraction methods from dried leaves were evaluated: maceration, microwave and stirring.

Method 1: maceration

One gram of the dried sample was chopped into small pieces and then extracted with 20 ml solvent (10% w/v) for 3 days at room temperature (25° C). Solvents used for extraction were distillated water and ethanol 70%.

Method 2: microwave (LG – Auto sensor diet, full power)

The suspension were irradiated three times under microwaves in pre-setting procedure (3s power on, 60s off) to achieve the desired temperature of 70° C.

Method 3: stirring

One gram of the dried sample was extracted with 20 ml water (then with ethanol 70%) for 60 min at 60° C.

Extracts were filtered in vacuum using Whatman filter. Aqueous as well as hydroalcoholic extracts were evaporated to dryness. The dried weigh was measured.. The yielding was defined: (crude extract weight / plant material

weight) x 100. The extract obtained by each extraction technique was analyzed by TLC and HPLC.

TLC analysis

Aliquots of standards and crude extracts were analyzed on silica gel 60 plate and developed in different mobile phases. Components were visualized under ultraviolet light (λ=254 nm). The following mobile phases were used:

1 ethyl acetat: ethanol: acetic acid: water = 16: 1,5:1:1
2 ethyl acetat: acetic acid: formic acid: water = 50: 11:11:20
3 ethyl acetat: formic acid: water = 50: 20:15
4 cloroform: formic acid: methanol = 50: 20:15

The flavonoid standards, rutin (Rf = 0,35) and kaempferol (Rf = 0,64) were verified in extracts after concomitant with standards. They were visible as yellow fluorescent spots.

HPLC analysis (Cacig, 2007)

Flavonoids were measured at 365 nm by a HPLC Agilent 1100. Separation was carried out on a Lichrospher 100-RP-18 column (5 μm, 250 x 4 mm). A gradient elution was performed with eluent: acetonitril:water = 1:1. The flow rate was 1 ml/min and the injection volume was 20 μl. Identification of the flavonoids was carried out by comparing their retention times to those of standards.

RESULTS AND DISCUSSION

Table 1. indicates significant differences in relation to extraction type

Table 1. Experimental conditions for obtaining extracts

	Maceration	***Microwave***	***Stirring***
Extraction solvents	Distilled water an ethanol 70%		
Temperature	25° C	70° C	60° C
Extraction time	3 day	3 x (3s)	60 min

The extraction yield obtained for extraction technique was shown in table 2.

Table 2. Extraction yield

Solvents for extraction	Extraction yield (%, w/w)		
	Maceration	*Microwave*	*Stirring*
Distilled water	2.5	5.5	-
Ethanol 70%	8	10	9

The important yielding was obtained after ethanol extraction compared with water extraction. The lowest yielding was obtained by maceration for both solvents.

The higher flavonoid content occured using 70% ethanol, for all methods. The microwave techniques provide a high flavonoid extraction, followed by stirring.

Compared with conventional extraction methods, microwave resulted in higher level of extracted flavonoids with the advantage of saving time and solvent.

Hydroalcoholic extracts analysis using HPLC revealed main compounds, among which the peak corresponds to rutin (t_R = 4,5) and kaempferol (t_R = 19,8) (Figure 1).

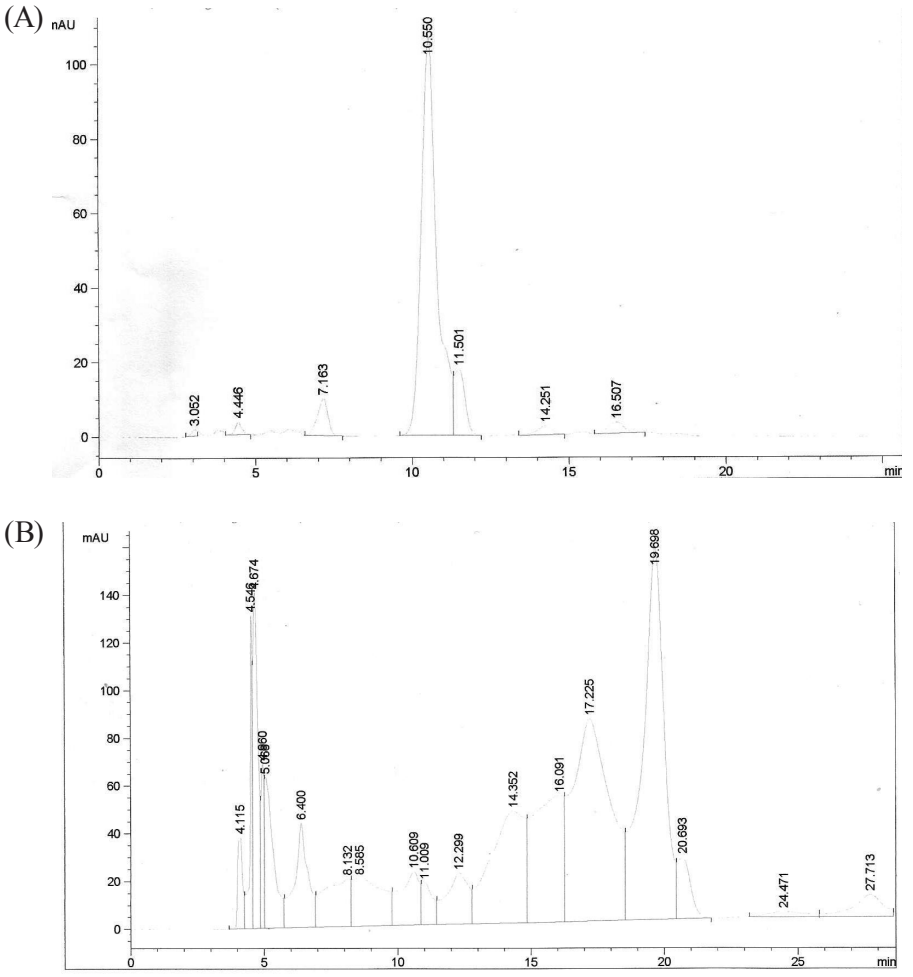

Figure 1. HPLC chromatogram of aqueous (A) and hydroalcoholic (B) extracts of *Ficus carica* obtained by microwave extraction: rutin (t_R = 4,5) and kaempferol (t_R = 19,8)

CONCLUSION

Microwave-assisted extraction of compounds is relatively new. Some reports showed its positive results for extracting phenolic compounds and flavonoids, more effective than conventional extraction methods.

Microwave and stirring may improve flavonoid extraction using as water and ethanol 70%. These methods besides higher temperature, reduced extraction time in comparation with maceration.

In the present study, the proportion of flavonoids was reduced by maceration in combination with ethanol 70% solvent were the efficient. It may suggest that microwave method using ethanol 70% is suitable for fast extraction of flavonoids.

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ЕКСТРАКЦИЈА ФЛАВОНОИДА ИЗ ЛИСТОВА *FICUS CARICA L.* УПОТРЕБОМ РАЗЛИЧИТИХ ТЕХНИКА И РАСТВАРАЧА

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РЕЗИМЕ: У раду су приказане различите методе за брзу и ефикасну екстракцију флавоноида из *Ficus carica* L. Екстракција из сувог лишћа урађена је са растварачима: дестилована вода и етанол (70%). Методе екстракције биле су: мацерација, микроталасна деструкција и мешање. Користећи TLC и HPLC технике одређени су рутин и кемпферол. Добијени резултати показали су да је етанол (70%) ефикаснији за екстракцију флавоноида од воде. Релативна концентрација рутина и кемпферола добијена је ако се користи микроталасни метод а као растварач етанол.

КЉУЧНЕ РЕЧИ: Ficus carica, флавоноиди, мацерација, микроталаси

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EFFECTS OF CULTIVAR AND YEAR ON LEAF NUMBER IN WINTER BARLEY

ABSTRACT: Leaf appearance in small cereals is the result of leaf primordium initiation and leaf primordium extension. Final leaf number (FLN) on main stem is determined by the number of primordia initiated up to the beginning of floral transition. The aim of this study was to determine the effect of growing season and cultivar on FLN in winter barley. Twelve cultivars differing in origin and time of anthesis (early, medium and late) were tested during six growing seasons (GS), from 2002/03 to 2007/08.

FLN across cultivars and GSs was 13.5. The highest FLN across GSs was in the late, six-rowed barley cultivar Kredit (14.7) and the lowest in the early, two-rowed barley cultivar Novosadski 581 (11.3). In regard to earliness, the lowest FLN was in the early groups of cultivars (12.9) and the highest in the late ones (13.9). The tested cultivars showed significant variability in FLN, which can be used for selecting most adaptable genotypes for specific growing conditions.

KEYWORDS: Barley (*Hordeum vulgare* L.), heritability, leaf number, polynomial regression

INTRODUCTION

The life cycle of cereals is divided into two main periods, period until anthesis and grain filling period. Period until anthesis can be divided into three phases: leaf initiation (vegetative phase), spikelet initiation (early reproductive phase) and spike growth (late reproductive phase) (Slafer and Whitechurch, 2001). Leaf appearance in small cereals is the result of leaf primordium initiation and leaf primordium extension. In normal growing conditions, both processes are mainly controlled by temperature (McMaster, 2005). The final number of initiated leaf primordia is proportional to the time from sowing to double ridge (Robertson et al., 1996). At the time of seed-

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ling emergence, the shoot apex has five to seven leaf primordia and this is considered to be the range in minimum final leaf number per wheat plant (Robertson et al., 1996).

Final leaf number (FLN) is determined by the number of primordia initiated up to the beginning of floral transition. Kirby (1992) showed that sowing date and location have an effect on the variability of the number of leaves produced by winter wheat. That variation can be explained, in part, by differences in exposure to low temperature during the phase when leaf primordia are being initiated. FLN depends upon the rate and duration of leaf initiation. The vernalization response is important for fitting the plant life cycle to the environment in which it is grown, so that it can make the best use of the seasonal opportunities for growth and avoid adverse climatic factors. The major effect of vernalization is to shorten the duration of the phase of leaf primordia production (Griffiths et al., 1985). Air temperature is the main factor affecting leaf number and phyllochron (Rickman and Klepper, 1991).

Researchers have concentrated on understanding how environmental factors, first of all temperature and photoperiod and then water and nutrition, affect the FLN. Only a few studies have evaluated cultivar effect on the FLN. In this research, we studied the effect of cultivar and year on FLN of winter barley.

MATERIAL AND METHODS

Cultivars and crop management. Twelve barley cultivars (Kompolti-4, Skorohod, Novosadski 525, Novosadski 581, Plaisant, Gotic, Sonate, Boreale, Novator, Kredit, Monaco and Cordoba) which differed in origin, pedigree and agronomic traits were used in this study. A 6-year experiment was conducted from 2002/03 to 2007/08 growing season (GS) at the experiment field of Institute of Field and Vegetable Crops in Novi Sad (45°20'N, 15°51'E, altitude 86 m) on a Chernozem soil and under rainfed conditions. The experiment was conducted in a randomized complete block design with 3 replications each year. Planting density in all GSs was 300 viable seeds per m^2 for six-rowed barley and 350 viable seeds per m^2 for two-rowed barley.

To determine the FLN on the main stem, recording was done according to the Haun scale (Haun, 1973) on three tagged plants in each replication. FLN was determined as the number of leaves on the main stem, including the flag leaf. GDD for leaf number development was calculated as $Tn=[(T_7+T_{14}+2T_{21})]/4$, where T_7, T_{14}, and T_{21} were temperatures at 7 AM, 2 PM and 9 PM, respectively (Pržulj, 2001). Base temperature was 0° C.

Statistics. All data were subjected to the analysis of variance using Statistica 9.0 (StatSoft, Tulsa, OK, USA). Barley cultivars and GS were supposed to be fixed factors. When differences among earliness groups (early, medium early, late), duration of developmental phases and agronomic traits were tested, four cultivars from each group were considered as replication for detection of developmental phases. Broad-sense heritabili-

ties were estimated using the variance components from ANOVA, as follows: $h^2=V_G/V_F$.

RESULTS AND DISCUSSION

Phenological development and phyllochron of small cereals result from genetics and numerous environmental factors (McMaster, 2005). In our study, FLN was controlled by cultivar, year and their interaction (Tab. 1). Year exhibited the highest contribution to FLN variation, about 74%. It means that the tested cultivars were genetically similar in leaf number. Low value of interaction showed stability of leaf number from year to year.

Tab. 1. – Mean squares of final leaf number (FLN) per main stem of winter barley

Source	Df	FLN
Cultivar	11	13.98**
Year	5	10.17**
C xY	55	0.98**
% components of variance		
Cultivar		17.20
Year		73.96
C xY		7.19
Heritability		0.93

** – significant at the 0.01 level

Considered across the GSs, the early cultivar Novosadski 581 had the lowest and the late cultivar Kredit the highest FLN (Tab. 2). In the cultivar Novosadski 581, early maturity was due to a reduction in FLN. Juskiew et al. (2003) found that, in spring barley, earliness was due to accelerated postanthesis growth rather than reduction in leaf number and phyllochron. Even though the cultivar x year effect on FLN was significant and participated in total variation with 7.2% (Tab. 1), the FLN variability due to interaction cultivar x year was rather small (Tab. 2).

Although differences in FLN were observed among the cultivars (Tab. 1, 2) they could not be ranked according to their FLN. For example, the early cultivar Kompolti-4 had one of the highest FLNs in 2002/03 GS and one of the lowest in 2005/06 GS (Tab. 2). The average FLN for the barley main stem was 13.5.

Across GSs and maturity classes, the early maturity group was the fastest in completing the FLN (Fig. 1I). The relationship between GDD requirement and FLN per main stem fitted the best the quadratic equation, with $R^2>0.99$. Also, quadratic equation was the most fitting for the relationship between leaf number development across cultivars in the same maturity group and GS, with $R^2>0.97$ (Fig. 1, II).

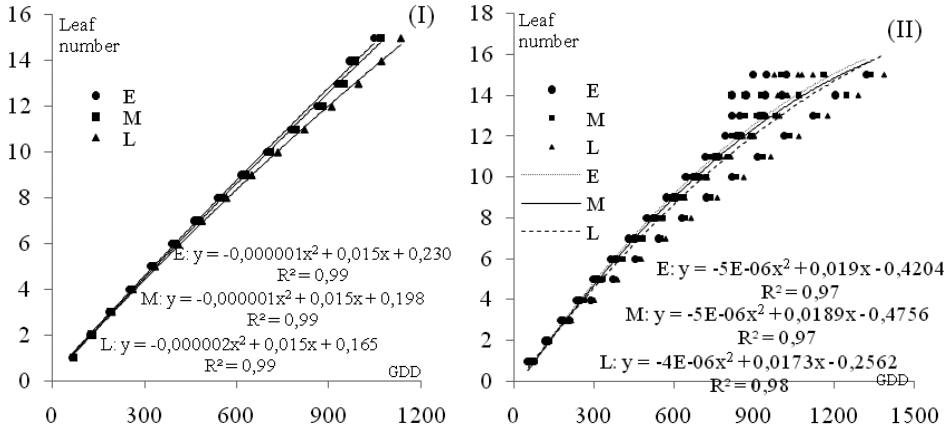

Fig. 1. – (I) Leaf number development in four early (E), four medium (M) and four late (L) winter barley cultivars across six GSs (II) Leaf number development of three winter barley maturity classes (E-early, M-medium, L-late) in different GSs. Each point represents the average of four cultivars belonging to a maturity group in individual GS. There are six points for each leaf per maturity group

Tab. 2. – Final leaf number per main stem of twelve winter barley cultivars during six growing seasons (GS)

Cultivar	GS						Average
	2002/03	2003/04	2004/05	2005/06	2006/07	2007/08	
Kompolti-4 (E, 6R)	14	15	13	13	15	14	14.0
Skorohod (E, 6R)	13	14	12	13	14	14	13.3
Novosadski 525 (E, 2R)	13	13	12	13	13	13	12.8
Novosadski 581 (E, 2R)	10	12	10	12	12	12	11.3
Plaisant (M, 2R)	15	13	13	13	15	14	13.8
Gotic (M, 6R)	15	13	14	13	15	14	14.0
Sonate (M, 2R)	13	13	12	13	14	13	13.0
Boreale (M, 2R)	13	13	12	13	14	13	13.0
Novator (L, 2R)	15	15	13	14	15	14	14.3
Kredit (L, 2R)	15	15	14	15	15	14	14.7
Monaco (L, 6R)	13	13	13	13	14	14	13.3
Cordoba (L, 6R)	14	14	13	14	14	15	14.0
Average	13.6	13.6	12.6	13.2	14.2	13.7	13.5
LSD		Cultivar	Year	CxY	CV		
	0.05	0.17	0.12	0.42	1.9%		
	0.01	0.23	0.16	0.56			

E – early, M – medium, L – late, 2R – two-rowed, 6R – six-rowed

In our investigation, the FLN was positively correlated with GDD accumulated until flag leaf completion, while the effect of precipitation was less important (Tab. 3).

Tab. 3. Simple correlation between final leaf number (FLN) and temperature and precipitation during some barley phenological growth stages (FGS)

FGS	GDD from E till DR	Precipitation from E till DR	GDD during DR	Precipitation during DR	GDD during J	Precipitation during J
FLN	0.35**	-0.25*	0.34**	-0.27*	0.79*	-0.16

GDD – growing degree days, E – emergence, DR – double ridge, J – jointing
*, ** – significant at the 0.05 and 0.01 levels, respectively

When difference among earliness groups was tested, i.e. four cultivars from each group were considered as replication, FLN was under control of maturity classes and years (Tab. 4). The interaction maturity class x year was not statistically significant (Tab. 4), i.e., the early cultivars usually have the lowest FLN and the late ones the highest FLN (Tab. 5). Across the studied GSs, the early cultivars had 12.9, medium early 13.5, and late 13.9 main stem leaves (Tab. 5).

Tab. 4. – Mean squares of final leaf number (FLN) per main stem for three maturity classes (early, medium, late) of winter barley

Source	df	FLN
Maturity class	2	8.56**
Year	5	3.39^{**}
Maturity class x year	10	0.58^{ns}
% components of variance		
Maturity class		14.90
Year		20.82
Maturity class x year		25.91
Heritability		0.71

** – significant at the 0.01 level, ns – non significant

The identification of genetic variability of leaf area is a crucial step in plant breeding. (R o y o et al., 2004). This is particularly important in species with a narrow genetic background, which may be a result of the selection pressure applied in breeding programs. Understanding of how changes in photosynthetic area may be affected by environmental conditions – particularly drought stress under field conditions – could provide a basis for developing superior high yielding varieties. Genotype by environment interactions should be taken into account to determine the optimum breeding strategy in a target environment.

Tab. 5. – Final leaf number (FLN) per main stem for three maturity classes (early, medium, late) across six GS

	GS						
Maturity class	2002/03	2003/04	2004/05	2005/06	2006/07	2007/08	Average
Early	12.5	13.5	11.8	12.8	13.5	13.2	12.9
Medium	14.0	13.5	12.7	13.0	14.5	13.5	13.5
Late	14.2	13.8	13.2	14.0	14.5	14.2	13.9
Average	13.6	13.6	12.6	13.2	14.2	13.7	13.5
		Year	Maturity cl.	Y x G	CV		
LSD	0.05	0.76	0.54	1.31	6.9%		
	0.01	1.01	0.71	1.75			

Since FLN is mainly defined by interaction between cultivar and growing conditions, the choice of appropriate cultivar for certain growing conditions is an important task for barley growers. The time of anthesis is an important physiological trait as a criterion of selection in barley breeding. Anthesis can be presented as a function of the leaf number produced by the main stem. Most of the environmental and genetic variation from seedling emergence to anthesis results from variation in the number of leaves produced by the main stem (He et al., 2011). Although variation in FLN was mainly affected by growing conditions, heritability for FLN was high in our study. Also, the early maturing cultivars had lower and late maturing cultivars had higher FLN on the main stem.

CONCLUSION

The average FLN on the main stem of winter barley grown under the conditions of the Pannonian Plain was 13.5. The early cultivars had one leaf less than the late cultivars. Although non-genetic factors were important in FLN, variation in FLN was rather a conservative trait, the early maturing cultivars having low and the late maturing cultivars high FLNs.

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УТИЦАЈ СОРТЕ И ГОДИНЕ НА БРОЈ ЛИСТОВА КОД ОЗИМОГ ЈЕЧМА

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РЕЗИМЕ: Појава листова код стрних жита резултат је формирања и издуживања примордија листова. Коначан број листова на главном стаблу зависи од броја формираних примордија до појаве примордија класића. Циљ овога истраживања је да се одреди утицај године и сорте на коначан број листова на главном стаблу код озимог јечма. Дванаест сорти јечма, дивергентних по пореклу и времену цветања (ране, средње, касне) тестиране су у периоду од шест производних сезона. Просечан број листова на главном стаблу за испитиване сорте и сезоне износио је 13,5. Највећи просечан број листова (14,7) имала је касна сорта шесторедог јечма „Кредит“, а најмањи (11,3) рана сорта дворедог јечма „Новосадски 581“. У односу на групе зрења најмањи број листова био је код групе раних сорти (12,9), а највећи код групе касних сорти (13,9). Испитиване сорте разликовале су се значајно у коначном броју листова, што се може искористити у избору адаптабилних генотипова за одређена подручја.

КЉУЧНЕ РЕЧИ: Јечам (*Hordeum vulgare* L.), број листова, криволинијска регресија

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EFFECT OF CULTIVAR AND YEAR ON PHYLLOCHRON IN WINTER BARLEY

ABSTRACT: Development and growth of leaves in cereals significantly affects grain yield since dry matter accumulation depends on the leaf area that intercepts light. Phyllochron (PHY) is defined as time interval between the emergences of successive leaves on the main stem. The aim of this study was to determine the effect of year and cultivar on phyllochron in winter barley. Twelve cultivars of winter barley differing in origin and time of anthesis were tested during six growing seasons (GS), from 2002/03 to 2007/08. The highest PHY across GSs was determined in the two-rowed cultivar Cordoba (81.6°Cd) and the lowest in the two-rowed cultivar Novosadski 581 (71.0°Cd). The early cultivars had fast leaf development, the medium cultivars medium and the late cultivars slow development, 72.5°Cd, 75.6°Cd and 78.9°Cd, respectively. The tested cultivars showed significant variability in the PHY, which can be used for selecting most adaptable genotypes for specific growing conditions.

KEYWORDS: Barley, *Hordeum vulgare*, phyllochron, heritability, variance

INTRODUCTION

The phyllochron (PHY) is defined as the thermal time interval between the emergence of successive leaf tips, expressed as growing degree days – GDD or degree days - °Cd (Johnen et al., 2012). It is a measure of plant development that could be used to assess how the plant has responded to environmental conditions or to predict how it is going to respond to them. To estimate the daily interval between growth stages, photothermal units or growing degree days are usually used. PHY is an alternative approach for measuring the period between growth stages. Its advantages are that it is more flexible than the other approaches and that it integrates developmental processes within the plant.

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PHY is a result of combination of genetic and environmental factors, which interact to produce plant leaves in a predictable manner. PHY has been widely accepted by crop modelers for predicting plant development and by producers for determining the timing of management practices such as irrigation, fertilization or pesticide application. The effect of environmental changes on the rate of leaf emergence in barley ought to be understood in order to make accurate predictions concerning the cropping technology.

Air temperature is the main factor affecting PHY (R i c k m a n and K l e p p e r, 1991). Other environmental factors (daylength, water stress, carbohydrate reserves and nutrient stress) have been shown to have little effect on PHY in grasses (B o r r à s - G e l o n c h et al., 2010). Long photoperiod increases the rate of leaf emergence, i.e., it decreases PHY in wheat and barley (M i r s c h e l et al., 2005). Water and nitrogen deficits decrease PHY. Leaf emergence rate in wheat depends on the cultivar and sowing date (M i r a l l e s et al., 2001), resulting in fewer leaves per plant in later sowing dates. A r d u i n i et al., (2010) found that the rate of leaf emergence is defined early in the life cycle.

The purpose of this study was to analyze the effect of cultivar and year on PHY of winter barley and to compare leaf emergence in cultivars with different thermal requirements until anthesis.

MATERIALS AND METHODS

Cultivars and crop management. Twelve barley cultivars which differed in origin, pedigree and agronomic traits were used in this study. A 6-year experiment was conducted from 2002/03 to 2007/08 growing seasons (GS) at the experimental field of Institute of Field and Vegetable Crops in Novi Sad (45º20'N, 15º51'E, altitude 86 m) on a Chernozem soil and under rainfed conditions. Planting density was 300 viable seeds per m^2 for six-rowed barleys and 350 viable seeds per m^2 for two-rowed barleys. To avoid negative effects of diseases and pests, the experiment was sprayed with the fungicide Tilt 250 EC in Zadoks phase 64 and with the insecticide Karate zeon. Weed control was performed by hand. Degree days with a T_b of 0° C were calculated according to P r ž u l j (2001).

Statistical analysis. All the data were subjected to the analysis of variance using Statistica 9.0 program (StatSoft, Tulsa, OK, USA). When differences among earliness groups (early, medium early, late) were tested, four cultivars from each group were considered as replication for detection of PHY duration.

RESULTS AND DISCUSSION

Phenological development and PHY in cereals are the result of genetic background and environmental factors (M c M a s t e r, 2005). PHY is controlled by two factors (cultivar and year), and their interaction (Table 1). Although differences in PHY were observed among the cultivars (Tables 1, 2), PHY-based ranking of cultivars could not be performed.

Table 1. Mean squares and percentage of variance components of the phyllochron (PHY) for winter barley

Source	df	PHY
Cultivar	11	176.78**
Year	5	1929.41**
C xY	55	41.54**
% of variance components		
Cultivar		23.09
Year		30.18
C xY		40.45
Heritability		0.77

** – Significant at the 0.01 level

If it is accepted that PHY is shorter in late sowing (M i g l i e t t a , 1991), one could conclude that sowing was late in 2003/04, 2004/05 and 2005/06, since PHY was 70.9, 71.7, and 70.9°Cd respectively (Table 2). However, sowing was in regular time and shorter PHY was a result of unfavorable growing conditions, first of all temperature and water, from sowing until flag leaf emergence. High soil temperature at the time of seed emergence only shortens the duration of germination and seedling emergence. It had no effect on either the PHY or phenological development in wheat and barley (M c M a s t e r and W i l h e l m , 2003).

The early cultivars Skorohod and Novosadski 581 had the lowest °Cd requirements, or the shortest PHY, and the late cultivars Novator, Kredit, Monaco, and Cordoba had the highest °Cd requirements or the longest PHY (Table 2). The early cultivars showed no consistency in the relationship between PHY and earliness, while the medium early and late two-rowed barley cultivars were found to have a longer PHY. While B o r r à s e t a l . (2009) determined that two-rowed spring barley cultivars have a short PHY, J u s k i w et al. (2001) found them to have a long PHY. PHY in spring barley varied in dependence of genotype and combination of temperature and daylength, but it invariably increased as temperature increased or daylength decreased.

In this study, the average PHY was 75.7°Cd, with a range from 71.0 to 81.6°Cd (Table 2). In relation to spring barley, the observed PHY was longer than the mean of 64.5°Cd for Alaska growing conditions (D o f i n g and K a r l s s o n, 1993) and shorter than the means of 77.2°Cd for North Dakota environment (F r a n k and B a u e r, 1995) and 107°Cd for winter wheat grown in Central Great Plains, USA (M c M a s t e r et al., 1992).

The time from sowing to emergence was 138 °Cd (P r ž u l j et al., 2012), which was about two PHYs. J u s k i w et al. (2001) attributed one PHY to the development of coleoptiles and another PHY to the first true leaf.

J u s k i w et al. (2001) found that PHY is prone to error because temperature and daylength are known to affect the leaf emergence rate. Our results confirm this statement, where error for PHY of a specific leaf was rather

Table 2. Phyllochron (°Cd) for twelve winter barley cultivars across six growing seasons (GS)

Cultivar	GS						Average
	2002/03	2003/04	2004/05	2005/06	2006/07	2007/08	
Kompolti-4 (E, 6R)	73.0	63.2	68.8	73.7	87.9	71.9	73.1
Skorohod (E, 6R)	70.1	68.0	68.1	63.9	86.0	71.3	71.2
Novosadski 525 (E, 2R)	72.3	71.9	72.9	70.7	90.0	70.5	74.7
Novosadski 581 (E, 2R)	70.7	65.4	68.6	68.4	83.5	69.4	71.0
Plaisant (M, 2R)	65.9	70.9	70.0	69.6	88.6	80.6	74.3
Gotic (M, 6R)	67.7	68.9	70.4	70.5	89.6	85.1	75.4
Sonate (M, 2R)	77.8	68.7	75.0	69.7	91.2	75.4	76.3
Boreale (M, 2R)	77.3	69.5	72.6	70.6	90.3	76.2	76.1
Novator (L, 2R)	71.8	73.3	70.4	70.7	90.8	85.4	77.1
Kredit (L, 2R)	76.8	72.3	69.8	72.0	94.3	86.9	78.7
Monaco (L, 6R)	82.1	78.0	70.7	74.2	92.8	75.6	78.9
Cordoba (L, 6R)	81.1	81.3	83.3	77.2	94.0	72.6	81.6
Average	73.9	70.9	71.7	70.9	89.9	76.7	75.7
		Cultivar	Year	CxY	CV		
LSD	0.05	0.9	0.7	2.7	1.9%		
	0.01	1.2	0.9	3.0			

E-early, M-medium, L-late, 2R- two-rowed, 6R- six-rowed

high, ranging from 13.63% to 33.47% of total variation (Table 3). Leaves 13th and 14th were exceptions, with the errors of 8.85% and 4.69%, respectively. There was a paradox associated with these two leaves: the lower the degree of freedom, the lower the error. It might be due to the lower variability for leaves 13th and 14th of the analyzed cultivars since those that had lower values of PHY (Novosadski 581, Novosadski 525, Scorohod, Sonate and Boreale) did not have 13th and 14th leaves and were not included in statistical calculations. The values of PHY heritability were rather high, although cultivar participation in total variation was less than 10% (Table 3).

When the cultivars were sorted according to earliness, PHY was determined by maturity class and growing season (Table 4). The interaction maturity class x year was not significant for PHY (Table 4), i.e., the early cultivars invariably had the shortest PHY and the late ones had the longest PHY regardless of the year (Tables 2, 5).

The rate of leaf emergence as a function of leaf number increased throughout the growing season from about 50°Cd for the first leaf to 100°Cd for the last leaves. The rate of change fitted the quadratic equitation with the R^2 value ≥0.93 (Figure 1). It greatly depended on GS which participated in the total variation with >30% (Table 1). Some studies indicated that the rate of leaf emergence or PHY was constant in both wheat and barley from seedling emergence to the emergence of the flag leaf (K a m a l i and B o y d , 2000; J u s k i w et al., 2001; J u s k i w and H e l m , 2003). Other studies showed

Table 3. Percentages of the components of variance and heritability for the PHY of individual main stem leaves across 12 winter barley cultivars and six growing seasons (2002/03-2007/08)

Leaf	Total df	Variance component				Percentage of variance components				h_b^2
		σ_G^2	σ_Y^2	σ_{GY}^2	Error	Culti-var	Year	C xY	Error	
1st	215	1.55	68.46	0.00	35.22	1.47	65.06	0.00	33.47	0.44
2nd	215	3.34	84.47	0.00	28.73	2.86	72.49	0.00	24.65	0.69
3rd	215	2.70	74.87	2.85	16.10	2.80	77.57	2.96	16.68	0.66
4th	215	4.81	79.06	5.56	17.74	4.49	73.77	5.19	16.55	0.72
5th	215	5.13	57.55	9.12	13.20	6.04	67.70	10.73	15.53	0.69
6th	215	5.51	49.41	8.45	16.29	6.92	62.03	10.61	20.45	0.70
7th	215	4.93	49.48	12.62	12.00	6.23	62.61	15.97	15.19	0.64
8th	215	5.44	50.86	11.49	13.70	6.67	62.42	14.10	16.81	0.67
9th	215	3.86	53.77	13.82	12.57	4.60	63.99	16.45	14.96	0.56
10th	209	5.74	50.51	13.33	14.50	6.83	60.07	15.85	17.25	0.65
11th	209	4.75	54.41	18.32	12.23	5.29	60.65	20.42	13.63	0.56
12th	182	6.56	72.76	19.85	16.61	5.67	62.84	17.14	14.35	0.61
13th	104	15.79	97.61	36.52	14.56	9.60	59.35	22.20	8.85	0.70
14th	53	11.67	316.73	5.81	16.43	3.33	90.33	1.66	4.69	0.84
Flag leaf	215	20.77	114.95	36.30	32.95	10.13	56.08	17.71	16.08	0.72

Table 4. Mean squares of phyllochron (PHY) for three maturity classes of winter barley

Source	df	PHY
Maturity class	2	257.88**
Year	5	643.35**
Maturity class x year	10	12.61^{ns}
% of variance components		
Maturity class		28.28
Year		30.21
Maturity class x year		0.00
Heritability		0.96

** – significant at the 0.01 level, ns – non significant

that PHY varied with plant development and that the pattern of leaf emergence was bilinear rather than linear. A change in PHY may occur between leaves 6 and 8 (M i r a l l e s et al., 2001). F l o o d et al. (2000) suggested that variation in PHY may be due to ontogenetic changes, the changes occurring around the double ridge stage. M i r a l l e s and R i c h a r d s (2000) found a linear relationship under long days and a bilinear relationship for leaf emergence under short days.

Table 5. Phyllochron for the three maturity classes (E-early, M-medium, L-late) across 6 growing seasons (GS)

Maturity class	GS						Average
	2002/03	2003/04	2004/05	2005/06	2006/07	2007/08	
E	71.5	67.1	69.6	69.2	86.9	70.8	72.5
M	72.2	70.5	72.0	70.1	89.9	79.3	75.6
L	77.9	75.3	73.5	73.5	93.0	80.1	78.9
Average	73.9	70.9	71.7	70.9	89.9	76.7	75.7
		Maturity cl.	Year	Mc x Y	CV		
LSD	0.05	2.2	3.1	5.5	5.1%		
	0.01	3.0	4.2	7.3			

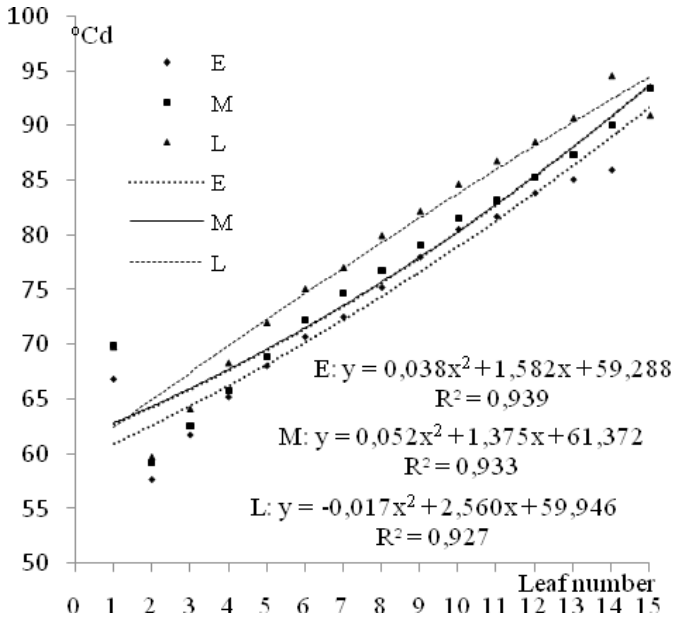

Figure 1. Phyllochron across four early (E), four medium (M) and four late (L) winter barley cultivars across six growing seasons

CONCLUSION

Under the conditions of the Novi Sad region, the average phyllochron of leaves on the main stem of winter barley was 75.7°Cd. The phyllochron in the early cultivars was shorter by 6.4°Cd than in the late ones. Since PHY is determined by the genotypes, growing conditions and interaction between cultivar and growing conditions, selection of appropriate cultivars for different growing conditions is important task of barley growers.

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УТИЦАЈ СОРТЕ И ГОДИНЕ НА ФИЛОХРОН ОЗИМОГ ЈЕЧМА

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РЕЗИМЕ: Развиће и раст листова жита значајно утиче на принос зрна јер акумулација суве материје зависи од лисне површине која апсорбује сунчеву светлост. Филохрон се дефинише као временски интервал између појаве сукцесивних листова на главном стаблу. Циљ овог истраживања је да се одреди ефекат године и сорте на дужину филохрона код озимог јечма. Дванаест сорти озимог јечма различитих по пореклу и времену до цветања тестирано је током шест производних сезона, од 2002/03. до 2007/08. на локалитету „Нови Сад“. Најдужи филохрон (81.6 °Cd) имала је сорта дворедог јечма „Кордоба“, а најкраћи (71.0 °Cd) сорта дворедог јечма „Новосадски 581“. Ране соте имале су најбржи, средње ране, средњи и касне сорте најспорији пораст листова. Код раних сорти просечна вредност филохрона износила је 72.5 °Cd, средње раних 75.6 °Cd и касних 78.9 °Cd. Тестиране сорте показале су значајну варијабилност у дужини филохрона, што може представљати основу у избору најадаптабилнијих генотипова у одређеним условима спољне средине.

КЉУЧНЕ РЕЧИ: јечам, *Hordeum vulgare*, филохрон, херитабилност, варијанса, филохрон

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VERTEBRATE FAUNA OF THE EARLY AND LATE IRON AGES IN VOJVODINA (SERBIA)

ABSTRACT: Based on current published and unpublished research results, a total of 34 vertebrate species from 4 classes have been registered at 9 archaeological sites from the Early Iron Age in Vojvodina (Serbia). The most numerous one is the mammal class (Mammalia) with 22 species, then osteichthyes class (Osteichthyes) with 10 species, while birds (Aves) and reptiles (Reptilia) are repsented with one species each. From the Late Iron Age, at 14 archaeological sites, a total of 21 species were registered, of which 16 belong to the mammal class (Mammalia), birds (Aves) are represented by 2 species, and osteichthyes (Osteichthyes) by 3 species.

KEYWORDS: Archaeological sites, the Early and Late Iron Age, Vertebrata fauna, Vojvodina (Serbia)

INTRODUCTION

On the territory of Vojvodina, there are archaeological sites from different periods, and the largest amount of data on animal remains has been collected from the Neolithic, but also from the Early and Late Iron Age. Systematic archaeological digs at most of sites from the Iron Age started during the 1970s and since then more than 35 000 samples have been collected (Blažić, 1993–1994; 1997; 2010; unpublished). These periods are interesting because, at the beginning of the Early Iron Age, a climate change occurred, therefore colder and more humid period had begun. Remains of the vertebrate bones also testify of this phenomenon (Bőkőnyi, 1981).

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MATERIAL AND METHODS

This paper features both published and unpublished results of the vertebrate fauna research from 9 archaeological sites in Vojvodina from the Early Iron Age, and 14 sites from the Late Iron Age (Map 1). Osteological material comes from the settlements and necropoleis. Determination was done by using the keys Driesch, (1976) and Schmid, (1972) and comparative osteological collections.

Map of Vojvodina with marked sites (1–19) with dating given for each site (V for the Early Iron Age and VI for the Late Iron Age).

1. Gomolava–Hrtkovci (V,VI); 2. Kalakača–Beška (V); 3. Feudvar–Mošorin (V); 4. Gradina–Vašica (V, VI); 5. Djepfeld–Doroslovo (V); 6. Čarnok–Vrbas (VI); 7. Turski Šanac–Bačka Palanka (VI); 8.Vrtlozi–Šimanovci (V, VI); 9. Tromeđja–Pećinci (VI); 10. Žirovac–Ruma (VI); 11. Bare–Voganj (VI); 12. Livade–Sremska Mitrovica (VI); 13. Mitrovačke livade–Sremska Mitrovica (VI); 14. Zabrana–Mandjelos (V); 15. Bregovi Atovac–Kuzmin (V, VI); 16. Velike ledine–Kuzmin (VI); 17. Gajić–Adaševci (VI); 18. Asfaltna baza–Zemun (V); 19. Židovar (VI)

RESULTS AND DISCUSSION

The period, from which the collected and processed material from archaeological sites in Vojvodina originates, is divided into nine phases, of which the **Early** and **Late Iron Age** should be singled out. The most important archaeological site in Vojvodina is **Gomolava**-Hrtkovci, where eight cultural layers have

been recorded. The Early Iron Age from this site has been dated to the period between 950 and 300 B.C., and the Late Iron Age to the period between 1st century B.C. and 1st century A.D.) (Petrović, 1984). The sites from the Early Iron Age are marked with 'V' in the paper, and the sites from the Late Iron Age with 'VI'.

At 9 archaeological sites in Vojvodina from the **Early Iron Age** (Hallstatt culture), a total of 34 vertebrate species from 4 classes were registered. The most numerous class is mammals (Mammalia) with 22 species that are classified in 5 orders. With 9 species, the most numerous order is Artiodactyla, and then comes order Carnivora with 7 species. Apart from this, from the Insectivora order only Erinaceus genus was determined, and from the Carnivora order only Mustela genus. In the period of the Early Iron Age, great diversity was registered also in the class of Osteichthyes in which 10 species belonging to 4 orders were determined, of which Cypriniformes order is the most numerous one with 4 species. Reptiles (Reptilia) and birds (Aves) are represented in this period by only one species each (Table 1).

Judging by the vertebrate fauna diversity at archaeological sites, it can be concluded that there is the richest diversity at **Feudvar**–Mošorin (Site No. 3) where 20 mammal species, 10 osteichthyes species and one reptile species were registered (Becker, 1991 [1]; Blažić, 1991 [6]). The diversity of the vertebrate fauna is, above all, the result of the fact that area around this archaeological site consisted of the river and coastal biotopes, very humid and dry habitats, dense forests, and bare lands (Becker, 1998). With 15 mammal species, 3 fish species and 1 bird species, **Gomolava** (Site No. 1) is the next in line for its faunal diversity (Blažić, 1986 [4]; 1988 [5]), and there is similar diversity at **Kalakača**–Beška (site no. 2) where 11 mammal species, one reptile species and one fish species was registered. This vertebrate faunal diversity is definitely in relation with the fact that this area was under forest and rich in water (Bőkőnyi, 1988 [17]). Slightly poorer diversity of vertebrate fauna (10 mammal species and one fish species) was recorded at **Asfaltna baza**–Zemun (Site No. 18) (Blažić, 2010 [13]). On the remaining 5 sites from this period, only mammals were registered, wherein at sites **Vrtlozi**–Šimanovci (Site No. 8) and **Bregovi Atovac**–Kuzmin (Site No. 15) 9 species were registered (Blažić, 1992 b [8]), and at **Gradina**–Vašica (Site No. 4), **Đepfeld**–Doroslovo (Site No. 5) and **Zabrana**–Manđelos (Site No. 14) 8 species were registered (Bőkőnyi, 1981 [16]; Blažić, 1992 b [16]; 1993–1994 [9]) (Table 1).

At all 9 sites from the Early Iron Age, the following species were registered: *Canis familiaris, Equus cabalus, Sus scrofa domestica, Sus scrofa, Cervus elaphus, Bos taurus, Ovis aries* and *Capra hircus* (Table 1). All of these animals were the base for the animal husbandry of this period (Becker, 1998; Blažić, 2005 a) and they were also bred in the settlements of the Early Iron Age in South-East Europe (Blažić, 1992 b). Stanc et al. (2010) discusses the significant presence of ox – *Bos taurus* in the economy of the Iron Age at several archaeological sites on the territory of Romania, stating that contribution of this species in the mammal fauna was between 30% and 60%, and between 30% and 70% in the fauna of domesticated mammals. Nevertheless, the presence of wild boar – *Sus scrofa* and red deer – *Cervus elaphus* at sites in Vojvodina, points to presence of large forests and areas rich in water (Bőkőnyi, 1981).

Table 1 – Fauna at some archaeological sites in Vojvodina from the **Early** (V) and **Late Iron Ages** (VI)

TAXON	DATING	SITE AND author
Classis MAMMALIA		
Ordo Insectivora		
Erinaceus sp.	V	3[1]
Ordo Rodentia		
(Citellus citellus) (L.1766) *Spermophilus citelus* Cuvier 1825	V	3[1]
Cricetus cricetus (L. 1758)	V	3[1]
Castor fiber L. 1758	V VI	1[4],[5]; 3[1] 13[7],[8]; 19[14]
Ordo Lagomorpha		
Lepus europaeus Pall. 1778	V	1[4],[5]; 2[17]; 18[13]
Lepus capensis L. 1758	V VI	3[1] 1[4],[9],[18]; 6[7],[9]
Ordo Carnivora		
Canis familiaris L.	V VI	1[4],[5]; 2[17]; 3[1]; 4,5[9]; 8,14,15[8]; 18[13] 1[4],[9],[18]; 4,6[7],[9]; 8,12,13,17[7],[8]; 11,15[7],[8],[9]; 9[8],[9]; 19[14]
Canis lupus L. 1758	V VI	1[4],[5]; 3[1] 19[14]
Vulpes vulpes (L. 1758)	VI	1[4],[9],[18]; 6[7],[9]; 19[14]
Ursus arctos L. 1758	V VI	1[4],[5]; 3[1] 6[7],[9]; 19[14]
Mustela sp.	V	3[1]
Martes martes (L. 1758)	V	1[4],[5]
Martes sp.	V	3[1]
Meles meles (L. 1758)	V VI	3[1] 19[14]
Lutra lutra (L. 1758)	V	3[1]
Felis silvestris Schreber 1777	V	3[1]
Ordo Perissodactyla		
Equus cabalus L. 1758	V VI	1[4],[5]; 2[17]; 3[1]; 4,5 [9]; 8,14,15[8]; 18[13] 1[4],[9],[18]; 4,6[7],[9]; 7[3],[7],[9]; 8,12,17[7],[8]; 11,15[7],[8],[9]; 9[8],[9]; 19[14]
Ordo Artiodactyla		
Sus scrofa domestica L. 1758	V VI	1[4],[5]; 2[17]; 3[1]; 4,5[9]; 8,14,15[8]; 18[13] 1[4],[9],[18]; 4,6[7],[9]; 7[3],[7],[9]; 8,12,13,17[7],[8]; 10,16[8]; 11,15[7],[8],[9]; 9[8],[9]; 19[14]
Sus scrofa L. 1758	V VI	1[4],[5]; 2[17]; 3[1]; 4,5[9]; 8,14,15[8]; 18[13] 1[4],[9],[18]; 4,6[7],[9]; 8,12[7],[8]; 15[7],[8],[9]; 9[8],[9]; 19[14]
Cervus elaphus L. 1758	V VI	1[4],[5]; 2[17]; 3[1]; 4,5[9]; 8,14,15[8]; 18[13] 1[4],[9],[18], 4,6[7],[9]; 7[3],[7],[9]; 8,12,13,17[7],[8]; 11,15[7],[8],[9]; 9[8],[9]; 19[14]

Dama dama (L. 1758)	V	3[1]
Capreolus capreolus (L. 1758)	V VI	1[4],[5]; 2[17], 3[1]; 8,15[8]; 18[13] 1[4],[9],[18]; 4,6[7],[9]; 8,12[7],[8]; 15[7],[8],[9]; 19[14]
Bos taurus L.	V VI	1[4],[5]; 2[17]; 3[1]; 4,5[9]; 8,14,15[8]; 18[13] 1[4],[9],[18]; 4,6[7],[9]; 7[3],[7],[9]; 8,12,13,17[7],[8]; 10,16[8]; 11,15[7],[8],[9]; 9[8],[9]; 19[14]
Bos primigenius (Bojanus 1827)	V VI	1[4],[5]; 2[17]; 3[1] 4,6[7],[9]; 19[14]
Ovis aries L. 1758	V VI	1[4],[5]; 2[17]; 3[1]; 4,5[9]; 8,14,15[8]; 18[13] 1[4],[9],[18]; 4,6[7],[9]; 7[3],[7],[9]; 8,12,13,17[7],[8]; 10,16[8]; 11,15[7],[8],[9]; 9[8],[9]; 19[14]
Capra hircus L. 1758	V VI	1[4],[5]; 2[17]; 3[1]; 4,5[9]; 8,14,15[8]; 18[13] 1[4],[9],[18], 4,6[7],[9]; 7[3],[7],[9]; 8,12,13,17[7],[8]; 10,16[8]; 11,15[7],[8],[9]; 9[8],[9]; 19[14]
Classis AVES		
Ordo Anseriformes		
Anser anser (L. 1758)	VI	1[4],[9],[18]
Ordo Galliformes		
Gallus domesticus (L. 1758)	V VI	1[4],[5] 1[4],[9],[18]; 4,6[7],[9]; 13[7],[8]; 11,15[7],[8],[9]; 19[14]
Classis REPTILIA		
Ordo Testudines		
Emys orbicularis (L. 1758)	V	2[17]; 3[1]
Classis OSTEICHTHYS		
Ordo Acipenseriformes		
Huso huso (L. 1758)	V	3[6]
Acipenser stellatus Pallas 1771	V	3[6]
Acipenser gueldenstaedti Brant et Ratzeburg 1833	V	3[6]
Ordo Salmoniformes		
Hucho hucho (L. 1758)	V	3[6]
Esox lucius L. 1758	V VI	1[4],[5]; 3[6] 1[4],[9],[18]; 4,6[7],[9]
Ordo Cypriniformes		
Aspius aspius (L. 1758)	V	3[6]
Abramis brama (L. 1758)	V	3[6]
Cyprinus carpio L. 1758	V VI	1[4],[5]; 2[17]; 3[6]; 18[13] 1[4],[9],[18]; 4,6[7],[9]; 15[7],[8],[9]; 19[14]
Leuciscus idus (L. 1758)	V	3[6]
Ordo Siluriformes		
Silurus glanis L. 1758	V VI	1[4],[5]; 3[6] 4,6[7],[9]; 15[7],[8],[9]

N.B. The number in the square brackets is the reference number; the number outside the square brackets is the site number

Archaeozoological researches at 14 sites in Vojvodina from the **Late Iron Age** (La Tène culture) have shown presence of 21 vertebrate species classified in 3 classes: mammals (Mammalia), birds (Aves) and osteichthyes (Osteichthyes). From the total number of species, 16 are mammals classified into 5 orders of which the most numerous one is Artiodactyla (8 species). The following were also recorded: 3 fish species members of 3 orders, as well as 2 bird species systematised into 2 orders (Table 1).

The largest number of mammal species (14) was recorded at **Židovar** (site no. 19), and besides these, carp – *Cyprinus carpio* was – also registered (Blažić, unpublished [14]). Disregarding the number of mammal species, **Čarnok**-Vrbas (site no. 6) stands out in terms of vertebrate diversity, where 13 mammal species, 3 fish species and one bird species were registered (Blažić, 1992 a [7]; 1993–1994 [9]). Then comes **Gomolava** (site no. 1) (Clason, 1979 [18]; Blažić, 1986 [4]) with 11 mammal species, 2 bird species and 2 fish species is, and similar situation was also recorded at **Gradina**–Vašica (site no. 4) where 10 mammal species, one bird species and 3 fish species were registered (Blažić, 1992 a [7]; 1993–1994 [9]). At **Turski Šanac**–Bačka Palanka (site no. 7) as well as at **9 sites along the highway through Srem**, with the exception of **Bregovi Atovac**–Kuzmin (site no. 15), only the presence of mammal species was recorded, the number of which was between 4 and 9 (Blažić, 1978 [3]; 1992 a [7]; b [8]; 1993–1994 [9]). Among registered mammal species, Eurasian beaver – *Castor fiber* should be mentioned, the presence of which at **Mitrovačke livade**–Sremska Mitrovica (Site No. 13) speaks of ideal life conditions in the nearby stream (Blažić, 1992 b [8]).

At all above mentioned sites from this period, the following species were registered: *Sus scrofa domestica, Bos taurus, Ovis aries* and *Capra hircus* (Table 1). This is entirely consistent with the picture of life in settlements from the Late Iron Age in Europe, where animal husbandry was more important than hunting (Blažić, 1992 b).

After comparing data on vertebrate fauna at archaeological sites from the Late Iron Age in Vojvodina with those in the neighbouring countries, it can be concluded that one site in south-east Romania and 3 sites in Bulgaria of the same period have much richer ornithofauna diversity (Gal et Kessler, 2002; Boev, 1993). Somewhat richer vertebrate fauna at sites in Vojvodina from the Late Iron Age was recorded compared with Kale–Krševica site in Serbia (Blažić, 2005 b).

CONCLUSIONS

Based on current published and unpublished research results from 9 archaeological sites in Vojvodina (Serbia) from the **Early Iron Age** and from 14 sites from the **Late Iron Age**, the following can be concluded:

- From the **Early Iron Age**, a total of 34 vertebrate species members of 4 classes were registered. The most numerous one is the mammal class (Mammalia) with 22 species, then osteichthyes class (Osteichthyes) with 10 species, while birds (Aves) and reptiles (Reptilia) are represented by one species each.

- From the **Late Iron Age**, a total of 21 vertebrate species were registered, of which 16 belong to the mammal class (Mammalia), birds (Aves) are represented by 2 species, and osteichthyes (Osteichthyes) by 3 species.

- From the **Early Iron Age**, the richest site in terms of vertebrate fauna is **Feudvar**–Mošorin.

- From the **Late Iron Age**, the richest site in terms of vertebrate faunal diversity is **Čarnok**–Vrbas.

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ФАУНА КИЧМЕЊАКА СТАРИЈЕГ И МЛАЂЕГ ГВОЗДЕНОГ ДОБА У ВОЈВОДИНИ (СРБИЈА)

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РЕЗИМЕ: На основу објављених и необјављених резултата истраживања, са девет археолошких локалитета у Војводини (Србија) из старијег гвозденог доба, детерминисано је укупно 34 врсте кичмењака припадника четири класе. Најбројнија је класа сисара (Mammalia) са 22 врсте, затим класа кошљориба (Osteichthyes) са 10 врста, док су птице (Aves) и гмизавци (Reptilia) заступљени са по једном врстом. У периоду млађег гвозденог доба на 14 археолошких локалитета регистрована је укупно 21 врста кичмењака од којих 16 припада класи сисара (Mammalia), две врсте припадају птицама (Aves) и три врсте припадају кошљорибама (Osteichthyes). Током старијег гвозденог доба највећим богатством фауне кичмењака одликује се локалитет Феудвар-Мошорин. У млађем гвозденом добу по диверзитету фауне кичмењака истиче се локалитет Чарнок-Врбас.

КЉУЧНЕ РЕЧИ: археолошки локалитети, старије и млађе гвоздено доба, фауна кичмењака, Војводина (Србија)

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ORNITOFAUNA FROM THE ARCHAEOLOGICAL SITES IN VOJVODINA (SERBIA)

ABSTRACT: After decades-long vertebrate fauna research, out of 42 archaeological sites in Vojvodina (Serbia) from different periods ranging from the Neolithic to the Middle Ages, remains of birds were registered at 17 sites (4 from the Neolithic, 1 from the Early Iron Age, 7 from the Late Iron Age, 5 from the Roman Period, 1 from the Migration Period, and 4 from the Middle Ages). A total of 14 species and 4 genera were registered for this vertebrate class. The richest ornithofauna is from the Neolithic, where 9 species and 3 genera were registered. The Migration and Medieval periods are next with 4 registered species and one genus each. There were 3 species registered from the Roman Period, and 2 species from the Late Iron Age. The poorest ornitofauna was registered from the Early Iron Age, only one species.

KEYWORDS: Archaeological Sites, Ornitofauna, Vojvodina (Serbia)

INTRODUCTION

Osteological material from the archaeological sites of different periods in Vojvodina is being collected since 1930s, but the more intensive fauna research has been done in the last forty years. Bird remains were found at many sites. First of all, the Neolithic site **Starčevo** should be mentioned. At this site, research began in 1932, and continued between 1969 and 1970 (Clason, 1980). **Nosa**–**Biserna obala** is the site that belongs to the same period. The research at this site was conducted in 1957 (Bőkőnyi, 1974). Research at **Donja Branjevina** site, near Deronje, was done in 1987 (Blažić, 1992 a). Systematic collecting

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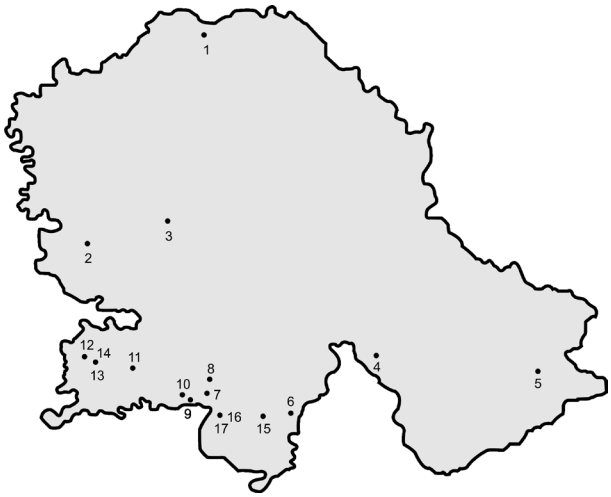
of osteological material at the multilayered archaeological site **Gomolava** near Hrtkovci began in 1971, although digging at this site began in 1953 (Petrović, 1984).

Bird bones were also found at many sites from the Early and Late Iron Ages: **Gradina**–Vašica, **Čarnok**–Vrbas (Blažić, 1992b, 1993-1994), **Bare**–Voganj, **Mitrovačke livade**–Sremska Mitrovica and **Bregovi Atovac**–Kuzmin (Blažić, 1992 c), and also at **Židovar** (Blažić, unpublished). Ornitofauna remains have also been found at sites from the Roman Period: **Malo Kuvalovo**–Krnješevci, **Kudoš**–Šašinci, **Prosine**–Prhovo (Blažić, 1992 c), **Sirmium site no. 85**–Sremska Mitrovica (Nedeljković, 2008) and **Vranj** (Blažić, 1993). From the Migration Period, bird remains were found only at **Sirmium site no. 85** (Nedeljković, 2008). Concerning the Middle Ages, bird remains were found at 4 sites: **Malo Kuvalovo, Gajić** and **Vračić**–Adaševci (Blažić, 1992 c), and at **Sirmium site no. 85** (Nedeljković, 2008).

MATERIAL AND METHODS

This paper features both published and unpublished results of the ornithofauna research from 17 archaeological sites of different periods in Vojvodina (Map 1). Material originates from the settlements and necropoleis from the Neolithic to the Middle Ages. Determination of vertebrates was done using the keys Driesch, (1976) and Schmid, (1972) and comparative osteological collections.

Map 1. Map of Vojvodina with marked archaeological sites



1. Nosa–Biserna obala (I); 2. Donja Branjevina (I); 3. Čarnok (III); 4. Starčevo (I); 5. Židovar (III); 6. Malo Kuvalovo (IV, VI); 7. Kudoš (IV); 8. Bare (III); 9. Sirmijum 85 (IV,V,VI); 10. Mitrovačke livade (III); 11. Bregovi (III); 12. Gradina (III); 13. Gajić (VI); 14. Vračić (VI); 15. Prosine (IV); 16. Gomolava (I, II i III); 17. Vranj (IV)
I – Neolit; II – Early Iron Ages; III – Late Iron Ages; IV – Roman period; V – Migration period; VI – Middle ages

RESULTS AND DISCUSSION

Period from which originates the collected and analysed osteological material from the archaeological sites in Vojvodina is divided into the following phases: Neolithic Age – New Stone Age (6000–3200 BC); Eneolithic Age – Copper Age (3200–2000 BC); Bronze Age (2000–950 BC); Early Iron Age (950–300 BC); Later Iron Age (4th century BC – 1st century AD); Roman Period (1st–4th century AD), Migration Period (4th–9th century AD) and Middle Ages (9th century – 1526 AD) (Cerović et al., 1997).

The most important archaeological site in Vojvodina is Gomolava-Hrtkovci, where 8 cultural layers were recorded (Petrović, 1984). Its stratigraphy is as follows: Late Neolithic Age – Early Eneolithic Age (3800–3400 BC); Middle Eneolithic Age (3400–2800 BC); Late Eneolithic Age (2800–2000 BC); Bronze Age (2000–900 BC); Early Iron Age (900–300 BC); Later Iron Age (1st century BC–1st century AD); Roman Period (1st–4th century AD), and Middle Ages. It should be noted that the osteological material from this site originates from the first, second, third, fifth and sixth layer.

At archaeological sites in Vojvodina, representatives of 7 bird orders were registered: Anseriformes, Accipitriformes, Galliformes, Gruiformes, Charadriiformes, Columbiformes and Passeriformes, of which the first order is the richest in terms of species (7), orders Galliformes and Gruiformes are represented by two species each, orders Charadriiformes, Columbiformes and Accipitriformes with one species each, taking into consideration that the last of the mentioned orders is also represented by 3 genera, while order Passeriformes is represented by only one genus (Table 1) (Blažić, 1986; 1988; 1992 a, b, c; 1993–1994; 1993; unpublished; Bőkőnyi, 1974; Clason, 1979; 1980; Nedeljković, 2008).

After comparing data from the territory of Vojvodina with those from the neighbouring countries, it can be concluded that the ornithofauna in Vojvodina is by far poorest, because at 23 sites in Bulgaria of the same period, 64 bird species which are representatives of 13 orders were registered (Boev, 1993). Gal et Kessler (2002) have researched orhnithofauna of the Eneolithic Age in south-eastern Romania and they have registered 32 species from 9 orders. At the territory of Vojvodina, there have not been found any ornithological remains from this period.

The above mentioned differences can be explained by the span of archaeological research, characteristics of sites, and by settlements' distinctiveness.

Based on the research results collected so far, at the **Neolithic** sites in Vojvodina (Serbia), 9 bird species classified into 4 orders have been registered, while from the Accipitriformes order determination could be done only to the genus. The greatest diversity of this vertebrate class, from the Neolithic (9 species), was recorded at **Starčevo** site, while at **Gomolava, Donja Branjevina** and **Nosa – Biserna obala** only one species was registered at each site (Gomolava *Anser anser*; Donja Branjevina and Nosa – *Otis tarda*) (Clason, 1979; Blažić, 1986, 1992 a, 2005; Bőkőnyi, 1974). At **Starčevo** site, 5 species from the Anseriformes order were found (*Anas clypeata, Anser anser, Anser fabalis, Cygnus olor* and *Cygnus cygnus*), while for the Accipitriformes order

Table 1. Ornitofauna of some archaeological sites in Vojvodina (Serbia) from the Neolithic to the Middle Ages.

TAXON	Neolit	Early Iron Ages	Late Iron Ages	Roman period	Migration period	Middle Ages
Ordo Anseriformes						
Anas clypeata L. 1758	+					
Anas domestica L. 1758				+	+	+
Anser anser (L. 1758)	+		+			
Anser fabalis (Latham 1787)	+					
Anser domestica L. 1758					+	+
Cygnus olor (Gmelin 1789)	+					
Cygnus cygnus (L. 1758)	+					
Ordo Accipitriformes						
Aquilla heliaca Savigny 1809				+		
Aquilla sp.	+					
Milvus sp.	+					
Circus sp.	+					
Ordo Galliformes						
Gallus domesticus (L. 1758)	+	+	+	+	+	+
Meleagris gallopavo L. 1758						
Ordo Gruiformes						
Grus grus (L. 1758)	+					
Otis tarda L. 1758	+					
Ordo Charadriiformes						
Numenius arquata (L. 1758)	+					
Ordo Columbiformes						
Columba domestica Gmelin 1799					+	+
Ordo Passeriformes						
Corvus sp.					+	+

determination could be done up to the genera Aquilla sp., Milvus sp. and Circus sp.; the Galliformes order was represented by only one species *Gallus domesticus*; two representatives of the Gruiformes were also recorded (*Grus grus* and *Otis tarda*), while the Charadriiformes order was represented by *Numeenius arquata* (Clason, 1980).

When comparing the ornitofauna among the sites in Vojvodina, other regions in Serbia, and the neighbouring countries, it can be concluded that there are certain differences. In Hungary, at Polgar-Csőszhalom site, apart from the species registered in Serbia, Bőkőnyi (1974) also lists findings of *Ardea purpurea* and *Bubo bubo*, and at Röszke-Lůdvár site there are 9 more bird species that were not registered in Vojvodina. In comparison with Neolithic sites of Crkvine and Belež in Kolubara basin in Serbia (Blažić and Radmanović, 2011), Divostin near Kragujevac, also in Serbia (Bőkőnyi, 1988),

Anza near Štip in FYR Macedonia (Bőkőnyi, 1976), Obre I and Obre II near Kakanj in Bosnia (Bőkőnyi, 1977) and Sitagroi in Greece (Bőkőnyi, 1986), greater diversity of vertebrate fauna was registered at sites in Vojvodina from the same period, although it should be stated that, in comparison with the last mentioned site, *Anas platyrhynchos, Mergus merganser* and *Coturnix coturnix* were not registered in Vojvodina. Absence of *Gyps fulvus* in Vojvodina is in relation with zoogeographical distribution of this species. Greater number of bird species was also registered at Padina (Clason, 1980).

The above mentioned differences were caused by geographical location, habitat conditions and span of archaeological research.

From the period of the **Early Iron Age** (Hallstatt culture), the remains of only one bird species – *Gallus domesticus* – were found at **Gomolava** site (Blažić, 1986, 1988), while from the **Late Iron Age** (La Tène culture), apart from the above mentioned species, *Anser anser* was registered, also at Gomolava (Clason, 1979; Blažić, 1986, 1993–1994). Apart from the Gomolava site, *Gallus domesticus* was also found at **Gradina** and **Čarnok** (Blažić, 1992 b, 1993–1994); then at **Bare, Mitrovačke livade** and **Bregovi Atovac** (Blažić, 1992 c, 1993–1994), as well as at **Židovar** (Blažić, unpublished).

After comparing data on vertebrate fauna at archaeological sites from the Late Iron Age in Vojvodina with those in the neighbouring countries, it can be concluded that at one site in south-eastern Romania there were 13 bird species from 6 orders registered (Gal et Kessler, 2002), and that 3 sites in Bulgaria of the same period have much richer ornitofauna diversity (Boev, 1993).

From the **Roman Period**, 3 bird species were registered: *Anas domestica, Aquilla heliaca* and *Gallus domesticus*. The first one was recorded at **Sirmium site no. 85** (Nedeljković, 2008), the second at **Vranj site** (Blažić, 1993), and the third was found at both of these sites, and also at **Malo Kuvalovo, Kudoš** and **Prosine sites** (Blažić, 1992 c).

The above mentioned differences can be explained by the span of archaeological research and differences between settlements.

From the **Migration Period** and **Middle Ages**, the presence of the same species was also registered: *Anas domestica, Anser domestica, Gallus domesticus, Columba domestica*, as well as the Corvus sp. genus. All of them were found at **Sirmium site no. 85** (Nedeljković, 2008), and *Gallus domesticus* was also found at **Malo Kuvalovo, Gajić** and **Vračić sites** (Blažić, 1992 c).

For one bird species – *Meleagris gallopavo*–turkey from the **site no. 85 Sirmium**, it could not have been determined whether it originates from the Roman Period, Migration Period, or Middle Ages (Nedeljković, 2008).

As it was mentioned above, at the sites in Vojvodina from the Roman Period, Migration Period and Middle Ages, 6 bird species and one genus were registered, unlike the territory of Bulgaria where, at 18 sites from the Roman Period and Middle Ages, 57 species were registered (Boev, 1993). Domestic hen *Gallus domesticus* was registered in Vojvodina at 5 sites from the Roman Period and at 4 sites from the Middle Ages. Presence of this bird species at sites in Hungary and Romania, also from the Roman Period, Migration Period and Middle Ages, is discussed by Gal (2008).

Nonetheless, the largest amount of data on fauna diversity at archaeological sites from all research periods, therefore from the Roman Period and Middle Ages also, was given in Bőkőnyi (1974) for the territory of Hungary. Concerning the Roman Period, differences in the composition of vertebrate fauna between Vojvodina and Hungary, according to the data of this author, exist for 12 sites in Hungary. Bőkőnyi, (1974) states that, at Tokod-Erzébetakna site, *Grus grus* was the recorded member of bird species, while at Tác archaeological site, 14 wild and 2 domestic bird species were registered. Nineteen archaeological sites from the Middle Ages at the territory of Hungary are also characterised by richer vertebrate fauna, because, apart from the species registered in Vojvodina, the presence of 16 bird species (*Ciconia ciconia, Buteo buteo, Haliaetos albicilla, Milvus migrans, Pavao cristatus, Perdix perdix, Phasianus coclchicus, Grus grus, Otis tarda, Bubo bubo, Strix aluco, Columba palumbus, Corvus frugilegus, Turdus pilaris, Turdus viscivorus, Upupa epops*) was also registered.

The identified differences among the archaeological sites from the Roman Period, Migration Period and Middle Ages in Vojvodina and sites of the same dating in neighbouring countries can be explained by the span of archaeological research.

CONCLUSION

At 17 archaeological sites in Vojvodina (4 from the Neolithic, 1 from the Early iron Age, 7 from the Late Iron Age, 5 from the Roman Period, 1 from the Migration Period, and 4 from the Middle Ages), the total of 14 species and 4 genera of birds have been registered. They belong to the following orders: Anseriformes, Accipitriformes, Galliformes, Gruiformes, Charadriiformes, Columbiformes and Passeriformes.

Ornithofauna is the richest in the Neolithic, where 9 species (*Anas clypeata, Anser anser, Anser fabalis, Cygnus olor, Cygnus cygnus. Gallus domesticus, Grus grus, Otis tarda* and *Numenius arquata*) and 3 genera (Aquilla, Milvus and Circus) were registered.

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ОРНИТОФАУНА АРХЕОЛОШКИХ ЛОКАЛИТЕТА У ВОЈВОДИНИ (СРБИЈА)

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РЕЗИМЕ: Током вишедеценијских истраживања фауне кичмењака на 42 археолошка локалитета у Војводини (Србија) различитих датовања од неолита до средњег века, остаци птица су констатовани на 17 локалитета (четири из периода неолита, један из старијег гвозденог доба, седам из млађег гвозденог доба, пет из римског периода, један из периода сеобе народа и четири из средњовековног периода). У оквиру ове класе кичмењака укупно је детерминисано 14 врста и четири рода припадника редова Anseriformes, Accipitriformes, Galliformes, Gruiformes, Charadriiformes, Columbiformes и Passeriformes. Орнитофауна је најбогатија у неолиту, у коме је регистровано девет врста и три рода, а затим, са четири констатоване врсте и једним родом следе период сеобе народа и средњовековни период, у римској епохи забележене су три врсте, у млађем гвозденом добу две, док је орнитофауна најсиромашнија у старијем гвозденом добу у коме је регистрована само једна врста.

КЉУЧНЕ РЕЧИ: археолошки локалитети, орнитофауна, Војводина (Србија)

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CONSTRUCTION AND TECHNIQUES OF WRITING A SCIENTIFIC PAPER IN NATURAL AND ENGINEERING SCIENCES

ABSTRACT: In science and scientific research there is a wide spectre of fields and subfields which are not always strictly confined. Different classifications of sciences are also known. Numerous books have been published regarding scientific research, types of scientific papers and manners in which their results are published. This literature is very detailed and precise within international academic circles, especially the literature that relates to publishing scientific books and doctoral theses. However, there are certain dilemmas and inconsistencies which can confuse a young scientist when writing an original research article. After a brief review of issues pertinent to scientific paper writing, methodology of scientific research and type of papers, this paper shows characteristics and construction of an original research article. It shows a technique of writing a paper in all scientific fields and subfields with special emphasis on natural and engineering sciences and in accordance with international and domestic standards. Wider practical guidelines can be found in the cited literature, so they can be additionally used, if needed.

KEYWORDS: Construction and technique of paper writing, methodology, natural and engineering sciences**,** scientific research, types of scientific papers

INTRODUCTION

Within international academic circles, there are numerous similar definitions of science and scientific research, some of which are provided in the text that follows.

Science represents a rational form of social awareness with the main objective to research and affirm the objective truth about the world as a whole or a part of it, as well as different instances within it and their legitimacies.

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Basic purpose of science is "to get to know the world so that it can be surmounted by human" and it primarily represents a " systemized code of knowledge" (Subotić, 2009). Today, scientific research in all branches of activities has been developing intensely and it represents a creative process which contributes immensly to the overall well-being of mankind (Folić, Kurtović-Folić, 2009). Scientist must be absolutely dedicated to his/her work that is to science: reading, researching, writing. Long time ago, it was said that success comprises 5% of talent and 95% of work (Filipović, 2004). This statement primarily relates to science, art and sport.

Systemised and tested knowledge which is acquired through thorough research and logical analysis is called science (Carić, O., Carić, M., 2011). Facts, scientific principles and regularities are acquired on the basis of performed research. Thus, scientific research commences with an unexplored or partially explored problem. Based on that, it leads to new findings, results and establishment of new inter-relations. No matter what field of science is in question, the results must be accurate, the experiments must be repeated in a sufficient number of times (natural sciences), apropos data must be gathered in a sufficient number (social sciences). There are 3 basic scientific-research methods which are most frequently used (Šamić, 1988): normative (renders result as a norm, standard, usually on the basis of statistics); experimental (experimental verification of natural regularities); historical (reaches conclusions using history – chronology; mainly present in historical sciences). Apart from the above mentioned, the following methods are also applied: case study, survey, interview, genetic method and comparative method.

In order to be displayed to the experts and critics, the results of scientific research are published in domestic and international scientific journals, at scientific meetings, in monographs, project reports, encyclopedias, patents, master thesis and doctoral (PhD) thesis.

This text will pay special attention to the construction of the original scientific paper.

TYPES OF SCIENTIFIC PAPERS

Basic characteristics of a scientific paper with regards to its categorisation are: original research paper, short communication, professional paper, review paper, conference proceedings, poster, plenary (introductory) lecture, scientific monograph, monograph of international importance, chapter in a monograph or thematic almanac, research project, encyclopedia, patent, technical solution, master thesis, magister thesis and doctoral thesis.

Text that follows provides examples of certain types of scientific papers published by the authors of this article, or other authors, some of which were published in Serbia and some abroad. Ranking and categorisation of scientific papers is done in accordance with ranking of journals and editors, both domestic and foreign depending on where the work was published.

Original research paper/article contains results of its own original scientific research. (e.g.: Nedučin, D., Carić, O., Kubet, V. (2009): Influences of gentrification on identity shift of an urban fragment: A case study, Spatium, 21, 66–75).

Short communication represents preliminary brief display of author's original scientific research results. (e.g.: Pieralice, M., Sergio, L., Di Venere, D., Venediktov, P. (2011): A brief note on thermoluminiscence analysis of photosystem II and lipid peroxidation during the shelf life of ready-to-use rocket (*Diplotaxis tenuifolia L.*), Eur Food Res Technol, 232, 919–923).

Professional paper processes and displays already known data. It requires author's pragmatic side, but it does not imply research originallity. (e.g.: Ignjatijević, S. (2011): The influence of Gross Domestic Product and income on private consumption, Economy – Theory and Practice, 4, 105–110.)

Unlike original research paper, **review paper/article** does not contain new research results. In it, the author provides the overview of the latest research done by himself/herself as well as by other authors worldwide on a chosen topic, analyses them, compares and possibly suggests new research courses. (e.g.: Kubet, V., Carić, O., Ristić, D. (2010): Werkbund Exibitions – Reading Modernism Today, Architecture and Town Planning, 28, 21–28)

Conference proceedings, unless they represent a review article, contain new, unpublished results. However, since reports are not reviewed always in entirety, they are not classified in scientific papers of the same level as papers published in scientific journals where review is mandatory. The same is true for posters, as well as for publishing the entire paper or abstract in conference preceedings or abstracts of papers at scientific conferences. (e.g.: Carić, O., Nedučin, D., Kubet, V. (2009): Cultural Street as a Result of Gentrification. Conference proceedings, The Eleventh National and the Fifth International Science Convention Planning, Projecting, Building and Renewal of Civil engineering, iNDIS 2009, Novi Sad, Faculty of Technical Sciences, pp.119–126)

Plenary (introductory) lecture represents an invited lecture. Even though they are reviews, these lectures contain results of authors original research as well, so they are usually published, and classified as original scientific papers. (e.g.: Caric, M., Milanovic, S. 1994: Advances in Kashkaval Cheese Technology. Proceedings of the Third California Cheese Symposium, University of California, Davis, San Francisco, February 14–15, 1994, pp. 1–17).

Scientific monograph represents a publication which independently and comprehensively elaborates on a given subject from a domain of some scientific area via methodological procedure appropriate to the topic and accepted in that science. A scientific monograph has to make a valid scientific contribution (e.g.: Carić, M., Milanović, S., 1997: Processed Cheese. Science, Belgrade, pp. 197).

Scientific monograph of international importance is dedicated to a theme which is wider than the one of national importance and is published in one of world languages. **Distinguished scientific monograph of international importance** must deal with a theme which is of utmost scientific interest and represents the top in its area. Publisher of this type of monograph has to be a recognized international publishing house with a long tradition in

publishing scientific literature (Ministry of Education and Science) (e.g.: Carić, M. (1994): Concentrated and Dried Dairy Products, VCH Publishers – Wiley, New York, p. 249).

Chapter in a monograph or thematic almanac is categorised in accordance with categorisation of the publication itself. (e.g.: Carić,M., Akkerman, C., Milanović, S., Kentish, E.S., Tamime, Y.A. (2009.): Technology of Evaporators, Membrane Processing and Dryers. Chapter 3. in: Dairy Powders and Concentrated Products, ed. Wiley-Blackwell, pp. 99–148).

Apart from the aforementioned forms of publishing scientific results, there are also the following forms of displaying the results of scientific research: **research project, encyclopedia, patent, technical solution.**

Special types of scientific research which has a specific text organisation, form and construction are: **masters thesis, magister thesis and doctoral (PhD) thesis.**

CHARACTERISTICS AND CONSTRUCTION OF AN ORIGINAL SCIENTIFIC PAPER

General characterisitics necessary for a high quality scientific paper, both in the field of natural and engineering (technical-technological) sciences as well as in medicinal and social sciences and humanities, are:

- Originality, which is basic and the most important characteristic of every scientific paper and contains the results of authorial research, thoughts and interpretations.
- Concise and clear defining and presentation, avoiding the elaboration of facts that are not directly connected with the research topic. Differentiating between important and unimportant and deleting the latter from the paper. Avoiding repetition. Avoiding explanation of things that are implied.
- The language of a scientific paper is specific. Its style needs to be clear and very concise and all the statements need to be documented by facts. Third person and passive are most commonly used verb forms in scientific texts.
- A good quality scientific work needs to comprise a coherent whole and the entire text needs to be in function of the basic idea that is the research theme. The aforementioned means that there is a logical connection among all parts of the paper.
- All the facts in the paper need to be adequately emphasized according to their importance.
- Every conclusion or thought present in the paper need to be explained and supported by evidence. The author must not fall under the influence of unconfirmed, apparent facts.

In his book *The Origin of Scientific Paper* M. Šamić (Sarajevo 1988) describes in greater detail the basic characteristics of a high quality paper, regardless of the field of science elaborated within it.

There is a general model used for construction of an original scientific paper, elaborated in detail later in this paper, which can be different in some details from journal to journal depending on the instructions given to the authors by certain scientific journals.

The value of a scientific paper depends on the category of the journal in the year in which the paper is published.

According to our current Law on Science (Sl. glasnik Republike Srbije, 2005, 2006, 2010, p.1) evaluation of national journals is performed annually for particular scientific fields by the corresponding Central Scientific Committee. The proposed list is afterwards accepted by Ministry of Education and Science and verified by the National Council. Journals are categorised as follows M51, M52 or M53. International magazines are ranked according to the ISI publications, Journal Citation Reports, SCI, SSCI (ISI list), and classified in the following categories M21, M22 or M23. In the process of journals evaluation, other ISI lists accepted by Ministry of Education and Science for every field and verified by the National Council can be applied as well. In technical sciences, the corresponding Central Scientific Committee can suggest one journal outside the ISI list to the category of internationally recognised journals – M24 for every scientific discipline it is in charge of. In social sciences and humanities, the corresponding Central Scientific Committee can suggest two journals outside the ISI list to the category of internationally recognized journals – M24 for every scientific discilpine that is within its competence. Suggestions made by the all Central Scientific Committees are accepted by the Ministry of Education and Science, and the final ranking is verified by the National Council annually.

Despite measurable, exact standars, such as number of scientific publications, citation rate, impact factor and h-index, an objective evaluation of the success of the scientific productivity and quality at social and international level is a very controversial issue; an interesting discussion on those topics is presented in the paper by Kastori et al. (2011).

Construction of an original research paper contains the following elements:

- Title;
- Author(s);
- Résumé (Abstract);
- Keywords;
- Introduction;
- Material and methods (Research description);
- Results and discussion
- Conclusions;
- Acknowledgements;
- References.

Title. The title of a paper needs to be concise with as less words as possible (of 12 words maximum acceptable), but also precise and clear in order to

best represent the essence of the research and the article content. The aforementioned characterisitics of the title of a scientific paper are also significant because of the Internet classification in international databases. Since they are mostly title based, a classification into adequate fields and subfields is performed, which enables high-quality search by professionals.

Author(s). In scientific paper writing, should be a team of multiple authors usually participates. A name of the author who gave the highest contribution to the paper realization should be listed as the first author. As a rule, the remaining authors are listed in descending order with respects to their contribution to all activities, from the idea, study and literature analysis, to planning and conducting the research and, finally paper writing. It is absolutely unethical to include the name of the author who did not participate in the activities of the paper realisation.

Résumé (Abstract). Résumé or Abstract is a short content of the scientific paper. Résumé sets the problem, the paper objective, lists the research methods and emphasises the most important results and conclusions reached. Scientific paper résumé usually contains 200 words maximum and is mainly limited by instructions given to the authors. It is written in the same language in which the paper is written, but also in English and sometimes in one more international language. Résumé represents a brief paper outline, thus it is advisable to write it once the paper is finished.

Keywords. Keywords are listed after the Résumé. They consist of several words – notions which need to be chosen so as to suit the content and the topic of scientific paper and they also need to be present in the text frequently. Keywords point to the essence of the scientific paper and facilitate easier Internet searches of scientific literature.

Introduction. In the introduction of a scientific paper, it is necessary to give an overview and explanation of the paper's theme, and to specify clearly and concisely the subject and object of the research. Reading the introduction, it is clear to the reader why that particular issue was chosen as the subject of scientific research.

Introduction also needs to comprise known and previously established facts, which are the outcomes of authors own research, as well as the results of other authors in the country and internationally, pertinent to the research topic. If the text, which relates to earlier researches of various authors is complex and comprehensive, contains numerous researches, discussions, confrontation of opinions and attitudes, it can be extracted from the introduction and placed under a separate title called „ Literature Overview".

Material and Methods (Research Description). In this part of the paper which describes the used material and work methods, applied processes and means during the course of the research are shown. By doing that, the possibility of result comparison with other similar researches is ensured as well as their repetition. If the research methods are widely known, they should not be described in detail, but their names and literature sources where they are described should be listed. New methods, but also the modifications introduced into the standard methods should be particularly carefully described. If

experimental research is in question, the number of experiment repetition needs to be provided. Both in natural and social sciences, a statistical data processing is used wherever possible. Software used for data processing should be cited in the paper.

Research Results and Discussion. Research results are the research essence and the most important part of a scientific paper. Results need to be representative and well organised. Along with the results, it is necessary to submit logical explanation of the results. The data, which can be displayed in graphs or tables, are not repeated in the text. Figures, graphs and tables are only commented, comparisons and explanations given. In recent papers, useful conclusions are reached by 3D graph application. As a rule, the results which are obvious are not explained, but the differences pertinent to the results obtained in similar researches by other authors, national and international, are discusssed.

Conclusions. Based on the discussion of the obtained results and comparison with the results obtained by other authors, new conclusions, findings and hypothesis are reached. There are general rules for forming conclusions in scientific paper. It seems that the most expedient and detailed instructions for conclusion formulation are given by Šomođi et al. (2004) in their book *Introduction into the Scientific Method*.

The following basic characteristics are considered as necessary parts of good conclusions:

Conclusions need to be brief, well formulated and clearly explained;

Conclusions need to be based on the data obtained in performed scientific research;

Counterarguments and the possibilities for alternative explanations and exceptions need to be commented on in conclusions;

The results need to be explained without their repetition and provided with comparison of other authors' earlier results;

Within conclusions, one needs to emphasise the scientific contribution of the performed researches and to describe their possible application – practical application of research results.

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Apart from the previously mentioned, it is also necessary to express gratitude to all associates who have aided the paper technically but whose contribution is such that they cannot be listed as co-authors of the paper.

Next to every acknowledgment, its reason must be specified.

References (Literature). Cited literature pertinet to the subject of scientific research the author deals with, enables the reader to get acquainted with the state of knowledge in the given research field in the country and the

world. Every citation needs to be brief, clear and directly assigned from the author. Only text from published publications of all types can be cited. As a rule, unpublished results are not used. The rules regarding citing the literature in the text vary depending on the references of certain journal editors. In international scientific journals and the world's recognized publishing houses, the most used ways of citing are Harward Reference System and the Latin style, which have proved to be the most practical ones. Harward Reference System enables simple perception of citation origin, since in the text, right after the citation, the author's name and the year of the publication are listed inside the brackets. The Latin Citation Style is less encumbering for the text, since the references are listed in footnotes and it is more precise because the page from which the citation is taken, even though paraphrased, is specified.

The remaining bibliographic data related to the paper, including the co-authors' last names and the initial letters of all the authors' names, are found in the reference list at the end of the paper, where all papers have been listed alphabetically starting from the last name of the primary author or differently if it was demanded by the publisher of scientific paper. Alphabetical system avoids confusion regarding ordinal numbers which were earlier often used to mark the paper in the text and in refernce list at the end of the paper. Namely, every subsequent text insertion and order change represented a problem and generated confusion with the numbers.

CONCLUSIONS

Even though there is a wide spectre of research fields and subfields in science, the construction and technique of writing an original scientific article, regardless of the field, is very similar. There is a particular degree of similarity in the field of natural and engineeering sciences where original scientific article implies experimental research. There are also general rules and standards regarding the writing of scientific paper worldwide, which have constanly been improving and have been subjected to minor changes, most often for the purpose of simplifying the writing process. Moreover, once a young scientist accepts and learns the scientific paper template, he/she uses it at all times for all the researches conducted and published it in our country and in the well-known journals worldwide, recognising the importance of continual development of this process.

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КОНСТРУКЦИЈА И ТЕХНИКА ПИСАЊА НАУЧНОГ РАДА У ПРИРОДНИМ И ИНЖЕЊЕРСКИМ НАУКАМА

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РЕЗИМЕ: У науци и научноистраживачком раду постоји широк спектар области и подобласти које нису увек строго разграничене. Постоје и различите класификације наука. Публиковане су бројне књиге о научноистраживачком раду, о врстама научног рада и начинима публиковања резултата научног рада. У међународним научним круговима ова литература врло је детаљна и прецизна нарочито литература која се тиче публиковања научних књига и докторских дисертација. Међутим, још увек су присутне извесне недоумице и неусаглашености које младог научника могу да доведу у забуну при писању оригиналног научног рада. У овом раду су, након кратког прегледа проблематике научног рада, методологије научних истраживања и врсти научног рада, приказане карактеристике и конструкција оригиналног научног рада. Приказана је техника писања научног рада за све области и подобласти наука, са посебним акцентом на природне и инжењерске науке, а у складу са светским и домаћим стандардима. Шира практична упутства налазе се у цитираној литератири, те се по потреби могу додатно користити.

КЉУЧНЕ РЕЧИ: методологија, научноистраживачки рад, врсте научног рада, конструкција и техника писања научног рада, природне науке, инжењерске науке

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

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

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

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

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

6. Јединице, имена, скраћенице и формуле

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

6.2. Називе живих организама на латинском треба писати италиком.

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

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

7. Илустрације

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

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

8. Табеле

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

8.2. Табеле се означавају арапским бројевима.

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

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

9. Копија рада у електронској форми

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

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

9.3. Аутори добијају 10 бесплатних примерака сепарата.

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