10th ANNIVERSARY

SEMINAR OF ECOLOGY - 2017

WITH INTERNATIONAL PARTICIPATION

Proceedings

27-28 April 2017, Sofia, Bulgaria

USB
UNION OF SCIENTISTS IN BULGARIA

IBER

10TH ANNIVERSARY
"SEMINAR OF ECOLOGY – 2017"
with international participation

Section „Biology“ – Union of Scientists in Bulgaria
Institute of Biodiversity and Ecosystem Research –
Bulgarian Academy of Sciences

10th Anniversary
Seminar of Ecology – 2017,
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Този сборник съдържа доклади, изнесени на 10ти Юбилеен „Семинар по Екология - 2017“, с международно участие, проведен на 27-28 април 2017 г. в Институт по биоразнообразие и екосистемни изследвания – Българска академия на науките, гр. София, България. Част от докладите са публикувани в пълен текст, а други като кратки съобщения. Семинарът е организиран от секция „Биология“ към СУБ и Институт по биоразнообразие и екосистемни изследвания – Българска академия на науките, гр. София, България, с любезната финансова подкрепа на фирмите БУЛГАП ЕООД и Л.К.Б - България ЕООД. Публикуваните в 10ти Юбилеен „Сборник по Екология - 2017” материали са рецензирани и редактирани. Част от представените пленарни доклади са публикувани в списание „Наука“, издание на СУБ (Брой 4 и 5/2017, том XXVII; http://www.bvu-bg.eu/nmvb/index.php?Clip=nauka).

Редакторите благодарят на рецензентите за проявените коректност и професионализъм.

От редакторите

These proceedings include the reports, presented at 10th Anniversary „Seminar of Ecology - 2017“, with international participation, 27-28 April 2017, Institute of Biodiversity and Ecosystem Research – Bulgarian Academy of Sciences, Sofia, Bulgaria. Proceedings include full text reports and short communications. The seminar was organized by section „Biology“, Union of Scientists in Bulgarian (USB) and the Institute of Biodiversity and Ecosystem Research – Bulgarian Academy of Sciences, Sofia, Bulgaria with the finantial support of the companies BULGAP EOOD and LKB Bulgaria EOOD.

All manuscripts have been reviewed and edited. Some of the presented plenary reports were published in NAUKA (SCIENCE) Magazine, USB edition (Issue 4 and 5/2017, Year XXVII; http://www.bvu-bg.eu/nmvb/index.php?Clip=nauka).

The editors thank the reviewers for their propriety and professionalism.

The editors

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До
Организационния комитет
на "Семинар по екология - 2017"

Уважаема Проф. СТЕФКА ЧАНКОВА
Уважаема Доц. АННА ГАНЕВА
Уважаеми участници, Скъпи гости, дами и господа,

Екологията все по-ясно се очертава като много важен сектор на научното познание за изследване на околната среда и човешкото влияние Изменението на климата, демографските промени, социалните и икономически фактори и глобалните процеси в земната биосфера са може би най-голямото предизвикателство на 21 век. Замърсяването на въздуха, деградацията на почвата, опустиняването и обезлесяването очертават необходимостта от намиране на решение за цялостен подход за преодоляване на глобалните климатични промени и редуцирането на вредните последствия от човешката дейност.

За мен е удоволствие да присъствам на този научен форум посветен на екологията, който днес отбелязва своето Десетото издание. За изминалите 10 години, Семинарът по екология се утвърди трибуна за изява и представяне на научни резултати в областта на управлението и устойчивото ползване на природните ресурси, на екологията и опазване на природната среда, на биоразнообразието и консервационната биология. Семинарът дава възможност за изява както на видни учени, така и на млади участници – докторанти и студенти да обменят опит и знания с утвърдените научни капацитети в различни области на екологията и биологията. С всяка година отбелязваме и увеличен интерес на международната научна общност.

Пожелавам на участниците и организаторите на Семинара успешна и ползотворна работа и много творчески успехи.

С уважение:

София
27.04.2017 г.

/проф. дбн Димитър Иванов,
Научен секретар, БАН/

СЪЮЗ НА УЧЕНИТЕ В БЪЛГАРИЯ

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ДО
УЧАСТНИЦИТЕ В X-ИЯ ЮБИЛЕЕН НАУЧЕН
„СЕМИНАР ПО ЕКОЛОГИЯ - 2017"
С МЕЖДУНАРОДНО УЧАСТИЕ

УВАЖАЕМИ ГОСПОЖИ И ГОСПОДА,

УВАЖАЕМИ КОЛЕГИ,

Имам удоволствието от името на Ръководството на Съюза на учените в България и лично от мое име да поздравя участниците в Десетия юбилеен „Семинар по екология - 2017" и да Ви пожелая ползотворна работа и творчески дискусии.

Съвременното общуване в научната сфера, чрез научни прояви като настоящия семинар или чрез модерните методи на комуникация ще допринесе за изграждането на глобална концепция за опазване на природното равновесие.

Висока оценка заслужават организаторите на този форум, тъй като последователно и настойчиво години наред поставят на вниманието на научната общност най-актуалните проблеми на екологията.

С постигнат успех в повишаване на екологична култура на обществото и създаване на неутрално обществено мнение значително ще се облекчи решаването на проблемите, свързани с опазването на околната среда, а с това ще се подобрят и условията за нормално и устойчиво развитие.

Уважаеми колеги, съидейници и съмишленици, надяваме се да не секват добрите идеи и творческите инициативи!

Адрес: София 1505, бул. Мадрид 39, ет. 2.
Телефони: офис: 02 444 36 44; списание "Наука": 02 444 37 44.
e-mail: president@usb-bg.org

Имайки предвид, че една от основните цели на семинара е да се стимулират младите учени, трябва да кажа, че интелектът на младото българско поколение е основен капитал за българското общество и той е ключът към развитието на българската държава.

Желая успех на семинара и конкретен принос за повишаване престижа на българските учени!

София, 27 април 2017 г.

проф. д.б.н. Диана Петкова,
председател на СУБ

Уважаеми колеги, драги участници и гости!

Имам удоволствието и честта за 10-ти пореден път да Ви приветствам с „Добре дошли“!

Актуалността на проблемите, които се поставят и обсъждат на нашия Семинар се определя от факта, че имаме само една Земя… и всички я споделяме, независимо къде се намираме. За значимостта на проблемите, които се поставят и обсъждат също няма да говоря. Самото Ваше присъствие и 10 годишния юбилей на Семинара, дават отговорите.

Бих искала днес да адресирам към всички нас два основни въпроса:

• Екология – празна дума или дума с огромна тежест и отговорност?

• Какво предпочитаме: Пеперуди или промишленост?

• Или и двете?

Надявам се в края на нашия научен форум да бъдем по-близо до верните решения!

Желая на всички успешна и плодотворна работа!

Проф., д-р Стефка Чанкова

REPORTS

Topic:
BIODIVERSITY AND CONSERVATION BIOLOGY

DEVELOPMENT OF WEB-BASED PORTAL AND INFORMATION SYSTEM FOR THE BIODIVERSITY OF SURFACE WATER BODIES IN BULGARIA

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Abstract

Aim: In Bulgaria there is no centralized information system on hydrobiological data. This paper presents part of the results from a project aimed at archiving, digitalizing and visualizing information of the biodiversity of the Bulgarian surface water bodies (SWB).

Materials and Methods: The database management system (DBMS) uses MySQL server.

Results: The database is a catalogue of the studied SWB and contains taxonomical and ecological information which can be assessed by different queries. In response to search queries geo-referenced maps of the species distributions could be created as well as graphs resulting from different statistical analyzes. Furthermore a virtual library of scientific papers what concerns hydrobiology studies is organized.

Conclusion: This product could collect the available information and serve as a tool for increasing the knowledge of scientists and students in the field of hydrobiology of surface water bodies in the country, including coastal marine waters.

Keywords: database, hydrobiology, biodiversity, Bulgaria, Oligochaeta, zooplankton

Introduction

Databases (DB) play very important role in science. They store, manipulate and share enormous sets of information on taxonomical diversity and distribution of species. All these data are critically important to be organized and available for all scientists and practitioners [1]. Hydrobiological studies in Bulgaria have already accumulated abundant information and the presented platform is designed to create a virtual space for exchange of scientific information, to improve communication between specialists and to serve as a starting point

for development of new topics for studying unexplored water bodies or taxa. The database behind the web site *www.limnoecology.com* will use information about the taxa diversity and distribution. It will serve as online tool for quick and easy access to results from hydrobiological studies in Bulgaria. Consequently, the role of databases in hydrobiology has increased in recent years and will become even more important in the future.

This paper presents the web-based information system *www.limnoecology.com* whose objectives are to archive, digitalize and visualize the biodiversity and environmental parameters of Bulgarian surface water bodies (SWB). Furthermore, it was designed to serve as an online tool for filling data during field work and for calculation structural parameters of the community on site. The current paper presents just a pilot part of the whole idea which brings together systematized information on biodiversity and ecological preferences of hydrobionts.

The data collection uses not only qualitative information (species variety) but also quantitative (number of species and/or biomass) to represent the taxa diversity and to calculate indices for each of the represented/studied communities in a SWB. This is the main difference between the currently presented database and the already known that uses only an index of the scientific names and geographical distribution. Furthermore, in Bulgaria there has never been such a working centralized information system on the hydrobiological biodiversity. For the current version of the database, data on zooplankton communities and Oligochaeta Limicola in Bulgaria were used. When finished, the "Limnoecology" project will provide a public web-service for all biological quality elements. All scientists are welcome to register and share their published data. The authors' rights are controlled at different levels of access and the current copyright laws. The users will have access to data from published materials, articles or reports, as well as to their own data or the data for which they have received permission to manipulate.

Following the idea of the largest taxonomical index Fauna Europaea the w*ww. limnoecology.com* contributes to the European Community Biodiversity Strategy of 2020 [2] by supporting one of the main themes of the Strategy: to identify and catalogue the components of European (Bulgarian in particular) biodiversity into a single database to serve as a basic tool for science and conservation policies [3].

Materials and Methods

The web-site *www.limnoecology.com* is based on the Codeigniter Framework, based on the PHP/MySQL Database. It uses JQuery JavaScript, HTML5 and CSS 3. The database management system (DBMS) uses a MySQL server. Different types of search are set in the project. The user can search for a specific taxon name, environmental information and a specific type of water body. The query results are visualized in a tabular view using Google Graph. Once the taxa list is filled-in, the user will be able automatically to calculate the structural parameters such as Shannon-Weaver, Margalef, Simpson's and Pielou's indices, pF, DF, DT [4, 5] and RCC [6].

The "Limnoecology" project began in 2010 when we designed the first version of the information retrieval system. At that time only the information about the zooplankton in

some Bulgarian standing SWB was used [7]. The data collection used not only qualitative information (taxa biodiversity) but also quantitative (correlation between number of species and biomass) to calculate the indices [8]. This is the main difference between the currently presented database and the already known that use only index of scientific names and geographical distribution of taxa.

Taxonomy database

The taxonomy, faunistic and environmental information is stored in a SQL Server relational database. The database is taxon-centric where each node is a taxon identified with a unique ID (taxid). The ID-s may be deleted if the taxa are removed from the database or merged when more than one taxon are synonymized in one record. The names are associated within nodes and each taxid is linked to its parent ID. The root node (ID 1) links to itself.

Public access to the taxonomy database is provided via the web page. The "Limnoecology" database will support Boolean queries, a wide range of search fields and a History function to track users' actions. These can be combined in Boolean expressions, e.g. Oligochaeta AND temperature AND date in order to visualize the results for a specific taxon, registered in a specific temperature range at a specific date. Some useful queries are shown in Table 1.

Table 1. Example of some of the predefined queries

all [filter]	Retrieves everything
family [rank]	Rank-based query
2017/07/20 21:2020 [date]	Date-bounded query
Oligochaeta [subtree]	All taxa within the Oligochaeta
Temperature between 10 and 20°C [property]	All records with this temperature

Data input

There are two ways to contribute data in the DB – importing it from excel tables in *.xls format (*ex situ*) or using the online platform (*in situ*) during the field work.

All scientists are welcomed to register and share their published or unpublished data. The membership in *www.limnoecology.com* family brings a number of benefits for science, for example, it stimulates experts to hand-over descriptive details on their groups, triggers new ways of community networking and participation motivates experts to update their data, supports a better documentation of their achievements. The authorship is controlled through different levels of access.

Classification

The first task to complete is the taxonomic hierarchy ranging from phylum to family. The hierarchy includes the universally accepted Linnean hierarchy categories: Kingdom – Subkingdom – Phylum – Subphylum – Infraphylum – Class – Subclass – Superorder – Order – Suborder – Infraorder – Superfamily – Family – Subfamily – Tribe – Subtribe – Genus – Subgenus – Species – Subspecies – Variety – Form.

Taxonomic data use the animal and plant codes of nomenclature [9, 10]. So far we have been working with data on the zooplankton and Oligochaeta Limicola in Bulgarian lotic and lentic ecosystems.

The format of names

Every registered member of the web-site can add information to the existing database. A group of experts in taxonomy has the obligation to check if the names are delivered correctly. The names provided by "Limnoecology" are scientific names. Where trivial names exist they also will be recorded.

A taxonomic framework is necessary to keep track of the thousands of taxa records incorporated into the database.

The scientific name of a species basically consists of two parts [3]:

(1) Genus group names (Generic name)

For each generic name, the following information was required:

- Containing family
- Containing subfamily, tribe and/or subtribe (optional)
- Author
- Year of publication

(2) Species group names (Specific name)

For each specific name, the following information was required

- Containing genus
- Containing subgenus (optional)
- Author
- Year of publication

When the information about "Author, Year" is presented in parentheses (Y/N) it indicates that this was not the original taxonomic placement.

Geographical coverage

The species presence should be registered at their sites of registration described with decimal unit degrees. The data could be entered directly in the database through the Data Entry Tool online or by importing a table in *.xls format. The species distribution is mapped by using the Google maps library; also maps generated by ArcGIS software are attached to every species on a regular basis.

Results

Visualization

At present the query results are visualized in a tabular view but in the future will be visualized using the Google Graph. For the map view of results the Google maps library will be used.

The studied water bodies are visualized using Google maps. When filling the protocol (entering data for the sampling point) the authors are encouraged to fill in not only the geographical coordinates of the sampling stations but also to provide more descriptive information about the water bodies. A built-in Google maps component on the site visualizes the standing water objects being explored. Each of them has basic passport data that is displayed when the object is tagged (Fig. 1).

Fig. 1. Surface water bodies from which the zooplankton was studied

When searching through the taxonomy database the result for species distribution could be displayed in a tabular form or in a map. The maps are visualized using Google maps or are static and pre-calculated in ArcGIS (Fig. 2).

Environmental (abiotic) parameters

The database collects data about the SWB, not only for the biotic, but also for the abiotic components such as physical and chemical parameters, hydromorphology etc. (Fig. 3a and b). The platform is designed different environmental parameters to be filled so the species environmental preferences could be identified.

Fig. 2 Visualization of some species distribution

One of the advantages of the web application is that all registered users can fill in the sampling protocol online during the field work using their laptops, tablets or smartphones. Most of the fields for filling are predefined as a drop boxes and restrictions for the data type are set. The filled data can be exported in an *.xls file suitable for further statistic calculations.

RECORD № for hydrobiological analysis of surface water bodies											
water body type:		code:		data: (dd/mm/yyyy)				time sart:		time stop:	
catchment:		name of the sampling point:									
type:											
ecoregion		exact sampling place:									
river basin:		geographic coordinates:	latitutde:		longitude:		altitude (in meters):				

Fig. 3 a). Part of the online field protocol (general information about the surface water body)

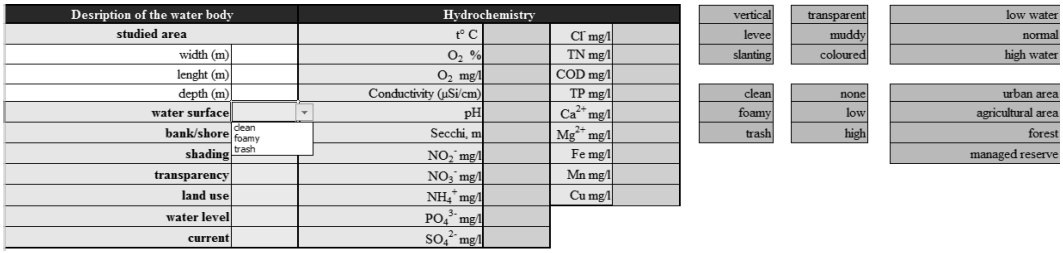

Fig. 3 b). Part of the online field protocol (information about the abiotic environmental parameters)

Index calculation

The application uses the data assigned to specific scientific research (added in the user account) to calculate indices for the community. Using some of the pilot data about zooplankton in Bulgarian surface water bodies some analyses are done automatically and the results are shown in Tabl. 2, Fig. 4a and b.

Tabl. 2. Dominant analysis of zooplankton community in Zhrebchevo Reservoir in October 2010 (Stanachkova, 2013)

N	Group	Taxa	st. 1 N ind/m^3	st. 2 N ind/m^3	st. 3 N ind/m^3	all N ind/m^3	pF,%	DF,%	DT,%
1	R	*Keratella cochlearis*	0	200	0	200	7.14%	0.00%	0.00%
2	R	*Kellicottia longispina*	450	450	200	1100	57.14	0.00%	0.00%
3	R	*Asplanchna sieboldi*	350	237	1200	1787	42.86	0.00%	0.00%
4	R	*Polyarthra dolichoptera*	0	0	200	200	7.14	0.00%	0.00%
5	R	*Polyarthra remata*	50	0	0	50	7.14	0.00%	0.00%
6	R	*Synchaeta* sp.	7900	5674	1800	15374	85.71	0.00%	0.00%
7	R	*Filinia* sp.	0	0	100	100	7.14	0.00%	0.00%
8	R	*Trichocerca* sp.	0	0	100	100	7.14	0.00%	0.00%
9	R	*Trichocerca capucina*	50	0	0	50	7.14	0.00%	0.00%
10	R	*Trichocerca similis*	100	0	0	100	7.14	0.00%	0.00%
11	CL	*Diaphanosoma lacustris*	750	6200	8400	15350	64.29	0.00%	0.00%
12	CL	*Diaphanosoma brachiurum*	1400	1300	2000	4700	57.14	0.00%	0.00%
13	CL	*Daphnia pulicaria*	300	0	0	300	14.29	0.00%	0.00%
14	CL	*Daphnia galeata*	0	450	0	450	21.43	0.00%	0.00%
15	CL	*Daphnia cucullata*	100	400	5200	5700	35.71	0.00%	0.00%

Sampling site (station)	st. 1	st. 2	all
Zooplankton abundance ind/m^3	8533	31684	33839
Zooplankton biomass mg/m^3	125.12	357.16	411.95
H (Shannon-Weaver, 1963)	1.74	1.75	1.93
C Simpson (1949)	0.27	0.26	0.22
E Pielou (1966)	0.58	0.61	0.58
RCC Kozuharov et al.	0.82	0.96	0.95

$$RCC = \frac{\sum (Ncl + Ncop)}{\sum (Ncl + Ncop + Nr)} * 100$$

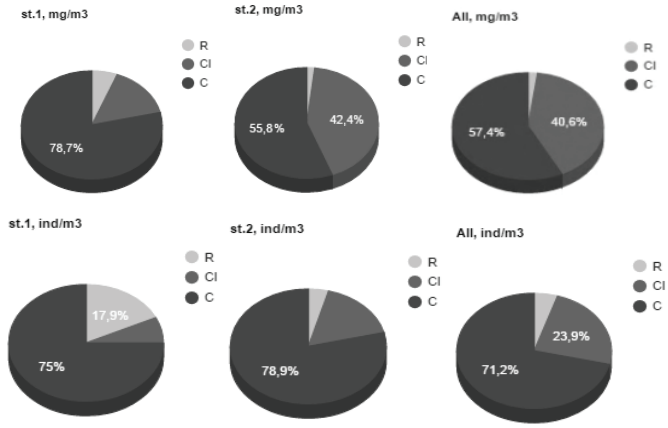

Fig. 4 a) Structural analysis of the main systematic groups of zooplankton in the Zhrebchevo Reservoir in October 2010 (Stanachkova, 2013)

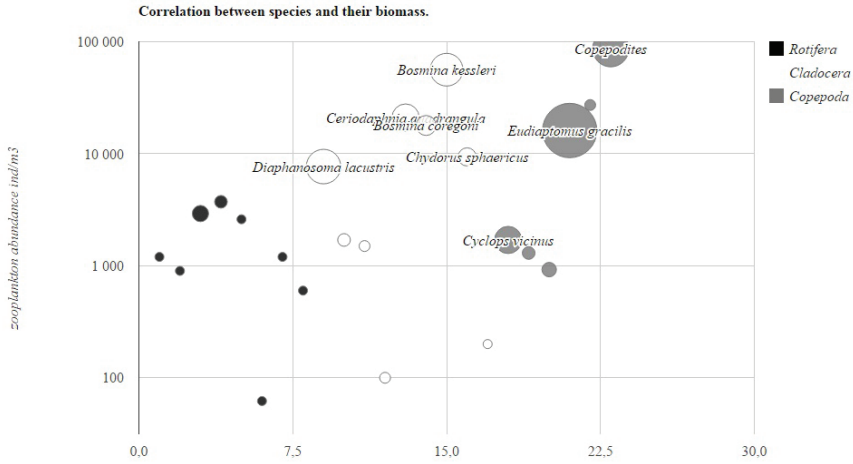

Fig. 4 b). Correlation between the total number of species and their biomass in October 2010 in the Koprinka Reservoir (Stanachkova, 2013)

Scientific papers/Repository

In addition to all the gathered data and produced calculation results the web application will be the first known online collection of Bulgarian papers which contains data for the water communities in fresh water bodies. It will comprise all available scientific papers by the Bulgarian hydrobiologists with a reliable mechanism for citation where each taxon is linked to a specific publication where it was reported for the first time or as a part of a corresponding community studied.

Discussion

On the scientific scene there are a lot of biodiversity databases that are used to store taxonomical information which provides data on the biodiversity of a particular area or group of living organisms [11]. In Bulgaria there is no such database on aquatic biodiversity. Furthermore, most of the known databases are used only for storage of some biological information at local/country [12, 13] or global level [14, 15 and 16]. The main difference between the presented database and other known is that it uses not only qualitative but also quantitative data to represent the taxa diversity and to calculate some predefined indices for the community.

Conclusions

Our product has the potential to combine the available information and to serve as a tool for increasing the knowledge of scientists and students in the field of hydrobiology. The online platform will systematize the available information on SWB and provide an accessible and free platform for the exchange of scientific information and could be used not only by scientists but also by school and university students interested in hydrobiology. It is open to everyday use by anyone interested and ready to contribute with the results of his/her works. In this aspect, the product is a "living document" which will continue to improve over the years. Our team is open to suggestions and collaboration with all colleagues who will share not only their information on the site but also ideas for its improvement.

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DISTRIBUTION OF THE TORTOISES *TESTUDO HERMANNI* AND *TESTUDO GRAECA* IN THE NATURA 2000 SITE „ORANOVSKI PROLOM – LESHKO" (BG0001022), BULGARIA

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Abstract

Aim: To supplement and summarize data about the distribution of the Hermann's Tortoise (*Testudo hermanni*) and the Spur-thighed Tortoise (*Testudo graeca*) in the Natura 2000 site BG0001022 „Oranovski Prolom – Leshko".

Materials and Methods: The specimens and the spot localities of the two species were registered during field trips in March 2010 – October 2016. The geographic coordinates have been determined with GPS. All the localities (published and new) have been summarized in tables and marked on maps.

Results: Totally 60 new spot localities and 74 specimens have been registered. The Hermann's Tortoise (54 specimens, 2 shells and eggs) have been found in 44, and the Spur-thighed Tortoise (19 specimens) in 16 localities. The proportion between the male and female specimens of *T. hermanni* has been 1.2:1, while the male specimens of *T. graeca* have outnumbered the female ones more than 3 times (3.3:1). The number of specimens of *T. hermanni* has been almost 3 times more than the one of *T. graeca*.

Conclusion: Both tortoises have been recorded in the study area. Data about the spread of the Spur-thighed Tortoise are reported for the first time, and the Hermann's Tortoise has been registered in many new spot localities. The distribution of both species is concentrated in the eastern part of the Natura 2000 site, mainly in its lower parts. *T. hermanni* has been more abundant and has got a wider altitude range.

Keywords: *Testudo*, new locality, Natura 2000 site BG0001022, Bulgaria

Introduction

The Natura 2000 site „Oranovski Prolom – Leshko" (code BG0001022) was approved as a Site of Community Importance (SCI) by the European Commission in 2008 under the terms of the EU Habitats Directive (Council Directive 92/43/EEC). The Area has been declared for conservation of 20 types of habitats and 28 species of wild fauna, including 3 reptiles: the Hermann's Tortoise (*Testudo hermanni* Gmelin, 1789), the Spur-thighed Tortoise (*Testudo graeca* Linnaeus, 1758), and the European pond turtle (*Emys orbicularis* (Linnaeus, 1758). The SCI has been surveyed during the realization of the project "Mapping and preservation of conservation status of natural habitats and species - Phase I". Seven specimens of the Hermann's Tortoise were reported from four localities. The Spur-thighed Tortoise has not been registered but the potential and the effective distribution of the species have been mapped [1].

Data about the spread of the Hermann's Tortoise in the study area and close to its borders are contained also in a publication of Pulev & Sakelarieva [2]. Only 2 specimens and a shell were reported from three localities.

The Hermann's and the Spur-thighed tortoises are protected according to the national and international legislation. Both species are included in the Appendix II (species whose conservation requires the designation of special areas for habitat protection) and in the Appendix III (species protected in the territory of the whole country) of the Biodiversity Protection Act of Bulgaria. Both tortoises are protected according to the Habitats Directive, they are listed in the Annex II (animal and plant species of community interest whose conservation requires the designation of special areas of conservation), and Annex IV (animal and plant species of community interest in need of strict protection) of the Directive. The species are protected according to the Bern Convention and are listed in the Appendix II (strictly protected fauna species). Both tortoises are objects of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). They are listed in Appendix II (species that are not necessarily now threatened with extinction but that may become so unless trade is closely controlled). Moreover they are included in the IUCN Red List of Threatened Species, the Hermann's Tortoise as near threatened and the Spur-thighed Tortoise as vulnerable. Both tortoises have been categorized as endangered species in the Red Data Book of the Republic of Bulgaria.

The aim of the present report is to supplement and summarize data about the distribution of the tortoises *Testudo hermanni* and *Testudo graeca* in the Natura 2000 site „Oranovski Prolom – Leshko".

Materials and Methods

The study site is located in southwestern Bulgaria. Its area is 13245.47 ha, and it falls within the territory of two municipalities – Blagoevgrad and Simitli.

The specimens and the spot localities of the Hermann's and the Spur-thighed tortoises were registered during field trips in March 2010 – October 2016, more intensively in May–October 2016. The species were determined visually after Arnold & Ovenden [3] and Stojanov et al. [4]. The animals were captured for a while and released in the same place. The geographic coordinates of the spot localities have been determined with GPS Garmin Dakota 20. The distributional data (published and new) have been summarized in tables and marked on maps. Gathered GPS data have been imported into ArcGIS. As a basis for the maps, a high resolution image from Google Earth has been used. It has been imported into ArcGIS and geo-referenced.

Results

Distribution and number of localities: Both tortoises have been recorded in the study area. They occur in open grassy areas (meadows), forest outskirts, forests, very often together. The total number of the spot localities of both species registered till now is 67. Only 7 of them were published [1, 2], and 60 are reported in the present report.

The Hermann's Tortoise has been recorded in 44 new localities: specimens (including a dead one) have been found in 37 spots, shells in 2, and eggs in 5 (Table 1, Fig. 1). They have been located between 331 m (Emeshen stream) and 916 m above the sea level (northeast of the village of Debochitsa), but most of them (66%) have been at an altitude of 300 – 500 m.

Table 1. Localities of *T. hermanni* in the SCI "Oranovski Prolom - Leshko"

№	Locality	Geographic coordinates	Altitude	Date and time of observation	Specimens observed	Data source
Published data						
1.	Dzhaleva Mahala neighbourhood (the village of Zheleznitsa)	N41°56′20″ E23°06′02″	310 m		1 specimen	[1]
2.	northeast of the village of Zheleznitsa	N41°56′22″ E23°06′20″	354 m		3 specimen	[1]
3.	northeast of the village of Zheleznitsa	N41°56′25″ E23°06′18″	333 m		2 specimen	[1]
4.	east of the village of Zheleznitsa	N41°55′56″ E23°06′18″	316 m		1 specimen	[1]
5.	east of the Dolno Tserovo neighbourhood (the village of Tserovo)	N41°56′42″ E23°06′40″	442 m	22.07.1997	1 shell	[2]
6.	Domozettsi neighbourhood (the village of Debochitsa)	N41°51′34″ E22°57′41″	1120 m		1 specimen	[2]
7.	Plyangovtsi neighbourhood (the village of Obel)	N41°57′37″ E22°53′21″	971 m		1 specimen	[2]
New data						
	Emeshen (Tserovsko Dere) stream	N41°58′17″ E23°06′02″	395 m	12.07.2014	1 ad.	
		N41°58′28″ E23°06′17″	377 m	25.05.2014 9:30 am	2 ad.	
		N41°58′32″ E23°06′23″	417 m	12.05.2015	1 ad.	
		N41°58′32″ E23°06′23″	417 m	03.08.2015 9:00 pm	2 ad. in copulation	
		N41°58′28″ E23°07′04″	496 m	18.04.2015 5:30 pm	2 ad. males	
		N41°58′17″ E23°05′50″	331 m	06.09.2016 2:12 pm	1 ad. male	
	north of the village of Tserovo	N41°58′03″ E23°07′33″	579 m	12.07.2014	1 ad.	
	northwest of the village of Tserovo	N41°58′19″ E23°06′42″	478 m	06.09.2016 2:20 pm	1 ad. male	
		N41°58′18″ E23°06′45″	476 m	06.09.2016 2:22 pm	1 ad. male	
		N41°58′16″ E23°06′46″	471 m	06.09.2016 2:24 pm	1 subad. female	
	east of the village of Tserovo	N41°57′35″ E23°08′20″	777 m	29.05.2016 10:35 am	1 ad. female	

	southeast of the village of Tserovo	N41°57′10″ E23°08′25″	781 m	29.05.2016 11:10 am	1 ad. female	
		N41°57′09″ E23°08′22″	780 m	29.05.2016 11:14 am	eggs	
		N41°57′04″ E23°08′08″	729 m	29.05.2016 11:51 am	1 ad. female	
		N41°57′06″ E23°08′03″	702 m	29.05.2016 12:03 pm	1 ad. female	
		N41°57′16″ E23°08′36″	797 m	11.06.2016 1:13 pm	1 ad. male, 1 subad. male	
		N41°57′10″ E23°08′33″	779 m	11.06.2016 1:34 pm	eggs	
		N41°57′06″ E23°08′28″	763 m	11.06.2016 1:43 pm	1 ad. female	
		N41°57′02″ E23°08′25″	753 m	11.06.2016 2:00 pm	1 ad. female	
		N41°57′17″ E23°08′37″	802 m	11.06.2016 3:44 pm	eggs	
	north of the Dolno Tserovo neighbourhood (the village of Tserovo)	N41°56′53″ E23°06′02″	369 m	05.09.2016 2:55 pm	eggs	
	northeast of the Dolno Tserovo neighbourhood	N41°56′55″ E23°06′20″	403 m	05.09.2016 1:59 pm	1 ad. female	
	Dolno Tserovo neighbourhood	N41°56′38″ E23°06′12″	323 m	10.06.2014 8:30 pm	1 ad. female	
		N41°56′45″ E23°06′09″	346 m	22.05.2016 11:27 am	1 shell	
	Dzhaleva Mahala neighbourhood (the village of Zheleznitsa)	N41°56′25″ E23°06′03″	324 m	26.08.2010 12:30 pm	1 ad. male	
		N41°56′19″ E23°05′53″	317 m	26.03.2010 5:00 pm	1 ad. male	
	east of Dzhaleva Mahala neighbourhood	N41°56′06″ E23°05′48″	321 m	10.06.2014 8:15 pm	1 ad. male	
		N41°56′10″ E23°06′25″	447 m	03.08.2014	1 ad. male	
		N41°56′02″ E23°06′04″	320 m	26.06.2014	1 ad. killed on the road	
		N41°56′10″ E23°06′16″	413 m	25.06.2014	2 ad.	
		N41°56′08″ E23°05′51″	339 m	03.08.2014	1 ad. male	
	northeast of the village of Zheleznitsa	N41°56′00″ E23°06′50″	391 m	17.07.2016 09:49 am	2 ad. male and female	
	west of the village of Zheleznitsa	N41°55′36″ E23°04′47″	437 m	19.06.2016 11:47 am	1 ad. male	
		N41°55′38″ E23°04′47″	461 m	19.06.2016 11:53 am	2 ad. male and female	
		N41°55′43″ E23°04′42″	524 m	19.06.2016 12:11 pm	4 ad. female, 3 subad. and 2 ad. male	
		N41°55′42″ E23°04′39″	521 m	19.06.2016 12:33 pm	1 ad. female	

	east of the village of Zheleznitsa	N41°55′19″ E23°06′48″	311 m	09.07.2016 11:05 am	1 shell subad.	
	Krastevski Dol stream	N41°55′17″ E23°06′58″	319 m	05.05.2015 11:45 am	1 ad. male, 1 juv.	
		N41°55′15″ E23°07′09″	347 m	05.05.2015 10:00 am	1 ad. female	
		N41°55′16″ E23°07′14″	364 m	05.05.2015 11:00 am	1 ad. female	
		N41°55′17″ E23°07′01″	329 m	09.07.2016 11:13 am	eggs	
		N41°55′16″ E23°07′07″	346 m	09.07.2016 11:20 am	2 ad. male	
	northwest of the village of Leshko	N41°56′13″ E22°57′59″	650 m	15.10.2016 2:13 pm	1 ad. male	
	northeast of the village of Debochitsa	N41°52′29″ E22°57′35″	916 m	28.08.2016 1:06 pm	1 ad. female	

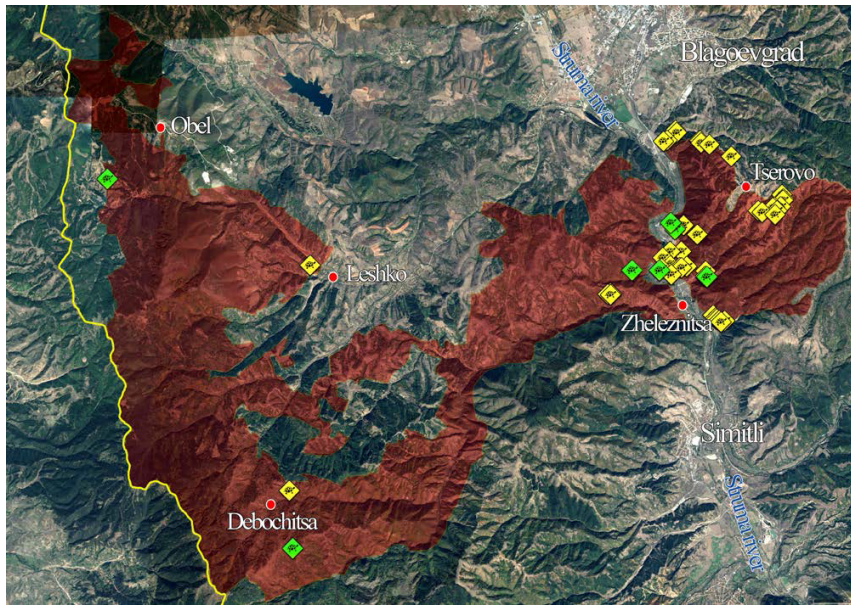

Fig. 1. Distribution of *T. hermanni* in the Natura 2000 site "Oranovski prolom - Leshko" (green colour – published localities; yellow colour – new localities)

Specimens of the Spur-thighed Tortoise have been found in 16 localities (Table 2, Fig. 2), most of which (81%) at an altitude of 320 – 400 m, and the highest one at 484 m (northeast of the village of Zheleznitsa).

In general the distribution of both species is concentrated in the eastern part of the Natura 2000 site, mainly in its lower parts.

Species abundance, sex and age composition: The total number of the unpublished till now specimens and shells of both species that have been recorded within the study site is 76

(74 specimens and 2 shells). Most of them have been Hermann's tortoises – 54 specimens and 2 shells. Only 19 individuals of the Spur-thighed Tortoise have been registered (Tables 1 – 2).

The proportion between the male and female specimens of *T. hermanni* has been 1.2 : 1, while the male specimens of *T. graeca* have been 3 times more than the female ones (3.3 : 1).

The proportion between the different age groups of *T. hermanni* has been 49 ad. : 5 subad. : 1 juv. The adult individuals of *T. graeca* have also prevailed over the subadults and the juveniles (15 ad. : 1 subad. : 3 juv.).

Table 2. Localities of *T. graeca* in the SCI "Oranovski Prolom - Leshko"

№	Locality	Geographic coordinates	Altitude	Date and time of observation	Specimens observed
New data					
1	Dolno Tserovo neighbourhood (the village of Tserovo)	N41°56′48″ E23°06′11″	360 m	22.05.2016 11:32 am	2 ad. male
	Dzhaleva Mahala neighbourhood (the village of Zheleznitsa)	N41°56′04″ E23°05′34″	320 m	26.08.2010 12:45 pm	1 ad. male
3	east of Dzhaleva Mahala neighbourhood	N41°56′09″ E23°06′10″	389 m	03.08.2014	1 ad. male
		N41°56′08″ E23°06′01″	374 m	03.08.2014	1 ad. male, 1 juv.
4		N41°56′01″ E23°05′56″	321 m	05.09.2016 3:24 pm	1 subad. male
	northwest of the village of Zheleznitsa	N41°55′46″ E23°06′04″	373 m	19.06.2016 1:45 pm	1 subad. male
7.	northeast of the village of Zheleznitsa	N41°56′01″ E23°06′42″	369 m	28.04.2016 12:19 pm	1 ad. female
8		N41°56′09″ E23°06′55″	420 m	28.04.2016 1:04 pm	1 ad. male
9		N41°56′15″ E23°07′06″	446 m	28.04.2016 1:44 pm	1 ad. male
		N41°56′01″ E23°07′05″	440 m	17.07.2016 10:10 am	1 ad. male
		N41°56′07″ E23°07′07″	484 m	17.07.2016 11:39 am	1 ad. male
	west of the village of Zheleznitsa	N41°55′23″ E23°05′56″	377 m	23.07.2013 9:00 pm	2 ad. in copulation
	the village of Zheleznitsa	N41°55′35″ E23°06′16″	314 m	19.06.2016 2:00 pm	1 juv.
10	Krastevski Dol stream	N41°55′17″ E23°06′58″	319 m	05.05.2015 11:45 am	1 ad. female
11		N41°55′16″ E23°07′14″	364 m	05.05.2015 11:00 am	1 ad. male
12		N41°55′15″ E23°07′11″	353 m	09.07.2016 11:28 am	1 ad. female

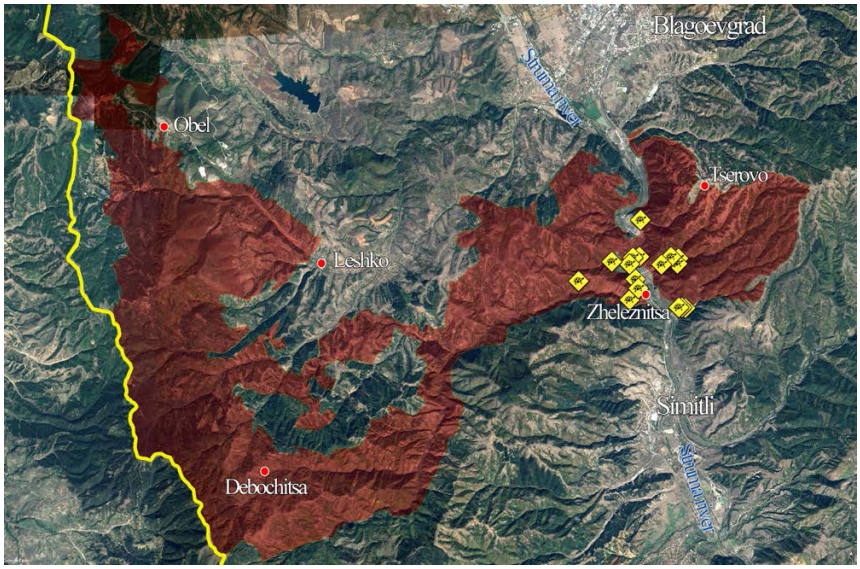

Fig. 2. Distribution of *T. graeca* in the Natura 2000 site "Oranovski prolom – Lesko"

Discussion

Data about the distribution of the Spur-thighed Tortoise have been reported for the first time, and the Hermann's Tortoise has been registered in many new spot localities, so the species find suitable habitats for living.

The Hermann's Tortoise has been more abundant and has got wider altitudinal range in the study area. As a whole the number of its localities and specimens has been almost 3 times as big as the ones of *T. graeca*. Similar and even more significant predominance of the number of localities and specimens of the Hermann's Tortoise (5:1) was recorded by Pulev & Sakelarieva [2] for the territory of the municipality of Blagoevgrad (in which a part of SCI "Oranovski prolom - Leshko"is included). Definitely *T. hermanni* is the species with larger distrubution (inhabiting higher altitudes), and with a higher abundance.

The altitudinal distribution of both species (limited mainly to 500 m a.s.l.) corresponds to that reported by Stojanov et al. [4] for the territory of Bulgaria.

Both cases of copulation (*T. hermanni* from Emeshen stream locality and *T. graeca* from the locality west of the village of Zheleznitsa) have been observed at dusk. Another such observation of Hermann's Tortoise, also from the territory of SW Bulgaria, was published by Pulev et al. [5].

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RECENT FINDS OF *LOBARIA PULMONARIA* AND *L. SCROBICULATA* IN BULGARIA

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Abstract

Aim: Additions to the data on the ecology and distribution of the foliose lichens from the *Lobaria* genus.

Materials and Methods: Field trips for collecting, locating and counting of the lichen thalli were arranged during 2015-2016, following the linear transect method. The newly and recent finds are shown on UTM-grid maps with 10 km^2 per cell.

Results: The most abundant populations of the lung lichen, spread on relatively large areas, were found in Rila Mts: Rilomanastirska Gora Reserve (on beech, maple, oak, scots pine and rocks) and Parangalitsa Reserve (on beech). New locations were reported from Balgarka Nature Park (near Balgarka chalet, on beech); Rila Mts (RMG Reserve, above Ilijna Reka, on oak, beech, rocks; along Bricheborski Dol, on beech, scots pine), Rila National Park (along Krayna Reka, on maple); The Rhodopes (vicinity of Devin, on oak; Chairski Ezera, on spruce). *Lobaria scrobiculata* was recorded with few local populations in Rila Mts (RMG Reserve, above Ilijna Reka, on mossy rocks, and Parangalista Reserve, on old trunk of beech).

Conclusion: New and recent localities are situated mainly in the mountain areas at altitudes between 1100 and 1700 m (Stara Planina, Rila Mts and the Rhodopes). Their substrata are bark of deciduous trees and mossy

rocks. Single find of *L. pulmonaria* is reported on bark of spruce. *Pinus sylvestris* and maple are found as new substrata of the tree lungwort in Bulgaria. The localities, exposed on the UTM-grid maps, along with the data obtained during the field work, are essential for future conservation strategies and bring additional information on their habitats and population ranges.

Keywords: distribution, ecology, *Lobaria*, tree lungwort

Introduction

The genus *Lobaria* (Schreb.) Hoffm. comprises large foliose lichen-forming fungi with known distribution generally in temperate and tropical regions of the world [1-6]. Its members are distributed in unpolluted areas, damp forest habitats with high rainfalls. The *Lobariaceae* in Bulgaria includes five species: *Lobaria amplissima* (Scop.) Forssell, *L. linita* (Ach.) Rabenh., *L. pulmonaria* (L.) Hoffm., *L. scrobiculata* (Scop.) DC. and *Sticta sylvatica* (Huds.) Ach. [7]. Numerous records and summarized information about the ecology and distribution of the lung lichen have been published so far in Bulgaria [8], while the known data about the distribution of *L. scrobiculata* could be obtained from [7]. Epiphytic lichens, as an integral component of the forest ecosystems, contribute to the nutrient cycles, and represent a major part of species diversity, while the associated epiphytic lichen communities develop very slowly in forest areas, dependent by particular microenvironmental conditions of their habitats [2].

Both species, subject of the present study, along with *L. amplissima* are regarded as indicators of ecological continuity, are considered as members of the *Lobarion* alliance Ochsner [9], and often are accompanied by other old forest indicators from the genera *Sticta* (Schreb.) Ach., *Nephroma* Ach., *Pannaria* Delise, *Parmeliella* Müll. Arg. The lung lichen was also found as a key species in primeval forest preservation matters [8, 9].

With the exception of already known findings available [6-8, 10-12], there are no published records confirming the old localities, or official reports of new ones about the tree lungwort and *L. scrobiculata* from our country. This work aims to expand the current knowledge about the substrata, distribution and populations of *L. pulmonaria* and *L. scrobiculata*, based on the results from field trips arranged in Rila Mts (2015), along with additional finds from the Balkan Mts (Nature Park Balgarka, 2016), the Rila Mts (Parangalitsa Reserve, 2004, 2011, 2016; Rilomanastirska Gora Reserve, 2016) and the Rhodopes (along Devinska Reka, 2009; Chairski Ezera, 2017). Some of them confirmed previously known data on the distribution [7, 8], but also new findings with essential information concerning their substrata and habitats, including microhabitats, were revealed.

Materials and Methods

Field trips for locating and collecting of the lichen thalli were arranged during 2015-2016, following the linear transect method. Single finds of *L. pulmonaria* were dated from 2004, 2009, 2011, 2016 and 2017. Information on the substrata and the number of individuals was noted in the field, the lichen thalli were documented in situ with the aid of Canon PowerShot

A1400HD, A710IS and A460 digital cameras. The findings from the field studies, made by the author, are supported with digital color photographs. Color photographs, supporting the presence of the lung lichen per location points, are designated in the text with an asterisk (*). Data of the geographic coordinates of the localities, cited in the text and shown on the presented UTM-grid maps with 10 km^2 per cell, were taken with the help of Garmin 62S or Garmin Etrex 10 receivers. Most of the studied materials are conserved at the Mycological Collection of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia (SOMF). The determination is justified generally after [13].

Results and Discussion

Lobaria pulmonaria (L.) Hoffm., Deutschl. Fl., Cryptogamie, p. 146 (1796), Figs 1-2; Fig. 5.

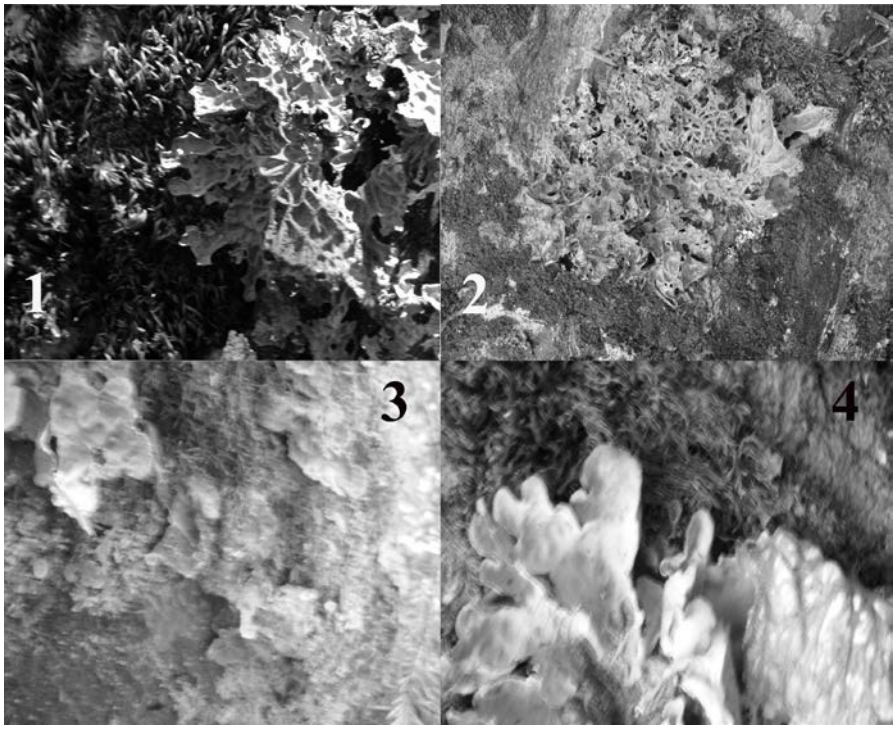
Materials examined: Balkan Mts: Nature Park Balgarka: below Balgarka chalet, above Suhata Reka river, 42°45'51.''N 25°30'57.4''E, alt. 1100 m, 22.06.2016, SOMF 28768, **LH-73** (Fig. 5), several thalli grown on the basal part of old *Fagus sylvatica* L. (*) – a new record; Rila Mts: Parangalitsa Reserve: ca 25 km from Blagoevgrad, above Blagoevgradska Bistritsa river, complex 'Bodrost' locality, 08.08.2004, leg. V. Goranova, SOMF 28776, **FM-95** (Fig. 5), intermixed by thalli of *Cladonia coniocraea* (Flörke) Spreng. & *Parmelia sulcata* Taylor (SOMF 26342); Blagoevgrad distr., Parangalitsa Reserve, nearby the river Bistritsa, bark of beech, 11.09.2011, M. Gyosheva, SOMF 28645, FM-95; on the south slopes above Blagoevgradska Bistritsa River, in the upper part, 42°09'15.8''N 23°38'15.4''E, alt. ca 1650 m, M. Gyosheva, 19.06.2016 (*), FM-95, on old beech; 7 localities in the reserve area (*): above Blagoevgradska Bistritsa River, located on a single or in groups up to 4 old beech trees, at alts between 1480-1545 m, bearing from 5 to 15 individuals of the lung lichen per tree, FM-95: along Bistritsa River, 42°02''29.5''N 23°22'04.3'', alt. 1483 m, 26.05.2015, SOMF 28767, on old beech (*); 42°02'30.7''N 23°22'11.7''E, alt. 1540 m, 26.05.2015, SOMF 28760, 8 thalli on old trunk of beech, adjacent to the thalli of *L. scrobiculata*; 42°02'28.7''N 23°22'11.7''E, ca 1486 m, 26.05.2015, SOMF 28761, fallen by the old beech, FM-95 (*); Rilomanastirska Gora Reserve: the buffer area, along the track to Ivan Vazov chalet, 42°07'48.4''N 23°19'50.8''E, alt. 1247 m, 30.04.2015, three thalli on old beech near the stream (*), SOMF 29635, **FM-96** (Fig. 5); buffer zone, with 3 individuals grown on rock over mosses (*), 42°07'58.5''N 23°19'56.1''E, alt. 1256 m, 13.05.2015, FM-96; 42°07'57.2''N 23°19'55.3''E, alt. 1257 m, large thalli in the basal part of old beech (*), 13.05.2015, FM-96; near Kalugerski Dol, 42°07'52.8''N E23°19'55.9'', alt. 1278 m, 30.04.2015, on beech, FM-96; idem., 42°07'54.8''N 23°19'32.2''E, alt. 1380 m (*), 30.04.2015 (*) & 13.05.2015, SOMF 26881, on bark of old *Acer* sp. – new substratum (*), accompanied by thalli of *Parmelina quercina* (Willd.) Hale, *Pertusaria albescens* (Huds.) M. Choisy & Werner and *Lepraria incana* (L.) Ach., FM-96; 42°07'52.8''N 23°19'55.9''E, alt. 1278 m, mature overlapping thalli, situated in the basal part of old beech, above the track towards Rila Monastery, 13.05.2015 (*), near the track towards Ivan Vazov chalet, 42°07'39.''N 23°19'15.''E, alt. 1512 m, 30.04.2015, M. Gyosheva (*), FM-96, with 20 individuals situated near the base and on the lower part of trunk of old *F. sylvatica*; in mixed deciduous forests - composed

predominantly of oak, hornbeam, hazel and beech, above Ilijna Reka River: 02.06.2015, FM-96, 16 location points in total, comprising from 1 to 13 individuals (including immature thalli) per point: at alts between 1133-1470 m, mostly on bark of oak trees, where often are accompanied by the thalli of *Anaptychia ciliaris* (L.) Körb, *P. albescens*, *Hypogymnia tubulosa* (Schaer.) Hav., *Ramalina farinacea* (L.) Ach., *Usnea hirta* (L.) Weber ex F.H. Wigg., *Parmelia sulcata, Parmelina quercina* (FM-96): 42°06'23.6''N 23°20''39.5'E, alt. 1314 m, SOMF 28764; 42°06'24.0''N 23°20'39.3'', alt. 1312, SOMF 28626, on oak; 42°06'24.5''N 23°20'38.7''E, alt. 1312 m, SOMF 28769, on oak, accompanied by thalli of *Physconia distorta* (With.) J.R. Laundon; 42°06'26.0''N 23°20'37.9''E, alt. 1325 m, SOMF 27658, on oak with juvenile thalli, accompanied by *Anaptychia ciliaris*; 42°06'26.2''N 23°20'37.7''E, alt. 1333 m, on oak, SOMF 26761; 42°06'26.1''N 23°20'37.5'', alt. 1338 m, SOMF 29584, juvenile thalli on oak bark, accompanied by *Pertusaria albescens, Parmelia sulcata* and *Parmelina quercina*; 42°06'32.8''N, 23°20'28.1''E, alt. 1409 m (*); it was observed mainly on oak, and from southwest slopes on beech, 42°06'23.8''N 23°20'39.4''E, alt. 1313 m, 42°06'24.0''N 23°20'39.3''E, alt. 1312 m (*); 42°06'25.7''N 23°20'11.4''E, alt. 1133 m, SOMF 28636, FM-96; above Ilijna Reka River, southwest slopes towards the river, on big rock, 42°06'28.7''N 23°20'21.5''E, alt. 1318 m, 02.06.2015, SOMF 28625; 42°06'31.7''N 23°20'28.3''E, alt. 1402 m, SOMF 27667, along with *P. albescens* on oak bark; 42°06'32.9''N 23°20'28.1'', alt. ca 1450 m, leg. R. Natcheva, SOMF 27328, big thallus fallen by the old oaks, along with thalli of *Anaptychia ciliaris* (SOMF 26757), accompanied also by *Physconia distorta*; on big mossy rock exposed to the sun, above Ilijna Reka, with up to 30 thalli of *L. pulmonaria*, alt. ca 1470 m (*); between 2 and 20 juvenile and mature thalli per tree were located above Rila Monastery, towards Vtoroto Pochivalo towards Brichebor peak, observed in six location points, most of them - recorded on bark of beech, and three immature thalli were found on *Pinus sylvestris* L. (FM-96): towards Brichebor peak, along Bricheborski Dol river, on old beech, 42°08'08.3''N 23°21'21.3''E, alt. 1395 m, SOMF 28672 (*); 42°07'53.3''N 23°21'27.8''E, alt. 1581 m (*); 42°08'08.1''N 23°21'21.4''E, alt. 1403 m (*); 42°08'06.3''N 23°21'19.8''E, alt. 1435 m, on bark of old *P. sylvestris* (new substratum), with juvenile thalli, SOMF 28770, accompanied on scots pine by thalli of *Leptogium gelatinosum* (With.) J.R. Laundon; 42°08'05.7''N 23°21'17.5''E, alt. 1424 m, SOMF 28701 (*), on mossy bark, adjacent to thalli of *Pertusaria albescens* (SOMF 28633), on beech; mature thalli on group of 3 single trees, 42°07'53.3''N 23°21'27.8''E, alt. 1581 m (*), FM-96; above Rila Monastery, 25.05.2015, between Kirilova Polyana locality and Rila Monastery, 42°09'25''N 23°23'19.1''E, alt. 1493 m, 03.06.2015, M. Gyosheva (*), **GM-06** (Fig. 5), with 36 predominatly juvenile thalli on two beech trees among *Abies alba* Mill. trees; Kirilova Polyana, near Rilska Reka river, 42°09'11.03''N 23°24'1''E, alt. 1470 m, 25.09.2016, GM-06, leg. B. Assyov, SOMF 28758, as *L. pulmonaria* f. *papillaris* (Delise) Hue - on old beech, a single thallus with about 20 red-brown to darkened apothecia, originated from local population of 15 individuals; National Park Rila: above Kostenets village, along Krayna Reka River, 42°13'29.9''N 23°47'37.9''E, alt. ca 1272 m, 09.06.2015, on old maple tree, **GM-27** (Fig. 5), small population comprised of 7 individulas (*) – a new record; The Rhodopes: along Devinska Reka river, 12.04.2009, 41°44'53.5''N 24°21'19.2''E, leg. R. Natcheva, on oak, SOMF 25719, **KG-72** (Fig. 5) – a new record; Chairski Ezera protected

area, Sinyoto Ezero locality, primeval forests, alt. 1424 m, 19.09.2017, leg. M. Gyosheva, SOMF 26344, on *Picea abies* (L.) Karst., with 10 mature thalli on mossy bark (*), **KG-81** – a new record.

Habitat: On *Acer* spp. (new substratum), *Fagus sylvatica, Picea abies, Pinus sylvestris* (new substratum), *Quercus* spp., rocks (including mossy rocks). Usually at altitudes between 1100-1700 m.

Known distribution in Bulgaria: Black Sea coast, Northeast Bulgaria, Forebalkan, Balkan Mts, West Frontier Mts, Mt Vitosha, Mt Belasitsa, Mt Slavyanka, Pirin, Rila, Sredna Gora and Rhodopi Mts, Mt Strandzha [7, 8].



Figs 1-2. *L. pulmonaria* from Rila Mts: 1. Rilomanastirska Gora Reserve, the area of Ilijna Reka (juvenile thallus on oak);
2. RMG Reserve, above Ilijna Reka (thallus grown on mossy rock in beech forest);
Figs 3-4. *L. scrobiculata* from Rila Mts:
3. Parangalitsa Reserve (thalli on bark of old beech);
4. RMG Reserve, near Ilijna Reka (thallus on mossy rocks). Photos by D. Stoykov

Comments. Only one specimen (SOMF 28758) bears at about twenty ± red-brownish apothecia, placed on the ridges and towards the edges of the thallus. Numerous small isidiate soralia persist along the ridges on the upper part. Disc above 1 mm in diam, red-brown, with thin thalline margin. Hymenium about 100 µm thick, turning blue in Melzer's Reagent (I+). Asci 60 × 12 µm, clavate. Ascospores (15-) 17-25 × 4.5-6 µm, fusiform, 3-septate, hyaline. The

thalli of the lung lichen produce apothecia extremely rare. During the previous field studies in the Rhodopes Mts, in the area of Cigov Chark (Mantaritsa Reserve), it has been collected with apothecia on a small piece of fallen tiny twig of beech only [8]. The fungal partner in *L. pulmonaria* is reproduced naturally with ascospores very rarely, while the vegetative propagation with symbiotic propagules (uniting the fungal and algal partners) is more frequent.

The tree lungwort has been reported occasionally on eruptive, syenite and mossy rocks, or on boulders in the river courses in Stara Planina, Mt Vitosha, Rila and Rhodopes Mts [8].

Lobaria pulmonaria is recorded in the Rilomanastirska Gora Reserve in June 2015, on the slopes above Ilijna Reka River, with numerous juvenile and single mature individuals growing on bark of oaks - on the southeast (Fig. 1) and exclusively on beech from the southwest slopes in the areas studied. These are the first reports of *L. pulmonaria* growing on rocks (including mossy rocks): a single point towards Kalugerski Dol River and three localities, detected on the slopes above Ilijna Reka River, documented with material or photographs taken (Fig. 2). The reserve area at the side of Bricheborski Dol River (above Rila Monastery) was investigated for the first time for the presence of the lung lichen on *Fagus sylvatica* and *Pinus sylvestris*. Previously the lung lichen was reported: on oak only in the region above the Rila Monastery at 1300 m; on beech, along the slopes of Droshlyavitsa River and at different altitudes from the regions above and in between Kirilova Polyana locality and Rila Monastery; along Rilska Reka River, on bark and mossy gneises in the valley of Rilska Reka – below 1300-1100 m [8]. All of the reports in the existing literature were given without exact locations, except the elevation data and substratum, where this information exists [8].

According to the published data, the tree lungwort was known in the country to occur on bark of oak, beech, *Corylus avellana* L., *Castanea sativa* Mill., conifers (*Abies alba* Mill*., Picea abies, Pinus* sp.), mossy ground, rocks in old woods, and boulders nearby mountain river beds in Bulgaria [8].

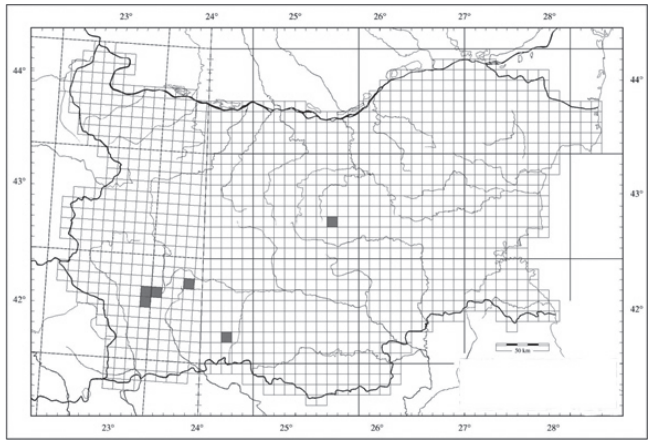


Fig. 5. UTM-grid map with recent localities of *L. pulmonaria* in Stara Planina, Rila and Rhodopes Mts

Remarks. *Lobaria pulmonaria* is characterized by relatively low dispersal potential [8], but the sexually produced fungal spores were thought to be important for long-distance

dispersal and require a suitable photobiont partner for reestablishing the symbiosis after dispersal [14]. However, despite its widespread distribution in Europe [6], the tree lungwort is considered as regionally threatened and its populations decline [14, 15]. The low frequency of sexual reproduction, characteristic for the lung lichen, is considered among the main factors limiting its local gene populations to adapt to changes in the particular environmental conditions. Mating system of *L. pulmonaria* requires the presence of individuals from different mating-type genes for sexual reproduction to occur, and this self-incompatibility (heterothallism) therefore affects the sexual process between individuals (especially the inhabitants of small areas) by the unbalanced spread of their mating-type genes.

Lobaria scrobiculata (Scop.) DC., Deutschl. Fl., Cryptogamie, p. 138 (1796), Figs 3-4; Fig. 6.

Thallus usually with broad, concave and rounded lobes, rather wider along ridges and on the margins; blue greyish and soft-textured when wet (Fig. 3), usually light grey and papery-textured when dried; the underside is covered by light brown tomentum, showing concave areas, corresponding to the depressions on the upper thalline surface (Fig. 4). Thallus lobes are more regular, rounded and flattened than those of the tree lungwort. Apothecia were not observed. Spot test: K (+) yellow.

Specimens examined: Rila Mts: Parangalitsa Reserve, along Blagoevgradska Bistritsa River, on old mossy trunk of beech, 42°02’30.7’’N 23°22’26.5’’E, alt. 1540 m, 26.05.2015, SOMF 27272, **FM-95** (Fig. 6) a local population of 20 individuals, adjacent to 8 thalli of *L. pulmonaria* (see Fig. 3), accompanied by podetia of *Cladonia fimbriata* (L.) Fr. (*); Rilomanastirska Gora Reserve, above Ilijna Reka river, on mossy rocks in the lower part above the river, 42°06’29.7’’N 23°20’16.3’’E, alt. 1121 m, 02.06.2015, SOMF 27354; southwest slopes, right above the river course, 42.10824°N 23.33787°E, alt. ca 1221 m (*), spot test: (K+) yellow, SOMF 26630, **FM-96** (Fig. 6), local population of 20 indivuduals at different size, living in the corrugations of big mossy rocks (see Fig. 4), accompanied by thalli of *Cladonia coniocraea* (SOMF 28719) and *C. fimbriata* (SOMF 28718).

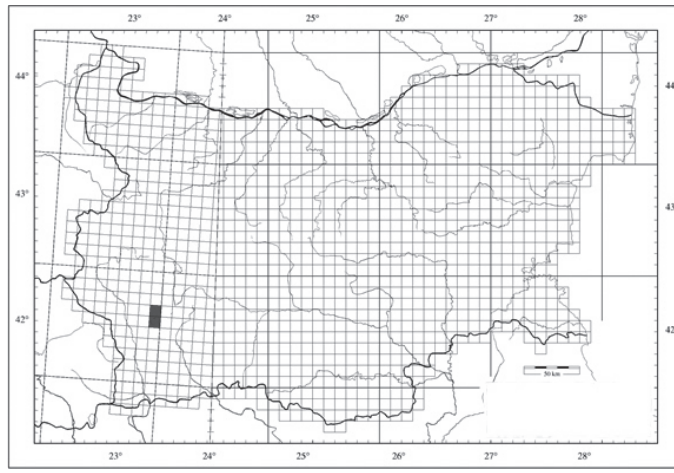


Fig. 6. UTM-grid map with the recent localities of *L. scrobiculata* (Rila Mts: Rilomanastirska Gora and Parangalitsa Reserves)

Known distribution in Bulgaria: Balkan Mts, Vitosha region, Mt Belasitsa, Pirin, Rila and Rhodopes Mts, Toundzha Hilly country [7].

Note. *Lobaria scobiculata* was known previously for the region of Rilomanastirska Gora Reserve only from Kirilova Polyana locality, on bark of beech and spruce and nearby the Rila Monastery on mossy gneises at 1200 m, without apothecia (GM-06) [8, 16]. In the region of Parangalitsa it was reported only from Kartula locality at alt. 1500 m, on beech [17].

Conclusion

During field trips arranged in the period of 2015-2016 in the studied areas of Stara Planina and Rila Mts, along with materials used from Parangalitsa Reserve (2004, 2011) and the Rhodopes Mts (2009, 2017), numerous new locations and recent finds were established, exactly located and well documented. The first find of the tree lungwort from Rilomanastirska Gora Reserve on maple (Kalugerski Dol), made during the spring of 2015, is located. Numerous thalli, growing also on oak and rocks (including mossy rocks) - from the region of Ilijna Reka and on beech - along Bricheborski Dol River, were reported. The lung lichen was found with additional new localities in the Nature Park Balgarka (Balgarka chalet) on beech, from the National Park Rila (Krayna Reka River) on maple (new substratum), from Rhodopi Mts (Chairski Ezera) on bark of spruce, and from Rilomanastirska Gora Reserve (Bricheborski Dol) on *Pinus sylvestris* (new substratum). *Lobaria scrobiculata* has been confirmed in Bulgaria from Rila Mts with local populations from the Rilomanastirska Gora Reserve (along Ilijna Reka River - on mossy rocks, and in Parangalista Reserve - on bark of old trunk of beech), representing the first precise locations of its local populations in this mountain since the time of the last mentioning in both areas, after a long period of more than 53 years. During 2016, the presence of single mature thallus from the lung lichen, bearing at about 20 apothecia, was detected in the Rilomanastirska Gora Reserve (Kirilova Polyana, along Rilska Reka River), thus confirming the last collection with apothecia from the same place [17].

Both species of *Lobaria* are of conservation concern, therefore our finds from Stara Planina, Rila and the Rhodopi Mts are essential for further protection activities in their habitats by bringing additional information on the ecology and distribution of those predominantly epiphytic foliose lichens.

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COMPARATIVE ANALYSES OF FLAVONOIDS IN ROOTS OF THREE BULGARIAN AND ONE UKRAINIAN *GLYCYRRHIZA GLABRA* L. POPULATIONS

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Abstract

Aim: The study was aiming at determination of the total flavonoid content in the commercially important medicinal plant *Glycyrrhiza glabra*, and screening for occurrence of flavonoid aglycones and glycosides.

Materials and Methods: Root samples were gathered from four populations of *Glycyrrhiza glabra*: Dolni Vit, Koilovtsi, and Beltsov, near Danube River in Bulgaria, and one Ukrainian, in 2015. Total flavonoids were determined spectrophotometrically according to the European Pharmacopeia 7. Methanolic extracts were examined by TLC for occurrence of nonpolar (aglycones) and polar (glycosides) flavonoids, and visual semiquantitative evaluation.

Results: Total flavonoids were similar for three of the tested populations: over 0.2%, while the sample from Dolni Vit contained only 0.04%. The TLC analysis showed that the extracts were richer in flavonoid aglycones than in glycosides. Eight spots with TLC data (Rf -values and color) of flavonoid aglycones and two of flavonoid glycosides were detected. Flavonoid profiles of the four samples relating to glycosides were the same in qualitative and quantitative terms. The profiles of flavonoid aglycones showed greater variability mainly in terms of quantity. One compound (aglycone) was determined only in the Dolni Vit sample.

Conclusion: The studied samples of *Glycyrrhiza glabra* showed a complex flavonoid profile concerning flavonoid aglycones. Additional identification of the components will be done with more precise chromatography method to accomplish the study.

Keywords: Licorice, Flavonoids, Medicinal plants

Introduction

Glycyrrhiza glabra (Licorice) is a medicinal plant which is used in both traditional and official medicine. It is a perennial plant species from the family Fabaceae. Its healing properties are due to the roots (Radix Glycyrrhizae). In folk medicine liquorice has been used for its anti-tussive, expectorant and carminative properties. In modern medicine it is used also to treat chronic hepatitis, ulcers, psoriasis, as sweetener of many drugs, and against some viruses like HIV, *Herpes simplex* and other [1]. Roots of 3-year old or older plants are used unpeeled or after the outer bark is peeled. Extracts are used separately or as component of the composition of many drugs.

The therapeutic effects of liquorice are due to saponins and flavonoids. Although the main biologically active substance of *G. glabra* is glycyrrhizinic acid, the content of flavonoids in this species is no less important. Flavanoids display important pharmacological activities such as antioxidant, antimicrobial, anti-inflammatory, and antiradical [2]. About 300 flavonoids

have been isolated from *Glycyrrhiza* species, belonging mainly to isoflavonoids as well as to flavanone and chalcone classes of flavonoids [3, 4]. Moreover, to avoid some negative side effects caused by the glycyrrhizinic acid, deglycyrrhizinated liquorice preparations, like Caved-S and Alcid V, are used in treating of various types of ulcer, haemorrhoids, liver, and skin eruptions like herpetic lesions and psoriasis [1]. The estrogen-like effect of liquorice is related to the isoflavene glabrene and the isoflavane glabridin [5], and caused one of the Bulgarian synonyms of the species: lady's herb.

Overexploitation of liquorice in the past led to depletion of its populations in Bulgaria and some of them are no more found out during the last decade. The species is protected by the Biodiversity Act (2002), and included in the Red Data Book of Bulgaria (2015) in the category "endangered" [6]. *G. glabra* is distributed in South and East Europe and South-West Asia, and it is naturalized in some places in South-West Europe. In Bulgaria the species occurs in a limited region in the Danube Plain, and the area of its main five localities has decreased from 66 dka in 1957 to 9.5 dka in 1996 [7]. Authors investigated the floristic composition of the communities and detected many anthropophytes among their species which suggested an adventitious nature of liquorice in the country.

The content of the important bioactive compounds was evaluated for the main five Bulgarian populations of *G. glabra* as well as for the cultivated plants in a small experimental plantation close to Sofia, originating from Russia [8]. Although all investigated populations contained glycyrrhizinic acid in higher concentrations then pharmacopoeia requirements, authors noticed significant differences between the distinct localities and recommended establishment of plantations using genetic material from the most perspective population. Our recent survey and contacts with the regional authorities of the Ministry of environment and waters confirmed the existence of only three of the mentioned liquorice populations: Dolni Vit and Koilovtsi, in Pleven district, and Beltsov, in Russe district, all of them situated in protected areas.

The present study was aiming at determination of the total flavonoid content in plants originating from the three populations of *G. glabra* confirmed in 2015, and from one Ukrainian population, and screening for occurrence of flavonoid aglycones and glycosides, as a part of our work toward establishment of high productive liquorice plantation with *in vitro* propagated plants.

Materials and Methods

Root segments were gathered from four populations of *Glycyrrhiza glabra*: three of them Bulgarian: Dolni Vit, Koilovtsi, and Beltsov, near Danube River in Bulgaria, and one Ukrainian. Roots from the Bulgarian populations were collected from two individuals per population, without destroying them, following the permission of the Ministry of environment and waters, while those from the Ukrainian population were supplied by Bioprograma EAD. Roots were washed with water, cut into small pieces, mixed, then dried in an oven at 55ºC, and ground.

Total flavonoids were determined spectrophotometrically (Jenway 6320 D) according to the European Pharmacopeia [9], in three samples per population, 1 g each.

Methanolic extracts were examined by thin layer chromatography (TLC) for occurrence of nonpolar (aglycones) and polar (glycosides) flavonoids, and for visual semiquantitative evaluation. Samples of 100 mg of plant material ground to a fine powder were macerated in Eppendorf tubes with 1 mL of methanol for 48 h at room temperature, than centrifuged. The supernatant was evaporated and the dry extract was dissolved in 200 μL methanol. Ten μL of each extract were applied on TLC plates for comparative analysis.

Statistical analysis of the results was done using Anova Single factor.

Results and Discussion

Liquorice populations were small, from 0.5 to 2.8 dka, and dense (Fig.1). The number of individuals used as source of root samples (Fig. 2) was limited to two per population according to the permission of the Ministry of environment and waters; however, to our opinion they were enough for the initial phytochemical screening because of the plants' vegetative propagation by horizontal stolon, forming an entire root net under the population.

Fig. 1. Population of *G. glabra* near the village of Dolni Vit

Fig. 2. Root segments of *G. glabra* and a sample of ground roots

The percentage contents of the total flavonoids expressed as hyperoside are presented on Fig. 3. Total flavonoids were similar for three of the tested populations, over 0.2%, while their content was 5 times lower in the sample from Dolni Vit and significantly differed from the others (P = 0.001) (Table 1, A & B). The dry matter of the unpeeled roots was in the range from 43.5% for Beltsov up to 50.6% for Koilovtsi. Flavonoids' content reported previously for cultivated plants originating from Russia showed some annual fluctuation, with an average of 0.19 ± 0.04% during the fruition phase [8]. These results are comparable with ours, concerning the populations of Beltsov, Koilovtsi, and the Ukrainian one.

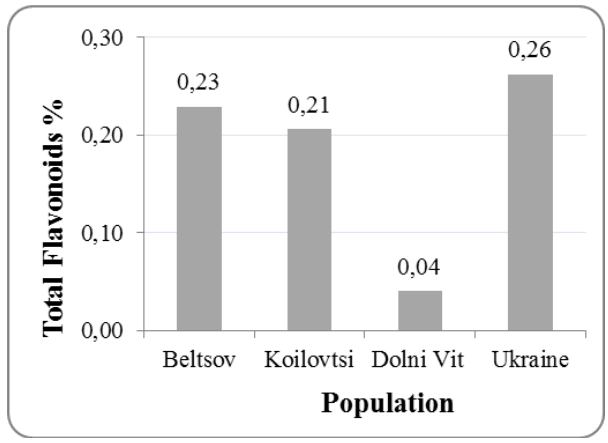

Fig. 3. Flavonoid content in the 4 tested localities of *Glycyrrhiza glabra*

Table 1. Similarity and differences in the content of the total flavonoids in the tested populations:

A) Comparison between all tested populations

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Dolni Vit	4	0.163525	0.040881	0.000733
Beltsov	3	0.68756	0.229187	0.005315
Koilovtsi	3	0.618743	0.206248	0.002725
Ukraina	3	0.78777	0.26259	0.002672

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	0.106682	3	0.035561	13.54839	**0.0011**	3.862548
Within Groups	0.023622	9	0.002625			
Total	0.130304	12				

B) Comparison between the three populations with values of the flavonoids over 0.20%

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Beltsov	3	0.68756	0.229187	0.005315
Koilovtsi	3	0.618743	0.206248	0.002725
Ukraina	3	0.78777	0.26259	0.002672

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	0.004816	2	0.002408	0.674425	**0.544246**	5.143253
Within Groups	0.021425	6	0.003571			
Total	0.026241	8				

More detailed TLC analysis showed that the extracts were richer in flavonoid aglycones than in glycosides. Eight spots with TLC data (Rf-values and color) of flavonoid aglycones and two of flavonoid glycosides were detected. Flavonoid profiles of the four samples relating to glycosides were the same in qualitative and quantitative terms. The profiles of flavonoid aglycones showed greater variability mainly in terms of quantity. One compound (aglycone) was determined only in the Dolni Vit sample (Fig 4). Thin layer chromatography was suitable for our purposes, as a basic method for initial screening of plant extract for biologically active substances.

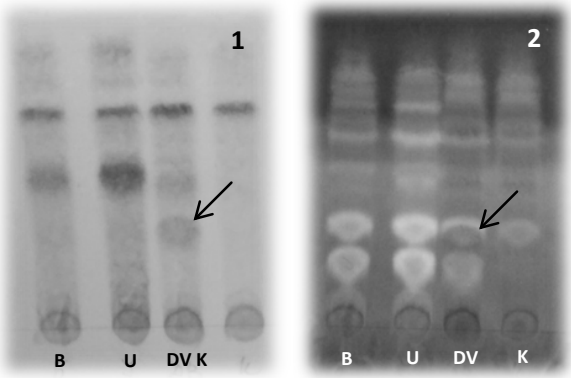

Fig. 4. TLC chromatograms of flavonoid aglycones in the 4 localities,under daily (1) and UV (2) light: (B) Beltsov, (U) Ukraine, (DV) Dolni Vit, and (K) Koilovtsi. Arrows indicate the spot of the compound specific for the population of Dolni Vit

Conclusions

In conclusion, we can assume that the population of *Glycyrrhiza glabra* close to Dolni Vit village differed from the other 3 populations, both by its lower flavonoid content and its aglycone profile. Several reasons could be related to these differences: One possibility is the suggested adventitious nature of liquorice [7] based on the floristic analysis of the communities and the high

number of anthropophytes, as well as on the memory of the local people saying liquorice had been introduced in the region in the beginning of the 20th century. Another possible factor could be the composition of the soil, which was sandy and reddish in the protected area "The red riverside" on the land of the Dolni Vit village, and quite different, clayey and humus black earth in the protected areas "Palaza" on the land of Koilovtsi village and "Locality of liquorice" on the land of Beltsov village. Soil analyses, in parallel with genetic diversity investigation, should be done to elucidate the observed differences in the flavonoid content and composition. To accomplish the study, additional identification of the components will be performed with more precise chromatography methods, and the concentration of glycyrrhizic acid will be determined as well. It is worth to mention that *in vitro* cultures have been successfully initiated with plant material gathered from the four studied populations of *G. glabra* [11], and a protocol for rapid plants multiplication has been established, thus preparing the future propagation of the most perspective liquorice plants.

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SHORT COMMUNICATIONS

Topic:
BIODIVERSITY AND CONSERVATION BIOLOGY

A GENETICALLY INFORMED PROPOSAL FOR CONSERVATION OF *CENTAUREA PSEUDAXILLARIS*, A RARE AND ENDEMIC PLANT IN BULGARIA

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Aim: *Centaurea* s.l. (Asteraceae) is one of the most endemic-rich genera in the Bulgarian flora. *Centaurea pseudaxillaris* Stef. & T. Georgiev is a Bulgarian endemic, protected by the national Biodiversity Act, included in the Red List of vascular plants and in the Red Data Book of Bulgaria with conservation status "Critically endangered" [1]. Among the main threats for *Centaurea pseudaxillaris* are: (i) its low reproductive potential, (ii) the extremely low number of individuals, and (iii) the destruction of its specific habitats [1]. In the course of previous studies, both *ex situ* and *in situ* measures were undertaken to preserve this endemic species, but with no prior knowledge of the genetic structure of the source populations [2]. Here, we aimed to provide more informed proposal for targeted conservation of *Centaurea pseudaxillaris* by supplementing the conventional conservation assessments with genetic data.

Materials and Methods: Fresh *Centaurea pseudaxillaris* leaves were collected randomly from the Besaparski hills locality (Pazardzhik district) and from near Dobrich village, a recently described locality in Haskovo district. Genomic DNA was extracted following a modified CTAB–procedure [3]. Eight ISSR primers (Microsynth, Balgach, Switzerland) were selected after the screening of 35 primers on a small subset of 10 individuals per population. Polymerase chain reactions were performed in a volume of 25 µl, containing a final concentration of 1 × PCR buffer (Fermentas, Vilnius, Lithuania), 1 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 100 µM of each dNTP, 1 µM of each primer and 50 ng of extracted DNA. PCR cycling conditions were as follows: 5 min initial denaturation at 95^{O}C, 35 cycles of amplification [45 s at 94^{O}C, 1 min at the annealing temperature (T_a), 2 min elongation at 72^{O}C] and a final elongation step of 5 min at 72^{O}C. PCR experiments were performed with a TC–5000 gradient thermal cycler (Techne, Staffordshire, UK). To determine the optimal annealing temperature for each primer, an interval of 10^{O}C around the

melting temperature (T_m) was tested. The PCR products were analyzed on 2% agarose gels (Fermentas, Vilnius, Lithuania) in 0.5 × TBE buffer. 100 bp plus DNA ladder size standard (Fermentas, Vilnius, Lithuania) was used to estimate the length of PCR products. The gels were stained by incorporating 1.5 µl of ethidium bromide (0.5 mg/ml) in 100 ml agarose. The electrophoresis was run for 1.5 h at 150 V, the ISSR–profiles were visualized with a UV transilluminator (TFP–M/WL, Vilber Lourmat, Eberhardzell, Germany) and further analyzed with a video image analyzer.

Results and Discussion: Here, we present data from our pilot studies of genetic diversity of Bulgarian endemic *Centaurea pseudaxillaris*. Among the 35 primers tested in the screening procedure, the following eight primers were used: $(GA)_8T$, $(GA)_8A$, $(CA)_8G$, $(AC)_8T$, $(AC)_8C$, $(AC)_8G$, $(AG)_8YC$ and $(AG)_8YT$. The reproducibility of the technique was tested by replicating each amplification reaction twice. The ISSR banding profiles were treated as dominant markers and each band was considered as a bi-allelic locus with one amplifiable and one null allele. The well-resolved and consistently reproducible amplified DNA fragment bands were scored concerning their presence (1) or absence (0). GenAlex (v. 6.5) software was used to calculate the genetic parameters (Shannon's information index *SI*, genetic diversity *h* and unbiased diversity h_u). Principal coordinate analysis was performed to reveal overall genetic variation. According to our results of analysis of molecular variance (AMOVA), the majority of the genetic variation occured within, rather than among the populations (unpublished data), In summary, the obtained pattern is typical for long-lived outcrossing plant species. From a conservation genetics perspective, the projected climate change could cause loss and redistribution of this genetic variation. As previous studies indicated, *Centaurea pseudaxillaris* is a typical ant dispersal plant [1]. More comprehensive studies on seed dispersal mechanism and the spatial genetic structure, i.e. non-random distribution of genetic variation among individuals within populations, may help to enhance our knowledge that is suggestive to any conservation strategy on this plant species.

Nowadays, only one population of *Centaurea pseudaxillaris* in Besaparski hills is under protection of the European ecological network Natura 2000 [1]. Recently, some *ex situ* acclimatized plants were transferred to the newly described population near Dobrich village in order to strengthen it [2].

Conservation genetics represents a key tool for measuring individuals and populations affected by habitat loss, exploitation and environmental changes. Genetic variation is involved in the survival and evolution of any species. Therefore, any project aimed at strengthening the populations of the species should be accurate by replicating the original gene pool. The genetically narrow populations will be able to survive only in a narrow range of conditions. If functionality is the main objective, then a range of genotypes should be introduced, thus allowing selection. Besides established *ex situ* collection, *in situ* measures should continue with strict respect to the observed genetic diversity scenario. Considering the high number of private alleles found in the present study, we underline that the smaller population in Besaparski hills also needs an urgent conservation efforts.

Anthropogenic disturbance of *Centaurea pseudaxillaris* habitat will undoubtedly threaten the viability of the surviving populations of the species by modification of ecological and genetic processes. It is worth to mention, that within-population genetic diversity may

be lost because of further fragmentation or local disturbance. Together with the high number of private alleles observed herein, the extinction of several localities of *Centaurea pseudaxillaris* in the past, due to human influence provides an evidence for existence of higher density populations before. Besides differences in the number of individuals, both investigated populations exhibited similar genetic coefficients. We consider that the smaller population may rapidly lose large amount of genetic variation due to isolation, bottlenecks in population size and genetic drift and thus may have reduced probability of long-term viability. This fact should be taken into consideration.

Conclusion: Given the presented results, the following important point arises from our survey. It is that bridging of conventional conservation with genetic data is essential for targeted conservation of *Centaurea pseudaxillaris* and any other endangered plant species. Random or unpredictable events such as the rapid environmental changes or genetic mutations can cause a sudden decrease in the surviving populations of the species. As already mentioned, they are too small and further decrease in individuals' number will sharply reduce the level of species' genetic diversity.

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CLIMATE FLUCTUATIONS IMPACT ON THE CRITICALLY ENDANGERED ORCHID *TRAUNSTEINERA GLOBOSA* (L.) RCHB. IN BULGARIA

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Aim: The aim of the study was to explore the effects of some of the factors related/ influenced by climate oscillations on the critically endangered orchid *Traunsteinera globosa* (L.) Rchb. [1] in Bulgaria.

Materials and Methods: The monitoring of the status of a selected population of the orchid was done from the spring of 2011 till the autumn of 2016. The monitored population (comprising of 35 up to 45 shoots depending on the year) was located in the Vitosha Mountain floristic region - N42°34'39"; E23°19'39". Several morphometric characteristics were recorded in the investigated plant individuals: A) *in situ:* stem height, flower/fruits number and number of flowering individuals, as well the ones successfully pollinated and reached seed dispersal phase; B) *in lab:* number of seeds per fruit. Due to the extreme rarity of the species only five mid-sized fruits per inflorescence were collected. Only the germinative individuals were counted and measured due to: A) similarities of the non-germinative individuals of *T. globosa* with the ones of other orchids found in the same meadows; B) to minimize the impact and damage from the monitoring procedures upon the habitat and the population.

Bulgaria falls within the transitory zone between the Eastern-continental and Mediterranean climate and thanks to its specific geomorphology it is divided into 5 climate zones according to Georgiev [2]. The monitored location is within the Mountain climatic zone: Vitosha Mountain. The values of the environmental variables were acquired from stations that are located within 1-4 km from the location and their altitude was 100-200 m lower than the monitored area (approx. 1100 m a.s.l.) from stringmeteo.com database. WorldClim and ECA&D databases were used additionally for the estimation of the prognostic trends.

The respective data sets were processed statistically with regression and correlation analysis. Results were expressed as means ± SEM. Differences were considered significant at $P < 0.05$.

Results: The regression analysis of the climate data for an extended period 2000-2016 showed consistent trend in the rise of the mean temperature for the months of active vegetation. This rise is most pronounced for the summer months - June, July and August with 0.036; 0.052; 0.043°C/year respectively for the monitored area.

The analysis of the precipitation showed a change from the patterns derived from the averaged data from the previous century [2]. During the period 2011-2016 the precipitation for the location although part of the Mountain zone had two maximums – May-June and September-October and two minimums January-February and July-August which is a pattern more typical for the transitional-continental zone. Additionally for half of the years

of the monitoring, the vegetation period (late May-August) was marked with local droughts (July-August) with duration exceeding 4-8 weeks and cumulative precipitation below 10mm/mm^2.

The averaged daily humidity during the observed period was generally above 50% throughout the year with less than 10% of the days falling at/or below the 40% level.

T. globosa is a polycarpic alpine plant found throughout the mountain meadows of Europe [1]. In Bulgaria it grows mostly in the mountain climate zone with vegetation period ranging from May-June till Jully-August (flowering – June; fruit maturation - Jully-August). Currently there are only 3 confirmed locations of the orchid in the country namely in Stara Planina Mt: Midjur Peak - <15 individuals; Vrachanski Balkan National Park - <10 individuals and the largest location – Vitosha Mt. - <50 individuals, which was monitored for the study.

At the monitored locations during the monitoring period minimal fluctuations were observed in the number of the shoots – nearly 90% of the monitored individuals flowered annually. The average proportion of the fruit set (the ratio fruits/all flowers) was rather high with some fluctuations (0.52±0.19). The average stem height for the monitored period was 47±8 cm and the average seed number per fruit was 2158±192.

The shifts in the temperature had most notable impact at the early stages of the vegetation period which would cause delay or acceleration of the vegetation onset. At the later stages the correlation between the temperature shifts and the observed morphometric parameters was negligible.

The occurrence of drier periods (with cumulative precipitation less than 20 mm and duration of 4-weeks and more) in the late spring and early summer was negatively related to the plant's height. Similar tendency was observed in regard to the flowers number. These trends seem to be some adaptation mechanisms to the water stress which is known to suppress the anabolic processes, photosynthesis, nutrition transport etc. In some plants (approx. 10%) it caused prolonged dormancy and delayed vegetation onset at the end of the drought time. The increased precipitation in the spring negatively impacted the fruit success of the orchid. This probably can be attributed to its deceptive non-rewarding strategy for pollinator attraction. It relies solely on the rewarding neighbors whose flower/inflorescence color and shape the orchid mimics and which serve as a kind of "pollinator-magnets" [3]. Half of the monitored individuals dwell in areas dominated by taller competing species with earlier vegetation onset and faster growth rate and the rest dwells among equal or lower height neighbors. Comparison between these two groups showed reduced pollination success in the non-favored group (with taller neighboring plants), which additionally drops and reaches over 30% difference (at $P<0.05$) when there's rise of the spring precipitation. This is probably because of the reduced visibility of the orchid flowers to the potential pollinators during the flowering phase.

The species managed to reach the fruit dispersal phase even when it fell in the drought periods, unlike many other temperate orchids who either drop off their fruits or the immature fruits dry on the stem under severe prolonged water stress conditions. This may be attributed to some of the following factors: A) the thick water accumulating/preserving layer of the soil was densely populated with dry grasses at the time of *T. globosa* fruit maturation; B) the relatively high air humidity (over 50%) accompanied by high temperature amplitudes

facilitated the moisturizing dew formation; C) the reproduction dominated over the self-preservation - the plant placed in unfavorable environmental conditions may derive resources from the bulbs for the fruit maturation thus endangering its long-term survival.

There was a decrease in the seed number with the occurrence of drought periods of 4 or more weeks at the mid and/or late phases of the vegetation. Compared with the year 2011 that had the most typical for the Mountain zone pattern the drop in the seed number could reach up to 20%.

Conclusions: The climate fluctuations impact the phenology and vegetation of this endangered orchid in a rather complex way as revealed in this study. Although the six years period of the study is relatively short for climate change generalizations and trends, we still do see a continuation of the global warming tendency expressed here as a switch to pattern typical for the transitional-continental zone observed in five out of six years of the monitoring period. The prognostic modeling of the long-term climate changes till the year 2050 showed that the climate in Bulgaria (and particularly in the Sofia area) will turn to more Mediterranean type with warm and moist spring and autumn and prolonged hot and dry periods in the summers (when using a "scenario with minimal reduction of the anthropogenic climate changing factors"). This is a very worrying tendency for the survival of this orchid in Bulgaria as it has a relatively late start of the vegetation cycle which falls in the unfavorable part of the annual pattern of the transitional-continental zone. Additionally it has the southernmost border of its areal which means that here it lives at the limits of its tolerance range to some of the environmental variables. So the further expected shift to a Mediterranean type of climate probably will cause directly or indirectly its complete extinction. Such signs have been observed in this study as: A) a reduced number of flowers per inflorescence and fruiting success in the hotter and drier years; B) lower fruit success and/or death of the plants due to the gradual occupation and invasion of the habitat territory by much taller species with higher rate of reproduction benefiting from the moister springs (approx. 10% of the monitored individuals were lost due to that).

Keywords: climate fluctuations, orchids, vegetation.

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REPORTS

Topic:
BIOTIC AND ABIOTIC IMPACT ON THE LIVING NATURE AND MECHANISMS OF ADAPTATION

PESTICIDES-PERSISTENT ORGANIC POLLUTANTS. IMPACT ON THE ENVIRONMENT AND HUMAN HEALTH (Review)

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Abstract

Persistent Organic Pollutants (POPs) are chemical substances that persist in the environment, bioaccumulate in agricultural products and in the food and pose a risk of causing adverse effects to human health and the environment. They are organic compounds that are resistant to natural degradation through chemical, biological, and photolytic processes. Many POPs are currently or were in the past used as pesticides, solvents, pharmaceuticals, and industrial chemicals. Humans can be exposed to POPs through diet, occupational accidents and from the environment (including indoor). Exposure to POPs, either acute or chronic, can be associated with a wide range of adverse health effects, including illness and death. Although their use as plant protection products has been banned in Europe and several other countries in the world many years ago, residues of POPs can still be found in agricultural commodities. One of the sources of their presence in the food is probably the storage of obsolete pesticides in the deserted and dilapidated warehouses. Adequate management measures are necessary, to find resources for the safe and environmentally sound destruction of this danger.

Keywords: Persistent Organic Pollutants (POPs), plant protection products, obsolete pesticides

Introduction

I. Common characteristics: Persistent Organic Pollutants (POPs) are organic compounds that are highly resistant to degradation by biological, photolytic or chemical means. These pollutants are compounds of natural or anthropogenic origin that possess a particular combination of physical and chemical properties such that, once released into the environment, they remain there for exceptionally long periods of time. POPs are chemical substances which persist in the environment, bioaccumulate in agricultural products and in the food and pose a risk of causing adverse effects to human health and the environment. Recently, POPs are a matter of concern because of their toxicity and tendency to accumulate in food chains [1].

POPs are also noted for their moderate volatility: this property allows relatively great amounts to enter the atmosphere and be transported over long distances; that is a property of their physico-chemical characteristics that permit these compounds to occur either in

the vapour phase or adsorbed on atmospheric particles. They have been measured on every continent, at sites representing every major climatic zone and geographic sector throughout the world [2].

Because of their persistence, POPs bioaccumulate with potential significant impacts on human health and the environment. The effect of POPs on human and environmental health was discussed, with intention to eliminate or severely restrict their production, by the international community at the Stockholm Convention on Persistent Organic Pollutants in 2001. Many POPs are currently or were in the past used as pesticides, solvents, pharmaceuticals, and industrial chemicals. The persistence in the compounds allows them to accumulate in animals and pass on more. When the pollutants progress into animals, they accumulate in the fat cells, organs and muscles [1].

In order to concentrate in organisms of the environment, POPs must also possess a property that results in their movement into organisms. This property is lipophilicity or a tendency to preferentially dissolve in fats and lipids, rather than water. High lipophilicity results in the substance bioconcentrating from the surrounding medium into the organism. Combined with environmental persistence and a resistance to biological degradation, lipophilicity also results in biomagnification through the food chain. Biomagnification results in much greater exposures in organisms at the top of the food chain [2].

Humans can be exposed to POPs through diet, occupational accidents and the environment (including indoor). Exposure to POPs, either acute or chronic, can be associated with a wide range of adverse health effects, including illness and death. Laboratory investigations and environmental impact studies in the wild have implicated POPs in endocrine disruption, reproductive and immune dysfunction, neurobehavioural and disorders and cancer. More recently some POPs have also been implicated in reduced immunity in infants and children, and the concomitant increase in infection, also with developmental abnormalities, neurobehavioural impairment and cancer and tumour induction or promotion. Some POPs are also being considered as a potentially important risk factor in the etiology of human breast cancer by some authors [2].

Pesticides are chemicals or biological substances used to kill or control pests. In the 1940s, many chlorinated hydro-carbon insecticides were developed though they did not come into widespread use until the 1950s. Common examples include aldrin, dieldrin, heptachlor, and endrin. However, in spite of their early promise, these organochlorine insecticides become much less used because of their environmental pollution impact [1].

II. Stockholm Convention: With the evidence of long-range transport of these substances to regions where they have never been used or produced and the consequent threats they pose to the environment of the whole globe, the international community has now, at several occasions called for urgent global actions to reduce and eliminate releases of these chemicals.

Pesticides have been widely used throughout the world since middle of the last century. About 1000 active ingredients have been employed and are currently formulated in thousands of different commercial products. They include a variety of compounds, mainly insecticides, herbicides and fungicides, with very different physico-chemical characteristics, and large differences in polarity, volatility and persistence. Pesticides are employed for many different

purposes. About 80% of the pesticides are used in agriculture and moved in the environment by means of volatilization, runoff, infiltration, transport along the food chain, etc. Although the application of POPs has been forbidden for a considerable period in many countries, the residues continue to induce a significant impact on the environment and its ecosystems [1].

The Stockholm Convention is based on the precautionary principle and seeks to guarantee the safe elimination of these substances, as well as reductions in their production and use. The Stockholm Convention on Persistent Organic Pollutants came into force on 17 May 2004. The Convention is a global treaty that aims to protect human health and the environment from the effects of POPs. The Convention has a range of control measures to reduce and, where feasible, eliminate the release of POPs, including emissions of unintentionally produced POPs such as dioxins. The Convention also aims to ensure the sound management of stockpiles and wastes that contain POPs. Convention is managed by the Environmental Program of the United Nations in Geneva, Switzerland. Bulgaria is party to the Convention since 20.03.2005 [3].

Under the Stockholm Convention, each country is required to develop, update and implement the National Plan for the management of POPs, in order to facilitate or undertake information exchange to promote and to support the public information and provide public access to existing information on POPs. The parties are also encouraged to carry out research studies, monitoring and cooperation on POPs, and where possible, in terms of alternatives to POPs and chemicals candidates for inclusion in the group of POPs. Countries report periodically to the Conference of the Parties on the measures they have taken to fulfill their obligations under it.

In 2004, 12 POPs were listed in annexes to the Convention. More recently, in 2010, nine additional POPs were added to the Convention. The Convention covers 23 priority POPs distributed both intentionally and unintentionally (e.g. by sources like waste incinerators), a great part of which are used as pesticides: aldrin, chlordane, chlordecone, dichlorodiphenyltrichlorethane (DDT), dieldrin, endrin, heptachlor, hexachlorobenzene (HCB), alpha hexachlorocyclohexane, beta hexachlorocyclohexane, lindane, mirex, technical endosulfan and its related isomers.

Although pesticides from the group of POPs have long been banned in Europe, remains of them can still be found in some agricultural products. A survey on the Rapid Alert System for Food and Feed (RASFF) for the period of years 2000 – 2015, determined some cases of pesticides, which are POPs in the agricultural commodities: 1 case of aldrin in chilled peppers from Turkey (in 2015); 2 cases of dieldrin in carrots from Albania – in 2013 and 2014; a case of endosulfan, fenarimol and endrin in roasted chickpeas, in 2002; heptachlor – in fresh carrots from Belgium (2010); hexachlorobenzene in long yellow peppers from Morocco, 2010; parathion-methyl and lindane and unauthorised substance alpha-HCH in chickpeas from India (in 2008); endosulfan in celery – in 2002. This inevitably leads us to a question, which are the sources of these banned pollutants and in which way they reach the foodstuff [4].

III. Influence of POPs on the environment and human health: Pesticides that belong to the group of persistent organic pollutants, exhibit a number of negative effects on human health and the environment. All of them have been banned long time ago, for the use in farms.

• Aldrin is an organochlorine insecticide that was widely used until the 1970s, when it was banned in most countries, mainly because of concerns over bioaccumulation and environmental effects, and possible carcinogenicity. Before the ban, it was heavily used as a pesticide for treatment of seed and soil to kill termites, grasshoppers, Western corn rootworm, and others; it is also known to kill birds and it is extremely toxic to fish. In soil, on plant surfaces, or in the digestive tracts of insects, aldrin oxidizes to the epoxide dieldrin, which is more strongly insecticidal. Humans are primarily exposed to aldrin through dairy products and animal meats; highly toxic by ingestion. It is immunotoxic, neurotoxic, damages liver and male reproductive function [2; 5]. According to International Agency for Research on Cancer (IARC), it is group 3 carcinogens [6]. The substance is banned from use for plant protection by the EU [7].

• Chlordane, an insecticide used to control termites and on a range of agricultural crops, is known to be lethal in various species of birds, including mallard ducks, bobwhite quail, and pink shrimp; it is a chemical that remains in the soil with a reported half-life of one year. Chlodane air pollution is believed the primary route of humane exposure; may be absorbed also from the gastrointestinal tract, by inhalation and through the intact skin. Chlordane has been postulated to affect the human immune system and is classified as a possible human carcinogen. The non-cancer health effects of chlordane compounds include diabetes, insulin resistance, migraines, respiratory infections, anxiety, depression, as well as permanent neurological damage. Damages also to the reproductive function and endocrine system [8]. Banned from use for plant protection in the EU [7]. Group 2B carcinogen [6].

• Dieldrin, a pesticide used to control termites, textile pests, insect-borne diseases and insects living in agricultural soils. In soil and insects, aldrin can be oxidized, resulting in rapid conversion to dieldrin. Dieldrin is highly persistent in the environment – it's half-life is approximately five years. Dieldrin is highly toxic to fish and other aquatic animals, particularly frogs. In humans, dieldrin has been linked to Parkinson's disease, breast cancer, and classified as immunotoxic, neurotoxic, with endocrine disrupting capacity, damages in liver and male reproductive function also [2; 9]. Classified in group 3 carcinogen [6]. Dieldrin residues have been found in air, water, soil, fish, birds, and mammals. Human exposure to dieldrin primarily derives from food. Since the early 1970s, registrations of aldrin and dieldrine have been resticted or withdrawn in many countries mainly because of concerns over bioaccumulation and environmental effects, or possible carcinogenicity. Since the early 1970s, the usage of aldrin and dieldrin has been progressively restricted. Dieldrin is banned in the EU [7].

• Endrin, an insecticide sprayed on the leaves of crops, and used to control rodents. Animals can metabolize endrin, so fatty tissue accumulation is not an issue, however the chemical has a long half-life in soil for up to 12 years. Endrin is highly toxic to aquatic animals and humans as a neurotoxin. Acute endrin poisoning in humans affects primarily the central nervous system. It can block the activity of inhibitory neurotransmitters. Endrin causes also allergic reactions, toxic hepatitis, damage to the central and peripheral nervous system. Human exposure results primarily through food [10]. Banned in the EU.

• Heptachlor, a pesticide primarily used to kill soil insects and termites, along with cotton insects, grasshoppers, other crop pests, and malaria-carrying mosquitoes. Heptachlor, even

at every low doses has been associated with the decline of several wild bird populations. Its half life can be about 2 years and residues could be found in soil 14 years after its initial application. Like other POPs, heptachlor is lipophilic and poorly soluble in water, so it tends to accumulate in the body fat of humans and animals. High dose heptachlor has lethal, with adverse behavioral changes at low-doses, neurotoxic and is classified as a possible human carcinogen (group 2B) and risk factor for breast cancer [2; 11]. Damages also to the reproductive function and endocrine system. Human exposure primarily results from food, but it can occur also by inhalation, ingestion of substances containing the compound, or by skin contact. Heptachlor is banned for use in the EU.

• Hexachlorobenzene (HCB), was first introduced in 1945–1959 to treat seeds because it can kill fungi on food crops. HCB is very toxic to aquatic organisms. It may cause long term adverse effects in the aquatic environment. Human exposure is primarily from food. Mothers who pass HCB to their infants through the placenta and breast milk had limited reproductive success including infant death. Negative effect on the nervous, endocrine and reproductive systems porphyria in humans. Chronic oral exposure in humans has been shown to give rise to a liver disease, skin lesions, thyroid effects, bone effects and loss of hair. Hexachlorobenzene may cause embryolethality and teratogenic effects; may cause lethal output [12]. HCB is considered to be a probable human carcinogen (IARC group 2B). Banned in the EU.

• Mirex, an insecticide used against ants and termites or as a flame retardant in plastics, rubber, and electrical goods. Mirex is one of the most stable and persistent pesticides, with a half-life of up to 10 years. Mirex is toxic to several plant, fish and crustacean species, with suggested carcinogenic capacity in humans (IARC group 2B); causes teratogenic effect, endocrine disrupter also. Liver, thyroid and kidney toxicant [13]. Humans are exposed primarily through animal meat, fish, and wild game. Banned in the EU.

• Dichlorodiphenyltrichloroethane (DDT) is a famous POP. Before the insidious effects of DDT on humans and wildlife were known, this potent nerve poison was widely used to control mosquitoes, black flies and other vectors that carry diseases such as malaria, typhus and yellow fever, during World War II. Farmers also used it to control insect damage to their crops. In the 1950s and 1960s, DDT was embraced as a cheap, effective, broad spectrum chemical pesticide. It was used worldwide and applied generously and indiscriminately to communities and crops alike [1]. DDT's persistence in the soil for up to 10–15 years after application has resulted in widespread and persistent DDT residues throughout the world including the arctic, even though it has been banned or severely restricted in most of the world. DDT is toxic to many organisms including birds where it is detrimental to reproduction due to eggshell thinning. DDT can be detected in foods from all over the world and food-borne DDT remains the greatest source of human exposure. Short-term acute effects of DDT on humans are limited, however long-term exposure has been associated with chronic health effects such as diabetes, carcinogenic (IARC group 2A) and mutagenic, reduced reproductive success, and has been linked to neurological disease; immunotoxic, damage of estrogen and endocrine systems [2]. Banned in the EU.

• Chlordecone, a synthetic chlorinated organic compound, is primarily used as an agricultural insecticide. Structurally, chlordecone is very similar to mirex and therefore these chemicals share main characteristics. Chlordecone breaks down slowly in the environment,

and it may stay for years in soil and water. Like mirex and other POPs, chlordecone can bioaccumulate in fish or other organisms that live in contaminated water or that eat other contaminated animals. Chlordecone is toxic to aquatic organisms, and classified as a possible human carcinogen – group 2B. The mechanism of toxicity to humans is not well understood. However, workers who were exposed to high levels of chlordecone over a long period (more than one year) showed harmful effects on the nervous system, skin, liver, and male reproductive system [14]. It is possible liver and kidney toxicant. May cause severe tremor. Banned in the EU.

• Pesticides from the group of Hexachlorocyclohexane (HCHs) are one of the most widely used and most readily detected organochlorine pesticides in environmental samples. The relatively high volatility of HCHs has led to global transport, even into formerly pristine locations such as the Arctic. Certain HCHs cause central nervous system, reproductive, and endocrine damage. α-Hexachlorocyclohexane (α-HCH) and β-Hexachlorocyclohexane (β-HCH) are insecticides as well as by-products in the production of lindane. Large stockpiles of HCH isomers exist in the environment. α-HCH and β-HCH are highly persistent in the water of colder regions. α-HCH and β-HCH has been linked Parkinson's and Alzheimer's disease. Because γ-HCH is rapidly metabolized, the β-HCH isomer is consistently found in higher concentrations in human fat, blood, and breast milk. In contrast, α- and γ-HCH are the most prevalent isomers in soil, water, and air samples [15]. They are banned in the EU and specified in Group 2B carcinogen.

• Lindane (γ-hexachlorocyclohexane), a pesticide used as a broad spectrum insecticide for seed, soil, leaf, tree and wood treatment, and against ectoparasites in animals and humans (head lice and scabies). Lindane rapidly bioconcentrates. It is immunotoxic, neurotoxic, carcinogenic, linked to liver and kidney damage as well as adverse reproductive and developmental effects in laboratory animals and aquatic organisms. Production of lindane unintentionally produces two other POPs α-HCH and β-HCH. Group 1 carcinogen. Banned in the EU.

• Endosulfans are insecticides to control pests on crops such coffee, cotton, rice and sorghum and soybeans, tsetse flies, ectoparasites of cattle. They are used as a wood preservative. Global use and manufacturing of endosulfan has been banned under the Stockholm convention in 2011, although many countries had previously banned or introduced phase-outs of the chemical when the ban was announced. Toxic to humans and aquatic and terrestrial organisms, linked to congenital physical disorders, mental retardation, and death. Endosulfans' negative health effects are primarily liked to its endocrine disrupting capacity acting as an antiandrogen. Banned in the EU.

• Toxaphen was one of the most heavily used insecticides in the United States, frequently applied with methyl or ethyl parathion, DDT and lindane, until 1982, when it was cancelled for most uses; all uses were banned in 1990. Causes damages of the central and peripheral nervous system. Specified in Group 2B carcinogen.

The weight of scientific evidence suggests that some POPs have the potential to cause significant adverse effects to human health. The developing fetus and neonate are particularly vulnerable to POPs exposure due to transplacental and lactational transfer of maternal burdens at critical periods of development. In the scientific literature a number of studies

are available, linking exposure to POPs in addition to many diseases in humans, also with impaired reproductive function [2].

Conclusion

It is known that in the tissues of living organisms inhabiting contaminated areas, measurable amounts of chemicals can be found. In some cases, the amounts can reach the so-called "dangerous levels" according to the legal norms. The advent of POPs in human body can be attributed to various sources, but found that nearly 90% of human exposure to them comes directly from food.

The availability of banned long time ago pesticides in food, inevitably draws attention to their possible sources. One likely source are warehouses, containing obsolete pesticides where certain quantities of them are stored for a prolonged period. Such warehouses left from former agricultural cooperatives are widespread in Bulgaria. Over time, due to violating the integrity of the packaging and washout by rain, dangerous substances falling into soil and groundwater from where easily reach cropland and cultivated in them agricultural crops. Although this problem has been written and discussed many times, it is not yet been solved for Bulgaria, due to lack of funds for safe and environmentally sound disposal of obsolete pesticides. Finding of adequate management measures is necessary, in order to reduce the risk.

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ASSESSMENT OF THE IMPACT OF SALINITY ON THE GROWTH AND ANTIOXIDANT ACTIVITY OF TWO *LYCIUM* SPECIES

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Abstract

Aim: *Lycium barbarum* and *Lycium chinense* are two species from which the goji berry is harvested. The impact of salinity on growth parameters and antioxidant activity in these species, grown *ex vitro* in soils with different salinity was studied.

Materials and Methods: Seeds and *in vivo* explants from *L. barbarum* and *L. chinense* were used for developing of *in vitro* multiplication protocol, licensed by Bio Tree Ltd., Bulgaria. Plant adaptability was traced after *ex vitro* cultivation of both plants in pots filled with non-saline (type 0 – peat) and two saline soils (type 1 and 2) under glass house conditions for two years.

The root and shoot dry mass of plants was measured gravimetrically after heating at 60^{O}C for 48 a constant weight. Spectrophotometric quantification of water- (WS – AOC) and lipid-soluble (LS – AOC) antioxidant capacity was performed through the formation of phosphomolybdenum complex. The total antioxidant potential of samples was determined spectrophotometrically by DPPH and FRAP assay.

Results: The ratios fresh mass/ dry mass of roots and shoots of *Lycium barbarum* rose in a great degree after cultivation on saline soil in comparison with *Lycium chinense*. LS – AOC antioxidant capacity was enhanced at the same conditions of cultivation in both plants, but total antioxidant activity measured by DPPH and FRAPS methods changed in a different manner.

Conclusions: Our results suggest that *Lycium chinense* was more salt tolerant than *Lycium barbarum* because it responded to high salinity level with increasing antioxidant activity and improved dry mass production.

Keywords: *Lycium*, salinity, growth, antioxidant activity

Introduction

Salinity in soil or water is one of the major negative influencing factors of the environment, and especially in arid and semiarid regions, can severely limit crop production. In recent decades, the investigations are focused on studying of biochemical and physiological traits [1], identification of key genes and/or discovering of distinctive molecular markers in a great number of agricultural crops for improving their salt tolerance [2]. By using salt tolerant species areas of degraded soils can be reduced and change their purpose for cultivation high-yielding plants producing bio-fuel or economic important bioactive products.

Lycium L. (Solanaceae) is a genus of approximately 80 species distributed mainly in Asia, America and Africa [3]. In Bulgaria, *Lycium barbarum L.* is widely cultivated as an ornamental shrub, for making hedges, and also for stabilization of the soil and landslides. *Lycium* species produce red or purple, fleshy berries which are rich in vitamin C. The plants are used in traditional Chinese medicine for improving eyesight, lowering blood pressure and cholesterol, and for treatment of diabetes and tuberculosis. In some Asian countries the fruits are used as a tonic and invigorating agent, stimulating the immune system. These effects of the fruits are widely popularised in Bulgaria during the past few years and the species is strongly recommended by some private companies for cultivation (under the name "Goji Berry") [4]. The physiological characteristics of drought-tolerance and salt-tolerance make also *Lycium* species ideal plants for preventing soil desertification and alleviating the degree of soil salinity. Overexploitation of *Lycium* natural habitats caused its deterioration and decreased existing populations [5]. That is why, the development of micropropagation technologies is necessary in future.

In the present study, pot experiments were carried out to investigate the differences in the growth between *Lycium barbarum* and *Lycium chinense* plants cultivated *ex vitro* on two types of saline soils for a period of two vegetative seasons. The effect of soil salinity on the antioxidant activity in the leaves of both species was traced in order to elucidate the role of antioxidants in adaptation of *Lycium* species differing in salt tolerance.

Materials and Methods

Plant material: Seeds and *in vivo* explants from the species of *L. barbarum* and *L. chinense* were used for developing of *in vitro* multiplication protocol [6]. Seedlings derived from *in vitro* micropropagation were cultivated in plastic pots filled with 1.5 kg non-saline (type 0) and saline soils (type 1 and 2).The experiment was set as 3 treatments, with 7 replications and

was conducted in a glass house (natural sunlight, temperatures 15^{O}C – 35^{O}C, relative humidity 40% - 65%) for two vegetation seasons. The soils used in this study were taken from an area located in the vicinity of the village Belozem, Bulgaria (42^{O} 20′N 25^{O} 3′E). A sampling strategy was carried out from the surface and at depths of 30-60 cm in different locations of the areas. As a control soil (type 0) peat moss-perlite (2:1, v: v) was used. The second type soil possessed exchangeable Na content about 5 times higher, Sodium Adsorption Ratio (SAR) about 6 times higher and electrical conductivity - 14.0 mS/cm about 2 times higher in comparison with the first type of soil [7]. The analysis of the soils was made by the Institute of Soil Science "Pushkarov", Sofia, Bulgaria.

Growth parameters: At the end of the experiment the plant samples were collected, washed with tap water and rinsed with distilled water before being separated into shoots and roots and fresh mass of each plant sample were measured gravimetrically. Dry mass of shoots and roots were determined after oven-drying (60^{O}C) for 2 days until constant weight was obtained.

Total antioxidant capacity: Spectrophotometric quantification of water- (WS – AOC) and lipid-soluble (LS – AOC) antioxidant capacity (expressed as equivalents of ascorbate and α-tocopherol, respectively) was performed through the formation of phosphomolybdenum complex [8]. The assay was based on the reduction of Mo (VI) to Mo (V) by the sample analysis and the subsequent formation of a green phosphomolybdenum complex at acidic pH. The dry leaf samples (0.1 g DW) were ground with pestle and mortar to a fine powder. 3 ml distilled H_2O was added and the suspension was homogenized, transferred to tubes and shaken for 2 h at room temperature in the dark. The suspension was filtered and extraction was repeated with 3 ml distilled H_2O. The pellet was washed again with 2 ml distilled H_2O. For lipid-soluble antioxidant capacity procedure was the same, but the extraction was carried out with hexane as a solvent. The method was optimized and characterized with respect to linearity interval, reproducibility and molar absorption coefficients for the quantitation of water-soluble and lipid-soluble antioxidant capacities expressed as equivalents of ascorbate, and α-tocopherol. Absorption coefficients were: (3.4 ± 0.1) × 103 M^{-1} cm^{-1} for ascorbic acid, and (4.0 ± 0.1)×103 M^{-1} cm^{-1} for α-tocopherol.

DPPH assay: Free radicals scavenging activity was measured from the bleaching of the purple-colored methanol solution of free stable radical (2,2-diphenyl-1-picrylhydrazyl, DPPH$^{\bullet}$) inhibition after [9]. DPPH$^{\bullet}$ radical is a stable radical with a maximum absorption at 517 nm that can readily undergo reduction by an antioxidant. The inhibition of free radical DPPH$^{\bullet}$ in percent (I%) was calculated in the following way: $I\% = (A_{blank}\text{-}A_{sampe}/A_{blank})\times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} is the absorbance of the test compound, i.e. leaf extracts. The reaction mixture was consisted of different concentrations from 15 to 180 μg ml^{-1} leaf methanol extract, 2.4 ml methanol and 0.1 mM methanol solution of DPPH$^{\bullet}$. Control and tested samples were incubated in the dark for 30 min before spectrophotometrically assay.

Ferric reducing power (FRAP assay): The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), ferri 2,4,6-tripiridyl-s-triazine(TPTZ) solution (10 mM TPTZ in 40 mM HCl) and $FeCl_3$-$6H_2O$ (20 mM) in a ratio 10:1:1 [10]. To perform the assay, 900 μl of FRAP reagent, 90 μl distilled water and 30 μl of leaf extract were mixed and incubated at 37 °C for 15 min. The absorbance was measured at 595 nm, using FRAP

working solution as a blank. The antioxidant potential of samples was determined from a standard curve plotted using the $FeSO_4.7H_2O$ linear regression. The results were corrected for dilution and expressed as μmol of Fe^{2+} g^{-1} of dried sample.

Statistical analysis: All data reported in this work were mean value from of at least five to six independent experiments. The significance of differences between control and each treatment was analyzed by Fisher`s LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

Results and Discussion

Seedlings growth is normally limited by increasing concentration of NaCl. Our results showed that with increasing salinity levels root and shoot dry mass of *L. chinense* increased, while root and shoot dry mass of *L. barbarum* decreased. The root and shoot ratios FM/DM are highest after cultivation of both plants on peat moss, but this ratio is enhanced for shoots of *L. chinense* after cultivation on saline soil (Table 1). Time-dependent changes of growth and development of plants exposed to salt stress have been reviewed [11]. In the first few minutes, cells lose water and shrink, whereas in a few hours they regain their volume, but its expansion rates are limited. In a few days and weeks, reduced cell elongation and cell division result in slower leaf appearance and inhibition of shoot growth. The shoot fresh mass, dry mass and its ratio rose in *L. chinense* grown on saline soil in comparison with non-saline soil. In spite of higher FM/DM ratio for the shoots of *L. barbarum* grown on saline soil in comparison with non-saline, its fresh and dry mass decreased drastically (Table 1).

Table 1. Changes in total fresh mass (FM), dry mass (DM) and the ratios (FM/DM) of shoots and roots of *Lycium* barbarum and *Lycium chinense*, cultivated for two vegetation seasons on peat moss (type 0), non-saline (type 1) and saline (type 2) soils in a glass house. Mean values ± SD (n = 5-6). Values with the same letter are not significantly different when means are separated by Fisher`s LSD test (P<0.05)

Variant	Total shoot fresh mass [FM] [g]	Total shoot dry mass [DM] [g]	Shoots [FM/DM] [g/g]	Total root fresh mass [FM] [g]	Total root dry mass [DM] [g]	Roots [FM/DM] [g/g]
Lycium barbarum						
Type (0)	**7.084**±0.724a	**0.808**±0.079a	**8.767**	**1.811**±0.211a	**0.276**±0.027a	**6.561**
Type (1)	**19.793**±7.124b	**2.728**±1.069b	**7.225**	**13.205**±1.422b	**4.148**±0.512b	**3.183**
Type (2)	**4.420**±2.056c	**0.588**±0.280c	**7.517**	**3.617**±0.346c	**0.900**±0.079c	**4.018**
Lycium chinense						
Type (0)	**2.702**±0.230a	**0.305**±0.022a	**8.859**	**1.533**±0.170a	**0.291**±0.022a	**5.268**
Type (1)	**1.842**±1.319b	**0.204**±0.131a	**9.029**	**1.181**±0.108b	**0.288**±0.044a	**4.101**
Type (2)	**2.649**±1.593a	0.**288**±0.169b	**9.198**	**1.505**±0.159c	**0.358**±0.039b	**4.203**

At saline conditions water deficit is a major constraint that leads to a restriction of CO_2 uptake (net-photosynthesis) and to the development of reactive oxygen species (ROS). Plant species differ in responsiveness of antioxidant enzyme activities, as well as abundance of nonenzyme compounds which rendered ROS. Our previous investigations obtained by hydroponic experiment showed that *L. barbarum* responded to increasing concentration of NaCl with increasing antioxidant enzyme activity of superoxide dismutase and improved dry mass production in comparison with *L. chinense* [12]. The water-soluble antioxidant capacity (WS-AOC) in the leaves of both plants increased after cultivation on non-saline soil, but negligible decreased after cultivation on saline soil (the differences between plants grown on saline and non-saline soils are not significant). The lipid-soluble antioxidant capacity (LS-AOC) in the leaves of both plants rose gradually after cultivation on non-saline and saline soils in comparison with peat moss (Fig. 1).

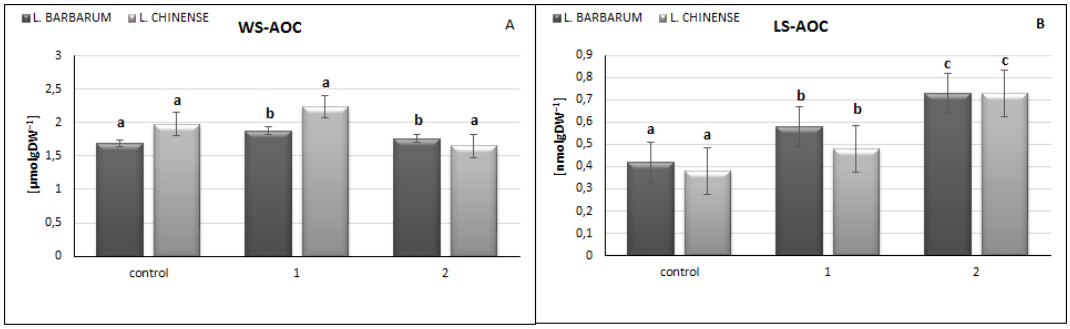

Fig. 1. Changes in leaf water-soluble antioxidant capacity (WS-AOC) – A and lipid-soluble antioxidant capacity (LS-AOC) – B of *Lycium barbarum* and *Lycium chinense*, cultivated for two vegetation seasons on peat moss (type 0 - control), non-saline (type 1) and saline (type 2) soils in a glass house. Mean values ± SD (n = 5-7). Values with the same letter are not significantly different when means are separated by Fisher`s LSD test (P<0.05)

Our previous results obtained from hydroponic experiments showed that the values of WS-AOC decreased in both plants with increasing salinity level from 50 to 200 mM NaCl, while for the second parameter highest value is established at 100 mM NaCl in *L. barbarum* [13]. Between hydrophilic compounds which scavenged ROS belong ascorbic acid, glutathione and polyphenols, while lipophilic compounds are α-tocopherols, carotenoids, etc. Our results showed an enriched lipophilic fraction after cultivation of plants on saline soil in comparison with non-saline soil (Fig. 1B). Detailed information about active compounds in the leaves of *Lycium* is scarce, but the analysis of the main components of *Lycium barbarum* fruits – polyphenols, polysaccharides and carotenoids showed that their content are significantly affected by environmental factors [14].

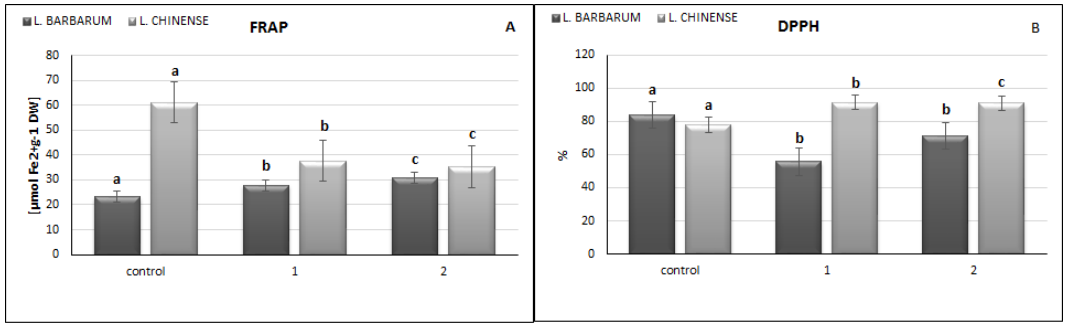

Fig. 2. Changes in leaf antioxidant activity measured by FRAP (A) and DPPH (B) assays in *Lycium barbarum* and *Lycium chinense*, cultivated for two vegetation seasons on peat moss (type 0 - control), non-saline (type 1) and saline (type 2) soils in a glass house. Mean values ± SD (n = 5-7). Values with the same letter are not significantly different when means are separated by Fisher`s LSD test (P<0.05)

Total antioxidant capacity measured by DPPH and FRAP methods changed in a different manner - the values measured by FRAP assay for *L. barbarum* changed contrary to *L. chinense*. The value measured by DPPH assay is highest for *L. chinense* grown on saline soil, while this value is the lowest for *L. barbarum* grown on non-saline soil (Fig. 2). Greater DPPH-radical scavenging capacity was chosen as an index characterizing more tolerant species to environmental conditions between encroachers in semi-arid grassland ecosystems [15]. Our results showed that the values measured by DPPH and FRAP methods for *L. chinense* grown on non-saline and saline soils are higher than these for *L. barbarum*, but the course of changes is opposite (Fig. 2).

In conclusion, *Lycium chinense* possessed better salt tolerance than *Lycium barbarum*, which could help to adapt to the increasing soil salinity. It responded to high salinity by the increase of the lipophilic antioxidant fraction (LS-AOC) and total antioxidant activity measured by DPPH and FRAP methods. The results suggested a possibility to improve saline soil by utilizing *Lycium chinense* because it enhanced dry mass accumulation in the roots and shoots.

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VARIABILITY OF PSII FUNCTIONALITY AND CHLOROPLAST MEMBRANE LIPIDS OF SOME HALOPHYTIC AND GLYCOPHYTIC REPRESENTATIVES FROM GENUS *LACTUCA* (ASTERACEAE)

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Abstract

The aim of this study was to investigate the structural-functional peculiarities of chloroplast membranes influenced by the environmental conditions in different natural habitats of some halophytic and glycophytic *Lactuca* species.

Materials and Methods: Leaves of *Lactuca* species were collected from their natural habitats. The pigment content was determined spectrophotometrically. Light microscopy was carried out by Nikon Eclipse 50i (Tokyo, Japan). Thylakoid membranes were isolated in Hepes, pH 7.6. The analyses of total lipophilic extracts were performed using thin-layer and gas chromatographic techniques. Specific changes in PSII functionality and recombination events were assessed by alterations in thermoluminescence (TL) emission parameters and oxygen-evolving patterns.

Results: Comparative histological analysis of the leaves showed specific anatomical characteristics of the *Lactuca* species. TL glow curves and oscillation pattern of isolated chloroplasts followed particularly well the TL parameters recorded with intact leaves. The observed species variations in the TL pattern and the kinetics of the initial oxygen burst might be a result of conformational changes of PSII due to some modification of membrane lipid composition. The comparative analysis of fatty acids composition of thylakoid membranes confirmed the existence of qualitative and quantitative differences.

Conclusion: The results of this study show specific structural and functional characteristics of photosynthetic membranes in halophytic and glycophytic *Lactuca* species, reflecting different adaptive strategies of the studied species to environmental conditions in their natural habitats.

Keywords: *Lactuca* species, histological analysis, fatty acid composition, PSII activity, thermoluminescence.

Introduction

In natural habitats, the environmental factors (temperature, water, light intensity, salinization, etc.) have a complex influence on the plants. Due to their immobile lifestyle, plant organisms are able to survive only by their ability to build rapid and highly adaptive responses to ever-changing environmental conditions. As a means of overcoming abiotic

and biotic limitations, plants have different adaptive and protective strategies. The plant response is complex because it reflects over space and time the integration of stress effects and responses at all underlying levels of organization [1]. Under field conditions the situation is complicated by the fact that the influence of abiotic stress is usually the manifestation of the superimposition of other stresses.

Constantly changing environmental factors induce physiological adaptation by influencing the activity of primary metabolic reactions such as photosynthesis, which is a major physiological process that determines plant productivity.

Perturbations by different stressful environments are first manifested in alterations in structure of thylakoid membranes and the photochemical efficiency of photosystems, especially photosystem II (PSII). PSII oxygen-evolving enzyme complex of thylakoid membranes appears main stress sensitive site in plants. The possibility of fast and reliable monitoring of the effectiveness of the operation of PS2 is the first prerequisite to solve site and mechanisms of stress injury and adaptation to biotic and abiotic environmental factors.

The physiological properties of the membrane such as permeability, selectivity, etc., are changed in dependence of environment conditions, resulting in changes in the physical orientation of membrane lipids and functional activity. The response of the cell in such cases is a series of quantitative and qualitative changes in the lipid composition of the membrane in order to restore the initial orientation and the physical properties of the membrane.

The aim of this study was to investigate the structural-functional peculiarities of chloroplast membranes influenced by the environmental conditions in different natural habitats of some halophytic and glycophytic *Lactuca* species.

The genus *Lactuca* L. (Asteraceae) comprises of about 100 wild species occurring in Europe, Asia, Africa and North America. Since ancient times, some *Lactuca* species have been well known as dietary and medicinal plants. In Bulgaria, seven *Lactuca* (lettuce) species can be found: halophyte *Lactuca tatarica* (L.) C.A. Mey and glycophyte species *Lactuca serriola* L., *Lactuca quercina* L., *Lactuca viminea* (L.) J.Presl & C.Presl, 1819, *Lactuca saligna* L., *Lactuca perennis* L., *Lactuca aurea* (Vis. & Pančić) Stebbin.

Plants of the genus *Lactuca* have been shown to produce sesquiterpene lactones (SL) [2] as their characteristic secondary metabolites. Included among these are guaianolides, germacranolides and some eudesmanolides [3]. Sesquiterpene lactones may play a highly significant role in human health, as pharmaceutical agents, due to their potential for the treatment of cardiovascular disease and cancer [4]. SL are responsible for a range of other effects such as prevention of neurodegeneration, antimigraine activity, analgesic and sedative activities and treatment of ailments such as diarrhoea, flu, and burns.

Materials and Methods

In the present study were selected three *Lactuca* species in flowering stage (July - August) from different natural habitats. The glycophyte *Lactuca serriola* L. is a drought-resistant species mainly found in solar micro-habitats, urban places, along railways, landfills, etc. The plants were collected around the city of Sofia from agricultural field. The glycophyte *Lactuca quercina* L. inhabits shadow and a semi-shade oak and beech forests.

The plants were collected in the Rila Mountain in the nearness of the "Rila Monastery" Nature Park in a beech forest. In nature, halophyte *Lactuca tatarica* (L.) C.A. Mey grows in meadows, in steppes and semi-deserts, as well as in salty soils, for example, along sea coasts. The plants were collected from the Bulgarian Black sea coast near Shabla.

The leaves were maintained in a hydration condition by being wrapped in wet paper at room temperature in darkness. Thylakoid membranes were isolated and re-suspended in a medium containing: 40 mM HEPES (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$ and 400 mM sucrose. The pigment content of the leaves was determined spectrophotometrically [5].

The structural organization of the leaves was determined by Nikon Eclipse 50i (Tokyo, Japan). The leaves are fixed in 3% (m/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 h at 4°C. Handmade transversal sections were mounted on slides in glycerol.

The total lipophilic extract from *L.* species thylakoid membranes was obtained and FAME analysed as described in [6].

The initial oxygen burst (induction curves) was measured using polarographic oxygen rate electrode (Joliot-type), 100 μl sample volume, without any artificial electron acceptors, as described in [7]. Deconvolution of the oxygen burst decay was performed by fitting of the function with two exponential components: $A_1 e^{(tk1)} + A_2 e^{(tk2)}$, where A_1, A_2 are amplitudes of the fast and slow components.

Thermoluminescence (TL) measurements were carried out in darkness using computerized equipment, described in detail in [8]. The samples were kept in the dark for 2 h before measurements. The excised pieces of a leaf with equal size and appearance were used in order to obtain reproducible results. After dark incubation the leaf samples were cooled to 2°C, illuminated with a single turn-over flash and warmed up to 70°C at a heating rate of $0.6°C.s^{-1}$. Alternatively, samples of isolated thylakoid membranes ware illuminated at 2-5°C to generate charge pairs within the PSII reaction centres and then rapidly cooled down in liquid nitrogen to trap those charge-separated states. Subsequent warming of the samples reveals several peaks of thermoluminescence emission [9, 10]. Decomposition analysis of TL glow curves was carried out using OriginPro 8.

Results and Discussion

The histological analysis of leaves (Fig.1.) in three *Lactuca* species showed morphological and morphometrical differences. Only in *L. tatarica* the leaf is ecvifacial, whereas in *L. serriola* and *L quercina* it is bifacial. *L. tatarica* and *L. quercina* are characterized by almost the same average thickness of the assimilation parenchyma, whereas in *L. quercina*, growing in shadow and a semi-shade oak and beech forests the thickness is approximately two times smaller.

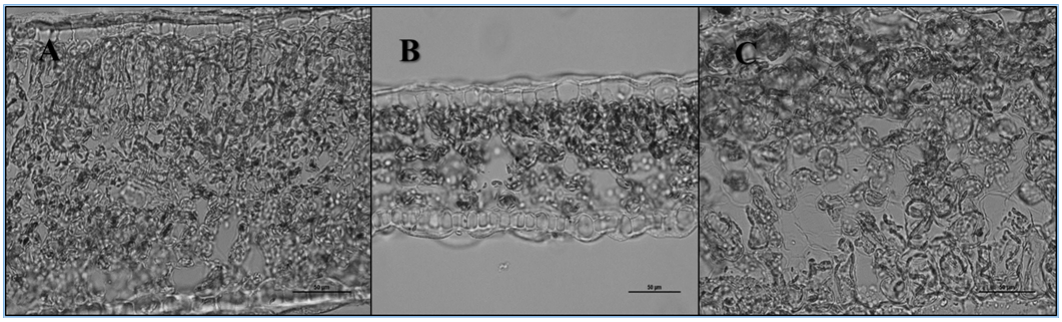

Fig.1. Comparative leaf anatomy in cross-sections of leaves in *Lactuca serriola* (A), *Lactuca quercina* (B), and *Lactuca tatarica* (C). Scale bar = 50 µm.

There are diffrences in the pigment content of the *Lactuca* species. It was shown that in leaves of glycophytes *Lactuca serriola* and *Lactuca quercina* the content of chlorophylls a (Chl *a*) and b (Chl *b*), and carotenoids (Car) was higher than that of the halophyte *Lactuca tatarica*.

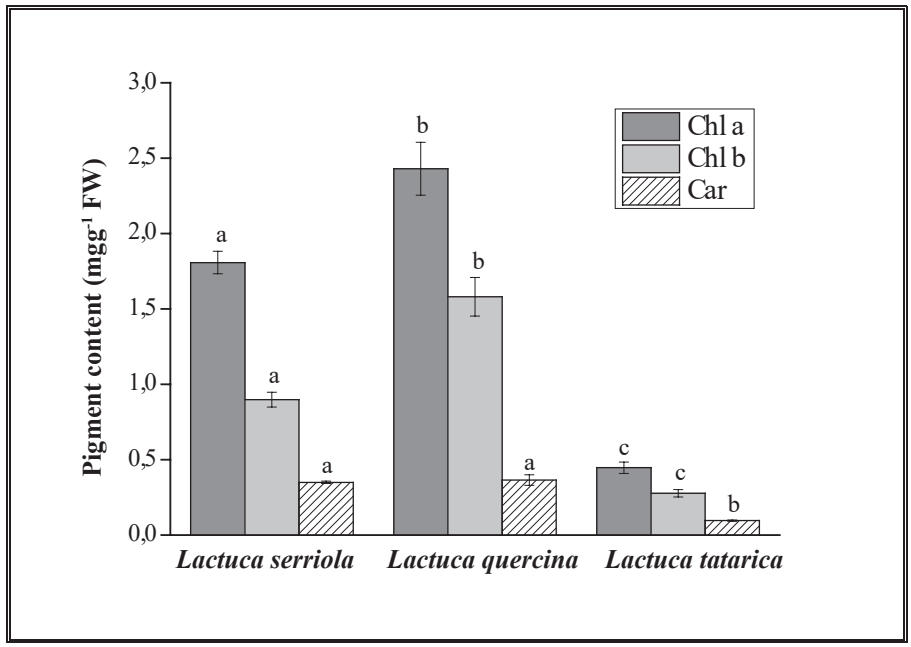

Fig. 2. Pigment content in leaves of *Lactuca* species

Thermoluminescence was used as a probe of the behaviour of PSII reaction centres, both in isolated chloroplasts and in whole leaves. Typical TL signals from the leaves are presented on Fig. 3. Upon illumination of leaf samples by single turn-over flashes at around 3°C, the leaves from the investigated species showed complex TL glow curves with different overall intensities.

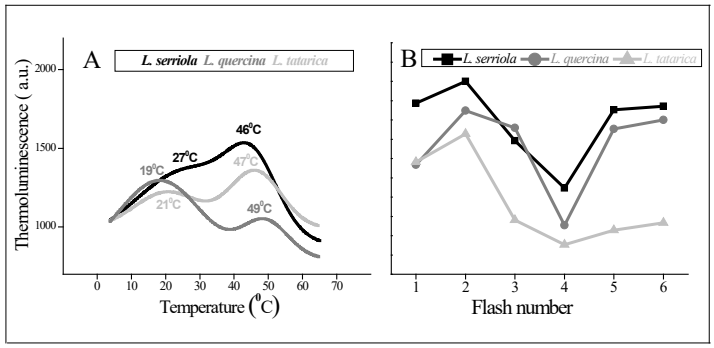

Fig. 3. Thermoluminescence of *Lactuca* sp. leaves after charging with two single turn-over flashes (A) and TL B-band oscillation pattern (B)

Decomposition analysis of the curves charged with two flashes showed the existence of two main TL components: TL B-band (from $S_2Q_B^-$ charge recombination) with T_{max} at around 19-27°C, reaching minimal values in *L. quercina* and AG-band at 46-49°C, reaching maximal value at the *L. tatarica*.

When leaves were illuminated by a flash series, the intensity of TL B-band exhibited a typical for active PSII period four-oscillation pattern with maximum on the second flash (Fig.3-B). TL glow curves and oscillation pattern of isolated chloroplasts followed particularly well the TL parameters recorded with intact leaves (Fig.4).

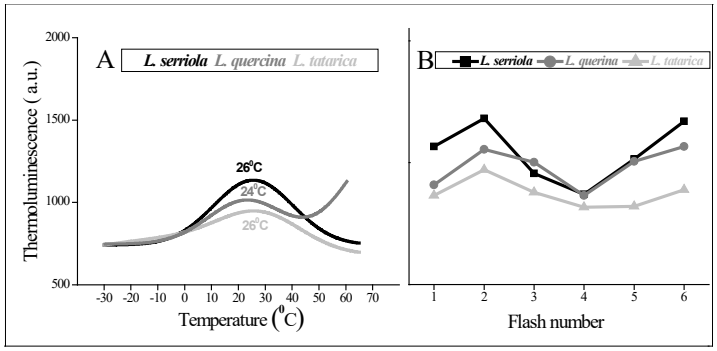

Fig. 4. Thermoluminescence curves of isolated chloroplasts of *Lactuca* sp. after charging with two single turn-over flashes (A) and TL B-band oscillation pattern (B)

Another reliable approach used to study the properties of PSII complex in *Lactuca* species was analysis of the kinetics of oxygen-evolving reactions under continuous excitation of isolated chloroplasts [11]. The induction curves after oxygen burst exhibit biphasic exponential decay. Kinetic parameters are given in Fig.5. These results suggest a decrease in the proportion of functionally active PSIIα centers in thylakoids in halophytic *L. tatarica* which could be attributed to the reduced grana formation and dominant operation of the cooperative mechanism of oxygen evolution in stroma situated PSIIβ centers.

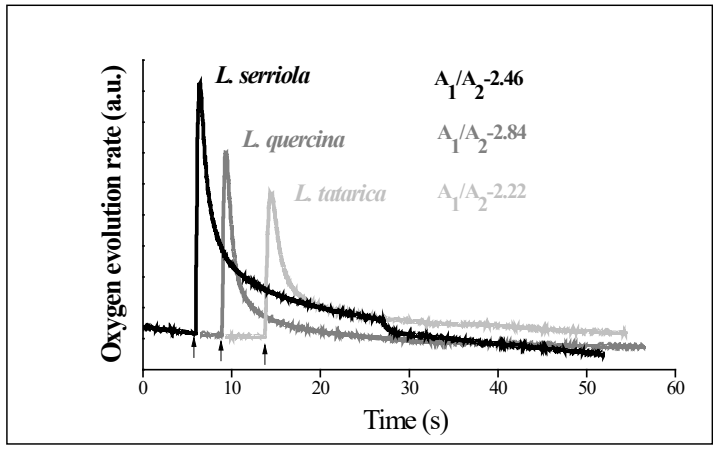

Fig. 5. Induction curves of oxygen evolution rate recorded after irradiation of *Lactuca* species thylakoids (chlorophyll concentration of 300 µg/ml) with continuous white light (450 µmol photons m^{-2} s^{-1})

The observed species variations in the kinetics of the initial oxygen burst and TL glow curve parameters might be a result of conformational changes of PSII due to some modification of membrane lipid composition.

Table 1. Fatty acid composition in thylakoid membranes from *Lactuca* species

	Fatty acids (wt% of total)								
	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C18:2/C18:3
Lactuca serriola	1.25	0.66	8.63	3.13	0.68	1.09	5.09	79.45	0.06
Lactuca quercina	3.83	0.95	15.04	3.45	1.50	2.09	10.05	63.08	0.16
Lactuca tatarica	2.57	2.05	16.13	1.70	1.36	1.20	7.16	67.82	0.11

Differences in fatty acid composition may be species-specific or due to varied environmental conditions. The plants respond to changes in the major environmental factors through lipid restructuring in the membranes, as well as changes in their fatty acid composition. The presence of a large amount of saturated fatty acids in the thylakoid membranes from *L. tatarica* leads to a reduction in membrane fluidity, which is an indicator of better resistance to increased soil salinity. The content of polyunsaturated fatty acids in the membrane lipids is one of the major factors determining the increase in their fluidity (permeability), until the maintenance in optimal degrees affects the resistance of photosynthetic apparatus to environmental stress. The amount of unsaturated fatty acids is consistent with the oxygen-evolution activity of studied species. We can assume that the unsaturated fatty acid of the membrane lipids could directly protect the oxygen-evolving device from stress-induced inactivation. This was most clearly observed in *L. serriola*.

The results of this study show specific structural and functional characteristics of photosynthetic membranes in halophytic and glycophytic *Lactuca* species, reflecting different adaptive strategies of the studied species to environmental conditions in their natural habitats.

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ADAPTIVE CHANGES IN THE STRUCTURE AND THE FUNCTIONAL PERFORMANCE OF THE PHOTOSYNTHETIC APPARATUS OF THE MEDICINAL PLANT *PETASITES HYBRIDUS* L. FROM DIFFERENT HABITATS

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Abstract

The aim was to investigate the influence of environmental conditions in different habitats of medicinal plant *Petasites hybridus* on structural organisation and functional characteristics of photosynthetic apparatus.

Materials and Methods: Leaves were collected from different florogeographical regions in Bulgaria. Light microscopy was carried out by Nikon Eclipse 50i (Tokyo, Japan). The pigment content was determined spectrophotometrically. The analyses of total lipophilic extracts were performed using thin-layer and gas chromatographic techniques. Specific changes in PSII photochemistry were assessed by alterations in thermoluminescence (TL) emission parameters.

Results: Histological analysis showed specific anatomical characteristics of the leaves from different locations. TL glow curves and oscillation pattern of isolated chloroplasts followed particularly well the TL parameters recorded with intact leaves. Presence of a substantial quantity of very-long-chain fatty acids was observed.

Conclusion: The comparative studies have shown that specific environmental conditions in different habitats of *Petasites hybridus* affect the structural-functional characteristics of photosynthetic apparatus.

Keywords: *Petasites hybridus* L., histological analysis, fatty acids, photosynthetic activity, thermoluminescence

Introduction

Petasites taxa have a broad distribution in the Northern hemisphere. In Europe, there eight native species are growing and two (*P. japonicus* and *P. fragrans*) have been introduced [1]. The most common species in Bulgaria is *Petasites hybridus* (L.) P. Gaertn., B. Mey. & Scherb. (Asteraceae), common butterbur, which is widespread to 1800 m above mean sea level on moist habitats along the banks of streams, ditches, ravines and rivers. The leaves are large with a diameter of 40–70 cm. It is a traditional herb in European phytotherapy. Extracts obtained from leaves and roots are in therapeutic use for more than 2000 years.

Petasites is a medicinal plant with a long history of use in respiratory, gastrointestinal and urogenital diseases, and it is also used for the treatment of hay fever and migraine [2].

To date, most of the drugs originate from *in situ* collections. Medicinal plants, containing useful secondary metabolites, have been reported to be under different abiotic stresses. Due to their sessile life style plant organisms are able to survive only by their ability to form fast and highly adaptive responses to constantly changing environment. Photosynthesis is a fundamental physiological process determing plant productivity. In this research we focused on the structure and functional performance of photosynthetic apparatus of *P. hybridus* plants from habitats in different florogeographical regions with various environmental conditions. This species is a hygrophyte and therefore drought intolerant. Although plants rarely encounter severe droughts in their native habitats, they can encounter temporal enhancement of drought stress due to increasing temperatures, decreasing precipitation, or both. Changes in water availability can reduce their stomatal conductance, making them susceptible to photo damage under conditions of excess light.

The leaf is the most adaptable organ in its response to impact of environmental factors [3]. For that reason we examined the leaf anatomy of *P. hybridus* from different habitats with specific environmental conditions. The effects of various stress conditions first appeared in changes in the structure of thylakoid membranes and photochemical efficiency of photosystems, especially of PSII. Both biotic and abiotic stresses alter the photosynthetic characteristics and metabolic status of photoautotrophs, which may be reflected in specific modifications of PSII [4]. During our studies of *Petasites hybridus*, using a highly sensitive TL technique, we observed some peculiarities of PSII redox reactions that can indicate specific adaptive characteristics of the photosynthetic system in dependence of the growth conditions. The physical properties of membranes are largely determined by chain length, polarity, and the degree of unsaturation of fatty acids that comprise their lipids. The fact that each membrane in the cell consists of a characteristic set of lipid classes and that each class has a distinct fatty acyl composition suggests that the lipid structure/composition is important for membrane function [5]. We examined the fatty acid composition of total lipophilic extracts from isolated chloroplast membranes of plants at various habitats.

Materials and Methods

Plant material and habitats: Leaves of *Petasites hybridus* were collected after the flowering period in June 2016 from plants growing in partial shade in various habitats in Bulgaria:

- Devil's bridge region - DB (in the vicinity of the village of Kokalyane, altitude 650 m);
- Saints Constantine and Helena - SC&H (Botanical garden in Saints Constantine and Helena, 10 km northeast of the center of Varna, altitude 80 m);
- Rila Monastery Nature Park – RMNP (2 km northeast of the Rila Monastery, altitude 1200 m).

The habitats were selected in different florogeographical regions: Vitosha region, Black sea cost and Rila, respectively. The leaves were maintained in a hydrated condition by being wrapped in wet papers at room temperature in the dark.

Histological analysis: For light microscopy, segments from the middle part of fully expanded leaves were fixed in 3% (m/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 h at 4°C and used for anatomical studies. Handmade transversal sections were mounted on slides in glycerol. Observations were carried out by using a light microscope Nikon Eclipse 50i (Tokyo, Japan).

Photosynthetic pigment determination: The pigment content of the leaves was determined as described in [6]. Spectra were recorded using a spectrophotometer UNICO 1100.

Isolation of broken chloroplasts (thylakoids): Petasites leaves (20 g) were homogenized by homogenizer type MPW-302 in cold 160 ml isolation buffer (pH 7.8) containing 0.4 M sorbitol, 50 mM tricine, 10 mM NaCl and 5 mM $MgCl_2$. The homogenated chloroplasts were filtered through nylon mesh and centrifugated at 3000 rpm for 5 min at 4°C. After removal of the supernatant solution, pellets were resuspended in the isolation buffer (pH 6.5) containing 0.3 M sorbitol, 40 mM MES, 10 mM NaCl and 5 mM $MgCl_2$. The content of chlorophylls in chloroplast suspensions was determined spectrophotometrically by measurements of A_{663} and A_{647} on 80% (v/v) acetone extracts [6]. The final chlorophyll concentration for the TL experiments was 1 $mg.ml^{-1}$.

Thermoluminescence (TL) measurements: TL measurements were carried out in darkness using computerized equipment, described in detail in [7]. In the experiments were used:

- discs (d =10 mm) from the middle part of fully expanded leaves with equal size and appearance in order to obtain reproducible results;
- 100 µl thylakoid suspension on filter paper discs.

After dark incubation the leaf samples were cooled to 3°C, illuminated with single turn-over flashes (1F to 6F) and warmed up to 65°C at a heating rate of $0.6°C.s^{-1}$. The samples of thylakoids were frozen after the illumination to -60°C and then warmed up. Decomposition analysis of TL glow curves was carried out using OriginPro 8.

Fatty acid analyses: The fatty acid composition was determined as methyl esters of the fatty acids as described in [8] with some modifications.

Results and Discussion

P. hybridus leaf is bifacial, hypostomatic, with well-structured adaxial and abaxial surfaces. The environmental conditions affect the thickness of assimilation parenchyma and the morphological characteristic of cells. The mean thickness of palisade parenchyma (36.08 ± 6.07 µm) is about two times smaller in SC&H leaves in comparison to the leaves, collected from DB (74.21 ± 1.38 µm) and RMNP (88.98 ± 7.38 µm) (Fig. 1). The shape of the photosynthetic cells is greatly rounded in SC&H leaves unlike in leaves from DB and RMNP.

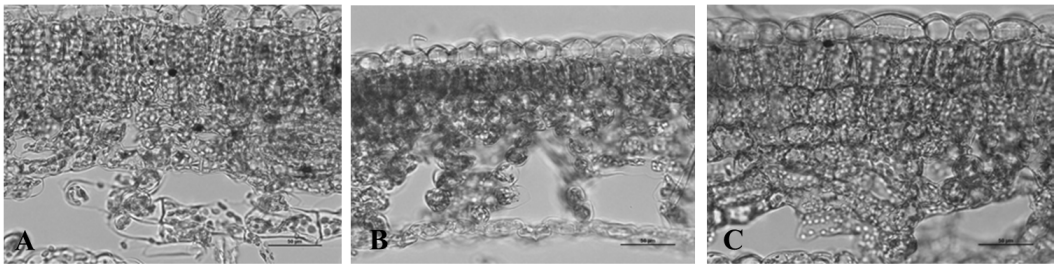

Fig. 1. Light microscope micrograph cross-sections of *Petasites hybridus* leaves from Devil's bridge region (A), Saints Constantine and Helena (B) and Rila Monastery Nature Park (C). Bar = 50 µm.

The total assimilatory pigment quantity, their partial quantity and the ratio between them give important information about the structural integrity and physiological condition of the photosynthetic system. SC&H leaves have high values of total chlorophyll (chl) content and the quantity of chlorophyll *b* (Fig. 2) is higher in comparison to the plants from the other regions. The pigment ratio for chl *a/b* is 2.37. It doesn't differ in DB leaves and RMNP leaves and amounted to 2.7. In comparison with plants developed under intensive illuminated conditions the total chlorophyll quantity and the quantity of chlorophyll *b* is higher in those plants grown under conditions with lower illumination [9].

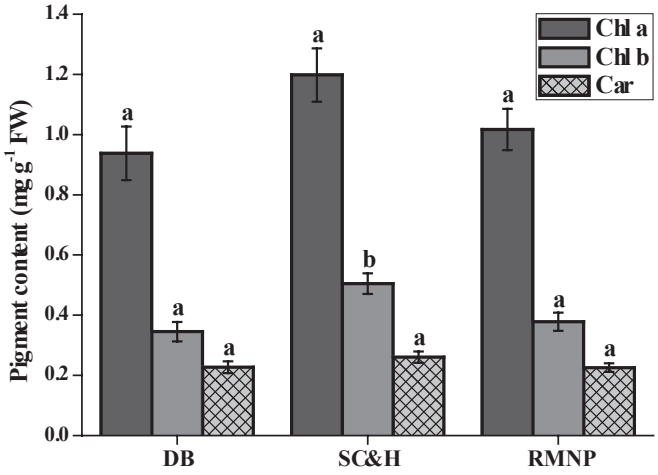

Fig. 2. Chlorophyll (Chl *a*, Chl *b*) and carotenoid (Car) content of leaves of *Petasites hybridus* plants growing at Devil's bridge region (DB), Saints Constantine and Helena (SC&H) and Rila Monastery Nature Park (RMNP). Different letters indicate statistically different means based on Tukey's test with P < 0.05

TL glow curve parameters were used to access the functional features of PSII. Thermoluminescence proved to be a very sensitive biophysical method for investigation of the functioning of PSII donor and acceptor side components. Upon illumination of leaf

samples by two flashes at around 3°C, the leaves from the investigated locations showed complex TL glow curves with different overall intensities and existence of two main TL components: TL B-band (from $S_2Q_B^-$ charge recombination) with T_{max} at around 28.5-31°C, reaching minimal values in SC&H location and AG-band at 46-49°C, reaching maximal value at the same location (Fig. 3A). When leaves were illuminated by a flash series, the intensity of TL B-band exhibited a typical oscillation pattern for active PSII (Fig. 3B). TL glow curves (Fig. 3C) and oscillation pattern (Fig. 3D) of isolated chloroplasts followed particularly well the TL parameters recorded with intact leaves. TL B-band in isolated chloroplasts from DB has T_{max} at around 27°C and 22°C at the other habitats.

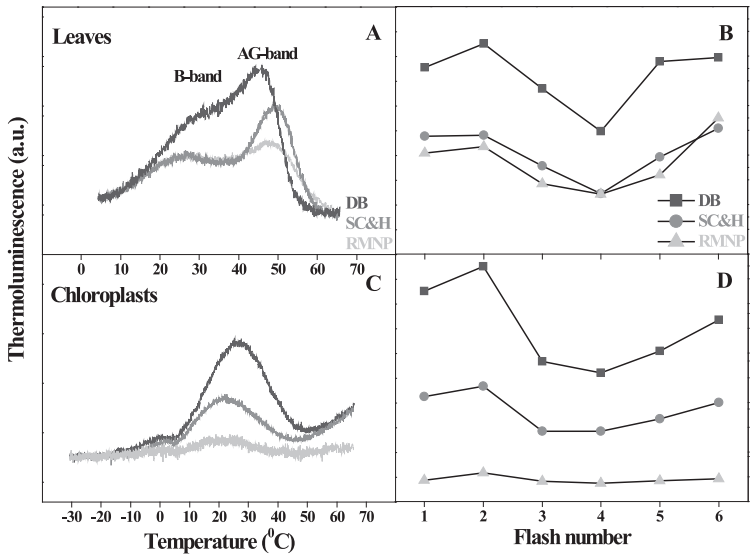

Fig. 3. Tl glow curves recorded after two flashes (A, C) and oscillations of B-bands intensity as a function of flash number (B, D) from *Petasites hybridus* plants growing at Devil's bridge region (DB), Saints Constantine and Helena (SC&H) and Rila Monastery Nature Park (RMNP)

TL B- and AG- bands in intact unfrozen leaves are related to dark distribution of the S_2 and S_3-states the of oxygen evolving complex and Q_B/Q_B^- ratio. It is generally accepted that the amplitude of TL B-band is proportional to the number of centres having $S_{2(3)}QB^-$ charge pairs after flash illumination, while the maximal emission temperature of this band is a measure for the redox span between the separate charges [10]. The AG-band is related to photosystem II centres initially in the $S_{2(3)}Q_B$ oxidation state, in which the electron acceptor Q_B becomes reduced either by reverse electron flow or reduction of the plastoquinone pool via an NAD(P)H plastoquinone oxidoreductase and is thought to be an indicator of the metabolic state of the leaf [11]. The cycling of the PSII charge pairs was typical for higher plants, thus suggesting no peculiarities in this parameter of *P. hybridus* from different habitats. The decline at T_{max} in chloroplasts can be explained by the removal of certain

protective substances from the stroma in the isolation procedure. In addition to the observed differences in T_{max} of the main TL B-band, the TL glow curve intensities were significantly different in dependence on the growing conditions. All this implies that the number of centers and the charge stability were lower in SC&H and RMNP samples in comparison to DB location.

Six common plant fatty acids (C16 and C18) are detected in thylakoids of investigated plants, including palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) (Table 1). The last one accounts for 58–61.5% of the total fatty acids in isolated thylakoid membranes from different habitats, followed by palmitic and linoleic acids. The content of palmitoleic acid is very low. It has been proved that polyunsaturated fatty acids in thylakoid lipids play important roles in stability of oxygen-evolving machinery and acclimation of the photosynthetic apparatus to fluctuations in environmental factors, and various forms of environmental stresses [12].

Table 1. Fatty acid profiles of chloroplasts isolated from leaves of *Petasites hybridus* plants growing at Devil's bridge region (DB), Saints Constantine and Helena (SC&H) and Rila Monastery Nature Park (RMNP)

Habitats	Fatty acids (% of total)											
	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	22:5	22:6
DB	3.46	0.43	6.69	0.53	0.61	0.78	5.61	61.52	3.61	1.44	2.48	11.22
SC&H	4.87	0.65	8.41	0.09	1.09	1.83	7.08	58.73	2.70	1.76	1.47	11.31
RMNP	4.24	1.58	11.20	0.95	1.16	1.94	6.26	60.41	2.23	1.57	1.51	5.36

Also, there is a presence of unusual fatty acids like lauric acid (12:0). Presumably, the accumulation of unusual fatty acids in membrane lipids would perturb the integrity of the bilayer [5].

In this study, we report on a substantial quantity of very-long-chain fatty acids (VLCFAs), with the docosahexaenoic acid (22:6) acid predominating. It is an omega-3 fatty acid and a primary structural component of the human brain, cerebral cortex, skin, and retina. However, the VLCFAs are uncommon in higher plants [13]. The biological meaning of their presence and levels in butterbur thylakoid membranes remains a question for further research. Although, the high contents may represent a key physiological characteristic that contributes to the considerable biotic and abiotic stress tolerance. Except for drought stress [14], no data can currently explain clearly the role of VLCFAs in certain specific abiotic contexts (like cold stress, mechanical injury and others).

Conclusions

Our comparative studies have shown that specific environmental conditions in different habitats of *Petasites hybridus* affect the structural-functional characteristics of photosynthetic apparatus. The plant response to environmental factors is complex because it reflects over space and time the integration of stress effects and responses at all underlying

levels of organization [15]. Under field conditions these responses can be synergistically or antagonistically modified by the superimposition of influences.

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ADAPTIVE POTENTIAL OF TWO *PHASEOLUS VULGARIS* L. GENOTYPES TO SINGLE AND COMBINED PEG AND UV-B TREATMENTS

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Abstract

Aim: to compare adaptive potential of two genetically closely related genotypes of *Phaseolus vulgaris* L. to single and combined polyethylene glycol (PEG) and UV-B treatments.

Materials and Methods: *P. vulgaris* L. genotypes were studied. Seed germination was performed in growth chamber at standard conditions for 3-7 days. Seedlings were grown to the cotyledon phase. Plants were separated into eight groups –controls, single treated with 16% PEG and UV-B - 100 $J.m^{-2}$, 250 $J.m^{-2}$; 500 $J.m^{-2}$; combined treated - 16% PEG+100 $J.m^{-2}$, 16% PEG+250 $J.m^{-2}$, 16% PEG+500 $J.m^{-2}$. PEG, Alfa Aesar, Germany, cat. № B21955; MW 10 000 was used for 24 h to induce osmotic stress in plants. At 12 h of the PEG treatment, cotyledons were irradiated with UV-B. After that cotyledons were kept for 12 h in PEG and grown to the first leaf phase. SOD and CAT were chosen as endpoints.

Results: Genotypes dependent response was found ten days after the removal of stress stimuli. The genotype Dobrudjanski 7 responded with 2.5-3 folds increased SOD levels ten days after the removal of single UV-B irradiation with 250 $J.m^{-2}$ and 500 $J.m^{-2}$. The most profound increase of SOD activity was found after the combined treatment with 16% PEG +100 $J.m^{-2}$ for Dobrudjanski 7. Higher levels of SOD (about 5-fold) were found after 250 $J.m^{-2}$ and after 16% PEG+500 $J.m^{-2}$ treatments for Dobrudjanski ran. Ten days after the removal of stress stimuli CAT activity in all Dobrudjanski 7 samples were not statistically proven. Interestingly, higher levels of CAT were measured after single treatments with PEG (about 2, 5 fold) and UV-B (about 2-3, 5 fold) and after combined 16% PEG+500 $J.m^{-2}$ (about 4, 5 fold) treatment for Dobrudjanski ran, ten days after stress removal.

Conclusion: Our finding illustrates that UV-B seems to be more stressful environmental factor than drought for the genotypes studied by us. Genotypes Dobrudjanski 7 and Dobrudjanski ran differ in terms of their adaptive potential. Dobrudjanski 7 can maintain elevated levels of SOD ten days after the removal of single and combined stress that could provide better adaptation of Dobrudjanski 7 comparing with Dobrudjanski ran. Here we would not ignore the statement concerning complexity of the tolerance mechanisms in plants and especially when stresses are in a combination. In our research, the role of SOD for plant adaptation to drought and UV-B irradiation seems to be a more determinant but that would be the subject of future research. We have not obtained clear evidence that pre-treatment with PEG that simulates drought may reduce the damaging effect of UV-B for both genotypes. Our experimental data demonstrated that pre-treatment with PEG probably increases the protective potential of Dobrudjanski 7 - significantly higher values of SOD were measured in combined samples.

Keywords: *Phaseolus vulgaris* L., polyethylene glycol, UV-B

Introduction

Recently, increased levels of ultraviolet-B (UV-B) irradiation and drought have received much more attention because of their potential to damage many economically important

plant species affecting their growth and metabolic processes [1; 2; 3]. Drought as one of the main environmental stress, results to series of morphological, physiological, biochemical and molecular changes in plants that can adversely affect growth, productivity and commonly can constitute serious threats to agriculture [4; 5]. In the recent years, gradual change in environment such as enhanced UV-B radiation on the Earth's surface as a result of ozone depletion has become an unavoidable fact and thus, causing real threat to the existing organisms.

Plants grown under field conditions are usually undergoing more than one stress at the same time. In literature studies are available indicating that plants response to the combination of two different abiotic stresses (drought and UV-B irradiation) is unique and cannot be extrapolated from plants response to the every single stress [6; 7; 8; 9]. The research concerning the relationship between UV-B and drought led to contradictory results. Both drought stress and UV-B irradiation could cause the accumulation of reactive oxygen species (ROS) and thus result in oxidative damage [10]. ROS are highly reactive and in the absence of effective protective mechanism/s, they can compromise normal metabolism through oxidative damage to pigments, lipids, proteins and nucleic acids [10; 11; 12].

During the evolution plants developed different strategies to cope with environmental stress such as: osmolyte accumulation, antioxidant system, absorbing compounds (flavonoids) and etc.

To use genotypes that differ in their resistance to oxidative stress is a commonly used strategy in genotoxicology and plant's adaptation to oxidative stress [13; 14; 15; 16]. Here we hypothetically presumed that using genetically closely related genotypes it could be possible to identify the most sensitive and reliable marker/s for the evaluation of genotype's adaptive potential evaluated ten days after the removal of stress. To check our hypothesis two biochemical markers were used: SOD and CAT activities.

As object of this study *Phaseolus vulgaris* L. (common bean) genotypes (Dobrudjanski 7 and Dobrudjanski ran) were used. Common bean is a very important, agro - economic widespread food legume, cultivated in the Balkan Peninsula and Bulgaria and convenient model in environmental mutagenesis. Based on molecular analyses it was previously found by us that these genotypes are genetically closely related [17; 18].

These markers were chosen because of their important role. CAT and SOD are one of the key enzymes of the antioxidant defense system. The SOD and CAT activities are also associated with UV-B exposure and other stresses such as drought. These enzymes act as antioxidant compounds reducing photo oxidative damage in plant leaves. SOD accelerates the conversion of superoxide to H_2O_2 and O_2, while CAT catalyse H_2O_2 breakdown [6; 19; 20; 21].

Aim: to compare adaptive potential of two genetically closely related genotypes of *Phaseolus vulgaris* L. to single and combined polyethylene glycol (PEG) and UV-B treatments.

Materials and Methods

Genotypes: the *Phaseolus vulgaris* L. genotypes (Dobrudjanski 7 and Dobrudjanski ran) were kindly provided by Prof. Genchev from the Dobrudja Agricultural Institute.

Seed germination was performed in growth chamber (GC 400) at standard conditions (t=23±0.2°C, 70% humidity, in continuously light) for 3-7 days. Seedlings were grown to the cotyledon phase.

Treatment with Polyethylene glycol (PEG): Based on our preliminary data 16% PEG (Alfa Aesar, Germany, cat. № B21955; MW 10 000) was used for 24h to induce osmotic stress in plants.

UV-B irradiation was performed in BLX UV Crosslinker (λ=302 nm).

Experimental design: Plants were separated into eight groups –controls, single treatment with 16% PEG and UV-B - 100 $J.m^{-2}$, 250 $J.m^{-2}$, 500 $J.m^{-2}$; and combined treatment - 16% PEG+100 $J.m^{-2}$, 16% PEG+250 $J.m^{-2}$, 16% PEG+500 $J.m^{-2}$. At 12 h of the PEG treatment, cotyledons were irradiated with UV-B. After that cotyledons were kept for 12 h in PEG. Then plants were grown at standard conditions to the first leaf phase.

SOD [22] and CAT [23] were chosen as endpoints.

Statistical Analysis of Data: Experiments were repeated at least three times. Data points in figures are mean values. The bars on figures show mean values calculated as percent of the control. Error bars represent standard errors of mean values. Where no error bars are evident, the errors were equal to or less than the symbols. One-way ANOVA with Tukey multiple comparison tests (GraphPad Prism 6.04 software, San Diego, USA) was used to assess differences among samples.

Results and Discussion

Today, it is believed that by improving our understanding on the antioxidant mechanisms developed in plants, it could be possible to improve the present state of knowledge concerning the mechanisms of plant adaptation to oxidative stress. During the evolution a lot of enzymatic and non-enzyamtic antioxidants have been developed in plants to scavange ROS and to restore the redox homeostasis of the cells. Many contradictory results about the antioxidant enzyme response to different type of stress have emerged due to the fact that stress response depends on the plant species, the developmental stage, plant organs, duration and severity of stress [7; 24].

SOD activity: SODs are located in the main cell compartments and play very important defence role against ROS due to SODs function to catalyze the dismutation of O_2^- to H_2O_2 which can then be converted to H_2O by ascorbate peroxidase (APX, EC 1.11.1.11), total peroxidases (POD) and catalase (CAT, EC 1.11.1.6) enzymes. The increase in SOD activity is frequently observed under stressful conditions [25; 26; 27]. The maintenance of high antioxidant capacity to remove toxic levels of ROS has been related to increased stress tolerance of crop plants [28].

Our data show increased SOD activities even ten days after the removal of single UV-B irradiation and combined treatment for the first studied genotype Dobrudjanski 7 (Fig. 1).

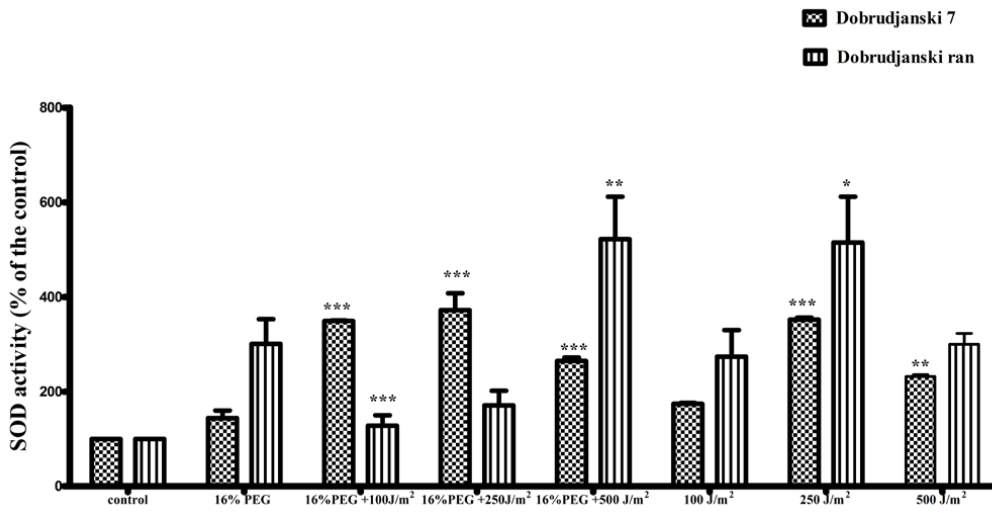

Fig. 1. SOD content (% of the control) depending on single and combined treatment with 16% PEG and UV-B irradiation in genotypes Dobrudjanski 7 and Dobrudjanski ran. Data presented are mean values ± SEM from at least three independent experiments. 100%-Control. For genotype Dobrudjanski 7, One-way ANOVA analysis reveals statistically significant differences between: 250 J.m^{-2} treatment vs control; 16% PEG+100 J.m^{-2} treatment vs control; 16% PEG+250 J.m^{-2} treatment vs control; 16% PEG+500 J.m^{-2} treatment vs control (***, $p < 0.001$); 500 J.m^{-2} treatment vs control (**, $p<0.01$). No statistically significant differences between: 16% PEG treatment vs. control; 100 J.m^{-2} treatment vs control (ns, $p>0.5$). Statistically significant differences were found between: 250 J.m^{-2} treatment vs control (*, $p<0.1$); 16% PEG+500 J.m^{-2} treatment vs. control (**, $p < 0.01$) for genotype Dobrudjanski ran. No statistically significant differences between: 16% PEG treatment vs. control; 100 J.m^{-2} treatment vs control; 500 J.m^{-2} treatment vs. control; 16% PEG+100 J.m^{-2} treatment vs. control; 16% PEG+250 J.m^{-2} treatment vs. control (ns, $p>0.5$). Where no error bars are evident, errors are equal or smaller than the symbols.

Single treatments (with 16% PEG and with UV-B irradiation): Looking at the data presented in Fig. 1, it could be seen that the SOD activities measured ten days after the removal of a single PEG treatment are very similar and not statistically different comparing with those in both controls ($p>0.5$). How to explain this finding? It could be due to the middle magnitude of drought stress induced by 16%PEG and ten days after the removal of stress factor both genotypes Dobrudjanski 7 and Dobrudjanski ran have overcome the effects of the PEG treatment (differences with controls measured ten days after the removal of single PEG treatment are not statistically proven ($p>0.5$). The question is still open.

The importance of the experimental design is demonstrated comparing our data with those of other authors where SOD activity was higher than that in the control 5 days after the removal of 20 days of drought [8]. How to explain such disagreement? At least two main reasons could be pointed out: different experimental designs and different species applied in these experiments.

As it is seen in Fig. 1 the SOD values were statistically significant higher than that in the controls depending on the genotype 10 days after the removal of UV-B irradiation. No

well expressed dose dependent effect was obtained for both genotypes. The response of Dobrudjanski 7 was the most pronounced.

Given that SOD activity depends on the experimental scheme, genotype, treatment time, time after the treatment etc. [9] our results are in a support of these statements – SOD induced by UV-B are likely to be species /genotype specific and dependent on the experimental design.

Enhanced SOD activity and other enzyme activities were reported after UV-B irradiation [26; 29]. The role of species and experimental design was shown again. Dose dependent enhancement of SOD activity was obtained in two *Vigna* species 24 h after UV-B irradiation [9]. Close to the control level of SOD activity was measured two days after nine days of stress in cucumber leaves [7].

Further we have tried to analyze how stressful were drought and UV-B irradiation for both genotypes. Statistically significant differences between SOD activities were calculated: 16% PEG vs. 250 $J.m^{-2}$ (208%; $p<0.05$) and 16% PEG vs. 500 $J.m^{-2}$ (88%; $p<0, 05$) for genotype Dobrujanski 7. No statistically significant differences for genotype Dobrudjanski ran were determined ($p>0.5$). Our finding illustrates well that genotypes respond differently to both types of stress and that UV-B seems to be more stressful than drought. Contradictory to our results are those described by Kubis and Zając [7]. This team found that the elevation of enzyme activities was higher under drought than under UV-B radiation two days after the removal of nine days of stress. Here the role of the experimental design and the magnitude of drought stress could be discussed.

It could be suggested that in our experimental conditions, genotype Dobrudjanski 7 could maintain enhanced SOD levels 10 days after the removal of stress and could be related to the genotypes with good stress tolerance to UV-B radiation. In this case we agree with the statement that due to the great complexity of the antioxidant system and plant stress tolerance, increased SOD levels are not the only factor that determines the level of tolerance, because other factors and several genes pathways are involved in the stress tolerance in plants [28].

Combined treatment (16% PEG + three doses UV-B irradiation): Based on this parameter some differences were obtained between the studied genotypes ten days after the removal of the combined treatment. Analysing the combined treatment data, it becomes clear that pre-treatment with PEG increases the protective potential of Dobrudjanski 7. Significantly higher values of SOD were measured - 349% for sample 16% PEG+100 $J.m^{-2}$; 372% for sample 16% PEG+250 $J.m^{-2}$; 265% for sample 16% PEG+500 $J.m^{-2}$ in comparison with that in untreated control. Interestingly comparing single PEG with combined treatment statistically significant differences were found between: 16% PEG vs. 16% PEG +100 $J.m^{-2}$ (205%; $p< 0,0 5$); 16% PEG vs. 16% PEG +250 $J.m^{-2}$ (228%; $p<0,05$); 16% PEG vs. 16% PEG + 500 $J.m^{-2}$ (121%; $p<0,05$).

The only increased SOD level (about 5-fold) for genotype Dobrudjanski ran was the one measured after the removal of the combined 16% PEG+500 $J.m^{-2}$ treatment. Comparing the single PEG treatment with the combined treatment statistically significant difference was measured between 250 $J.m^{-2}$ vs. 16% PEG + 250 $J.m^{-2}$ (344%; $p<0, 05$). Kubis and Zając reported for higher SOD activity two days after nine days of combined treatment with UV-B and drought in cucumber leaves [7] where SOD activity was increased 2 days after the

removal of stress. The authors found no protective effect of drought - the elevation of enzyme activities was higher under drought than under the combined treatment two days after the removal of stress.

It can be assumed that elevated levels of SOD maintained ten days after the removal of combined stress could provide better adaptation of Dobrudjanski 7 comparing with Dobrudjanski ran. Here we would not ignore the statement written above concerning the complexity of the tolerance mechanisms in plants and especially when stresses are in a combination.

CAT activity: It is well known that catalases are responsible for the removal of H_2O_2 by reducing H_2O_2 to $2H_2O$ and CAT activity could increase under stress conditions [25; 26; 27]. Plants possess multiple CATs encoded by specific genes, which respond differentially to various stress factors known to generate ROS. Although CAT showed an up-regulation in UVB treatment, the activity was more discrete compared with the other antioxidant enzymes measured.

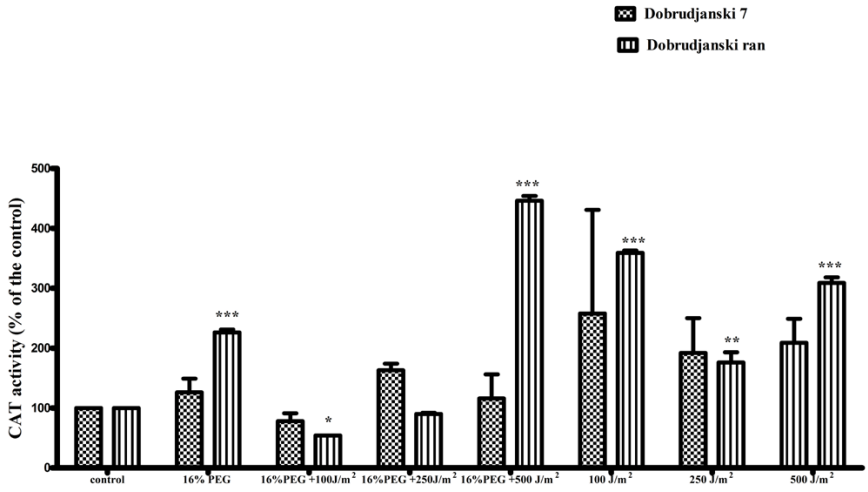

Fig. 2. CAT content (% of the control) depending on single and combined treatment with 16% PEG and UV-B irradiation in genotypes Dobrudjanski 7 and Dobrudjanski ran. Data presented are mean values ± SEM from at least three independent experiments. 100%-Control. One-way ANOVA analysis reveals no statistically significant differences for genotype Dobrudjanski 7, between: 16% PEG treatment vs. control; 100 $J.m^{-2}$ treatment vs. control; 250 $J.m^{-2}$ treatment vs. control; 500 $J.m^{-2}$ treatment vs. control; 16% PEG+100 $J.m^{-2}$ treatment vs. control; 16% PEG+250 $J.m^{-2}$ treatment vs. control; 16% PEG+500 $J.m^{-2}$ treatment vs. control (ns, $p>0.5$). For genotype Dobrudjanski ran One-way ANOVA analysis reveals statistically significant differences between: 16% PEG treatment vs. control; 100 $J.m^{-2}$ treatment vs. control; 500 $J.m^{-2}$ treatment vs. control; 16% PEG+500 $J.m^{-2}$ treatment vs. control (***, $p<0.001$); 250 $J.m^{-2}$ treatment vs. control (**, $p<0.01$); 16% PEG+100 $J.m^{-2}$ treatment vs. control (*, $p<0.1$). No statistically significant differences between: 16% PEG+250 $J.m^{-2}$ treatment vs. control (ns, $p>0.5$) for Dobrudjanski ran. Where no error bars are evident, errors are equal or smaller than the symbols.

Single treatments (with 16% PEG and with UV-B irradiation): Not so clear results in terms of CAT were obtained in our experiments. Data are available that various types of

environmental stress can cause either enhancement or depletion of CAT activity, depending on the species, intensity, duration, and type of the stress factor. It is well discussed in the review of [30], that stress factors capable to reduce the rate of protein turnover also reduce CAT activity. It is well known that at the cellular level, UV-B radiation inducing reactive oxygen species (ROS) can damage proteins, lipids, and other biomolecules.

Analyzing the CAT values presented in Fig. 2 it is obviously that genotypes differ substantially on this parameter measured on the 10th day after the removal of both stress factors. No statistically significant differences from the control were calculated for Dobrudjanski 7 depending of the experimental design ($p>0.5$).

The response of the other genotype Dobrudjanski ran is quite different. Well expressed enhancement of CAT levels was measured ten days after the removal of UV-B irradiation (2-3.5 fold) and about 2.5 fold after the removal of PEG induced drought stress. There is a lot of experimental evidence that UV-B can elevat CAT activity in irradiated plants [6; 31; 32]. What is different in our experiment? What needs to be addressed here is that Dobrudjanski ran has maintained these high levels of CAT for a long time, probably as a defense mechanism? The question is still open.

Comparing both single treatments statistically significant differences ($p<0.05$) were found between 16% PEG vs. 100 J.m^{-2} (133%); 16% PEG vs. 250 J.m^{-2} (50%) and 16% PEG vs. 500 J.m^{-2} (83%) for genotype Dobrudjanski ran. This finding gives us a reason to suppose that UV-B radiation is a stronger stress factor for Dobrudjanski ran than drought.

Combined treatment (16% PEG + three doses UV-B irradiation): Similar CAT activity was measured to that in the controls after the removal of all combined treatments for genotype Dobrudjanski 7 (Fig. 2). There were no statistically proven differences with control and combined treatments ($p>0.5$) after the removal of the stress for that genotype.

Results obtained for Dobrudjanski ran ten days after the removal of combined treatment show not statistically proven data and differences comparing with the control for most of the samples. The only well expressed enhancement was measured for combined treatment with 16% PEG+500 J.m^{-2}.

Data in the literature are very contradictory. Bassahi et al [31], found that UV-B and combined stress increased CAT activities after long-term stress conditions (measured just after stress removal) in lettuce. On the other hand combined application of both stress factors did not lead to any significant changes in the enzyme activities in comparison with the UV-B application [6] in pea and wheat. The data of [6] illustrated that UV-B stress exceeded the effect of drought in relation to the enzyme activities when applied together. Other authors have obtained an increased CAT activity after the removal of combined treatment in Vicia faba [33]. In our conditions, higher CAT activity was obtained for genotype Dobrudjanski ran ten days after single UV-B irradiation and in combined sample 16% PEG+500 J.m^{-2}.

Conclusion

Here an attempt was made to compare the adverse effects of drought and UV-B irradiation in two closely related genotypes of *Phaseolus vulgaris* and to throw some light whether these

genotypes would express similar/or different adaptive potential to single and combined PEG and UV-B treatment.

Our finding illustrates that UV-B seems to be a more stressful environmental factor than drought for the genotypes studied by us. Genotypes Dobrudjanski 7 and Dobrudjanski ran differ in terms of their adaptive potential. Dobrudjanski 7 can maintain elevated levels of SOD ten days after the removal of single and combined stress that could provide better adaptation of Dobrudjanski 7 comparing with Dobrudjanski ran. Here we would not ignore the statement concerning the complexity of the tolerance mechanisms in plants and especially when stresses are in a combination. In our research, the contribution of SOD for plant adaptation to drought and UV-B irradiation seems to be more determinant but that would be the subject of future research. We have not obtained clear evidence that pre-treatment with PEG (mimic drought) may reduce the damaging effect of UV-B for both genotypes. Our experimental data demonstrated that pre-treatment with PEG probably increases the protective potential of Dobrudjanski 7 - significantly higher values of SOD were measured in combined samples.

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ENHANCEMENT OF SEED GERMINATION AND GROWTH OF *ECHINACEA PURPUREA* (ASTERACEAE)

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Abstract

Aim: The present study deals with stimulation of seed germination of the medicinal plant *Echinacea purpurea* (purple coneflower) and enhancement of the seedlings growth, using gibberellic acid, potassium nitrate, and aeroponic system.

Materials and Methods: Mature seeds from the *ex situ* collection of IBER were either sown on a bench with soil in the greenhouse, or triple dipped consecutively in hot and ice-cold water, then soaked for 1 h in GA_3 (0.1% or 0.35%) or 0.2% KNO_3; control seeds in distilled water. Seed germination was tested in terrines, 30 shelled and 30 intact seeds per variant, or on aeroponic system, 30 shelled seeds per variant. All rosettes were transferred to the open field plot.

Results: Best germination rate and seed survival, 46% and 40% respectively, were noticed in the variant with intact seeds soaked in 0.35% GA_3. Seedlings growth was twice faster in the aeroponic system due to the fast root development. Rosettes were easily adapted to open field plot. All plants were in blossom in their second year.

Conclusion: First results on seed germination and growth enhancement were promising and could be further improved.

Keywords: Purple coneflower, Seed germination, Aeroponics, Medicinal plants

Introduction

Echinacea purpurea (L.) Moench is a perennial plant belonging to the family Asteraceae, commonly known as "purple coneflower" due to its vivid pink flowers. It is native to the mid-western region of North America and used for centuries as medicine by the Indians and the traditional herbalists, along with *E. angustifolia*, for topical applications and healthy immune system [1, 2]. Traditionally used as fresh juice or chewed on, also as a topical remedy for horses, and as herbal smudge, nowadays it is one of the most widely used medicinal plants, with increasing market demand. The roots, leaves, and flowers are used fresh or dried, as a tea or tinctures and extracts, or as powder in tablets, capsules, and ointments, for healing of many ailments, from common flu and colds, insect and snake bites to cancer treating. Herbal actions are immune supporting, depurative, vulnerary, lymphatic, etc.

E. purpurea was introduced in Europe in the 1900's, first as an ornamental plant, later on as medicinal herb, and currently it is cultivated in many European countries [3]. In Bulgaria, initial studies on cultivation of the species were conducted in the period 1985-1991 with seeds originating from Germany, in the Sofia field conditions [4]. Although the results were

promising, no large-scale plant cultivation was set up. According to the special assessment report of the European Medicines Agency, on *E. purpurea* herba recens, four products based on *E. purpurea* (herba succus recens) were authorized in Bulgaria: 3 for oral use (oral drops, in 1999 and in 2002, for supportive treatment of recurrent infections of respiratory and urinary tracts; syrup, in 2007, for supportive treatment of recurrent infections of respiratory tract), and 1 for topical use (ointment, traditionally used as a mild acting medicine for the promotion of wound healing, was registered in 1999) [5]. The monograph in the European Pharmacopoeia (2008) defines only the dry herbal substance "Purple coneflower herb"; the US Pharmacopeia (2004) – "*Echinacea purpurea* Aerial Parts"; and the German homoeopathic pharmacopoeia (2011) described fresh herbal drug in the monograph *Echinacea purpurea* [5]. The effective compounds of the species, listed in the report, belong to different groups of constituents: caffeic acid derivatives, alkamides, polysaccharides, melanins, lipopolysaccharides, and lipoproteins. However, *E. purpurea*, altogether with *E. angustifolium* and *E. pallida*, were listed as "plant species with roots or rhizomes commonly used in the medicinal and phytopharmaceutical industries", for their roots [6], so as traditional use and experimental research concern plant tops and/or plant roots [7, 8, 9].

The growth of the purple coneflower seedlings was reported to be slow, and the rhizomes needed long time to develop; intensive plant growth was noticed only from the third year, and the biomass accumulation depended strongly on the annual climatic fluctuations [4]. The new, totally controlled conditions offered by the hydroponic cultivation techniques, including aeroponic systems, are suitable for growing of high-quality raw materials for industrial use of medicinal plants, especially for species which are cultured for their roots and rhizomes [6, 10]. Presently, a few experiments have been reported on application of these techniques on species from genus *Echinacea* [7, 8, 9]. The present study was aiming at stimulation of seed germination of *Echinacea purpurea*, and enhancement of the seedlings growth.

Materials and Methods

Mature seeds from *E. purpurea* plants from the *ex situ* collection of the Institute of Biodiversity and Ecosystem Research (IBER) were stored in a cool chamber during the winter. Several hundreds of them were sown on a bench with soil in the greenhouse in October 2014 and their growth was observed periodically; in May 2015 seedlings were transferred to open field plot. Other seeds were treated by different means to stimulate their germination: seeds were triple dipped consecutively in hot and ice-cold water, then soaked for one hour in GA_3 (0.1% or 0.35%) or 0.2% KNO_3; control seeds were soaked in distilled water for the same time. Each variant consisted in 30 intact seeds and 30 seeds shelled before the treatment (2 repetitions 15 seeds per sub-variant). Seeds were put in terrines with soil mixture in a room phytotron. The numbers of the germinating and surviving seeds were counted after one month. In addition, seed germination was tested on vertical aeroponic system "Green Diamond" (General Hydroponics) with 120 shelled seeds using the same stimulating agents and a control variant (2 repetitions 15 seeds per variant). In this case seeds were put in organic cubes centered in plastic gridded pots, filled in with inert material (keramzit), one seed per pot. Water was scattered in fine drops through the nozzles of a vertical pipe inside the system, 15

min every 3 hours, simultaneously with the system rotation, thus ensuring regular moistening of the substrate. Water was supplemented with rooting agent Root It® first feed, 15 ml/L (General Hydroponics) at the end of the first week, at the beginning of the germination, and the nutrient solutions Flora gro®, Flora micro®, and Flora bloom® (General Hydroponics), were added 5 weeks later, in concentration 0.7 ml/L each. The solution was recycled in a 40 L plastic tank at the base of the system, and moved up by a pump. The pH was maintained between 5.4 and 6.5 while the electrical conductivity (EC) was in the range 1.8 ± 0.6 $dS \cdot m^{-1}$. The diurnal ambient conditions in the phytotron were: light regime of 18 h light / 6 h dark (Metal Halide lamp, 600 W, full spectrum), temperature 23±4°C, and relative air humidity between 32 and 52%. After 74 days on the aeroponic system, root length was measured, and plants were potted in soil mixture and left in the room phytotron (1 month), greenhouse (1 month), and finally acclimated to the open experimental field of IBER. Results were analyzed statistically using ANOVA single factor and ANOVA two-factor with replication.

Results

Seed germination in the terrines began in the second week and continued until the end of the third week. One-month aged seedlings reached height of about 2 to 6 cm and had one or more true leaves (Fig. 1). First germs emerged on the ninth day in the variants treated with gibberellic acid. In the control variant, the shelled seeds began to germinate three days before the intact ones. In all variants there were noticed germs dying immediately after their appearance or several days later, and the total percentage of the dropped out germs reached 36.7%; in the control they were a little bit less: 33.3%. The effect of the stimulating agents seemed to be toxic for the shelled seeds in the applied concentrations, causing necrosis of 66.7% of the germs in these variants versus 18.5% for the intact seeds in the same variants (Fig. 2). The interaction between the presence of seed coat and the effect of the stimulating agents was proved to be significant concerning survived seeds ($p = 0.02$, ANOVA two-factor with replication). The removal of the thick seed coat resulted in significantly higher germination rate in the control variant: 33.3% versus 16.7% for the intact seeds. Best results were obtained in the variant with intact seeds soaked in 0.35% GA_3 where 46% of the seeds germinated in the terrine, and 40% survived (Fig. 2).

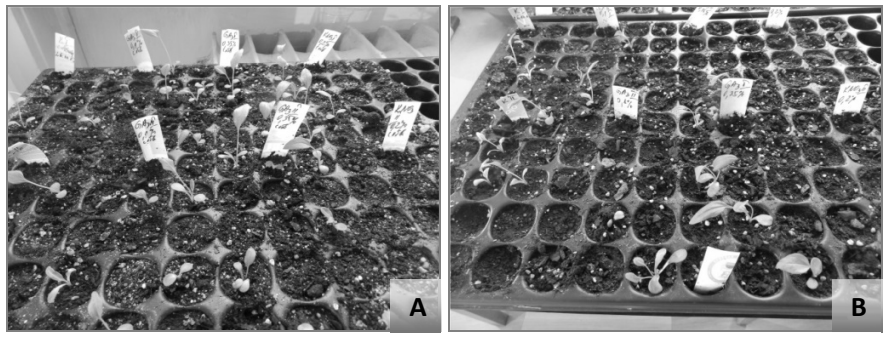

Fig. 1. Germination of seeds in terrines with soil mixture:
A) intact seeds; B) shelled seeds

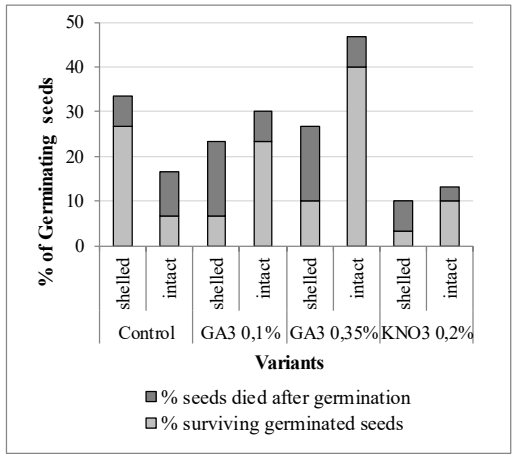

Fig. 2. Germination rate of intact and shelled seeds in the terrines (control and stimulated variants)

On the aeroponic system, seed germination began on the sixth day and lasted two weeks. There were no dropped out germs except for the seeds treated with the high concentration of gibberellic acid (variant GA_3 0.35%), where one third of the germs necrotized. Survival of germinating seeds was probably due to the solution which rinsed regularly the seeds. The influence of the stimulating agents (gibberellic acid and potassium nitrate) on the germination rate of the shelled seeds depended on the applied method: terrines or aeroponic system ($p = 0.03$, ANOVA two-factor with replication). Thus, among stimulated seeds, the best results were obtained in the variants treated with gibberellic acid on the aeroponic, reaching 26.7% healthy germinated seeds in variant GA_3 0.1% and 30.0% in variant GA_3 0.35% while the control variant was better in the terrines (Fig. 3).

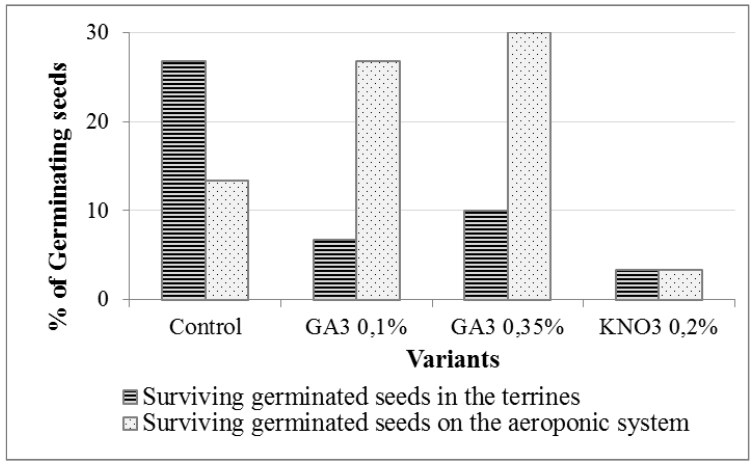

Fig. 3. Percentage of surviving germinated shelled seeds in the terrines and on the aeroponic system

Seedlings were grown on the aeroponic system until they formed well developed leaf rosettes and roots (Fig. 4-A). Root length of the plants measured 10 weeks after the start of the experiment, was 13.2 ± 5.6 cm (Fig. 4-B, C). Plants were potted and adapted to soil mixture in the room phytotron for one month (Fig. 5). They were transferred to the greenhouse for another month, and finally acclimated to the open field plot.

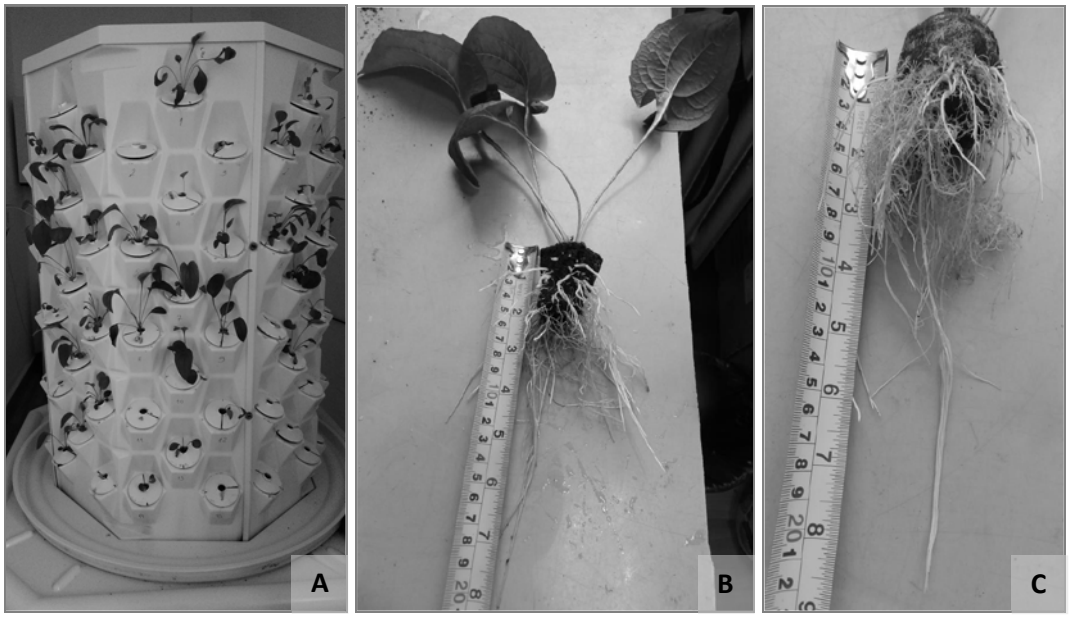

Fig. 4. Ten-week old seedlings of *E. purpurea* obtained on the aeroponic system: A) Seedlings on the system Green Diamond; B) Entire seedling; C) Well developed roots

Seeds sown on the bench with soil in the greenhouse at the end of October 2015, germinated in March next year, and were growing in the conditions of thick seedlings for two months. They were transferred on the open field plot in May 2016, keeping a distance of 20 cm between plants in the row and 40 cm between the rows.

Seedling growth was twice faster on the aeroponic system, most probably due to the fast development of their roots. Plant rosettes of equal size were obtained for 9 months by direct sawing in soil and for 4.5 months when the aeroponic system was used (Fig. 6-A, B). Most of the plants did not bloom in the first season; however, there were some individuals in flower in August 2015, only 3 months after their transfer to the open field plot (Fig. 6-C). All plants obtained either by direct sowing in soil or on the aeroponic system, formed many leaves and usually one flower in summer 2016 (Fig. 6-D). During their third spring on the field plot in 2017, they often developed several flowers per plant, those obtained by germination on the aeroponic system reaching up to 4 flowers (Fig. 6-E).

Fig. 5. Seedlings growing in pots with soil mixture after germination on the aeroponic system

Fig. 6. Plants growing on the open field plot: A) 9-month old, obtained by direct sawing in soil on a greenhouse bench; B) 4.5-month old, germinated on the aeroponic system; C) Flowering of single individuals in the first autumn, obtained by direct sowing in soil; D) Well developed individual in flower in the second year, obtained on the aeroponic system; E) Plants obtained by direct sowing in soil (s) and by germination on the aeroponic system (a), in their third spring

Discussion

Published data concerning seed germination of *Echinacea* species is contradictory: some authors classified germination as difficult and variable between 2 and 50% [8], while others reported high germination rate of 86 up to 92% [4]. In spite of the seed storage in a cool chamber during the winter, the application of temperature stress and stimulating agents prior to the experiments, our results did not exceed 46% of germinating. During the trials we respected the recommended light regime; however, it is possible that the ambient temperature and humidity were of great importance. According to the above mentioned authors the optimal temperature was between 18 and 22°C i.e. lower than in our experimental premises. The careful management of the environmental conditions was emphasized as crucial for the transplants quality of *E. angustifolia* which was successfully grown under relative humidity 60 – 80% [8].

Our results confirmed the use of hydroponics as an efficient tool for plant growth enhancement. Aeroponics is one of the most commonly used greenhouse systems for plant root production, along with deep flow solution culture and hydroponic production system with pots containing either sand, or easy-to-wash ProMix [7]. Authors tested the three mentioned hydroponic systems and reported the last of these techniques as the best one for producing roots and total biomass of *E. angustifolia* and *E. purpurea*, starting with seedlings at the 3-4 true leaves stage. They obtained 6.5 g dry root biomass per *E. purpurea* plant for three months. The important parameters of the nutrient solution were similar to those in our aeroponics: pH of 6, and moderate EC of 1.8 $dS \cdot m^{-1}$. In addition, authors tested the effect of the periodic water stress, expecting root growth enhancement; however, this resulted in reduction of the leaf area of both species and decrease of the total *E. purpurea* biomass. The effect of the insufficient water during drier seasons was reported to significantly affect the root biomass yield in the field crops of *E. angustifolia* (up to twice) although the species was defined as tolerant to dry conditions [8]. Lack of adequate watering was critical especially for the newly planted seedlings.

A large review on *E. purpurea* world production compared the yield and the quality of field-grown plants in many countries from all continents cultivated under various ecological conditions, and hydroponically-grown plants [9]. Regarding the dry matter yield (kg/ha), 8-month old hydroponically-grown plants produced 7840 kg/ha and took second place after plants cultivated in California, US (8500 kg/ha); however, filed-cultivated plants were 3-year old. The content of cichoric acid was also high (2.10% dry matter in the tops and 2.21% dry matter in the roots). Authors calculated that root yield of hydroponically-grown plants was about 2.3 times as high as average of North American field production. Besides, their roots were easy to clean due to the absence of soil, stones, and weeds, and had minimum microbial contamination. No loss of fine roots containing highest amounts of cichoric acid occurred.

Similar advantages were reported about cultivation efficiency of *E. angustifolia* [8]. Despite the slow growth of the young seedlings, the roots of 10-month-old plants grown in greenhouse hydroponics contained total alkamides in levels comparable with those of 3 to 4 year old field-grown plants.

In addition, aeroponics were proved to be more effective for *Echinacea* root production than hydroponics using soil-less mix, calculated on the base of the average root dry weight, and on the ratio root vs shoot biomass [8]. The common feature of the aeroponic systems is the lack of soil-less substrate; plants are growing suspended and their roots are periodically sprayed with nutrient solution. Other authors recommended an A-framed aeroponic system as the most suitable for multi-product crops cultured for both their aerial and aboveground parts, especially for production of herbs and roots, and less efficient for rhizomes [6]. This kind of aeroponics could be perfect for cultivation of purple coneflower as well, because it allows more than one root harvest from the same plants, thus increasing root biomass yield.

In conclusion, we can resume that our results concerning stimulation of *Echinacea purpurea* seed germination as well as enhancement of seedling growth could be applied in the practice. Bearing in mind that seed peeling is labor-consuming, it's better to apply soaking of intact seeds in 0.35% gibberellic acid prior to germination. Using of aeroponic system stimulated the formation of well-developed roots and shortened the time for plant germination and growth. Further experiments based on our results and on the literature data should be carried out to additionally improve root growth using different aeroponic systems, and to apply root harvest without transfer of the plants to the field.

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CONTEMPORARY CONCEPTS IN THE ECOTOXICOLOGY OF CADMIUM (CD) IN TERRESTRIAL ECOSYSTEMS AND ITS BIOMONITORING IN TERRESTRIAL VERTEBRATES

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The present study is a review of the most current perspectives on the general toxicity and ecotoxicity of cadmium and the contemporary achievements in the biomonitoring of this trace metal in terrestrial animals.

Aims of the study a review and assessment of the toxicity and ecotoxicology of cadmium

Materials and Methods: Since this is a review article, materials and methods are not applicable

Results and Discussion: Cadmium is a well-established toxicant and carcinogen in mammals. Recent years have seen a decrease in the production and applications of the metal within the European Union. Nevertheless, due to the problems of disposal, and its presence in polymetallic ores, it still remains a contaminant of concern for the environment.

The current article discusses the most contemporary achievements in the study of the toxicity of cadmium, exploring novel data on the mechanisms of deregulation and inhibition of key mammalian DNA repair systems. In addition, ecological aspects are discussed: the ecotoxicity of cadmium in terrestrial ecosystems, the kinetics of the toxicant in the bodies of mammals, and past and current achievements in the biomonitoring for Cd in terrestrial ecosystems, including the use of monitor species and non-invasive techniques.

Conclusion: Cadmium is confirmed as a powerful, relevant to the contemporary world toxicant, its genotoxicity is explored in detail, and examples of its biomonitoring are given.

Keywords: ecotoxicology, genotoxicity, cadmium, biomonitoring, review

Introduction

Cadmium is a relatively rare, silvery-white metal with atomic number 48, belonging to the chemical group IIB in the periodic table; despite its rarity, due to its presence in

polymetallic ores, it was discovered relatively early, in 1817, as an impurity in zinc carbonate [1]. Like zinc, Cd possesses valencies of +2 and +1, with the +2 state being the predominant chemical form [1]. Over the course of the last century or so, the metal has found many industrial applications, including electroplating, the fabrication of nickel-cadmium batteries, and the production of corrosion-resistant metal alloys and paints. Since the 1940s there has been an increasing concern about the toxicity of Cd to living organisms, including its carcinogenicity and gene toxicity in complex organisms [2, 3]. The year 1912 saw the emergence of a pollution-associated disease, called "itai-itai" in the prefecture of Toyama, Japan, which was later, in the 1950s, identified as cadmium poisoning. During the 1950s and 1960s workers, employed in cadmium smelters started showing an increased incidence of lung and skin cancers, providing evidence that the metal is carcinogenic to mammals [3]. Subsequent experiments in laboratory animals confirmed the metal's toxicity, and, later, genotoxicity in rodents [2, 4]. Since 1993, Cd has been classified as a Group 1 carcinogen by the IARC [5]. The metal accumulates primarily in the liver and kidneys, and these organs contain the bulk of the body burden of Cd in mammals [2, 5]. Classical cadmium poisoning occurs when sufficient amounts of the metal (above 4 μg/g dry weight) are accumulated either in the kidneys, liver, or bone of any given mammalian species [2, 5]. The symptoms include joint pain, osteoporosis, chronic nephropathy and acute kidney failure, acute hepatic failure at high doses, and hematopoietic effects [2, 3]. Until now there is no specific effective therapy against cadmium poisoning; however, chelation therapy and dietary supplementation with zinc can reduce the risk and severity of intoxication with Cd [2, 5]. Cadmium is primarily excreted from the body through the kidneys, when bound to metallothionein (Cd-MT complexes). Despite the fact that this mechanism reduces the body burden of the metal, it is nonspecific and easily saturated at high doses; Cd-MT complexes tend to form plaques in the kidneys and cause renal failure, which means that the kidney is the primary target organ for Cd intoxication [2, 6].

Although cadmium can produce genotoxic and mutagenic effects, until recently it was thought that this only occurs at very high doses [5]. Mutagenicity assays using the Ames test in the late 1980s and early 1990s showed that Cd, by itself, is only weakly mutagenic in prokaryotic test systems [5, 7]. Nevertheless, it's co-mutagenicity with several known mutagens (MNU, MNNG, MMS, UV light and benzo(a)pyrene) has been established in both prokaryotic and eukaryotic test systems, and leading scientists have hypothesized that the genotoxicity of cadmium is due to interactions with DNA repair systems [4, 7]. Recent studies have confirmed this hypothesis and shown concrete interactions of the metal with zinc-finger DNA-binding proteins in general, and double-strand break (DSB) repair systems in particular [8, 9].

The eco-toxicity of cadmium is still an active area of research. Cadmium can be released into the environment by volcanic emissions, or through the combustion of fossil fuels (lignite coal and several grades of petroleum being particularly rich in Cd). Nevertheless, the main concern for ecologists is local contamination with Cd due to anthropogenic enrichment of the metal in lead-and-zinc mining and smelting. In Europe two of the most cadmium-contaminated areas are Szopienice and the Bukowno metallurgical complex in Poland [10]; in Bulgaria, cadmium release into the environment is an issue of concern in the immediate

vicinity of the lead-zinc smelting factory between the towns of Plovdiv and Asenovgrad, since Cd is present in polymetallic dust emissions, containing also lead and zinc micro aggregates [11]. Biomonitoring for cadmium contamination has been well-developed, but continues to be a current issue and a productive area of research, with scientists establishing correlations between Cd body burden and biological endpoints (metallothionein (MT) levels, glutathione levels, apoptosis, cytogenetic abnormalities, DNA integrity and other parameters), and looking for non-invasive sampling methods to monitor for pollution and environmental effects [10, 11, 12]. The current article is a brief review of the most up-to-date achievements in the study of cadmium in the context of the eco-toxicological biomonitoring of terrestrial small vertebrates. We hereby discuss 1) the classical concepts of cadmium toxicity to mammals; 2) new and emerging concepts, including genotoxicity and co-genotoxicity of Cd in complex organisms, 3) cadmium detoxication, and 4) the biomonitoring of Cd contamination and its effects in terrestrial small mammals and birds. Conclusions have been drawn about the ecotoxicity of the metal and the significance of continuing efforts to limit and monitor cadmium as a hazard to the environment.

Results and Discussion

1. Ecotoxicity of cadmium: Cadmium is found most often in polymetallic ores containing zinc, which means most of its anthropogenic enrichment, occurs during the smelting of lead-zinc ores [3]. Worldwide the most heavily contaminated areas are the region of Toyama, Japan (historic), as well as certain parts of China and India, which are engaged with active mining and extraction of the metal. In the EU, local contamination with cadmium is most pronounced in industrial areas in Poland such as in the vicinity of Szopienice and Bukowno [10, 12].

The ecotoxicity of cadmium is still a subject of much debate, because it rarely occurs as a contaminant by itself – during mining and processing of lead-zinc ores, the occurring pollution is most often polymetallic. In the combustion of coal and oil products containing Cd, other pollutants such as sulfur dioxide and radionuclides are most often released in the atmosphere along with Cd and other toxic elements. Although the toxicity of cadmium is relatively well understood, its role in the environment is still an area of active study and discussion. Biomonitoring for Cd has taken place both in Europe in general and in Bulgaria [10, 11, 12, 13, 14]. The accumulation of cadmium in plants and mammals has been observed for a long time [2, 3]. For instance, the tobacco plant accumulates cadmium preferentially, contributing to cancer risk in smokers. Mann et al. have presented evidence for biomagnification of Cd along terrestrial food chains by showing that the insectivore *Sorex araneus* accumulates up to ten times more of the metal than the rodent *Myodes glareolus* for comparable soil concentrations of Cd (100 mg/kg dry weight in *S. araneus* compared to 10 mg/kg for *M. glareolus*) [13]. Biomonitoring for the metal continues both independently [10, 12] and as a component of polymetallic contamination [11, 13, 14].

2. The toxicology of cadmium – classical concepts: The toxicity of Cd has been established in the period following 1945, first by observing increased incidences of lung cancer in cadmium workers, and then by identifying it as the causal agent of the itai-itai

disease in Japan [2, 3, 5, 6]. In terrestrial mammals, the toxicity of the metal is characterized first by damage to the kidneys, then, at higher doses, damage to the liver, pancreas and nervous system [2, 3, 15]. Generally, Cd acts as a toxicant by displacing zinc (Zn) from different mammalian tissues, and disrupting a variety of enzymes, which utilize zinc as a cofactor and bind Zn stably or transiently [3, 5, 6].

Cadmium has no identified biological roles in terrestrial birds and mammals, which means that no specific detoxication system exists [2, 3, 6]. Chronologically, the course of acute and chronic cadmium intoxication is the following: 1) Cd enters the bloodstream from the GI tract or via inhalation; 2) it binds to metallothionein (MT) in the liver, forming stable Cd-MT complexes; 3) Cd-MT complexes enter the kidneys, from where they are partially excreted through the urine [3, 6]. Both acute and chronic cadmium poisoning occur when the metal concentrations, defined by blood levels in excess of 40 μg/dL, exceed the capacity of the body to bind and excrete cadmium [3, 6]. Metallothionein expression is inducible by cadmium, indicating that mammals have the capacity to mount an adaptive response against the toxicant; nevertheless this response is saturated at higher doses; recent research indicates that Cd-MT complexes accumulate in the kidneys, forming plaques and contributing to nephronal tubular degeneration [6, 10].

There is no specific treatment for cadmium intoxication. In animals from areas with higher-than-average cadmium levels, two main methods exist to abate Cd toxicity: 1) dietary supplementation with zinc (this leads to lower absorption and retention of Cd), 2) administration of zeolites together with the food (the zeolites, if properly activated, act as sorbents, reducing absorption of dietary Cd) [3, 5, 6, 15]. In humans suffering from Cd poisoning, nonspecific chelation therapy (with EDTA, British anti-Lewisite (BAL) and other chelators) is sometimes used to reduce blood concentrations of the metal [5, 6].

3. Emerging concepts – carcinogenicity and genotoxicity of cadmium: Early evidence of the toxicity of cadmium indicated that it is a powerful inhalation carcinogen in both humans and laboratory rats and mice [2, 3]. Initial experimentation with the Ames test in bacterial test systems suggested that Cd 2+ is either non-mutagenic or very weakly mutagenic [4, 5]. Nevertheless, in the late 1980s and early 1990s evidence emerged that cadmium is both clastogenic and leading to accumulation and retention of small amounts of repairable DNA damage in mammalian cells [4, 5, 7]. These findings have lead scientists to hypothesize that the mechanism of Cd-induced DNA damage is indirect [5, 7, 8]. In the same time period, tests were conducted for cadmium as a co-mutagen with other types of proven mutagens, such as DNA alkylating agents, UV light and ionizing radiation [4, 8, 9]. These tests indicated that the metal enhances toxicity and mutation frequency, leading to a hypothesis of DNA repair inhibition [5, 7]. A summary of some of the test systems and effects observed is given in Table 1.

Table 1. Co-mutagenicity of Cd 2+ in different test systems [7 and references cited within]

Cadmium (II) in combination with:	Cell line:	Dose, μM:	Effect:
Bacterial test systems:			
MNU	*E. coli*	10-500	Enhanced mutation frequency
MNNG	*S. typhymurium*	250-500	Enhanced mutation frequency
MMS	*E. coli*	250-500	Enhanced mutation frequency
Mammalian cells:			
UV light	V79	0.5-2	Enhanced mutation frequency (hprt)
	human fibroblasts	5	Reduced colony-forming ability
	human fibroblasts	4	Inhibition of DNA synthesis
	human fibroblasts	4	Accumulation of DNA strand breaks
	HeLa	5	Inhibition of thymine-thymine dimer removal
benzo(a)pyrene	SHE	1.9	Increase in morphological transformations

In all the test systems observed, cadmium acted as a co-mutagen, even at the comparatively low doses of 4-5 μM [7]. Nevertheless, concrete evidence for the mechanisms, in which the metal acts on DNA was not provided until recently [8, 9]. Several experiments have demonstrated that Cd 2+ inhibits non-homologous end-joining (NHEJ) by interfering with DNA-PK catalytic activity and the kinetics of DNA DSBs as measured by γH2AX foci kinetics [8, 9]. In addition, it has been established that cadmium at higher concentrations reduces glutathione concentrations in organisms, as well as their overall antioxidant status, leading to promotion of oxidative DNA damage [10, 12]. Although there is still much to be done concerning the concrete mechanisms of cadmium's effects on DNA, the fact that it is genotoxic and carcinogenic is no longer in question.

4. Cadmium in biomonitoring studies of terrestrial small mammals: Cd has been the subject of biomonitoring studies since the 1970s [10, 13]. At first, scientists were primarily concerned with the Cd body burden, then sought to establish correlations with biological parameters, initially in laboratory-based experiments, then, at a later stage, in monitoring of pollution gradients around major sources of atmospheric emissions of the metal [10]. Laboratory-based studies on cadmium-induced liver and kidney injury have been conducted until the recent past [15]. Today, the main focus on current studies is: 1) the correlation of biological

endpoints (such as DNA damage, cytogenetic abnormalities, apoptosis, glutathione levels, redox status, metallothionein levels and the activities of different enzymes) with cadmium body burden in animals, as well as the development and implementation of new sampling techniques, including non-invasive sampling methods [10, 12, 14, 15]. Among the recent advances in the study of cadmium as a xenobiotic and disruptive factor in ecosystems has been the establishment of correlation between Cd levels in an organism and the histopathological changes [15], as well as the introduction of European magpies (*Pica pica*) as a monitor species, owing to the possibility of obtaining samples non-invasively (feathers and eggs), as well as the sensitivity of magpies to Cd pollution, expressed by the clear correlation of redox status, and overall cytotoxicity and apoptosis in this bird species to the specific body burden of the metal [10, 12].

Conclusion

Cadmium (Cd) has already been established as a strong toxicant and carcinogen in terrestrial small mammals and birds at body concentrations exceeding 30 μM [7, 8, 9, 12]. It is beyond doubts that local contamination with Cd poses a hazard to terrestrial as well as aquatic ecosystems. A number of sources have provided evidence for the biomagnification of the metal along terrestrial food chains, underscoring the importance of biomonitoring studies, and the selection of suitable bioindicator species in order to estimate the effects of Cd pollution in complex terrestrial ecosystems [10, 12, 13, 15]. While debate existed regarding the genotoxicity of cadmium in the late 1980s and early 1990s, the element is now widely accepted to be genotoxic and carcinogenic, even at doses below the threshold for deterministic effects such as acute or chronic intoxication [7, 8, 9]. The concrete mechanisms of Cd-induced DNA damage are still an area of active research, with recent studies underscoring the role of the metal as an inhibitor of DNA repair, in particular the NHEJ pathway of DNA DSB repair [8, 9].

Despite being relatively well-studied as a toxicant, cadmium represents an active area of research in ecotoxicological biomonitoring. Recent advances include the correlation of biological parameters with metal burden in organisms, as well as the introduction of different sampling techniques for measuring body burden of the element [10-15]. In spite of decreasing production and atmospheric emissions across the European Union, Cd continues to cause concern among scientists and policymakers. Its continuing mining, application and disposal across the globe present ongoing problems; it is estimated that biomonitoring studies for cadmium pollution will continue to be an active and productive area of ecological research for the foreseeable future.

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THE IMPACT OF SEA WATER IMMERSION ON THE VIABILITY OF PSAMMOPHILOUS SPECIES *GALILEA MUCRONATA* (L.) PARL.

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Abstract

Aim: This study aims to determine the effects of flooding stress on whole plants of *Galilea mucronata* and how long its rhizomes can remain viable in sea water in order to investigate its capacity as dune stabilizer.

Materials and Methods: Two simulated flooding experiments were conducted. Whole plants and rhizomes were immersed in sea water, in order to establish visible morphological changes of leaves, stems and roots, rhizomes viability, biomass and root/shoot ratio.

Results: Whole plants stay viable longer than the flood with a maximum duration along the Bulgarian Black Sea Coast, and rhizomes were able to regenerate after 30 days in seawater. Statistical analysis of experimental data demonstrates that immersion in sea water increases rhizomes viability, biomass and allocation to root biomass.

Conclusions: *G. mucronata* is less tolerant to water immersion than other psammophytes, but demonstrates a high potential to be a key species for dune stabilization.

Keywords: Immersion tolerance, viability, *Galilea mucronata*.

Introduction

Coastal areas are very fragile ecosystems that are sensitive to global climate changes, sea level rise and frequent storm surges [1]. These areas provide habitats for many rare and endangered species, which will be lost due to the combination of negative consequences of flooding and erosion [2] as well as strengthen anthropogenic impact.

Dune plant species form an extensive system of horizontal and vertical rhizomes that reduced wind speeds across the surface, trapping and holding a great amount of sand [3]. Thus, they support sand stabilization and increase the dune's ability to act as a buffer. Furthermore, they can effectively minimize erosion and reduce storm damages with minimal negative impacts to natural ecosystems [4]. Ecosystem services require searching for well adapted plants with extensive root systems and studying their ability for erosion and flooding control in order to replace the artificial coastal protection and stabilization structures with "soft" transplanting techniques of native, salt-tolerant plant species.

Recorded damages from extreme meteorological events over the Bulgarian Black Sea Coast [5] show the high potential of the root system of native psammophytes to accumulate sands and prevent from washout [6, 7]. Although, the Bulgarian Black Sea Coast is relatively protected from sea floods due to the small amplitude tides [6], some extreme meteorological events, such as unusual storms may cause flooding and erosion of dunes. Storm waves carry away sandy sediments and cause destruction of communities of dominant sand stabilizers *Leymus racemosus* (Lam.) Tzvelev subsp. *sabulosus* (M. Bieb.), *Ammophila arenaria* (L.) Link [7] and *Carex colchica* J. Gay. In such cases *Galilea mucronata* (L.) Parl. become a major dune stabilizer and colonizes territories from the dune pioneers (Fig. 1).

Data about the impact of storms and flooding to *G. mucronata* are insufficient unlike those about *L. racemosus* subsp. *sabulosus* [7], *A. arenaria* [7, 8, 9] and *C. ligerica*, which show high viability of psammophytes to the stress of flooding in short-term intervals.

This study aims: 1) to determine the effects of flooding stress caused by storms on whole plants of *G. mucronata* and how long its rhizomes can remain viable in sea water and 2) to investigate post-immersion changes in plant biomass and allocation to above- and belowground biomass in order to determine the capacity of *G. mucronata* as dune stabilizer.

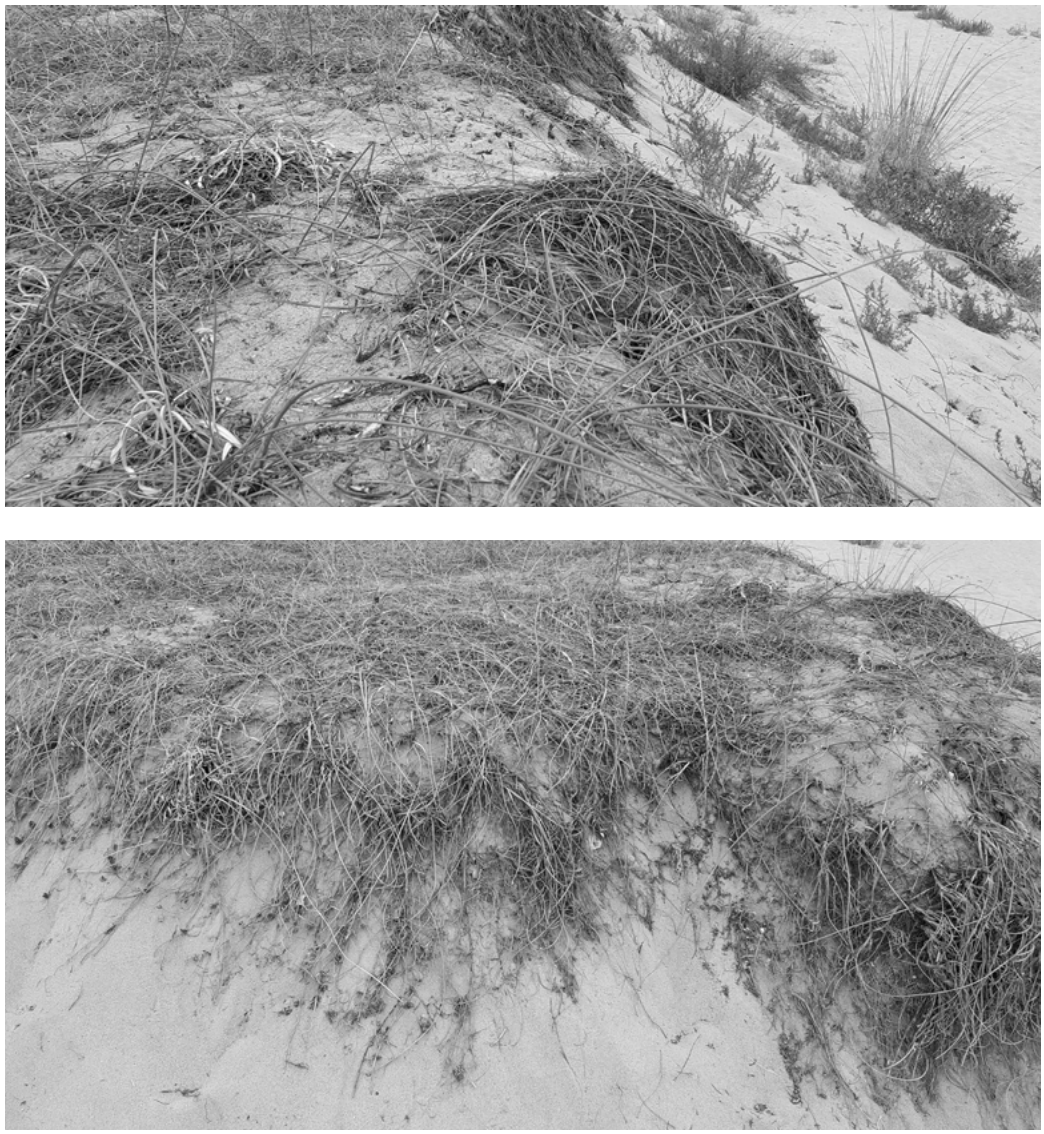

Fig. 1. *Galilea mucronata* communities three months after storm (photos S. Vergiev)

Materials and Methods

Study species: Galilea mucronata (L.) Parl. (syn. *Cyperus capitatus* Vand.) is a perennial plant from the family Cyperaceae with creeping rhizomes, up to 50 cm high, triangular or nearly rounded stem and basal revolute leaves [10]. The species is included in the Red Data Book of the Republic of Bulgaria as endangered [11]. In the investigated area, *G. mucronata* forms fragmented monodominant communities consisting of small number of individuals with high abundance and poor floristic composition [11].

Simulated flooding experiments: Two simulated flooding experiments were conducted in the Botany Laboratory of the Varna Museum of Natural History. Rhizomes and whole plants of *G. mucronata* were collected at the sandy beach (42º19′48.12″ N, 27º44′03.73″ E) in April 2016 for the first experiment and in January 2016 for the second experiment.

In the first experiment, ten whole plants were planted in washed and sterilized sand in plastic pots and immersed in glass tanks (40 l), filled with sea water at a depth of 2–4 cm below the water surface with constant maintained temperatures (4±1ºC, 13±1ºC and 23±1ºC) for 480 hours. The water was changed several times per day. Visible morphological changes of different parts of the specimens (leaves, stems, roots) are recorded and assessed in 12 parameters [7].

In the second experiment, rhizomes were immersed for 720 hours in similar conditions [7]. Ten rhizomes per treatment were removed every fifth day and were planted in washed and sterilized sand in plastic pots in the glasshouse with controlled air temperature [8]. Control rhizomes were planted directly. Rhizomes were watered with fresh water daily and allow growing for one month before harvesting [7]. All plants were cleaned and oven-drying at 60° C for 24 hours.

Statistical analyses: Mean bud viability was measured as the percentage of rhizome nodes that produced vegetative shoots and roots [8]. Maximum rhizome bud viability is defined as the bud viability of the rhizome replicate with the highest bud viability for each treatment [8]. Mean bud viability as well as mean dry weight biomass per plant replicate and R:S ratio (Root mass/Shoot mass) were analyzed with one- and two-way analyses of variance (ANOVA). P-value less than 0.05 was considered as statistically significant. Where necessary, data were transformed in order to obtain homogeneous variances.

Results and Discussion

The most important factor in assessments of negative consequences of flooding to natural plant communities is the exposure time to sea water in flooding simulated experiments. In the present study, direct methods were conducted in order to establish the changes and adaptations of *G. mucronata* to the stress of flooding in certain short-term intervals and the period of which whole plants, submerged in sea water still maintain viable buds capable of producing new plants, without taking into consideration the direct mechanical effect of storm waves. Previous study [7] shows that experimental methods based on the direct submergence of psammophytic species are more appropriate than the experiments of studying substrate salinity and salt spray due to their regular exposure to sea water and specific mechanisms of neutralizing sea water salt.

Results from the first simulated flooding experiment showed that submergence in sea water did not have a lethal effect on the specimens. Twelve visible parameters for morphological changes of different parts of the specimens (leaves, stems, roots) were assessed (Table 1). Beginning of the decomposition of leaves of the submerged plants started on the 140th hour. On the 168th hours, a growth of stems and root sprouts was observed. There was no visible decompositions of newly grown stems, roots and rhizomes till the end of the experiment (480 hours). All investigated parameters were unrelated to water temperature.

Table 1. Results from simulated flooding experiment. Visible morphological changes of different parts of the specimens (leaves, stems, roots) assessed in 12 parameters

Parameter	Hours in seawater		
	4ºC	13ºC	23ºC
Beginning of the decomposition of			
leaves	140	140	140
stems	360	360	360
roots	480	480	480
Complete decomposition of			
leaves	460	460	460
stems	n/a	n/a	n/a
roots	n/a	n/a	n/a
Growth of			
stems	168	168	168
root sprouts	168	168	168
Beginning of the decomposition of newly grown			
stems	n/a	n/a	n/a
roots	n/a	n/a	n/a
Complete decomposition of newly grown			
stems	n/a	n/a	n/a
roots	n/a	n/a	n/a

As a result of this test, the Critical Decomposition Time (CDT) for *G. mucronata* was obtained. CDT is defined as the time point at which each plant shows signs of irreversible decomposition of vegetative organs [12] and indicates that the plants will not survive and their communities will not be able to recover. This parameter is crucial in models for vulnerability assessments of flooding impacts to coastal plant communities [13]. CDT for *G. mucronata* is 140 hours, which is longer than the flooding with a maximum duration for the Bulgarian Black Sea Coast [7, 12]. This value of the CDT is similar to values of other psammophytes *L. racemosus* subsp. *sabulosus*, *A. arenaria* and *C. colchica* [12].

Results from the second experiment show that *G. mucronata* rhizomes remain viable immersed in sea water for 720 hours, which is the maximum duration of the second simulated flooding experiment. This is in agreement with studies of other psammophytes (e. g. *A. arenaria*) which evaluate their rhizomes viability from 13 to 70 days in sea water [7, 8, 9, 14].

Mean bud viability gradually increased to the maximum of 61.3% (treatments with 4°C) on the 480th hour of sea water submergence, followed by a slight decrease at the end of the immersion (Fig. 2). All the treatments had higher values than the mean viability of the untreated control (58.6%) and appeared to be enhanced slightly by sea water ($P = 0.037$). Despite different temperatures, all treatments demonstrated identical trends (Fig. 2). The same trend was observed for other psammophytes mean bud viability [7]. The values are similar to the mean viability of *C. ligerica* [14], and lower than psammophytes from the family Poaceae [7, 14].

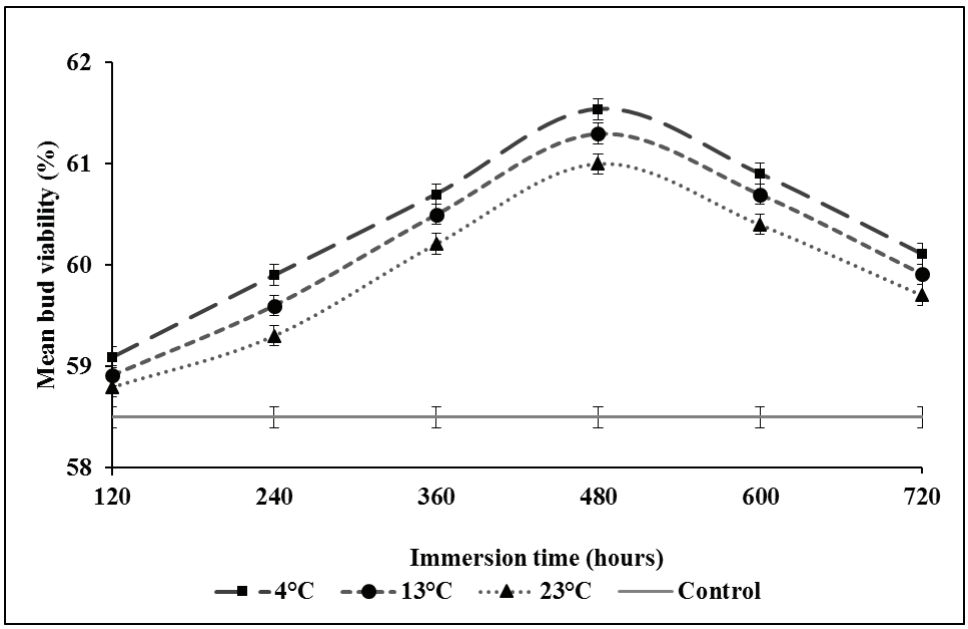

Fig. 2. Mean bud viability following sea water immersion

All treatments had a maximum rhizome bud viability of 100%. At the end of each immersion period, 80% of the rhizomes still had at least one viable bud and 5% of the rhizomes had maximum bud viability.

Storm events on the Black Sea Coast occur during winter and early spring when average surface sea water temperature is about 4ºC [17]. In order to study the relation between temperature and *G. mucronata* viability, two other treatments with temperatures of 13ºC (average surface sea water temperature) and 23ºC (average summer surface sea water temperature) were included in the simulated experiments.

The different temperatures influenced rhizome viability in similar levels (P = 0.089), and average differences between coolest and warmest temperatures were only 0.44% (Fig. 3). Replicates in all treatments demonstrate a higher viability than the untreated replicates (P = 0.038). This is contrary to the results of the study of *A. arenaria* rhizomes [9], which retained viability for longer in cooler water. So it can be concluded that the water itself as a defining factor impacts viability more than the temperature of water.

While crucial for rhizomes viability is the cumulative effect on the durability of flooding and sea water temperature, defining factor in ability of psammophytes to fix loose sandy substrates and contribute for dune stabilization is the size of their root systems. In order to measure how immersion affected the root system, the mean dry weight biomass per replicate was taken as well as R:S ratio (Root mass/Shoot mass).

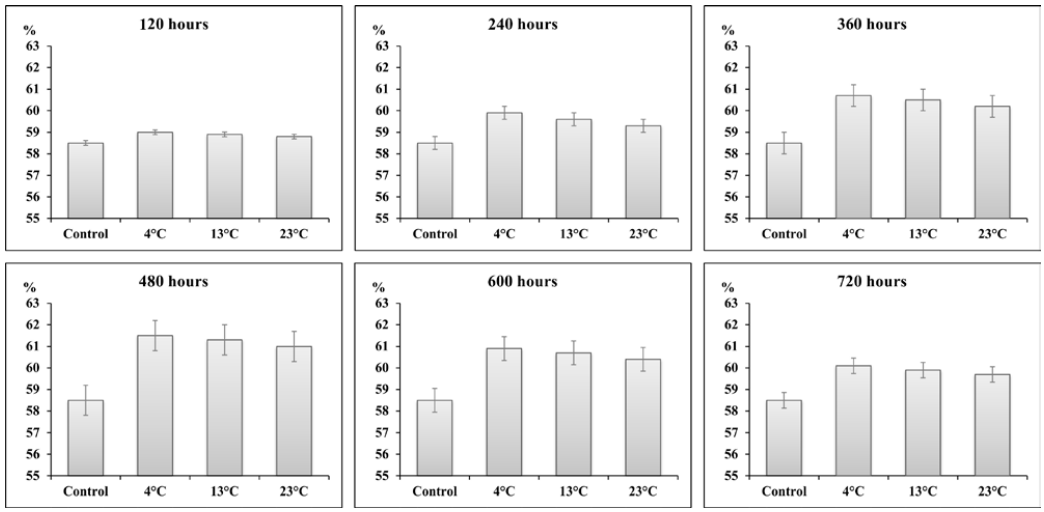

Fig. 3. Effects of different temperatures of sea water on mean bud viability

Dry weight biomass was increased by immersion in sea water till the 480th hour of the experiment (Fig. 4) and remained unchanged till the end of the experiment. The water temperature had no significant effect (P = 0.077) on the biomass of the treated groups. Replicates in all treatments demonstrated higher biomass than the control replicates (Fig. 4).

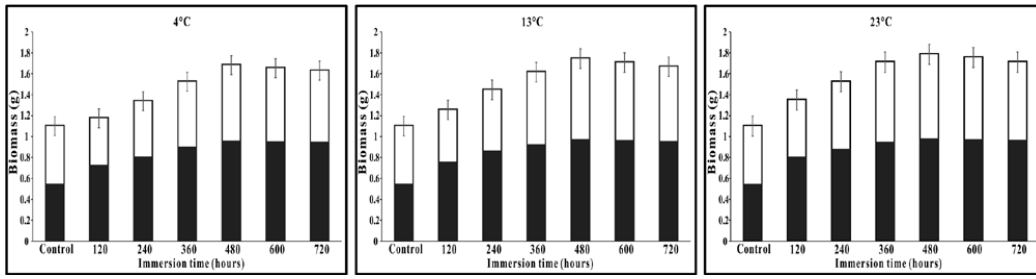

Fig. 4. Effects of sea water immersion on dry weight biomass. The shaded portion of the bars represents belowground biomass; blank portion represents aboveground biomass

The R:S ratio measures the plant allocation to above and belowground biomass [15]. This variable was not affected significantly by immersion duration (P = 0.072). Biomass allocation to roots in plants exposed to sea water was slightly increased. The increased water temperatures tended to decrease the R:S ratio, but the effect was insignificant (P = 0.059) (Fig. 5). Replicates in all treatments demonstrated higher biomass than the control replicates.

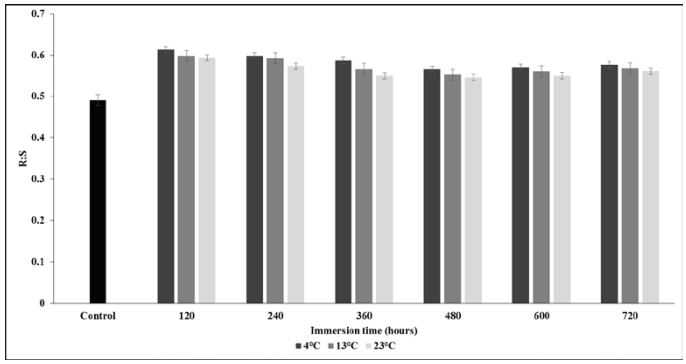

Fig. 5. Effects of sea water immersion on R:S ratio

Conclusion

G. mucronata shows high tolerance to sea water immersion and high viability during the simulated flooding experiments. Statistical analysis of experimental data shows that the immersion in water increases rhizomes viability, biomass and allocation to root biomass. Other factors, such as duration of immersion and temperatures of sea water have not significant effect. *G. mucronata* are much less tolerant to water immersion than other psammophytes *L. racemosus* subsp. *sabulosus*, *A. arenaria* and C. *ligerica*, but demonstrates high potential to be a key species for dune stabilization.

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ACCUMULATED HEAVY METALS AND OXIDATIVE STATUS IN TISSUES OF THE BLACK SEA MUSSEL (*MYTILUS GALLOPROVINCIALIS* LAMARK, 1819)

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Abstract

Aim: To assess the oxidative status of tissues of *M. galloprovincialis* in relation to levels of accumulated metals.

Materials and Methods: The digestive gland (DG), gills (G) and foot (F) were studied regarding: 1) determination of Cu, Pb, Zn, Cd and Ni by using ICP-OES and 2) spectrophotometric measurement of

oxidative stress markers: lipid peroxidation (LPO), glutathione (GSH) levels and the activities of antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD).

Results: 1) In G highest amounts of Pb and Zn, together with highest LPO and lowest GSH were observed; 2) In DG highest Cu and Cd content, along with medium LPO and GSH, were found; 3) The F had the lowest metal content along with lower LPO and the highest GSH. Reciprocal relationship between CAT and SOD activities in tissues was observed.

Conclusion: The studied tissues of *M. galloprovincialis* accumulated different amounts of metals, as the content of Pb and Zn appeared to be related with high LPO and low GSH levels as sensitive markers of oxidative stress.

Keywords: Black Sea, *Mytilus galloprovincialis*, heavy metals, oxidative stress.

Introduction

Many xenobiotics accumulate in seawater, deposited as sludge or suspended in the environment. These substances present a particular risk to the environment and human health due to their bioaccumulation in food chains of ecosystems, reaching higher trophic levels, including humans. An increasing number of studies offer as a possible option for objective assessment of the state of the marine environment, the use of the biological response of the naturally occurring marine organisms to monitor changes in the environment.

Shellfish are globally recognized as good biomonitors and are widely used as control organisms in a number of international programs such as the Global Program for Monitoring of Chemical Contamination (Mussel Watch) and others. Due to their attached, low-mobile lifestyles, the ability to feed themselves by filtration and the lack of excretion mechanism, sea mussels have a significant capacity to bio-accumulate different xenobiotics from the environment and concentrate them in easy to measure quantities in their tissues.

In shellfish, as in all aerobic organisms, reactive oxygen species (ROS) are generated continuously. Being highly reactive, ROS are capable to modify all biomolecules – lipids, proteins and nucleic acids. In physiological conditions, the antioxidant defense system neutralizes ROS by enzymatic and non-enzymatic antioxidants and in this way the cells are protected from their harmful effects. However, impairment of the antioxidant defense system and/or excess ROS production may result in oxidative modifications of biomolecules causing damages of the subcellular structures, altering their function and ultimately leading to cell death [1].

The oxidative status of the organism reflects the intensity of the prooxidation processes and the state of the antioxidant defense system. The imbalance toward the pro-oxidative state is referred to as “oxidative stress”. It is well known that the fine balance between pro- and antioxidant processes could be disrupted by a huge variety of pressures, including metal contamination [2]. The alterations in oxidative status could be used to evaluate stressful environmental conditions, especially the toxic effects of metal pollutants [3].

The current pilot study aims to assess the oxidative status in tissues of Black Sea *M. galloprovincialis* and the corresponding levels of metals, as persistent pollutants, accumulated from the marine environment.

Materials and Methods

Forty-seven specimens of one year grown *M. galloprovincialis*, 4.9 – 6.0 cm length, were used for analysis. They were obtained from the "Black Sea Mussels" farm, located in conditionally clean water (FAO region 29) near the town of Sozopol. Samples were collected from the farm's "long lines" at 1 to 6 m depth, 50 m distance off the shore of the island Saint Ivan (N 42.439176, E 27.685076). The mussels were cleaned in seawater, placed in plastic nets and transported to the laboratory in refrigerated box.

The digestive gland, gills and foot were isolated from the mussels on ice (0-4°C) with stainless steel instruments. The tissues of 12 mussels were rapidly frozen in liquid nitrogen and stored in freezer at -80ºC until biochemical analysis. On the day of measurements, the samples were homogenized in potassium phosphate buffer (pH 7.5). The homogenates were centrifuged at 600 g for 10 min in order to obtain a "post-nuclear" fraction for determination of lipid peroxidation (LPO) and glutathione levels (GSH). Part of this fraction was re-centrifuged at 10 000 g for 20 min to obtain a "post-mitochondrial" supernatant for determination of the activities of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), gluthatione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD).

The lipid peroxidation was determined by the method of Hunter et al. [4] and was expressed as nmoles malondialdehyde/mg protein using a molar extinction coefficient of $1.56 \times 10^5 M^{-1}cm^{-1}$.

Total glutathione was measured according to Tietze [5].

The catalase activity was determined according to Aebi [6] and was expressed as A240 per minute per milligram of protein.

The Cu, Zn – superoxide dismutase activity was determined according to Beauchamp and Fridovich [7] and was expressed as units per milligram of protein. A unit of SOD activity is considered the amount of the enzyme producing 50% inhibition of nitro-blue tetra-zolium (NBT) reduction.

The glutathione reductase activity was measured by the method of Pinto and Bartley [8]. The values, measured at 340 nm were expressed in nmoles NADPH oxidized per min per mg protein, using a molar extinction coefficient of $6.22 \times 10^6 M^{-1}cm^{-1}$.

The glucose-6-phosphate dehydrogenase activity was determined according to Cartier et al. [9]. The values, measured at 340 nm were expressed in nmoles NADP+ reduced per min per mg protein, using a molar extinction coefficient of $6.22 \times 10^6 M^{-1}cm^{-1}$.

Protein content was measured by the method of Lowry et al. [10]

The tissues from the remaining 35 specimens were dried at 60°C to an air-dry state and digested using a concentrate nitric- and perchloric acid mixture (2:1). They were used for determination of Cu, Pb, Zn, Cd and Ni total content by ICP-OES. The results were presented in μg/g dry weight of mussel's tissue.

The obtained results were statistically analyzed by one-way ANOVA with Dunnett post-test; $p < 0.01$ was accepted as the minimum level of statistical significance of the established differences. The mean values are presented in the text with standard deviation (± SD).

Results

The results for the measured metal content of the studied tissues of *M. galloprovincialis* specimens are presented in Table 1. The concentration of the studied metals in the mussel tissues was different. The gills showed at an average higher metal content in comparison to the digestive gland and the foot. The digestive gland was characterized by intermediate accumulation of metals. The lowest metal content was present in the foot.

Table 1. Metal concentrations (µg/g dry w) in tissues of the Black Sea *M. galloprovincialis*

Metal\Mussel tissue	Foot	Gills	Digestive gland
Cu	8.27	12.43	17.39
Pb	6.17	9.17	7.13
Zn	53.85	131.82	106.67
Cd	1.60	3.77	11.84
Ni	2.00	1.65	0.30

The most common metal pollutants Pb and Cd were found to have different concentrations in the studied mussel tissues. The digestive gland had 1.3 times lower Pb contents than the gills. On the other hand, Cd had much higher concentration (3.14 times higher) in the digestive gland than in the gills. The concentration of Cd (as well as Cu) was highest in the digestive gland and lowest in the foot. On the opposite – the measured Ni concentrations in mussel's foot was 6.67 and 1.21 times higher than in the digestive gland and gills, respectively.

The assessed markers of oxidative stress in tissues of *M. galloprovincialis* are presented in Table 2. Higher LPO level compared to the other tested tissues was observed in the gills, along with the lowest total GSH concentration. An intermediate value for both LPO and glutathione levels was measured in the digestive gland. The lowest LPO content and the highest GSH content was detected in the foot.

Table 2. Markers of oxidative stress in tissues of Black Sea *M. galloprovincialis* (mean ± SD)

Mussel tissue\Markers of oxidative stress	LPO nmoles MDA/mg protein	GSH ng/mg protein	CAT ΔA240/ min/mg protein	SOD U/mg protein	GR U/mg protein	G6PD U/mg protein
Digestive gland	1.33 ± 0.41	537.64 ± 215.90	2.40 ± 0.53	8.69 ± 3.11	11.64 ± 3.60	15.84 ± 3.47
Gills	2.02* ± 1.22	258.04 ± 132.31	1.66 ± 0.66	11.41 ± 7.50	4.28* ± 1.51	25.97* ± 6.29
Foot	1.08 ± 0.42	1428.31† ± 905.4	0.44*† ± 0.19	17.66* ± 7.29	9.51 ± 5.40	25.12* ± 5.30

***p < 0.01 vs digestive gland; † p < 0.01 vs gills**

In regard to activities of the antioxidant enzymes the digestive gland had higher CAT and GR activities in comparison to the other tested tissues. Moreover, in the digestive gland the lowest SOD and G6PD activities were established. The highest G6PD activity was found in gills along with the lowest GR and intermediate SOD and CAT activities. The foot muscle was the tissue that showed the highest SOD and the lowest CAT activities, along with intermediate values for the GR and G6PD activities.

Discussion

As expected, for a one-year growth even in conditionally clean waters, the studied mussels have accumulated metals significantly exceeding the maximum permissible levels in sea water. The established quantities of metals and measured markers of oxidative stress in *M. galloprovincialis* showed clear tissue specificity, likely due to their structural and functional characteristics. Gills are in direct contact with the water and because of their structure, they are highly susceptible to the impact of different factors from the marine environment, which could impair the pro-/antioxidant balance. Regarding the measured indicators, the gills appear to be the most affected by oxidative stress tissue. LPO has already been used successfully as a biomarker of metal-induced oxidative stress in *M. galloprovincialis* [11]. It has been demonstrated that the exposure of *M. galloprovincialis* to sub-lethal concentrations of Cu(II), Cd(II), Pb(II), and Fe(II) for a period of 10 days lead to increase of LPO in gills and the mantle [12]. In this study we found a positive relation between the level of both Pb and Zn in mussel tissues and LPO and a negative relation between the level of Pb and Zn in mussel tissues and GSH.

Regarding the activity of the antioxidant enzymes, it has been suggested that under metal contamination they initially activate as an adaptive response in order to overcome oxidative stress in the polluted environment. However, the prolonged exposure to pollutants may result in antioxidant enzymes' inactivation. Induction of the antioxidant enzyme CAT has been documented in copper exposure to low doses in *Perna perna* and *Mytilus galloprovincialis* [13]. However, high concentrations of copper lead to reduction of antioxidant enzyme activities [14].

In this study we found high activity of G6PD in gills. Since G6PD activity is the primary source of NADPH for the cell, it's high activity measured in gills may be associated with the low values found for GR, needed for the regeneration of GSH in this tissue – 2.7 and 2.2 times lower respectively for the digestive gland and the foot. Evaluated in gills relatively high values for SOD (1.3 higher than digestive gland) and CAT – 3.8 times higher than foot, showed the development of oxidative stress in this tissue and active reaction of cellular enzymatic antioxidants for handling with ROS. The intermediate values of GSH and LPO levels measured in the digestive gland, suggested a relative stability of this organ with respect to the oxidative stress induction. Although the oxidative status of the digestive gland may depend not only on the metal content in tissue but also on a number of external (temperature, radiation, other than metal contamination etc.) and internal factors (nutrition, spawning etc.). The established low SOD and high CAT activities in the digestive gland are hard to be explained. Probably, other processes than enzyme-catalyzed dismutation of superoxide,

generated the hydrogen peroxide that is actively neutralized by CAT. The mussel's foot seems to be the least affected organ with the lowest values of accumulated metals and respectively low indicators for the peroxidation processes (LPO) and high antioxidant levels (GSH). Relatively low CAT and intermediate GR and G6PD activities measured in this tissue, supports the valuation. The highest SOD activity measured in the foot is likely to be determined by the functional specifics of this organ. Thus, the removal of damaged byssus is associated with active depolymerization of collagen, for which it is known that is accompanied by activation of transcription of a respective locus in the mussel genome, which triggers the synthesis of SOD [15]. The elevated SOD activity in this tissue may be associated with the mechanical damage of the byssus threads during the sampling and sample proceeding.

In conclusion, the results of this pilot study indicated that the tissues of *M. galloprovincialis* accumulated different amounts of metals. The observed relation between the high Pb content and high LPO respectively, along with low GSH levels allowed us to assume that the accumulation of Pb was mainly responsible for induction and development of oxidative stress in the tissues.

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DISTRIBUTION OF BOTTOM FAUNA UNDER CAGE FISH FARMS IN THE KARDZHALI RESERVOIR

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Abstract

Aim: To investigate the influence of fish farming cages on distribution of benthic community of Kardzhali Reservoir.

Materials and Methods: The sampling was conducted in the summer and autumn of 2016. Bottom sediments and macrozoobenthos were collected using standard bottom sampler under and at a distance of cage fish farms. At the same time water depth and current direction were analyzed. In laboratory the abundance of different macrozoobenthos taxa and the organic content in the sediments were determined. Spatial and temporal parameters of the benthic community were analyzed against a set of environmental variables.

Results: Under the fish cages a decreased species richness and significant abundance of class Oligochaeta contrary to the control station were registred. A negative relationship was established between the direction of the cumulative loading and biotic variables as number of taxa, total abundance and species richness.

Conclusion: The impact of the cage fish farming on benthic community structure was expressed as reduced species richness and an increased abundance of class Oligochaeta. Water flow rate seems to be an important factor for mitigating the strength of that impact.

Keywords: fish farming cages, impact assessment, benthos community response, Kardzhali dam.

Introduction

The main impact caused by cage fish farming which affects the environment is nutrient loading caused by dissolved food waste and fish excrements in the water column [1] as well as accumulation of suspended organic material in the sediment under fish cages [2]. Benthic communities successfully register the impact of containment crops on organic load of sediments [3, 4, 5]. The increased amount of organic matter in sediments increases the overall benthos abundance [6] or disproportion of some indicator and tolerant groups such as Mollusca, Chironomidae [7], Copepoda, Nematoda and Polychaeta over other groups, as well as overall reduction in biodiversity is established [8]. In some cases after loading cessation, a recovery process occurs [9], while sometimes it is less pronounced [8]. In order of a reliable assessment, the importance of regionally validated measures as well as expert judgment is considered as important [10]. However most research focuses on the impact of fish cage cultures over benthic communities in marine environment [1, 2, 4, 5, 9, 10, 11] and quite a few are the studies regarding the inland waters in particular deep reservoirs [6]. Organic deposition largely depends on factors like speed of the local currents that respectively determine the rate and extent of the sedimentation processes [12]. Deep reservoirs are characterized by high magnitude of water level fluctuations often caused by high rates of water accumulation and exchange. Although low current speed allows the increase of organic matter [4], higher current speed could be sufficient to disperse the nutrients and prevent water quality impact [1].

In the present study we consider that several structural parameters like diversity, abundance and distribution of the bottom fauna in Kardzhali Reservoir should reflect the specific environmental conditions determined by the anthropogenic impact like fish farming and water flow regulation.

Materials and Methods

The field research was conducted in the late summer and autumn of 2016 (in the beginning of August, September and October) at four sampling stations (Fig.1). Three of them were selected as monitoring stations situated under the fish cages, and one control station was situated upstream. Totally twelve samples were collected and analyzed. Sampling was conducted according to ISO 10870:2012. Standard bottom sampler "*Ekman Berge*" was used for collecting macrozoobenthos and bottom sediments. The samples were immediately stored in refrigerator at 4^{o}C and analyzed in laboratory within few days. Laboratory treatment consisted of washing and separating the samples through a set of sieves with different mesh size (2 mm, 0.5 mm, 0.2 mm, 0.15 mm and 0.063 mm). All specimens were fixed in 95% ethanol. Each obtained fraction was examined under a stereomicroscope and compound microscope if necessary. Taxonomic determination of the bottom invertebrates was made to the lowest possible level, but finally several main groups were identified and used for the analyses.

Mapping was processed in ArcGIS 9.3.1 while data representation and statistical analyses were performed by Primer software. Total abundance of macrozoobenthos, total

abundance of Oligochaetes, percentage of Oligochaetes and Shannon's Diversity Index were calculated in order to obtain information about spatial and temporal dynamics of the structure and dominant groups of bottom fauna. Mean values and standard deviation of indices were represented for each site and sampling period. Distribution of the benthic community was represented by Non-metric multidimensional scaling (MDS). The analyzed data were calculated according to Bray-Curtis similarity matrix and tested for similarities between control station and the stations under the cage facilities with One-Way Analysis of Similarities (ANOSIM) tests.

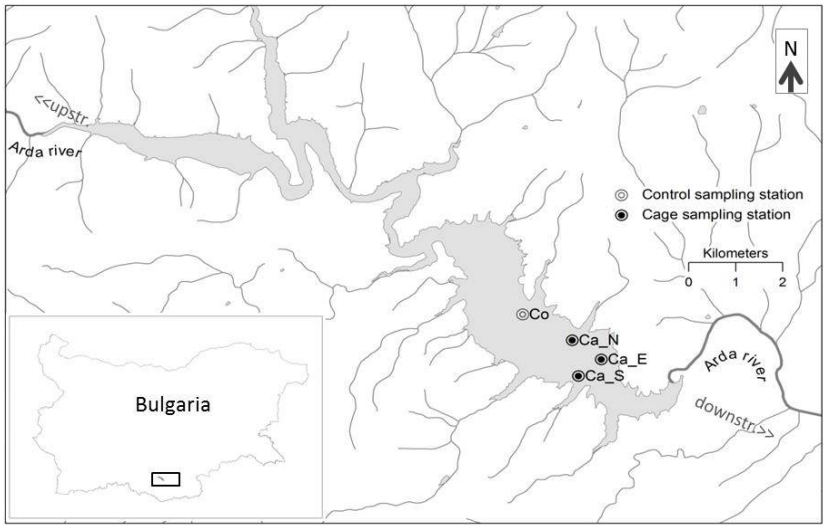

Fig. 1. Kardhali Reservoir – location of the sampling sites: Ca_N – north fish farming cages, Ca_E – east fish farming cages, Ca_S – south fish farming cages (sampling next to cages) and Co – control sampling site

Several environmental variables were observed in order to identify those that determined the community structure: water depth, organic content in sediments (%), approximate number of cages per farm, farm "age" (the time of the exploitation of the site measured in years snapped by Google earth images) and water current location. The estimation of the last three variables (number of cages, farm age and location) were obtained by Google Earth application. Water current location was created as a variable with a categorical scale from 0 to 3. Score 0 is given to the control station (Co) situated upstream where no impact of a fish farm was expected. Score 1 was given to the closest farm (CaN) downstream to control station considering impact by its own. Score 2 was given to the next farm (CaS) located downstream of (CaN) considering possible impact by (CaN) and by its own. Score 3 was given to the most distant farm (CaE) downstream to control station. This variable intended to scale the cumulative loading effect from the upstream fish farms. Rank correlation coefficient was used to test the environmental variables against the obtained biotic variables.

Results and Discussion

The spatial distribution of macrozoobenthos represented on an MDS plot (Fig. 4) showed that the structure of the bottom invertebrate communities in August and September was clearly different between the control site (bottom right) and under the cage stations (left side of the diagram) (Fig. 4-a). However, in October the dissimilarity between the sites decreased (Fig 4-b). Two of the under cage samples from October showed community structure that differed from the main group of under cage samples and was more similar to the control samples group.

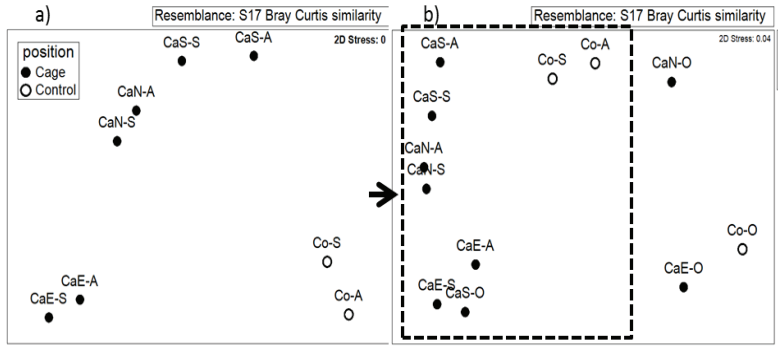

Fig. 4. MDS plot represents the distribution of benthic communities in Kardzhaly dam. a) August and September; b) August, September and October. CaS – sample under south cages, CaN – samples under north cages, CaE – samples under east cages, Co – control station samples. -A – August, -S – September, -O – October

ANOSIM test provided the significant differences (R = 0.72, p = 0.035) between the groups of control samples and under cages samples (Tab. 1) for the August and September period (Fig. 4a). In September, significant changes seemed to occur in the bottom community structure expressed as a partial recovery of the species richness under the cage facilities (Fig 3). As a consequence the ANOSIM test did not provide significant differences (R = 0.236, p = 0.095) between the groups of total control samples and total under cage samples for the whole period of investigation (Tab.1-b).

Table 1. ANOSIM test for similarities between Cage stations and Control site. Colunm a) represent results for August and September; column b) represents results for August, September and October

Global Test	a)	b)
Sample statistic (Global R)	**0.719**	0.236
Significance level of sample statistic	**3.6%**	9.5%
Number of permutations (All possible permutations)	28	220
Number of permuted statistics greater than or equal to Global R	1	21

Two main taxonomic groups were dominant in the benthic community – class Oligochaeta and lower crustaceans of phylum Crustacea. Two different taxonomical ranks from lower crustacean were identified – superorder Cladocera and subclass Copepoda. Furthermore, rotifers and Cladocera's ephippia were rarely encountered. Considering that the average size of the registered specimens was relatively small (smaller than oligochetes), the majority of the community could be classified rather as meiofauna.

The spatial distribution revealed the stations of under cage facilities as more affected than the control site. The control site was characterized with the highest species richness and the lowest number and percentage of oligochetes, despite the higher variability in the total abundance, which seems to be driven by seasonal changes. Total abundance was highly variable parameter (Fig. 2-a). Spatial distribution showed higher fluctuations in total abundance of control station (Co) and East cages station (CaE). In addition, Shannon's diversity showed higher values at the control station (Co) and lower diversity under the cage structures (Fig. 2-b). Oligochetes were represented in all samples in most of which dominated by numbers (Fig. 2-c; 2-d). Far fewer numbers of Oligochaeta were reported from the control stations (Mean = 6088 ind.m^2, SD = 856 ind.m^2) versus these situated next to the fish cages (Mean = 16862 ind.m^2, SD = 9814 ind.m^2). Copepods and cladocerans equally occurred in more than half of the samples. Copepoda was numerically dominated over Cladocera in most of the cases where the two species co-occurred.

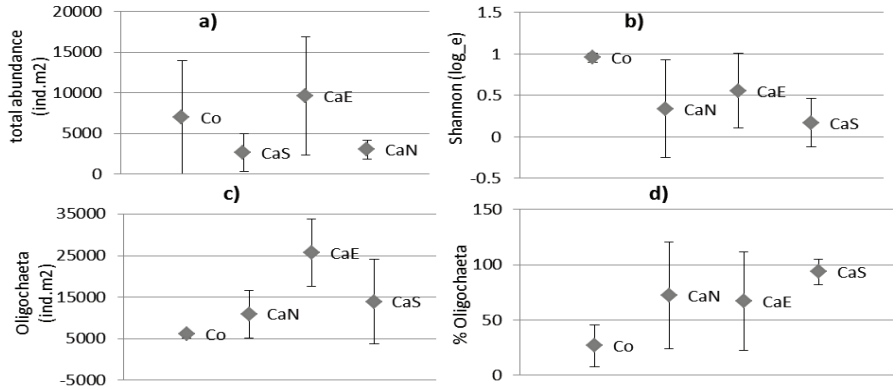

Fig. 2. Mean values and standard deviation of total benthic abundance, abundance of Oligochaeta, percent Oligochaeta and Shannon diversity index for the investigated stations. Co – control, CaS – South farm, CaE – East farm, CaN – North farm

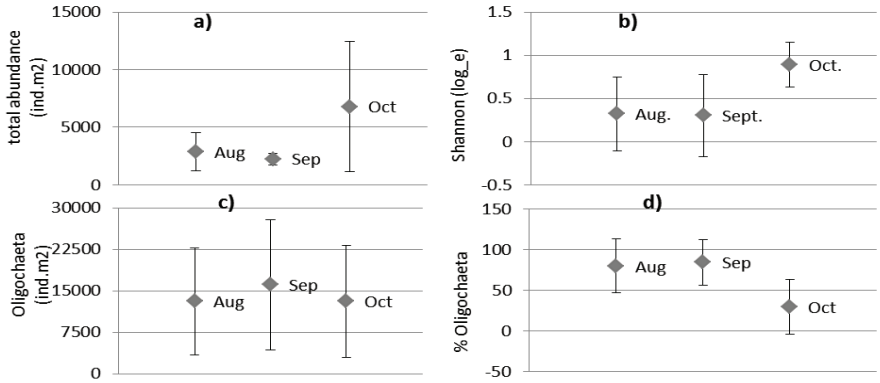

Fig. 3. Mean values and standard deviation of total benthic abundance, abundance of Oligochaeta, percent Oligochaeta and Shannon diversity index for the period - August, September and October 2016

The impact of fish cage facilities on the benthic community was analyzed based on a set of environmental variables. The fish farm age showed that the north farm (CaN) was the oldest fish farm snapped on Google Earth image since 1984, considering more than 32 years of exploitation (Tab. 2). The east (CaE) and south (CaS) farms were not observed by Google Earth until 2012, considering age of about 5 to 7 years each. The size of the farms determined in 2016 by Google Earth was approximately 420 cages for the Northern farm (CaN), 190 cages for the Southern farm (CaS) and about 140 cages detected in the Eastern farm (CaE) with an average diameter between 5-12 meters. The age (time of exploitation) and the size (number of cage facilities) cumulate the highest potential impact to the Norhtern farm (CaN) having the highest number of cages – 420 and the longest time of exploitation (age) – over 32 years.

Table 2. Calculated years of exploitation and relevant number of cages according to Google Earth images in 2016

Fishfarm	number of cages in 2016	considered age (years of exploitation)
CaN	420	32
CaE	140	6
CaS	190	6

The correlation analysis between the environmental variables and the target biotic parameters showed that the abundance of Oligochaeta increased proportionally (significant positive correlation) to the size ($r = 0.76$, $p < 0.05$), as well as the "age" ($r = 0.76$, $p < 0.05$) of the farm (Tab. 3). The hither number of the biotic variables correlated negatively with the environmental variable "water flow location". Nevertheless, significant correlation was determined only with the abundance of Copepoda. Taking in account the effect of loading

accumulation downstream the cage structures, the analyzed indices (total number of taxa, total abundance, Shannon's diversity, abundance of Copepoda and abundance of Cladocera), respectively, decreased downstream. Inversely, the "percentage of Oligochaeta" increased indicating the deteriorated environmental conditions.

Table 3. Spearman rank correlation between the environmental variables and the biotic variables. The correlations in bold has significance level < 0.05

correlation coeff.	Depth (m)	% organic content	Number of cages (size)	Google earth age (y)	Water flow location
Number of taxa	0.10	-0.14	-0.05	-0.05	-0.46
Total abundance	-0.01	-0.07	0.24	0.24	-0.45
Shannon (log e)	0.15	-0.17	-0.18	-0.18	-0.48
% Oligochaeta	-0.15	0.02	0.26	0.26	0.57
Oligochaeta (ind/m^2)	-0.32	-0.04	**0.76**	**0.76**	0.06
Copepoda (ind/m^2)	0.18	-0.07	-0.15	-0.15	**-0.59**
Cladocera_(ind/m^2)	0.20	-0.07	-0.11	-0.11	-0.56

The initially detected difference between the benthic communities in the control area and the areas under fish cages suggests different environmental conditions between both studied habitats. Both the farm's "age" and size seems to be significant factors determining the loading process and determining the environmental conditions registering a positive relationship with the Oligochaeta abundance and reduced species richness, although the stations that were situated downstream experienced a higher impact due to the cumulative loading as compered with the upstream situated farms. Usually in the late summer, the reservoir registers its lowest water levels, due to water abstraction and decreased surface inflow. This process could be associated with the more intense velocity at the bottom of the reservoir, which could result in entrainment, transportation and mixing of bottom sediments and organisms downstream. Taking into account the seasonal dynamics or human induced regulation of the water flow rate, it seems to be an important factor for mitigating the strength of fish farming loading.

Conclusion

The spatial distribution revealed the stations under cage facilities as more affected than the control site. The control site was characterized with highest species richness and lowest number and percentage of Oligochetes.

Shannon's diversity showed higher values at the control station and lower diversity under the cage structures. Oligochetes were represented in all samples in most of which dominated by numbers. However, fewer numbers of Oligochaeta were reported from the control stations. Correlation analysis between the environmental variables and the target biotic parameters showed that the abundance of Oligochaeta increased proportionally (significant positive correlation) to the size ($r = 0.76$, $p < 0.05$), as well as the "age" ($r = 0.76$, $p < 0.05$) of the farm.

Water flow rate appears to be an important factor mitigating the strength of fish cage farming on benthic community structure.

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SHORT COMMUNICATIONS

Topic:
BIOTIC AND ABIOTIC IMPACT ON THE LIVING NATURE AND MECHANISMS OF ADAPTATION

CYTOTOXIC ACTIVITY OF *SIDERITIS SCARDICA* EXTRACTS AND FRACTIONS ON HUMAN BREAST ADENOCARCINOMA CELL LINE MCF7

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Sideritis scardica Griseb. (Lamiaceae) is a Balkan endemic species. It is traditionally utilized as a pulmonary treatment, as well as anti-flu and wound healing remedy [1]. Recent research has brought insight also into other pharmacologically relevant activities of different preparations of the plant. Different types of its extracts have been shown to possess cytotoxic effect on murine melanoma B16, human leukaemia HL-60 cells, as well as C6 rat glioma cells, these effects being attributed to reactive oxygen species induction by the chemical constituents present in the studied preparations [2 and references cited within].

The aim of the present work was to investigate the cytotoxicity of extracts and fractions of the plant on human breast adenocarcinoma cell line MCF7.

Materials and Methods:

Plant material

Commercial sample of herbal tea (consisting of the flowering aerial parts of the plant) was purchased from a pharmacy in Sofia.

Extraction and fractionation

Plant material was ground in a mill and was consecutively subjected to ultrasonic extraction (three times per ten minutes with fresh solvent) with hexane, chloroform and methanol. Then the methanol extract was fractionated with ethyl acetate, butanol and water.

Cytotoxicity assay

MTT-colorimetric method for the detection the cytotoxic activity of the extracts and fractions on MCF7 human breast adenocarcinoma cell line in concentrations of 200, 100 and 50 μg/ml (dissolved in 0.1% DMSO) was observed after 24 h treatment. Cells grown in media with 0.1% DMSO, showed no deviation in their viability as compared with control cells grown the culture medium without any treatment. Results were recorded by ELISA reader, as values represent the mean of five measurements per concentration. Cell viability values were expressed as percentage of the non-treated control.

Preliminary chemical characterization of the methanol extract and polar fractions of the plant

Thin layer chromatography and co-chromatorgraphy with reference compounds previously isolated from the plant was performed on the methanol extract, ethyl acetate and butanol fractions.

Statistical processing of results

Comparison of means was conducted by the Student t test for unequal variances. The differences were compared at $P \leq 0.05$.

Results:

Extraction yields

The following yields of the respective preparations were obtained: hexane extract – 2.08%, chlorophorm extract – 1.12% and methanol extract – 13.3% of the dry plant material. Methanol extract fractionation resulted in 13.27% ethyl acetate, 36.71% butanol and 43.33% water fractions (percentage is calculated on the basis of the methanol extract).

Cytotoxicity of obtained Sideritis preparations

At the end of the 24-h period, the highest concentration of the hexane (cell viability 14.6% ±1.9 as compared with the control) and chloroform (40% ±4, respectively) extracts, and the ethyl acetate fraction (8% ±0.3) of *Sideritis scardica* were observed to exhibit remarkable cytotoxicity on the MCF7 cells. No negative effect on cell growth as compared with the control non-treated cells was recorded upon methanol, butanol and water preparations treatments.

Chemical profiling of the methanol extract and active polar fractions

The TLC characterization of the samples showed that while a mixture of phenyethanoid (verbascoside) and flavonoid (isoscutellarein and hypolaetine type) diglycosides were characteristic for the total methanolic extract and butanol fraction, the ethyl acetate fraction exhibited predominantly the presence of only the two flavonoid diglycosides.

Conclusion: The results are indicative of higher cytotoxicity of the flavonoid glycosides, as compared with phenylethanoids, towards the tested cell lines and might provide evidence

on the mechanism of action of the different polyphenolics present in the aerial parts of Balkan endemic *Sideritis scardica*. Further research is needed to test the toxicity and evaluate the possible selectivity of tested compounds towards normal cell lines.

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Keywords: *Sideritis scardica* Griseb., cytotoxicity, adenocarcinoma cell line

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SCREENING FOR GENOTOXITY OF AMARYLLIDACEAE PLANTS EXTRACTS ON THE *CHLAMYDOMONAS REINHARDTII*

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It is known that pesticides could be of natural or synthetic origin. Synthetic pesticides are some of the commonly used chemicals for plant protection in agriculture. It is established that synthetic pesticides can have harmful effects on the human and animals' health and on the environment. In the last decades it is of a great importance to find alternative sources for crop protection. The bio-pesticides can be one of these alternatives.

The Amaryllidaceae family contains 73 genera and at least 1,600 species that are perennial herbs. The Amaryllidaceae plants contain an exclusive group of alkaloids. In addition, some of these alkaloids express a phytoncide effect and could be applied as bio-pesticides.

In the field of pharmacy biological activities of alkaloids of the Amaryllidaceae plants are investigated in detail. Additionally, it is known that the Amaryllidaceae components have different biological activities - antitumor, antiviral, antiparasitic, anti-inflammatory, psychopharmacological; interactions with human cytochrome P450 3A4; acetylcholinesterase (AChE) inhibitory activity, etc. [1].

Currently, little is known concerning the toxicological properties, genotoxicity, mutagenicity and carcinogenicity of alkaloids isolated from Amaryllidaceae plants [2]. The evaluation of bioactivity of compounds derived from plants and their application in plant protection is a long process. In this process several stages are included - screening of biotoxicity; genotoxic potential; antimicrobial properties; anti-parasitic activity of different total plants extracts; their fractionating, analyzed by GC-MS to establish their alkaloid profile, biological activity of alkaloids, etc.

Our aim is to analyze the potential genotoxic capacity of 10 Amaryllidaceae plant extracts.

Materials and Methods: Ten total leaf extracts of Amaryllidaceae plants were prepared by prof. Berkov and his team using the following procedure: dried leaves were extracted with CH_3OH for 24h in proportion 1:10. The extracts of the following plants were tested: *Hippeastrum papilio* (Ravenna) Van Scheepen, *Zephyranthes grandiflora* Lindl., *Crinum* spp., *Leucojum aestivum* L., *Galanthus elwesii* Hook.f., *Clivia miniata* Regel, *Spikelia formosissima*, *Eucharis amazonica*, *Amaryllis belladonna* L., *Narcissus triandrus* L.

Clamydomonas reinhardtii strain 137 C+ (WT) was applied as a test-system. Cell suspensions at the end of exponential and the beginning of stationary growth phase were treated for 2h and 24h with concentrations in the range of 5µg/ml to 1000µg/ml. Spot test was used as a preliminary test to clarify the range of concentrations with potential genotoxic effect. The genotoxicity of extracts was evaluated on the basis of spot intensity [3] and compared with those in control cells – DMSO (0.1%), Sager-Granick liquid medium (SG) and positive control PQ (5µM).

Results: Similar spot intensity was observed in control samples and those in samples treated with extracts of *Zephyranthes grandiflor*a Lindl., *Crinum* spp., *Leucojum aestivum* L., *Galanthus elwesii* Hook.f., *Clivia miniata* Regel,. *Eucharis amazonica*, *Amaryllis belladonna* L. at both expositions - 2h and 24h. We established that regardless of the exposure time and the concentrations applied, extracts of these 7 plants possess no genotoxic effect on *Chl. reihardtii.*

Further, our preliminary data demonstrated that the genotoxic potential of *Hippeastrum papilio*; *Spikelia formosissima* and *Narcissus triandrus* depends on the specificity of the extracts, exposure time and concentrations.

For example, both concentrations and exposure time can affect spot intensity of samples treated with extract of *H. papilio* comparing with control samples. No genotoxic effect was found when exposition of 2h was applied at all concentrations' range. The only concentration with mild genotoxic capacity was 1000 µg/ml applied for 24h.

Genotoxic effect was established after the treatments of cells with *Spikelia formosissima* extract at concentration 1000 µg/ml and exposition 2h. Additionally, effect of exposure time was demonstrated after the treatments of cells with concentrations in the range of 5µg/ml to 1000 µg/ml and exposition 24h.

The present study clearly indicates that *Narcissus triandrus* L. total leaf extracts show significant genotoxic capacity depending on the exposure time and applied concentrations. The strongest reduction of spot intensity is established in samples treated with 1000µg/ml concentration and expositions of 2h and 24h. Based on this it could be suggested that the genotoxic effect is exhibited at concentrations higher than 500µg/ml. In this regard we conducted a second experiment where the following concentrations were applied - 250 µg/ml; 500 µg/ml; 750 µg/ml and 1000 µg/ml and exposition of 2 and 24h. Decrease in spot intensity of the samples treated with *Narcissus triandrus* L. extract was obtained after treatment with concentrations equal or higher than 500µg/ml and exposition of 2h in comparison with control samples. A dose-dependent genotoxic effect was observed at exposition of 24h. The spot intensity decreases with the increase of extracts' concentrations.

Spots intensities in the samples treated with extracts of the 10 Amaryllidaceae plants for 2h and 24h were not so well expressed as in the positive control 5µM PQ (positive control). Interesting is the fact that similar spot intensity in cells treated with 5µM PQ and 1000 µg/ml *N. triandrus* L. extract at 24h exposition is observed.

Conclusion: The genotixic activity of 10 extracts was checked. Three of them – *H. papilio; S. formosissima* and *N. triandrus* demonstrated some genotoxic potential. Further experiments must be done to elucidate the mode of action.

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Keywords: Amaryllidaceae plants, *Chlamidomonas reinhardtii*, extracts, genotoxity, spot test

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DNA SUSCEPTIBILITY OF *CHLAMYDOMONAS REINHARDTII* AND *SACCHAROMYCES CEREVISIAE* TO NURELLE D

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Today, environmental pollution is considered as one of the most serious global problems concerning human health, genetic consequences in next generations as well as the future of natural and agricultural ecosystems.

According to the Agency for Toxic Substances and Disease Registry (ATSDR) 1275 species are accepted as priority hazardous substances. Chemicals known as mutagenic are listed in the Environmental Mutagen Information Center database. Unfortunately, this list is annually expanded as a result of human activity, including pesticides usage in agricultural practices. Today's 890 synthetically chemicals are approved as pesticides throughout the world and the number of marketed products is estimated to be 20700.

Organophosphorus insecticides (OPIs) are still the biggest group of insecticides with 67 active ingredients on the market [1]. One of the most commonly used ones is chlorpyrifos. It is major active compound in the commercial product Nurelle D together with the pyrethroid cypermethrin. In agricultural practice Nurelle D is commonly applied in concentrations up to 0.1% depending on the target insects. Limited data exist concerning the possible DNA damaging effect of such concentrations on non-target organisms.

Short-term tests involving unicellular organisms or cell cultures are frequently applied in toxicology, in order to provide some information on the DNA damaging potential of chemicals. *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae* could be considered as two appropriate test systems for such studies due to their genotype characteristics. They are chosen because of their cell similarities with plant – *Chlamydomonas reinhardtii* and animal cells – *Saccharomyces cerevisiae*.

The aim of the study was to compare the DNA susceptibility of *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae* towards the insecticide Nurelle D.

Materials and Methods: The yeast *S. cerevisiae* diploid strain D7ts1 (*MATa/α; ade2-119/ade2-40; trp5-27/trp5-12; ilv1-92/ilv1-92; ts1/ts1*) and the unicellular green algae *Ch. reinhardtii* strain 137 C+ were chosen as test systems.

DNA susceptibility of both species was compared based on the response towards treatment with different concentrations of the commercial product Nurelle D. Nurelle D is a commonly used insecticide containing two active substances – the organophosphate - chlorpyrifos and the pyrethroid - cypermethrin.

Our preliminary data showed that Nurelle D concentrations higher than 0.05% resulted to 100% lethality of *Chlamydomonas reinhardtii* based on clonal assay. Due to this high toxicity of the product on *Ch. reinhardtii*, two different concentration ranges were applied. *Ch. reinhardtii* cells were treated with Nurelle D at concentrations: 0,01%; 0.02%; 0.03%;

0.04% and 0.05%. The concentrations chosen for the treatment of *S. cerevisiae* were 0,01%; 0,1% and 1%. The DNA susceptibility was evaluated based on the induction of double strand breaks (DSBs), detected by constant field gel electrophoresis (CFGE).

Briefly, cell suspensions of both species with a density $1x10^6$ cells/ml were treated for 30 min. After that, cells were centrifuged, the pellet was embedded into agarose plugs and CFGE was performed as described in [2, 3]. Results are presented as mean fraction of DSB released (FDR).

Results: Treatment with the lowest tested concentration – 0,01% Nurelle D did not result in a statistically significant induction of DSBs in both test systems.

Very pronounced DNA damaging effect on *Chlamydomonas reinhardtii* was obtained after the treatment with Nurelle D in a concentration range 0.02 - 0.05%. Around 4 – 5-fold higher DSB levels were measured compared to the spontaneous DSB levels in untreated cells. No effect of concentration was found - no statistically significant difference among the results obtained after the treatment with those concentrations was shown.

On the other hand, treatment of *S. cerevisiae* with a 2-fold higher Nurelle D concentration - 0.1% led to only 3-fold increase in DSB levels (0.281±0.033) in comparison with the control untreated cells.

As expected, 1% Nurelle D resulted to very pronounced DNA fragmentation in *Saccharomyces cerevisiae*.

Conclusion: In agricultural practice Nurelle D is commonly applied in concentrations up to 0.1% depending on the target insects. Our results demonstrate that such concentrations are DNA damaging not only for the insects but also for some non-target organisms. The most pronounced toxic effect depends on the genotype. *Chlamydomonas reinhardtii* strain 137 C+ is found to be more DNA susceptible than *Saccharomyces cerevisiae* diploid strain D7ts1. Further experiments will provide significant evidence concerning the toxic effect of low doses Nurelle D on non-target organisms.

Keywords: DNA DSBs, *Chlamydomonas reinhardtii*, organophosphate insecticide, *Saccharomyces cerevisiae*

Acknowledgements: This study was funded by the projects „Strategies for overcoming the genetic risk of anthropogenic pollution with organophosphorus pesticides“ - Joint Research Project between BAS and National Research Centre, Arab Republic of Egypt; and „Ecological and genetic assessment of the environment, management and strategies for risk overcoming“ – Bulgarian Academy of Sciences.

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BIOMASS OF PINE FORESTS IN THE NORTH-EAST OF THE EUROPEAN PLAIN IN DEPENDENCE ON SOIL MOISTURE CONDITIONS

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Aim: Biomass is an important resource of forest ecosystems. It is recognized as an Essential Climatic Variable [1] and an Essential Biodiversity Variable [2]. Remote sensing is one of the most common approaches to estimate forest biomass and its dynamics over large areas but it requires ground measurements for more reliable data using this method [3]. Thus, studies of forest biomass are an actual in present day. The aim of study was to estimate biomass of pine forests in North-East of European plain in depend on soil moisture conditions.

Materials and Methods: The objects under study were eleven pine stands located in the North-East of the European plain. The stands belonged to three groups of forest types: *Lichen, Myrtillus* and *Sphagnosa* type. They differ in stage of development: three are middle-aged, two are ripening, three are mature and three are over mature stands. The pine forests are almost pure in composition and formed by *Pinus sylvestris* with little admixture by other species. The biomass of stands was evaluated using equations that derived from the analysis of 5−10 sample trees on each plot. The principles and method of selection are described in literature [4]. The close correlation was observed between the mass of the separate fraction and tree diameter height 1.3 m (DBH). The biomass of shrub and moss layers was determined by cutting 30−50 samples of 625 cm^2. The data are presented in absolutely dry weight. Regression analysis and ANOVA were used for estimation of influencing soil moisture conditions on biomass.

Results: Total biomass in pine forests varies from 79.6 to 182.5 $Mg.ha^{-1}$. The highest values were observed in overmature *Myrtillus* type pine forest, the lowest – in middle-aged pine forest of *Sphagnosa* type.

The pine stands of *Lichen* type contained124–150 $Mg.ha^{-1}$. The ecosystems in the mesic soil moisture conditions (*Myrtillus* type) had 100–182 $Mg.ha^{-1}$, and in over wetting conditions (*Sphagnosa* type) – 79–125 $Mg.ha^{-1}$.

The most of the biomass concentrates in trees whose share is changed from 89 to 99%. Stem and roots are the main fractions in the pine stands that concentrate 55–71% and 16–25% respectively from trees biomass. The role of the belowground organs is higher in the pine stands on wet soils. Input of needless/leaves varied from 3 to 4%, branches – from 4 to 13%, bark of stem – from 4 to 8%.

The biomass of the ground cover plants varies in a wide range from 2.1 to 11.0 $Mg.ha^{-1}$. The middle-aged and mature pine forests of *Sphagnosa* type are characterized by the highest values and the ripening pine forest of *Lichen* type – by the lowest values. The belowground organs of shrubs and grasses are the main fraction in the ecosystems on the mesic and over wet soils. The aboveground organs of shrubs and mosses dominate in the aboveground biomass in these communities. The role of grasses is insignificant. Above ground lichens forms more than half of ground cover plants biomass in pine forests of the *Lichen* type. The share of shrubs varies from 5 to 42% and mosses - from 1 to 23%.

The analyses of the database showed that our estimates of biomass are comparable with other data [4]. On the East European plain biomass varied from 62 to 160 $Mg.ha^{-1}$, from 120 – 290 $Mg.ha^{-1}$ and from 66 to 215 $Mg.ha^{-1}$ in pine forests of *Lichen, Myrtillus* and *Sphagnosa* type respectively. Wide range of estimates is explained by the age of the stands. The highest values in a similar stage of development are observed in the pine forests of *Myrtillus* type.

Conclusion: The soil moisture conditions determine the role of ground cover plants in the total biomass. We found a positive correlation ($R=0.64$; $p=0.03$) between soil moisture and the biomass of ground cover plants. This impact was confirmed by ANOVA ($F=34.9$; $P<0.05$). The biomass is an important component of the present climate change investigations. It is the base for further studies of the carbon cycle in pine ecosystems (occupying about 7.1×10^6 ha) located in the North-East of European plain.

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REPORTS

Topic:
ECOSYSTEM RESEARCH, SERVICES AND ECOLOGICAL AGRICULTURE

THE CASE OF AIRSOFT GAMES IN MALA PLANINA AS AN UNCONVENTIONAL CULTURAL ECOSYSTEM SERVICES USE

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Abstract

Aim: The main aim of the current research is to investigate the provision of ecosystem services in the western part of Mala Planina. The paper examines the cultural ecosystem services in particular.

Materials and Methods: The study is based on cameral and terrain research and the focus is a specific type of sport in the form of live action role-playing game (LARP), called Airsoft. The game is based on the elimination of your opponents by firing non-harmful Airsoft pellets launched via replica weapons. The specific area of the LARP falls within the boundaries of the abandoned military unit near Ponor village. A thorough open-ended interview with events arranger, called a game master is applied, as a qualitative research method for gathering the necessary information, which is processed in order to fulfill the aims of the investigation.

Results: Around 640 players (a major share of them participate more than once) per season take part in the game, organized at least one time per month and the fact that a lot of the players come back again proves undoubtedly the quality of the natural capacity of Mala Planina.

Conclusion: The study is demonstrating the fact that the western part of Mala Planina attracts visitors by the cultural ecosystem services that it provides.

Keywords: Mala Planina, cultural ecosystem services, live action role-playing game, Airsoft

Introduction

The fundamentality and importance of ecosystem services in our present is indispensible and our well-being would be unimaginable without the quality and quantity of natural resources. The direct and indirect contributions of ecosystems that can be transformed in different commodities used for the cultural, economic, scientific etc. development of humanity can be described as ecosystem services. At the same time they provide wealth for the society and the provision of ecosystem goods and services depends mainly on the functional capabilities of organisms [1]. The cultural services, which are in an integral connection with the other types, are the main focus of this paper. The BISE [2] postulates, that they represent nonmaterial benefits that people obtain from ecosystems through spiritual enrichment, intellectual development, recreation, and aesthetic experiences. On Fig. 1 it can be see the place of cultural ecosystem services, alongside the other three types of services.

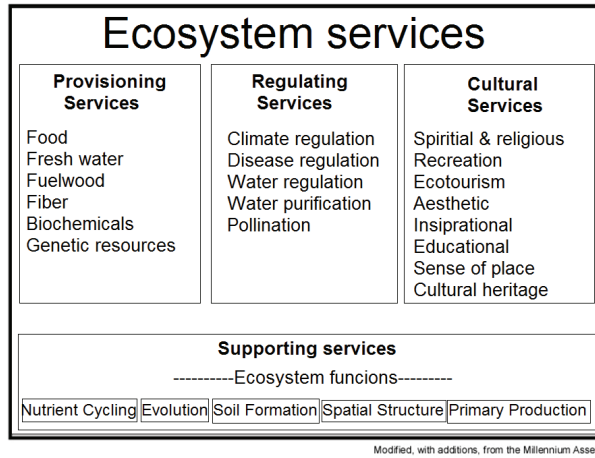

Fig. 1. Ecosystem services (modified by Millenium Ecosystem Assessment, 2005)

The global information pool, concerning ecosystem services as a whole, has been filling up in the last decade, as the studies by Figueroa-Alfaro & Tang [3], Milcu et al. [4] and Schirpke et al. [5] show. Bulgaria has also contributed for the expansion of ecosystem knowledge and some of the research ideas that deserve our attention were published by Assenov [6] and Assenov & Borissova [7]. These papers provide the necessary basis for the deeper understanding of the ecosystem services and develop the idea the term should be considered in the form of ecosystem/landscape services. Nedkov [8] and Nedkov & Burkhard [9] add more weight on this matter.

This research examines the provision of cultural ecosystem services represented by a specific kind of sport in the form of a live action role-playing game (LARP), called airsoft. LARP is a type of role-playing game where players physically act out the movement and actions of their characters, who can be fictional, as well as historical or modern heroes and even from a far distant futuristic era, for example. The real world is the arena of the LARP, where the participants act in a fictional setting and try to accomplish their targets. The setting and the

rules of a particular game are a mission of game master/s, who deals with all the arrangements. The LARP mania started in the end of 1970s and the beginning of 1980s when tabletop role-playing games begun to gain popularity. At the same time Airsoft is a specific sport with some typical characteristics for LARP – use of costumes, equipment, gameplay in a fictional setting etc. The historical roots of Airsoft dig deeply in the 1980s and the country of Japan, where it is illegal for private citizens to own a firearm, following a nationwide ban. However, Airsoft guns, which represent replica firearms, are legal. They closely emulate real firearms and are available on many store shelves in Japan. The Airsoft guns are becoming more and more popular among the police and the military, because they can be used as training tools. During the 1980s manufacturers managed to create a gun that can project pellets (the "Airsoft bullets") through the release of a compressed gas, kept in a canister. With the scientific and technical progress at hand, the development of plastic pellets decreased the possibility of severe injuries among the players. This led to the rapid popularity of Airsoft games, for more and more people are easily impressed by the military battles and active hobbies. There was no internet during the 1980s so the epicenter was Japanese and remained as such for years, while in the 1990s the rumors reached America and Europe and this mania continues to the present [10].

Materials and Methods

The area of the current research – the area near Ponor village (Fig. 2) is located in the western part of Mala Planina and the complete boundaries of the mountain are published by Grigorov & Assenov [11]. Mala Planina is situated in western Bulgaria, with the Iskar river gorge to the east, the Sofia valley and the capital city of Sofia to the south, and the mountains of Ponor and Chepan to the north. This landlocked territory is a part of the biogeographic region of the Balkans, Assenov [12]. The area of the former military unit of the Bulgarian army near Ponor village, where the Airsoft games are organized, is chosen by the game masters due to its perfect location near the capital city of Sofia and its background ideal for Airsoft battles.

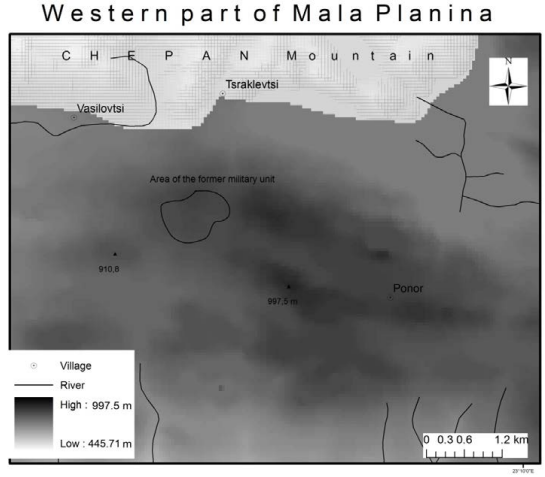

Fig. 2. A map of the western part of Mala Planina and the location of the Airsoft polygon (Borislav Grigorov)

The cartographic materials are prepared by the application of the software ArcGIS 9.3 and the presented detailed maps illustrate better the study, while the terrain research adds more depth. A main tool, used for the paper as a qualitative research instrument, is the semi-structured interview with one of the organizers of the game, called game masters. It consists of key questions, which provide an opportunity for pursuit of the main idea in more detail. This is its strongest point compared to the structured and unstructured interview. Moreover, the approach of the semi-structured interview is flexible and allows for the discovery of important information [13]. Interviews in general are most appropriate where our knowledge about the study phenomenon is restricted. At the same time, they are also particularly applicable for exploring sensitive topics, where participants may not want to talk about such issues in a group environment [13], although this is not the case in the study. The current semi-structured interview consists of questions, based on several fulcrums: the number of players per game and for a whole season, the frequency of the Airsoft battles, the duration of the games etc. and it provides enough information for the identification of a specific cultural ecosystem service in the western part of Mala Planina.

Results

Following the answers of the semi-structured interview, it became clear that the former military unit near the village of Ponor in the western part of Mala Planina is one of the favorite and most popular arenas in Bulgaria for organizing live action role-playing games, such as the Airsoft battles. Apart from the individuals who come to play, team buildings, organized for famous companies like Porsche and Vivacom, are also present, which may eventually lead to the increase of the popularity of the Ponor polygon. An important note which is necessary to be made is that this is not the only territory game masters use for their weekend activities. Similar Airsoft games are organized in the cities of Varna, Ruse, Plovdiv and Veliko Tarnovo, but the number of participants there is nearly the half of those who play in the area around Sofia. However, it turns out that the location near the Ponor village has some unique characteristics to offer. It is situated in a natural environment and not in an urban area, which provides different kinds of shelters and places for making ambush. Furthermore, the abandoned military buildings provide hideouts like no other. The presence of a bunker, which can be used as concealment or a target place, makes Ponor even more attractive, therefore more and more participants come for the battles. The zone is even more interesting, because it is situated near the territory with the highest conservation priority in Mala Planina - the Ranislavtsi Field, where three protected areas, home of three critically endangered plant species (*Lathyrus palustris, Plantago maxima, Salix rosmarinifolia*), are located. Following the information, presented above, it can be stated that the cultural ecosystem services that the western part of Mala Planina presents, are adopted in an untraditional way.

The Airsoft live action role-playing games are organized by “Airsoft Sofia” and “Airsoft Warriors” during the playing season between April and November (Table 1). The event arrangers of the battles have decided upon the duration of a standard game to be 5 hours, but there is a special battle three times per season in the area around Ponor. This game continues for more than 24 hours, with different rules and even foreigners come to participate. At

least 40-50 participants take part in all kinds of battles. The number reaches 110-115 at the height of the season, which means that the average count of the players is 75-80 per battle and around 640 players (a major share of them participate more than once) per season. According to the game master 80% of the participants in each battle have already taken part in the Airsoft LARPs and 20% are totally new.

There are four polygons present, which they use for the battles. They play every Sunday in the tank division in the town of Slivnitsa, located in an immediate closeness to the western boundary of Mala Planina, near Bosnek village (called "the southern Ponor" by them), in the forest around Novi han and, of course, in the former military unit near the village of Ponor. They also organized battles near the town of Bankya until recently. The LARP near the Ponor village are at least once a month. "Airsoft Warriors" plan to expand their events and play on Saturdays, as well, when the number of the participants in the battles will be of around 40 players and the polygon, which will be used for Saturday sports, is the one near Ponor village. The expected increase in the number of the attenders in the western part of Mala Planina leads to the conclusion that the cultural ecosystem services will be used in the future even more intensively.

Each Airsoft game in the area around Ponor gives the participants a sense of inspirational and spiritual enrichment, as the semi-structured interview confirms. The players also come to the polygon for a recreational purpose – to escape from their busy working weekdays. They relief the daily pressure by playing Airosft near Ponor and it is a perfect example for a cultural ecosystem service. The highest share of the games are of fictional character, in the form of defending a base, armed with a bomb or a virus, guarded by a group with a specific fictional features.

The Airsoft battles near Ponor can also be viewed as a tool for ecotourism, because the games are played in the open space and the participants get familiar with the natural environment in the area. They learn how to appreciate the aesthetic service, because the karst terrain and the views of Sofia valley provide a unique experience. Last, but not least, the educational cultural ecosystem service is also present in the form of studying about the flora and fauna of the area, as well as the geology and relief. The participants also learn how to survive in natural environment, because some of the battles are going on for 24 hours.

A central moment in the provision of ecosystem services in general is their cost. In this case study the participants do not pay for the game, because the organizers do not own the polygons. However, if you want to be a part of a battle near Ponor, you should spend some money on equipment. There are two options: you can rent or you can buy it. Airsoft Warriors offer a gun, a battery and protective glasses for rent at the price of 30 BGN. If you desire to own the equipment, than you have to broaden your perspectives. A middle class shotgun costs 360-460 BGN, a pistol – 210-280 BGN, a protective mask - 40 BGN and protective glasses – 20 BGN. A lot of the players own their armory, so a quick reckoning shows that each one of them has spent 630-800 BGN to shoot in the area around Ponor.

The provision of cultural ecosystem services in the western part of Mala Planina in the form of Airsoft games comes at a certain environmental price. Every player carries with himself at least one fully loaded magazine of 400 non-harmful pellets. According to the interviewed game master, a player fires an average number of 500 pellets per battle. The

size of the polymeric pellet is 6 mm and its weight is 0.28 grams. If we multiply the count of the shots per person with the weight of the pellet, we conclude that every participant releases 140 grams of plastic in the Ponor area per game. Multiplied by the number of players per battle, the weight of the plastic, left all around the territory, equals 11.2 kg. A quick calculation leads us to the estimation that the Airsoft LARPs leave behind 89.6 kg of plastic in the environment. However, it has to be noted that a part of the pellets are released in the bunker of the former military unit, but the total count is not clear.

Another environmental problem is that participants inevitably step on grassland species during their games, which turns into a problem, especially when humidity after raining has increased. Some of the typical grassland species for this part of Mala Planina, representative for two habitat types: Rupicolous calcareous or basophilic grasslands of the *Alysso-Sedion albi* (6110) and Eastern sub-mediterranean dry grasslands (62A0), such as *Satureja Montana, Chrysopogon gryllus, Artemisia alba, Teucrium polium, Sedum* spp., *Sempervivum* spp. etc., are damaged and it is difficult for them to recover. Players also place some of their equipment on the grass and although there is an asphalt road from Ponor village to the area of the former military unit, which can be used as a parking lot, as well, sometimes participants park their vehicles on the grass.

The interviewed game master shared some of their future plans, as well. They plan to prepare a project in the sphere of youth and sport, which will give them the opportunity to buy a terrain near the village of Ponor and develop it for more elaborate Airsoft battles. They intend to build shelters, embankments, barriers etc. and this undoubtedly will attract even more participants to the western part of Mala Planina. Another part of their future planning is the replacement of the polymeric pellets, fired by the replica weapons, with biodegradable ones. The ammunition they use now decomposes for years, while the decomposition of biodegradable pellets, depending on their composition, takes around two or three months. And last but not least, there are ongoing plans for increasing the number of 24 hour games in order to get more involvement and appreciation.

Table 1. The Airsoft live action role-playing game balance near the village of Ponor

Type of game	Playing season	Duration of a standard game	Average number of participants	Cultural services gained by the participants	Environmental cost per game in the form of plastic contamination
Airsoft live action role-playing game	April-November	5 hours	75-80	Inspirational Spiritual Recreation Ecotourism Aesthetic Educational	11.2 kg

Discussion

The current study, concerning the provision of cultural ecosystem services in the area of Ponor village in the western part of Mala Planina is an attempt to understand in a deeper context the interaction between natural environment and a live action role-playing game,

like Airsoft. The location of the Airsoft polygon in Mala Planina is more than attractive, following its former function as a military unit, surrounded by perfect natural environment for a LARP. The increasing popularity of Airsoft games expands their activities in another three polygons in Sofia's surroundings and similar battles are conducted near other major Bulgarian cities, such as Plovdiv, Varna, Ruse and Veliko Tarnovo. The seasonal character of the games entices around 640 players, who get involved in battles near Ponor at least one time a month on Sundays. They readily spend decent amount of money to get supplied with better equipment for the battles, which represent the monetary expenses people are willing to pay in order to enjoy the cultural ecosystem services in Mala Planina. However, from environmental point of view, Airsoft and pellets, in particular, have to be reckoned as a pollution factor, because of their polymeric origin. The matter should be regarded more seriously, because of the proximity of the territory with the highest conservation priority in Mala Planina - the Ranislavtsi Field, where three protected areas, where three critically endangered plant species (*Lathyrus palustris, Plantago maxima, Salix rosmarinifolia*) are located. Fortunately, the game masters realize that this fact should not be taken lightly and plan to change the pellets with biodegradable ones. Their plans of purchasing land for making their own polygon, as well as sending invitations for Saturday games, prove the importance and attractiveness of this particular area in the western part of Mala Planina for the development of Airsoft LARPs. As expected by the authors, the cultural ecosystem services will be used for a long period of time and there is an urgent necessity of a preservation of the regulating services. The ecosystem's state have to be stable and functioning so there is a need of the application more conservation measures. A possible example of such a measure is the instalation of a monitoring system, in the form of a remote sensing instrument, in order to guarantee the positive results of the preservation efforts in the area. We are convinced that this study can be used as a base for other studies, concerning the use of cultural ecosystem services in a mountainous territory in an unorthodox way. Our final implication is that the presented work shows promising results and it can be used as an example for more comprehensive and elaborate future investigation of the cultural ecosystem services in Mala Planina.

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THE EFFECT OF FOLIAR TREATMENT OF *DIANTHUS CARYOPHYLLUS F. SPRAY* HORT. WITH THE BIOMINERAL FERTILIZER PLANTAGRA

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Abstract

Aim: The objective of the present study was to explore the effect of the different number of treatments with the biomineral fertilizer Plantagra on the growth and development of spray carnation mother plants.

Materials and Methods: In 2013-2014, a pot trial with two Bulgarian spray carnation cultivars – Russalka and Naslada was carried out in glasshouse conditions at the Institute of Ornamental Plants, Sofia.

Results and Discussion: The positive effect of the biomineral fertilizer was proven in all variants of the trial in both cultivars; the plants were better developed with regard to height, bigger number of lateral branches and more productive cuttings, compared to the untreated controls.

Conclusions: The best result was reported for the variant with four treatments of mother plants with 0.14% solution of Plantagra, where the production of cuttings exceeded that of the untreated plants with 15.4% in cv. Russalka and 25.0% in cv. Naslada.

Keywords: spray carnation, leaf treatment, fertilizer, growth, productivity of cuttings

Introduction

The conservation of the environment and human health require a careful choice of the types of fertilizers, application rates and timing in order to keep the residues in the soil within the admissible limits, ruled by the international standards [1].

A wide range of organic fertilizers have been manufactured and released on the market in the recent years but their effects have not been investigated in all crops [2]. Flowers need a rational fertilization system that ensures a balanced nutrition during their development [3, 4].

The scientific research of the biological tests of organic fertilizers in flowers is much scarcer than in other crops. The Institute of Ornamental Plants in Sofia has conducted such studies on a number of flower species (spray-carnation, chrysanthemum, lilium, gypsophila, aster and calendula, etc.) in order to establish the effect of the complex mineral fertilizers such as Vege, Lactofol, Crystalon and HortiGrow [5, 6, 7, 8, 9]. The effect of organic fertilizers was studied as Biostim, Humustim, Lumbricol and Baykal in cut flower cultures (spray-carnation, chrysanthemum and gypsophila), flowering pot species (petunia, impatiens, miniature roses, carnation and chrysanthemum) and annual flowers [10, 11, 12, 13, 14, 15].

The objective of the present study was to explore the effect of the different number of treatments with the biomineral fertilizer Plantagra on the growth and development of *Dianthus caryophyllus* f. *spray* Hort., Caryophyllaceae (spray carnation) mother plants.

Materials and Methods

In 2013 – 2014, a pot trial was carried out at the Institute of Ornamental Plants – Sofia, with two Bulgarian spray-carnation varieties – Russalka and Naslada that studied the effect of the biomineral fertilizer Plantagra on the initial growth and development phases of mother plants for the production of cuttings.

Plantagra is essentially a biomineral humic product with immobilized enzyme systems and live cells of fungi, bacteria and other microorganisms that are beneficial for the plants. The biomineral fertilizer Plantagra is a mineral based well balanced microfertilizer composition of organic substances. The manufacturer of the product is the company Romb Ltd., Sofia.

In vitro obtained and *ex vitro* adapted plants were used for the trial. They were planted on January 10 in glasshouse in pots No. 9 in a substrate of soil, peat and perlite in a ratio of 2:2:0.5.

Two pinchings were performed at the beginning of April and in the middle of June for the formation of mother plants.

The experiment was carried out in four variants with a different number of foliar treatments with 10 plants per variant including untreated control plants:

Variant I – untreated plants (C);

Variant II – double treatment with 0.14% solution of Plantagra;

Variant III– triple treatment with 0.14% solution of Plantagra;

Variant IV– four times treatment with 0.14% solution of Plantagra.

Four foliar treatments were performed every 10 days, the first one being on June 20 at the start of the trial.

The concentration of the working solution (0.14%) used in the variants of the trial was proved optimal for foliar treatment in our previous studies.

The initial measurements of the height and number of lateral branches were taken prior to the treatment of the plants with the biomineral fertilizer; the first index report was done 10 days after the treatment with Plantagra and the subsequent ones – every 20 days.

The cuttings were harvested 3 times, first on July 5 (15 days after the first treatment) and the subsequent ones – every 30 days.

Statistical data processing was done with ANOVA Single factor test. The significant difference between the control and variants was presented as * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$) and the non-significant – as ns.

Results

Table 1 shows the results on plant height following foliar treatment with the biomineral fertilizer Plantagra. The effect on plant height was positive in all trial variants and both spray carnation cultivars. The growth rate increased with the increase of the number of treatments and varied within 5.6% – 25.9% in cv. Russalka and 15.8% – 19.3% in cv. Naslada. The differences in the growth rate of the plants in the variants with triple and four times treatment vs. the control were significant at $P \leq 0.01$ and $P \leq 0.001$ except for Variant II (double treatment) of cv. Russalka (ns).

Table 1. The effect of different number of treatments with the biomineral fertilizer Plantagra on spray carnation height

Variant	Average plant height							Total growth	
	20.06	30.06.		20.07.		10.08.		cm	% vs. C
	initial, cm	cm	%	cm	%	cm	%		
cv. Russalka									
I – untreated plants (C)	14.8	15.7	100.0	18.9	100.0	20.2	100.0	5.4	100.0
II – double treatment with 0.14%	14.8	15.9	101.3	19.4	102.6	20.5	101.5	5.7 ns	105.6
III – triple treatment with 0.14%	14.8	16.0	101.9	19.2	101.6	21.0	104.0	6.2 **	114.8
IV – four times treatment with 0.14%	14.8	16.5	105.1	19.5	103.2	21.6	106.9	6.8 ***	125.9
cv. Naslada									
I – untreated plants (C)	14.2	15.8	100.0	17.0	100.0	19.9	100.0	5.7	100.0
II – double treatment with 0.14%	14.2	15.5	98.1	16.4	96.5	20.8	104.5	6.6 ***	115.8
III – triple treatment with 0.14%	14.2	15.7	99.4	16.5	97.0	20.7	104.0	6.5 ***	114.0
IV – four times treatment with 0.14%	14.2	15.9	100.6	16.7	98.2	21.0	105.5	6.8 ***	119.3

* ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), non-significant – ns

The number of treatments also had a positive effect on the development of the lateral branches in spray carnation in all the trial variants (double, triple and four times treatment) (Table 2).

The effect was expressed better in cv. Russalka with the highest growth rate reported in Variant IV (four times treatment) and in cv. Naslada – in Variant III (triple treatment) with the growth rate values exceeding those of untreated plants with 28.6% and 15.0%, respectively. The results were significant only in cv. Russalka ($P \leq 0.05$, $P \leq 0.01$).

Table 2. The effect of different number of treatments with the biomineral fertilizer Plantagra on spray carnation lateral branches

Variant	Average number of lateral branches per one plant							Total growth	
	20.06	30.06.		20.07.		10.08.		no.	% vs. C
	initial, no.	no.	%	no.	%	no.	%		
cv. Russalka									
I – untreated plants (C)	3.1	4.0	100.0	5.0	100.0	5.2	100.0	2.1	100.0
II – double treatment with 0.14%	3.1	4.2	105.0	5.4	108.0	5.6	107.7	2.5 *	119.0
III – triple treatment with 0.14%	3.1	4.3	107.5	5.6	112.0	5.6	107.7	2.5 *	119.0
IV – four times treatment with 0.14%	3.1	4.2	105.0	5.3	106.0	5.8	111.5	2.7 **	128.6
cv. Naslada									
I – untreated plants (C)	2.8	3.4	100.0	4.2	100.0	4.8	100.0	2.0	100.0
II – double treatment with 0.14%	2.8	3.9	114.7	4.3	102.4	5.0	104.2	2.2 ns	110.0
III – triple treatment with 0.14%	2.8	4.0	117.4	4.6	109.5	5.1	106.3	2.3 ns	115.0
IV – four times treatment with 0.14%	2.8	4.4	129.4	5.0	119.0	5.0	104.2	2.2 ns	110.0

* ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), non-significant – ns

Table 3 shows the results for the productivity of cuttings, obtained following different number of treatments with the biomineral fertilizer. The positive effect of foliar treatment in both spray carnation cultivars was expressed better with more applications (triple and four times) with higher relative percentage vs. the control, namely 15.4% and 10.2% in cv. Russalka, respectively, and 25.0% and 21.9% in cv. Naslada. The differences with the control were significant only in Variants III and IV ($P \leq 0.05$ and $P \leq 0.001$).

Table 3. The effect of different number of treatments with the biomineral fertilizer Plantagra on spray carnation cuttings productivity

Variant	Total productivity of cuttings/variant		Productivity of cuttings per harvest					
	no.	% vs. C	05.07.		05.08.		05.09.	
			no.	%	no.	%	no.	%
cv. Russalka								
I – untreated plants (C)	39	100.0	14	35.9	10	25.6	15	38.5
II – double treatment with 0.14%	41 ns	105.1	17	41.5	6	14.6	18	43.9
III – triple treatment with 0.14%	43 *	110,.2	17	39.5	8	18.6	18	41.9
IV – four times treatment with 0.14%	45 * * *	115.4	18	40.0	7	15.6	20	44.4
cv. Naslada								
I – untreated plants (C)	32	100.0	15	46.9	7	21.9	10	31.2
II – double treatment with 0.14%	35 ns	109.4	17	48.6	8	22.8	10	28.6
III – triple treatment with 0.14%	39 * * *	121.9	19	48.7	9	23.1	11	28.2
IV – four times treatment with 0.14%	40 * * *	125.0	21	52.5	8	20.0	11	27.5

* ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), non-significant – ns

Discussion

The positive results of the foliar treatment of spray-carnation with Plantagra came from the total functional effect of the microbial complex on plant development that stimulated the root system, improved the plant habitus and increased the yield of cuttings. The good results from the application of the new organic and mineral fertilizers were due to the balanced formulas with rich content of organic matter, macro and micro elements, vitamins, humic acids and hormones, on the one hand and the easily assimilated form of the nutrients, on the other.

Our investigations on the biomineral fertilizer Plantagra once again confirmed the advantages of the modern organic and mineral fertilizers. The application of the organic fertilizers Humustim and Lumbricol, tested in gypsophila, potted (carnation and chrysanthemum) and annual flowers (petunia and impatiens) also showed a positive effect on plant growth and development [11, 14, 15]. The study of the new complex mineral fertilizer HortiGrow also showed a positive effect on the total habitus and the separated phases of the development of spray-carnation, cyclamen and gypsophila [3, 8, 9]. The positive effect of the organic fertilizers on flowering cultures is a solid proof of the improved plant growth and development, environmental safety and human health protection.

Conclusions

• The plants of the Bulgarian spray carnation cultivars Russalka and Naslada developed better following the foliar treatment with the biomineral fertilizer Plantagra – they were

higher, with more lateral branches and higher cuttings productivity compared to the untreated plants.

• The best results were obtained after four times treatment of the mother plants with a 0.14% solution of Plantagra, where the productivity of cuttings exceeded that of the untreated plants with 15.4% in cv. Russalka and 25.0% in cv. Naslada.

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A RAPID METHOD FOR VULNERABILITY ASSESSMENT OF COASTAL PLANT COMMUNITIES FROM FLOODING CAUSED BY UNUSUAL STORMS

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Abstract

Aim: This paper proposes a rapid method for vulnerability assessment of the coastal plant communities from flooding caused by unusual storms over the Bulgarian Black Sea Coast.

Materials and Methods: In order to create a dynamic GIS model, data from experimental results and detailed GIS mapping on the Kabakum beach (Varna) were incorporated. As a result of a simulated flooding experiment, the Critical Decomposition Time (CDT) was obtained.

Results: Linking flood duration with CDT and altitudinal spreading of plants determines that *Artemisia vulgaris* L., *Eryngium maritimum* L. and *Crambe maritima* L. are vulnerable to storms.

Conclusions: The plant communities in Kabakum beach are not threatened by complete destruction even during a storm with a return period of 100 years. Habitat recovery is likely within a season and does not require human intervention.

Keywords: Vulnerability assessment, plant communities, GIS, floods, Black Sea coast

Introduction

The Varna city coastal area in Northeastern Bulgaria is relatively protected from floods. The main reasons for this are the small amplitude tides in the Black Sea and the lack of big rivers flowing into the Varna Bay. The only risk comes from extreme meteorological events

such as unusual storm surge levels in combination with reinforced wave upon the shore [1]. Recorded damages from storms over the Bulgarian Black Sea Coast [2] showed the negative impact to the dunes and the high potential of the root systems of some of the native psammophytes to accumulate sand and prevent from washout [1, 3].

Although the coastal plant communities are well adapted to salt stress due to their regular exposure to sea water some of the species are vulnerable and sensitive to the impact of flooding [3]. Plants could be affected by direct storm-wave damage during which they are uprooted or buried by sediments [1]. These factors in combination with the increasing human impact may lead to various negative consequences to coastal plant communities in the long term.

The necessity to assess and quantify these negative effects on natural habitats requires the development of rapid models for vulnerability assessment. Different flood scenarios and models were introduced in order to assess the possible negative consequences to the coastal areas from storms [1, 4]. However, most of them are focused only to the socio-economic dimensions [5, 6].

This paper proposes a rapid method for vulnerability assessment of the coastal plant communities from flooding caused by unusual storms over the Bulgarian Black Sea Coast. This model allows experimental results to be applied directly to the present situation to predict the effects of future storm events on the dune vegetation.

Materials and Methods

Simulated flooding experiment. Ten specimens of each investigated species were collected at Kabakum Beach, Varna (43°18′20.92″N, 28°03′13.51″E) in April 2016. The plants were planted in washed and sterilized sand in plastic pots and immersed in glass tanks (40 l), filled with sea water at a depth of 2–4 cm below the water surface with constant maintained temperatures (4±1ºC, 13±1ºC and 23±1ºC) for 20 days. The water was changed several times per day. Visible morphological changes of different parts of the specimens (leaves, stems, roots) were recorded and assessed in 12 parameters [3].

Intensity Duration Frequency (IDF) vs. altitudinal spreading. Various datasets and models were used in order to estimate the possible negative consequences of flooding to plant communities. To implement the hydrodynamical modelling, detailed topography and bathymetry surveys were carried out and 2 typical cross-shore profiles were constructed along the Kabakum Beach. The topography surveys were accompanied by detailed GIS mapping of the plant communities. Flood duration was calculated for four return periods (RP) – 5, 20, 50 and 100 years. IDF (Intensity Duration Frequency) functions were constructed for each representative cross-shore profile [1].

Results and Discussion

A flowchart of the rapid method for vulnerability assessment of coastal plant communities from flooding caused by unusual storms over the Bulgarian Black Sea Coast is presented in Fig. 1.

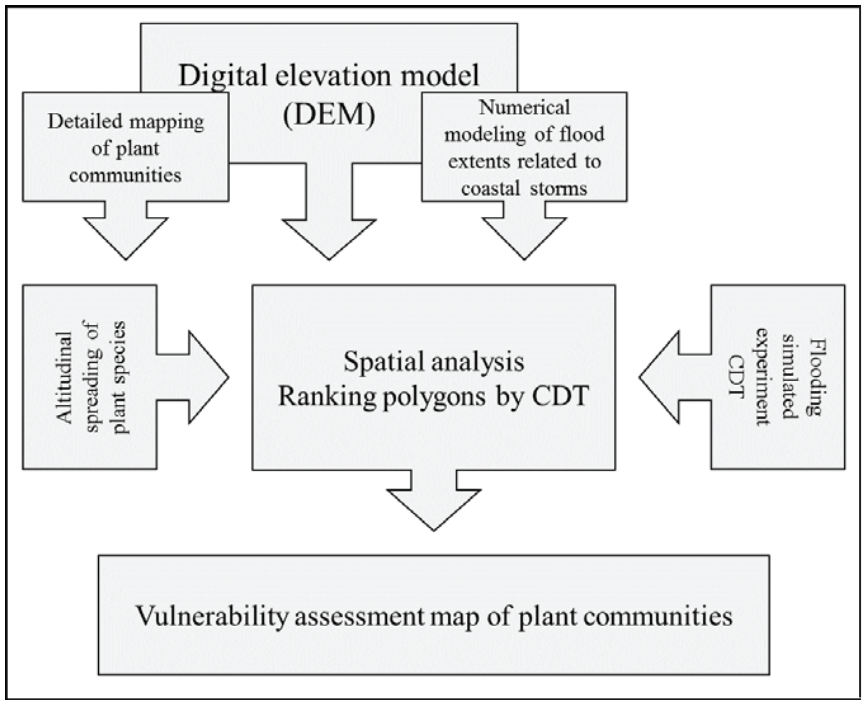

Fig. 1. Flowchart of the rapid method for vulnerability assessment of the coastal plant communities to flooding caused by unusual storms over the Bulgarian Black Sea Coast

Different flood patterns were explored by means of morphodynamical modeling [1]. Forcing relevant for present climate conditions was obtained using statistical analysis of surge data and numerical modeling of wave action. The interpretation of the results links flooding duration and elevation above the mean sea level due to surge events for four return periods: 5, 20, 50, and 100 years [1]. The flood durations from a number of scenarios were ranked by flood elevation in order to produce intensity–duration–frequency (IDF) curves [5].

A full inventory of the vascular plants was conducted in the investigated area between May and October 2011 and 2015. Overall 14 plant species were recorded and mapped but according to flood maps [1] only 8 of them are threatened by flooding: *Crambe maritima* L., *Artemisia vulgaris* L., *Eryngium maritimum* L., *Salsola ruthenica* Iljin, *Xanthium strumarium* L., *Cynodon dactylon* (L.) Pers., *Leymus racemosus* subsp. *sabulosus* (M. Bieb.) Tzvelev and *Ammophila arenaria* (L.) Link.

The proposed model is based on the experimental methods of simulating sea water floods without taking into consideration the direct mechanical effects of storm waves. A previous study [3, 7] demonstrated that experimental methods of direct submergence were more appropriate than the experiments of studying substrate salinity and salt spray due to the regular exposure of dune plants to sea water and their specific mechanisms of neutralizing sea water salt.

Crucial for the development of the method is the experimental results. As a result of the test, the Critical Decomposition Time (CDT) was obtained (Table 1). It is defined as the time point at which each plant, submerged in sea water, shows signs of irreversible decomposition of vegetative organs [5] and indicates that the plants will not survive and their communities will not be able to recover after floods. CDT is a parameter that is subjectively determined on the basis of visible morphological changes and shows the smallest degree of decay of plant organs (most often the leaves).

Although the storm events at the Black Sea Coast occur during winter and early spring when average surface sea water temperature is about 4ºC [8] two other treatments with temperatures of 13ºC (average surface sea water temperature) and 23ºC (average summer surface sea water temperature) were included in the simulated experiment in order to study the relation between temperature and CDT [9]. Most of the investigated parameters were unrelated to water temperature except those concerning leaves decay of *C. maritima, E. maritimum* and *X. strumarium*.

Table 1. Results from the simulated flooding experiment. Visible morphological changes of different parts of the specimens (leaves, stems, roots) assessed in 12 parameters. Data in shaded cells are accepted as CDT

Plant	T ºC	Parameters											
		Beginning of decomposition of			Complete decomposition of			Growth of		Beginning of decomposition of newly grown		Complete decomposition of newly grown	
		leaves	stems	roots	leaves	stems	roots	stems	roots	stems	roots	stems	roots
C. maritima	4	48	168	192	96	312	336	n/a	n/a	n/a	n/a	n/a	n/a
	13	42	168	192	96	312	336	n/a	n/a	n/a	n/a	n/a	n/a
	23	42	168	192	96	312	336	n/a	n/a	n/a	n/a	n/a	n/a
A. vulgaris	4	48	192	240	114	336	360	n/a	120	n/a	216	n/a	312
	13	48	192	240	114	336	360	n/a	120	n/a	216	n/a	312
	23	48	192	240	114	336	360	n/a	120	n/a	216	n/a	312
E. maritimum	4	48	168	192	96	312	336	n/a	n/a	n/a	n/a	n/a	n/a
	13	48	168	192	96	312	336	n/a	n/a	n/a	n/a	n/a	n/a
	23	42	168	192	96	312	336	n/a	n/a	n/a	n/a	n/a	n/a
S. ruthenica	4	72	264	312	216	384	408	n/a	120	n/a	384	n/a	408
	13	72	264	312	216	384	408	n/a	120	n/a	384	n/a	408
	23	72	264	312	216	384	408	n/a	120	n/a	384	n/a	408
X. strumarium	4	96	240	288	240	360	384	240	240	288	288	432	432
	13	96	240	288	234	360	384	240	240	288	288	432	432
	23	88	228	288	234	336	384	240	240	282	288	432	432
C. dactylon	4	144	264	360	408	n/a	n/a	120	144	n/a	n/a	n/a	n/a
	13	144	264	360	408	n/a	n/a	120	144	n/a	n/a	n/a	n/a
	23	144	264	360	402	n/a	n/a	120	144	n/a	n/a	n/a	n/a
L. racemosus subsp. *sabulosus*	4	168	n/a	n/a	480	n/a	n/a	168	168	n/a	n/a	n/a	n/a
	13	168	n/a	n/a	480	n/a	n/a	168	168	n/a	n/a	n/a	n/a
	23	168	n/a	n/a	480	n/a	n/a	168	168	n/a	n/a	n/a	n/a
A. arenaria	4	168	n/a	n/a	456	n/a	n/a	168	168	n/a	n/a	n/a	n/a
	13	168	n/a	n/a	456	n/a	n/a	168	168	n/a	n/a	n/a	n/a
	23	168	n/a	n/a	456	n/a	n/a	168	168	n/a	n/a	n/a	n/a

The integration of results from the altitudinal spreading of the investigated species and IDF functions, as well as linking of flood duration and CDT for these species, shows that the communities of *A. vulgaris, E. maritimum* and *C. maritima* are vulnerable to storms in the investigated area (Fig. 2).

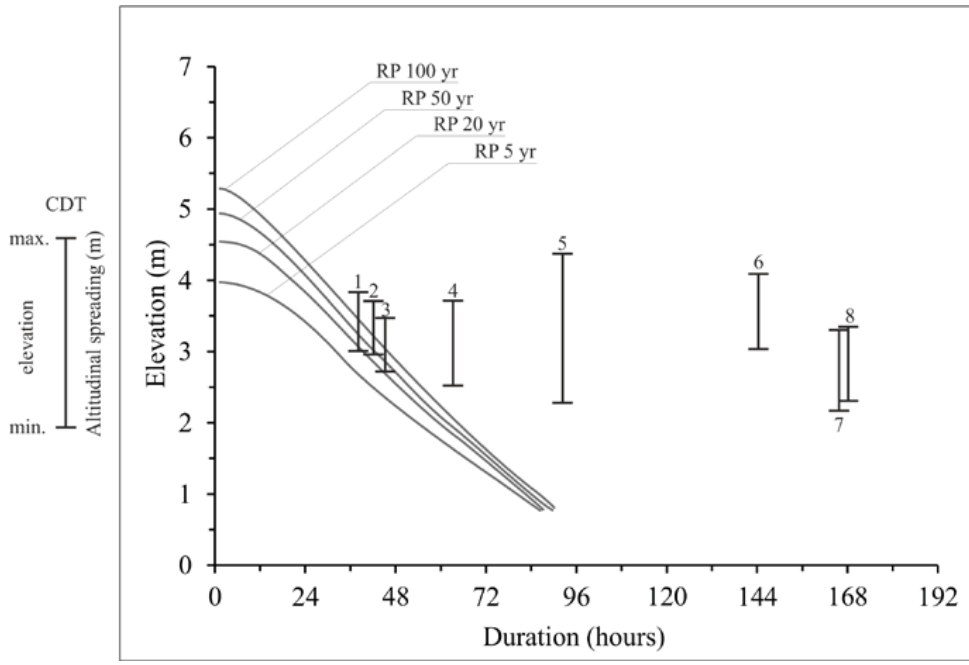

Fig. 2. IDF (Intensity Duration Frequency) functions and spatial distribution vs. CDT.
1. *C. maritima*, 2. *A. vulgaris*, 3. *E. maritimum*, 4. *S. ruthenica,* 5. *X. strumarium*, 6. *C. dactylon*, 7. *L. racemosus* subsp. *sabulosus*, 8. *A. arenaria*

According to the experimental results the investigated species can be divided into 3 groups: CDT < 48 h, CDT = 48 – 96 h, and CDT > 96 h.

The potentially threatened plant communities were mapped and ranked by CDT (Fig. 3) in order to produce vulnerability assessment maps. The maps in the present case study were constructed for most significant floods with greatest extents related to eastern storm events with RP 100 yrs. [1].

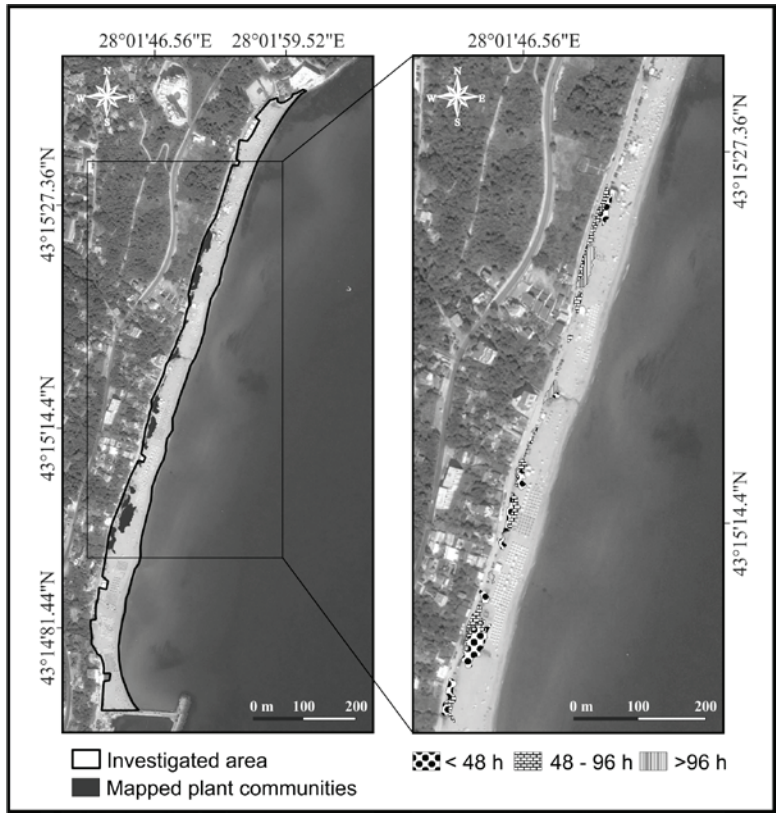

Fig. 3. Map of the investigated area and mapped plant communities at Kabakum beach (left) and vulnerability assessment map with ranked polygons by CDT (right) for floods with greatest extents, which are related to eastern storm events with RP 100 yrs. (Satellite images: Google Earth 2017, TerraMetrics; CNES/Airbus)

The plant communities in Kabakum beach are not threatened by complete destruction, even during a storm with return period of 100 years. The recovery of the habitat does not require human intervention and full recovery is likely within a season, therefore the Environmental Vulnerability Index [10] could be estimated to be equal to 1.

Conclusions

The proposed GIS-based rapid method for vulnerability assessment is applicable and has allowed the experimental results to be applied directly to a present situation to predict the effects of future storm events on the dune vegetation. The integration of the results from the inventory, distribution and altitudinal spreading of the plants and the flooding maps of Kabakum Beach shows that only 8 species are potentially threatened by flooding during storm events. Linking flood duration and CDT for these species determines that *Artemisia vulgaris*, *Eryngium maritimum* and *Crambe maritima* are vulnerable to storms. The plant

communities in Kabakum beach are not threatened by complete destruction even during a storm with a return period of 100 years. The recovery of habitat does not require human intervention and full recovery is likely within a season. Therefore, the Environmental Vulnerability Index could be estimated to be equal to 1.

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REPORTS

Topic:
LANDSCAPE ECOLOGY

CHALLENGES IN BIOGEOGRAPHY DEVELOPMENT

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Abstract

Aim: The aim is to reveal the challenges that research methodology in Biogeography faces today.

Materials and Methods: The interdisciplinary nature of biogeography as a synthetic science creates a prerequisite for the use of a wide range of methods that are typical of the main nature sciences – biology, geography, ecology, paleontology, physics, chemistry, mathematics, even medicine. The emerging new sub-disciplines in biogeography are analyzed as they are a challenge for the development of biogeography's subject of research.

Results: Periodization of the development of biogeography based on the analysis of the progress of biogeographic sub-disciplines and changing philosophical views in the development of science is made. The parallel comparison of achievements in different biogeographical sub-disciplines globally is carried out demonstrating the wide-aspect horizontal interactions of biogeographers in Europe, North and South America, Australia, New Zealand and many of the Commonwealth countries.

Conclusion: Determining the subject of biogeography is a complex issue arising from the interdisciplinary nature of science itself, and the performed analysis of modern biogeographic methodology and emerging sub-disciplines shows a shift of the subject to biodiversity as a scientific category.

Keywords: biogeography, subject, paradigm, sub-disciplines

Introduction

Biogeographic studies from this century reflect a change in the subject of biogeography, characterized by expanding and refining the methodology. The famous American geographer and ecologist Glen Michael MacDonald in his book "Biography: Space, Time and Life" [1] uses examples of key biogeographical theories that appoint space and time as a prerequisite for the distribution of living organisms. McDonald accepts that the synthetic nature of biogeography is a precondition for the many different jobs positions in which biogeographers have been employed - faculties of geography, biology, geology, paleontology and anthropology. One way to deal with the complexity of the subject of biogeography McDonald [1] sees in the emergence of new divisions and sub-disciplines.

At the end of the 19th century Ratzel made a remarkable theoretical contribution to geography by introducing the term "biogeography" [2]. Influenced by the ideas of Darwin,

Haeckel, and Wagner, he formulated the thesis of common biogeography as a link between space and human activity, establishing a connection between physical and human geography. His sudden death in 1904 hindered the completion and publication of his great synthesis of general biogeography. The achievements of biogeography at the end of the 20th century are related to the thematic expansion in the science diversification, which is the most important driving force for the thriving growth in the number of publications, leading to increased awareness of biogeography as a strict science.

Materials and Methods

The aim of the paper is to reveal the contemporary challenges to biogeography research methodology.

The interdisciplinary nature of biogeography as a synthetic science creates the precondition for the use of a very wide range of methods that are inherent in the main nature sciences - biology, geography, ecology, paleontology, physics, chemistry, mathematics and even medicine. An analysis of the emerging new sub-disciplines in biogeography has been carried out, which is a challenge for the development of the biogeographic subject of research. In-depth analysis of existing disputable issues with regard to the new sub-disciplines in biogeography has been made and a review of their critical perceptions has been carried out, compared over time in the context of their transformation with the corresponding changes and innovations through the rapidly evolving computer technologies. The most important studies relevant to biogeography in the 19th, 20th and 21st centuries have been highlighted and new definitions have been proposed to the theoretical foundations of the science.

Results and Discussion

Development of Biogeography in the 19th Century: After Darwin the development of biogeography shows a number of scientific trends - genetic, analytical and ecological, whose achievements reflect the pre-paradigm stage, characteristic for most natural sciences during this period. After the introducing of the term "biogeography" by Ratzel (1888) [2] this field of science began its existence. Although the philosophical views in that period were still of a pre-scientific nature, in the 19th century the works of Unger [3] could be attributed to the genetic trend, and those of de Candolle – to the analytical trend. The latter spatially divides the Earth's vegetation into 20 flora or areas, using data for his analysis from geological history [4]. Wagner enriches biogeography methodologically with his concept on the species migration [5]. Humboldt is considered to be the founder of biogeography, but his merits are mainly in phytogeography [6]. Darwin's theory also contributes to the rapid advancement of the zoogeographic studies [7]. Using statistical methods, Sclater [8] developed a system of faunistic areas, which with some changes, is widely accepted today. In 1868 Huxley [9] proposed a zoogeographical land zoning, reflecting the centers for classes' development among mammals. Based on that idea, Lydekker separated three kingdoms: Notogea, Neogeia and Arctogua [10]. The historical principle of zoogeography is endorsed by Wallace, who used the evolutionary theory and accumulated paleontological data to build a system of zoogeographic

areas similar to Huxley [9] and Lydekker [10] but also introduced the category of sub-regions [11]. A deficiency in both Wallace's and Darwin's perceptions is the concept of continents and oceans constancy. The ecological principles in land zoogeographic zoning were applied by Severtsov and also by his disciple Menzbir, who included in the faunistic zoning not only the systematic categories of order, family and genus, but also species [12]. This is the era of gradual clarifying (use another word) of pre-scientific approaches in the various branches of nature sciences, and the gradual formation of specific sciences' paradigms, which in the 20th century underwent rapid development and significant changes.

Development of Biogeography in the First Half of the 20th Century: At the beginning of the 20th century the predominant part of the phyto- and zoogeographic studies accepted the organisms' paradigm, and biogeography was very closely related to ecology. At the same time, Berg defined the law of longitude zonality [13] and developed the science of geographic landscape, while the German geographer Carl Troll set the scientific foundations of landscape ecology [14]. The dominant organisms' paradigm determined the still weak link between phytogeography and zoogeography. A considerable number of books were published, including the "Plant Geography" of the German scientist Diels [15]. Other significant publications include: "Fundamentals of Physical Geography", volume III - "Biogeography", by the French scientist de Martonne [16], "Plant Communities on Earth" by Rübel from Switzerland [17], and "Geography of Plants and Animals" by Newbigin [18] from Scotland. Between the First and the Second World War Russian biogeography also developed under the influence of the organisms' paradigm. The most important publications of Russian geographers include the "General zoogeography" by Geptner [19] and the "Historical Geography of Plants" by Wulff [20]. Other substantial contributors from this period were the English biogeographer Good with his "Geography of Flower Plants" [21], and the Canadian biologist Dansereau [22], who interpreted biogeography within its relations with ecology in the publication "Biogeography in Ecological Perspective". The last two authors, and many others since the end of the Second World War, were aware of the rapid changes in the development of science and gradually, through their work, they abandoned the organisms' paradigm and orientated towards new philosophical views.

New Methods and Approaches in Biogeography from 1950 until now: In the 1950s, the ideas of the continuum (continuity) started to make their way as a paradigm, following the publications of Curtis and McIntosh [23] and Whittaker [24]. In the second half of the last century the development of biogeography was based on the continuum namely, resulting in forming a unified methodological nature of the biogeographic science. Most significant was the development of the toolkit of historical biogeography, which can be presented in scientific and methodological terms by the following approaches and methods:

1. Studying the centers of origin and dispersal. This approach assumes that spreading and extinction are the only historical events that are involved in the distribution of taxa. This approach has been compromised with the adoption of global tectonics in the second half of the XX-th century, but in recent years has been re-used in biogeography, for example as an approach to ancestral areas.

2. The phylogenetic approach that provides the basis for the phylogeography sub-discipline also considers dispersal and extinction as key factors in the distribution of species.

Many studies have been published over the past few years (especially with molecularly-based phylogeny) in which explicitly (Knox and Palmer) [25] or implicitly this method is applied in the part of its assumptions. Studies are often conducted with molecular cladograms in which taxa have been replaced in the areas they occupy to postulate the event of spreading, which is supposed to have a direction from the base to the top of the cladograms. The new sub-discipline phylogeography has an explosive growth and is considered to play a dominant role in modern historical biogeography [26, 27]. Based on mitochondrial DNA analysis (animals) and chloroplast DNA analysis (plants), phylogeography has developed tools to reconstruct phylogeny and population genetic structure through gene traits within species and between closely related species, thus generating new insights of evolutionary and geographic responses for species and populations related to the dynamics of Earth's history [28, 29]. The impact of phylogeography is revolutionary in both perspectives - geographic and biological. Biologists are able to match evolutionary models of divergence in organisms with patterns of environmental change over the last few million years, thus being able to overcome the lack of integration between historical biogeography and ecological biogeography lasting until 1990 [30]. Phylogeography works with the spatial and temporal parameters of genealogy and can thus be considered as a branch of biogeography. Through its innovative genealogical perspectives and its significant links with various areas of micro- and macro-evolutionary theory, biogeography through ecogeography, phylogeography and molecular genetics will continue to provide a robust empirical and conceptual bridge between major evolutionary disciplines that have limited communication with each other. An integrated method of geographical diversification is proposed within the scope of the species, which allows conclusions to be drawn about the geographical location of the ancestors. This method, called phytoene desaturaze [31], has been used to model the migratory processes of the phylogenetic tree, which allows the historical geographic position of the ancestors to be restored over time. For example, the method has been applied for diversification in two non-tropical birds genera - Psophia tubers (Aves: Gruiformes, Psophiidae) and Sparrow Cinclodes (Aves: Passeriformes, Furnariidae).

3. The method of ancestral areas duplicates the idea of a center of origin in phylogenetic biogeography. Its main focus is to postulate the hypothesis of distribution as a history of individual groups. The areas of the individual taxon are considered to be a valid part of the study of the natural history of a particular group, which can be determined by topological information from the cladograms.

4. Panbiogeography is based on the notion that land and life are developing together. It aims to re-introduce the spatial significance or the geographic dimension of life diversity into the understanding of evolutionary models and processes. This method allows to identify the ancestral biotas and suggests the possibility of spreading, vicariance and extinction, and the focus is the biotas' history. Panbiogeography is criticized for the uncritical use of systematics.

5. The cladistic biogeography is an "areal biogeographic" research program. Its purpose is to search for a model in the relationship between the areas of endemism that arise repeatedly in the phylogeny of various taxa, which may correspond to events in the history of the Earth. The ultimate goal of the cladistic biogeography is to interpret the history of the areas

by means of distribution and phylogenetic information on taxa. Cladistic biogeography suggests that correspondence between phylogenetic links and spatial relationships is biogeographically informative. Comparisons between the cladogram ranges obtained from different taxa appearing in a particular region allow common patterns of explanation. Such patterns are most likely caused by allopatric speciation, originating from vicariance events. On the other hand, the uncertainty associated with these models may be due to sympatric taxa that do not make allopatric speciation or dispersal events. The cladistic biogeography is criticized by Donoghue and Moore [32], because its methods completely ignore the time of diversification of constituent traits (the ancestry components). These authors argue that neglecting time information conceals the relationship between biogeographic patterns and the reasons causing them.

6. Parsimony analysis of endemicity (PAE). This approach is based on the ideas of Rosen [33]. PAE classifies areas, quadrates or regions (analogous to taxa compared to the analysis of phylogenetic systematics) according to their shared (common) taxa, using the most economical solution (parsimony principle). The purpose of this approach is to clarify the history of the regions or localities. Crisci et al. [34] distinguish three variants of the PAE which they have classified as follows: PAE based in localities - Rosen [33], PAE based in areas of endemism - Craw [35], and PAE based on quadrates - Morrone [36]. The latter method has a different purpose than the other two methods. Rosen and Craw's two variants of parsimony analysis aim to clarify the history of areas, while Morrone's method aims to identify endemic areas based on shared presence of taxa. Although the methodology of PAE is analogous to cladistic systematics, this approach is not related to cladistic biogeography because its objectives and assumptions are quite different. Some authors assume that the PAE is conceptually related to panbiogeography [37]. The main criticism of parsimony analysis is that it ignores the cladistic (i.e. phylogenetic) relationship between taxa and only considers their distribution [38]. This critique is only applicable to the variants of parsimony analysis which aim to clarify the history of areas, i.e. those of Rosen and Craw.

7. Event-based methods. According to Ronquist [39], this approach is the starting point for explicit models of processes that affect the geographic distribution of living organisms. The main focus is the distribution and history of specific taxa. However, recent uses of the method are related to solving the problems with biogeographical regions [40, 41]. Different types of processes (dispersal, extinction and vicariance) are identified with cost-benefit target values. A conclusion is drawn on the distribution and history of the taxon based on its phylogenetic information by applying the criterion of maximum benefits and minimal costs in keeping with biogeographic processes (e.g. maximizing vicariance and minimizing dispersal and extinction). According to some authors [42, 43], the main argument against this approach is the commitment to explicit models and the possibility of their infinite combination.

8. The phylogeographic approach was originally proposed by Avise et al. [44] and can be defined as studying the principles and processes governing the geographical distribution of genealogical traits at the intra-species level using mitochondrial DNA (mtDNA) for animals and chloroplast DNA (cpDNA) for plants. Its purpose is to understand the distribution and

history of the populations and subsequently the history of the regions. This approach uses individuals whose genotype originates from maternal traits and the resulting phylogeny is linked to geographic distribution patterns. This approach implies the possibility of dispersal and vicariance, and implicit extinction. The statistical methods it includes are developed as demographic-phylogenetic methods in phylogeography. Among them is an inserted monophyletic group for phylogeographic analysis (analysis of the inserted monophyletic groups) [45]. Critics consider phylogeography as an isolated and limited approach within the harmonious and interdisciplinary biogeography, which fails to address the context of pre-history [46].

9. Experimental biogeography was proposed by Haydon et al. [47], who, through computers, have repeatedly modeled faunistic accumulations against a fixed vicariance background on ecological and evolutionary time scales. Experimental biogeography allows the biogeographer to know both the two vicariance stories and the actual phylogeny. Additionally, history can be replayed repeatedly to accumulate a sample of several phylogeny to assess the function of probability density of biogeographic variables. The roles of stochastic, historical and ecological processes in adaptive dispersal can be assessed. This approach implies the possibility of dispersal, vicariance and extinction. The main problem of this approach is the history of the regions and for now only theoretical applications are made.

One of the most important problems in biogeography is the lack of integration between historical and ecological biogeography. Ecology and history are inextricably linked, and long-established separation between them is an obstacle to the advancement of biogeography. Efforts have been made to overcome the duality between historical and ecological biogeography by Haydon et al. [47], Avise [48], Grehan [49] and Crisci et al. [50].

In terms of methods, biogeography has to deal with two issues that could be defined as "plurality" and "non-recognition". Every attempt to link historical biogeography with a distinctive and unique method is doomed to failure. Based on the many available methods in the discipline, some authors consider that historical biogeography is a "mess" [51]. The proliferation of methods that shape biogeography is the result of competing ideas such as pan-biogeography over cladistics or major events as compared to model-based methods. Crisci et al. [34] consider that the spread of historical biogeographic methods in the last two decades is a symptom of a scientific revolution (in the sense of Kuhn) [52]. Each of the historical biogeographic methods has been developed within a certain approach. Each approach consists of a central core of concepts and assumptions, and has different goals. This means that not all historical biogeographic methods have the same objectives, for example, the reconstruction of the distribution in the taxon's history compared to historical reconstruction in areas of endemism. Therefore, some methods are more appropriate than others in different situations. Despite the lack of a unique method, the increased accuracy and rigor of the latest methodological developments offers an opportunity to abandon biogeographic theories with which the state of science cannot be verified and to preserve those that generate verifiable hypotheses of general importance.

Non-recognition in many cases is associated with molecular trees that are used in the context of a phylogenetic study for additional assumptions of distribution and vicariance without explicit application of a biogeographic historical method. Synchronicity as a historical

biogeographic problem has challenged Hunn and Upchurch [53] to advocate the need for a "chrono-biogeographic paradigm". These authors accept that changing the paradigm in biogeography is a logical development rather than a substitute for the current paradigm. Donoghue and Moore [32] emphasize that cladistic biogeographic methods remain susceptible to stirring effects from historical events on the spread of organisms, mainly due to the fact that these methods do not take into account the absolute time of genealogical diversification. In other words, ignoring the timing information is a blurring of the relationship between biogeographic models and their possible underlying causes (i.e. vicariance and dispersal events). In recent years, more and more historical biogeographic studies have found that time information and molecular clock have become a regular source of nodes breakdowns (points of genealogic diversifications). However, Heads [54] considers that the use of obsolete nodes of phylogeny as absolute ages is incorrect and that such knowledge is minimal for phylogenetic events from past epochs. According to Heads [54], only the nodes of the young geological events may be inappropriate for phylogeny, whereas those geological events that are earlier than the dated nodes cannot be rejected as they affect the history of the analyzed taxon. Heads [54] focuses his conclusion on the way the molecular clock is calibrated and states that "whether or not there is a strict clock it is assumed that at least one node of the tree must be calibrated for to give the tree a dimension in time".

The source of the calibration data (mainly, the age of the earliest known fossil for the group or its associated groups) only allows to determine the minimum ages, as the oldest known fossils do not necessarily indicate the timing of the taxon's origin. In research, there are many examples in which dated nodes are considered to be absolute ages and then the earlier geological events are discarded as causative events of biogeographic models, which in many cases have led to preferred dispersalist explanations against possible vicariance explanations [54]. Currently, there are two main sources of time information about taxa: the fossil and the molecular clock. Unfortunately, the relevance of fossil record data is underestimated by most neo-ontologists. Fossils are reduced to calibration tools for the molecular clock, although fossils can provide more than purely chronological information. For example, they can be used to reconstruct the paleoecological conditions and draw conclusions for the paleoclimate. The paleoecological evidence together with the geological knowledge can in turn be used to create a framework for the biogeographical evolution of existing taxa, and thus, this core of independent evidence could help postulate hypotheses affecting the physical and paleoecological reasons that have formed the distribution patterns of living organisms. Historical biogeography in the future should analyze not only the distribution of taxa models but also their time patterns.

The loss of global biodiversity is the result of complex impacts caused by changes in the global environment. Sala et al. [55] consider that most of these changes are anthropogenic and the magnitude of the change in biodiversity is so great and so strongly related to ecosystem processes and the use of natural resources by humans that the depletion of biodiversity is currently seen as an important global problem. In this scenario, new strategies for biodiversity conservation are mandatory and the prospect of success in the new strategies is by focusing on the species that currently live, who lived in the past and who will live in the future. This is a uniform biogeographic position and several

biogeographic historical methods are used to define conservation strategies. These include parsimony analysis of endemicity (PAE), panbiogeography and phylogeography, as well as environmental approaches to biogeography, as the island biogeography theory that is useful in designing environmental networks. The benefits of biogeography will be complete in conservation, only if there is a new conceptual framework that reflects the spread of organisms, changes in environmental factors such as climate change, land use change and the creation of new historical events such as human-mediated dispersal events as a result of biological invasions.

A new method in conservation biogeography is aimed at studying the homogeneity patterns of biota [56]. Olden concludes that the increase in invasive species and the extinction of other taxa from the local biota followed by biotic differentiation over time leads to a reduction in the genetic, taxonomic or functional similarity between biotas. In the 21st century conservation biogeography will play a particularly important role in the global re-assessment of the state of our planet. Biogeography has its share in the realization of sustainable development not only as a part of geographic research but in direct connection with ecology and biology. In addition to the already mentioned sub-disciplines in biogeography, i.e. Ecological Biogeography, Analytical Biogeography, Biogeographic Modelling, Tropical and Neotropical Biogeography, Island Biogeography, Marine and Freshwater Biogeography, Anthropogenic Biogeography, and Molecular Biogeography have to be added. In 2007, the Systematic and Evolutionary Biogeographical Association (SEBA) [57] produced and ratified the first draft of the International Code of Area Nomenclature (ICAN). An example of the names in the ICAN can be the study of Morrone [58] on biogeographical regionalization in the Andean region, which offers a hierarchical classification of sub-regions, provinces, sub-provinces and districts. It is based on biogeographic analyses of terrestrial floristic and faunistic taxa and aims to ensure universality, objectivity and stability of perception. The Andean region currently consists of the Central Chilean, Sub-Antarctic and Patagonian sub-regions and the South American transition zone, 15 provinces, 5 sub-provinces and 81 districts. It should be noted that these are biogeographic regional taxonomic units with their authors' names, as there exist authors' names in the Linnaeus taxonomic system and in the syntaxonomic classification of plant communities. By its very nature, the creation of the ICAN is a revolutionary development in biogeography giving standardization to the names of biogeographic chorological spaces. However, this process implies some competition and a race in the definition of names, which may be subject to selfish motivations connected with self-realization in science. Such a way of thinking could be a reflection of the opinion of many taxonomists who do not accept the detailed differentiation in taxonomy with the separation of new genera, or may result from the inertia of the changing paradigm.

Conclusion

The subject of biogeography is a complex issue arising from the interdisciplinary nature of science itself, and the performed analysis of modern biogeographic methodology and emerging sub-disciplines shows a shift of the subject to biodiversity as a scientific category.

The subject of biogeography can be defined as: “Biogeography studies the regularities in the origination, dispersal and distribution of all categories of the hierarchical systems of biodiversity in the geographic space”. Despite the different research approaches and divisions in biogeography it is much unified as a science because living organisms are uniquely dispersed and distributed in the planetary space. The unveiling of this diversity remains a fundamental goal of biogeography.

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REPORTS

Topic:
ECOLOGY AND EDUCATION

MODEL FOR ORGANIZING ENVIRONMENTAL EDUCATION THROUGH "REGION FOR KIDS"- A SUCCESSFUL BRAND AT REGIONAL LEVEL

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Introduction

One of the global objectives of the Ministry of Education and Science is the formation of such a value system in the younger generation that leads to responsible behavior towards the environment. The principles of sustainable development formulated at the Rio de Janeiro Conference in 1992 add a new aspect to environmental education. Ministry of Education and Science, through the Access to Education and Development Support Directorate, implements, manages, organizes and controls the activities of one of the projects stimulating the development of extracurricular activities through the project: BG051PO001-4.2.05-0001 "School for self-validation and preparation for European Horizons "(SUCCESS). The overall objective of the project is to make the free time of the students from the state and municipal schools meaningful and productive, including students with identified need for specific support, as well as pupils at risk of dropping out (and/or showing aggression and/or violence) through participation in extracurricular and out-of-school activities [1].

Specific objectives are defined as follows: through specific extracurricular and out-of-school forms of work, to increase the motivation of the pupils to participate in the educational process according to their interests and needs; Students to develop additional knowledge, skills, competencies; To reconcile students' leisure time by focusing on their preferred personal expression; To reduce the number of early school leavers and the number of pupils with destructive behavior (aggression).

It is important to note that extracurricular and out-of-school activities under the SUCCESS project is funded by the Operational Programme Human Resources Development - "Making the school attractive to young people"; they are not funded by the school budget, do not allow themes and activities provided in curricula for the subjects; are not included in the school curriculum and do not duplicate the content characteristics of the subjects taught in

the Bulgarian school and grouped in the cultural and educational areas by classes, stages and degrees of education.

Conducting extracurricular activities in Biology using the Regional Environmental Center for Central and Eastern Europe – Bulgaria educational toolkit

Unlike curricular teaching, which is regulated by standards and curricula for each subject, extracurricular activities due to their specificity and peculiarities are carried out without strictly fixed normative frames. This, in turn, is a challenge for the biology teacher. On one hand, these challenges are related to the content provision of the extracurricular activities and, on the other hand, to its resource provisioning. Often the subjects of the extracurricular activities are very similar to the curricula of the compulsory teaching material without overlapping entirely with it. Here, the teacher has to take into consideration that the children's interests and requirements are important when drawing up and implementing the extracurricular activities [2].

By definition, extracurricular activities and out-of-school activities are a form of organization and interaction between the teacher/lecturer's activity and students in order to accomplish specific tasks related to meeting the interests and creativity of students outside of the state educational requirements for learning content. Out-of-class activities are carried out on a voluntary basis, at the request of the students, with the consent of the parents.

Structural and functional analysis of educational toolkits

Educational tool kits for extracurricular activities from 4th to 8th grade - "Western Balkan for Kids" and "From Osogovo to Belasitsa for Kids" as well as "Pirin for Kids" were developed as teacher tool kits, created by REC for CEE - Bulgaria in cooperation with teachers and experts from Bulgaria. The package of each handbook includes: Teacher's Guide, A Little Guide to Biodiversity in the Region and Material CD-ROM. Structurally, the manuals contain a set of biological and methodological information, as well as all necessary resources for a given topic [3].

Topics are divided into modules, part of which relate to the biodiversity of the region, information on traditions and customs as well as topics directly related to the region's pressing environmental problems.The Teachers' tool kits structured in this way provide the teacher with the opportunity to organize extracurricular activities on both a modular and a weekly lesson hours distribution method. Each topic includes information modules and a system of teaching activities that allow the content included in the topic to be processed. Content is organized on the principle of "science" which is based on the interdisciplinary approach to the presentation and study of concepts, laws and regularities [4].

Each module also includes a topic related to the field activities through which students acquire practical knowledge and skills. The main methods through which the activities are performed are interactive (brainstorming, case studies (dilemmas), visualization and associative cloud, interactive demonstration, interactive study (role-playing), role play, situation discussion, situational games, interactive tests, fairy tales and parables, development of small projects) and in line with the new educational paradigm, which assigns an active

role to the child in the educational process. Methods for each method present information about its nature, way of organization in different age groups. This eliminates the uncertainty experienced by the teacher in the applying this group of methods.

Using the educational toolkits allows teachers to conduct extracurricular activities mainly related with 1 year extracurricular activities or 2 years extracurricular activities. Partial using of activities and topics from the toolkits in the curricular classes. Extracurricular and out-of-school activities. Here is a specific example of the Osogovo to Belasitsa Toolkit in terms of the distribution of the teaching content and the main learning activities included in it: Modules: 6; Topics: 24; Activities: 219, of which 66 field activities [5]

Through the Educational Toolkits of REC, the biology teacher generally receives a specific-objective, biological and methodological information to organize an optimal TIM version of the extracurricular activities. When determining the content, the teacher can rank the modules, topics and activities in it according to the goals, interests, age of the students, and last but not least according to the resources available to the school.

This unified structure of the educational toolkits allow the performing of the following educational functions:

– Information function – mastering specific subject and general knowledge and skills through different ways of learning; Passing on the acquired knowledge through the use of various methods and tools tailored to the students' age characteristics.

– Communication and educational function – Shaping skills to establish rational and emotional relationships with individual students and work relationships with other teachers and parents; Through the various interactive methods, the personal relationships in the group are improved and developed.

– Educating function – to update, expand and deepen the theoretical knowledge and its application in solving specific pedagogical tasks; to form skills and habits for organizing and managing the pedagogical activity; Expanding the knowledge of modern pedagogical technologies and their application in the field of environmental education.

– Management function – form class management skillsduring various curricular an dextracurricular activities.

– Diagnostic function – An opportunity to form and evaluate the personal achievements of each student in a diverse educational context; To predetermine their future professional choices.

In resume the system of tutorials provides the teacher with a complete pedagogical model and the toolkit included in it for organizing a diverse extracurricular activities giving them the choice and additional qualification related to the specifics of the toolkit. These toolkits could be considered as a means of mastering professional-pedagogical skills, habits, experience and way of developing pedagogical thinking and applying the theoretical knowledge in practical work.

Materials and Methods

In order to establish the real status of the pedagogical practice regarding the implementation of the extracurricular activities in biology and the degree of applicability of the two

educational/teachers toolkits - "Western Stara Planina for Kids" and "Osogovo, Ograzhden and Belasitsa for Kids" a study was conducted in the target regions, which focuses on answers to two groups of key questions: identifying the real problems and barriers when organizing the extracurricular activities and the pedagogical effectiveness of the teachers' toolkits.

For this purpose, the following scientific research tasks were formulated:

How applicable are the toolkits "Western Stara Planina for Kids and "Osogovo, Ograzhden and Belasitsa for Kids" in different forms of student preparation in schools in the target regions. Which are the best practices for organizing extracurricular activities using the teachers' toolkits in the target area. What are the subjective and objective barriers when we use the toolkits to organize the extracurricular activities in the target regions? Identifying the teachers' need for professional help (methodical, specific-subject).

In the context of the goals and objectives of the study, we organized focus groups with teachers from the target regions who are using the tools for 1-2 years now. By its very nature, organizing focus group research is a qualitative method of gathering information and opinions on a relevant issue. In the focus group survey, 70 teachers took part, who represent the typical age and social profile of the active extracurricular activities teachers. Each of the participants was interviewed through a semi-structured interview. In this type of interview, the formulations of our questions and the order of their assignment were strictly fixed. The groups of interviewed people participate in pedagogical practice in different ways, with common educational tasks and different competences and therefore can give evaluation from different professional angles.

Results

The summary interview includes questions related to the main issues and barriers that teachers encounter in organizing, conducting, management of extracurricular activities, including through these educational toolkits. Questions related to sharing their own pedagogical experience with regard to the effectiveness of individual modules, topics and activities from the toolkit, including the ability to change students' attitudes and motivation to learn through the toolkits. Questions related to their experience with participation in different projects and their need for further qualification. Here I will present the results obtained on the questions and their analysis.

Regarding how applicable the toolkits “Westers Stara Planina for Kids”/”From Osogovo To Belasitsa from Kids” are when organizing extracurricular activities for your students?

On this question the interviewees point out the following: about 30% of the teachers use the toolkits to organize extracurricular activities (and more specifically in organizing of one school club and two different extracurricular activities under the project: BG051PO001-4.2.05-0001 SUCCESS. 20% of them have been using ”From Osogovo To Belasitsa from Kids” and 10% - “Western Balkan for Kids” - for the organization of the extracurricular activities for students 4th, 5th to 8th grade. The topics that are used for the extracurricular activities are relevant to the region in which the children live. This is quite understandable given the regional principle of selecting the content of the teachers toolkits and one of their main objectives, namely to explore local biodiversity.

Rest of the teachers (70%) partly use the teachers‘ toolkits in the curricular activities in the school subject – Man and Nature, Biology and Health Education (7th to 9th class), Geography, Mathematics, Physics and Teachers’ class.

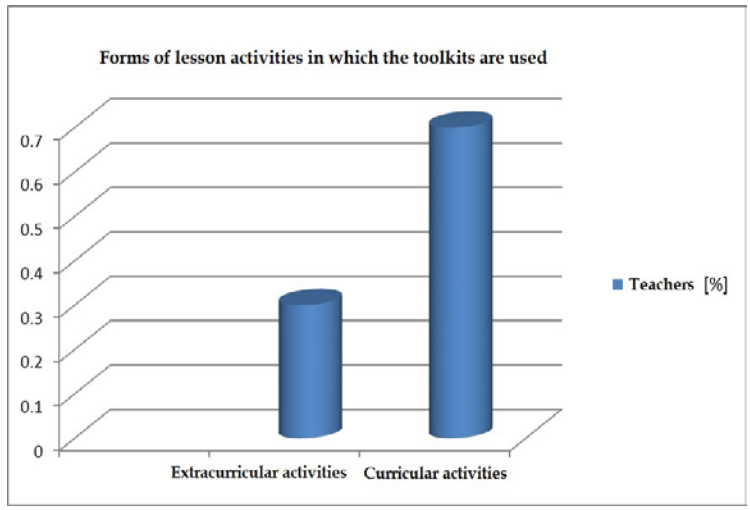

Fig. 1 Applicability of teachers toolkit

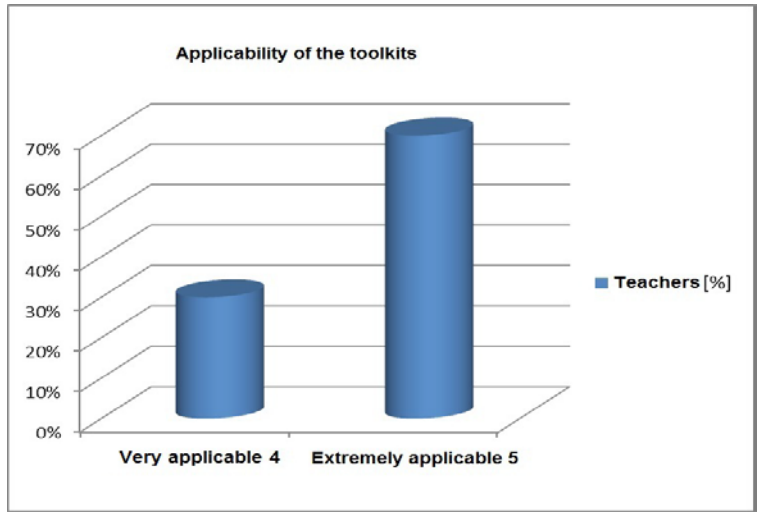

Fig. 2 Applicability of the toolkits

Regarding the question what will you change in the content of the teachers’ toolkit?

According to the teachers' interviews regarding additions or changes that they would make to the content of the teaching toolkits, teachers indicate that the tools are sufficiently comprehensive and rich in both information and teaching resources for the various activities. According to some of the teachers (about 10%) it is necessary for younger students to

have more visual materials - films and interesting games with costumes, etc. According to their opinion: "There is definitely more need for additional visual material. It is also necessary to put in more materials educating moral values. To distinguish between good and evil. "According to some teachers, it is necessary to introduce new technologies into the classroom.

Discussion

The additional visualization of the content included in the toolkits especially for students of grades 4 and 5 is extremely important as they have a predominance of visual thinking and encoding of information in images is easier to be processed and comprehend compared to students from the next age group. Used in the toolkit, images have decorative and well as educational function, they are linked in a logical fashion and seem to "tell a story". The images here depict meaningful units of information and generally complement the main text included in the themes.

It is necessary to consider the possibility of including static and dynamic images within the CD, for example. Especially this is needed for children with special educational needs, such as dyslexia.

The need to include activities that require the use of modern technology is indispensable. Modern technologies have great potential in terms of attracting attention, enhancing motivation, facilitating access to knowledge of children with special needs. For example, using an interactive whiteboard, designing educational apps created with the help of students. Some of the teachers identified it as necessary to include learning activities related to the formation of empathy, improvement of interpersonal relationships, formation of civic consciousness etc.

After analyzing the results obtained with regard to proposals for future improvement and popularization of the toolkit:

- Adapting it to a wider educational context by including separate thematic parts and modules in the curricula;
- The need to create such tools in the secondary education phase;
- Providing practical teacher training and financial support for better implementation of the model.

This can be explained by the fact that most educational toolkits are addressed to young students (4-8 grade). In the higher educational level such educational toolkits are rare to be found in our educational system and this has its subjective and objective explanation - different design of the tools with regard to interactive methods adequate for this age group, the lack of extracurricular activities for this age group; Targeting students' efforts towards successful final exams, lack of established lesson hours/programs. Some of the teachers point out that extracurricular activities are usually organized only for Bulgarian language and Mathematics.

When asked the question of the possibility of working with such kind of educational toolkits, and specifically with this teachers' toolkit having led to a change in students' attitudes and motivation towards biology education, the teachers share the following:

The use of such kind of educational toolkits, which leads to a change in students' attitudes and motivation towards biology education, all teachers agree that this is a fact and students who have used the toolkits have raised their interest and motivation for learning A new type of behavior and attitude towards nature has been formed. Some of the children have taken the initiative to create an eco-club at school to have more time working with the toolkits. In part of the schools where teachers had been using the "Natura 2000" toolkit in education, a group of students has been formed that have been conducting open lessons and at this stage part of the children continue their higher education in the field of ecology. This proves the high pedagogical efficiency of the tools and their ability to predetermine the future professional orientation of the children. The ability these toolkits to lead to a change in attitudes, motivation to learn, and student behavior towards the environment, and towards each other is a proven fact. Particularly in areas with real problems related to the conservation of natural resources, children are particularly sensitive to human activities and what can be done to sustainable development. The same applies to minority children where work with the toolkits has led to a change in attitudes towards protecting the environment, health and relationships. In this context, it would be good to include separate headlines related to health education, for example on issues related to environmental pollution and human health.

Regarding the participation of teachers in training related to the implementation of environmental education, the following results were obtained: the majority of teachers receive support from the directors for organizing extracurricular activities. Opportunities for active work on projects have been created. Some of the teachers (20%) point out that school principals are to tell what extracurricular activities should be organized at school, which is not in agreement with teachers and the interests of students.

A large number of teachers have been involved in training related to various teachers' toolkits: "From Osogovo to Belasitsa for Kids", "Green Pack Junior"; "Plants and Animals in Osogovo", Training for Project Preparation – "Logical Framework Approach"; Biodiversity of Pirin organized by Bulgarian Biodiversity Foundation.

About 80% of the interviewed teachers receive the support from school proncipals for conducting extracurricular activities at school. The support is expressed in moral empathy, freedom in the choice of subject, financial support through the inclusion of teachers in different programs. Almost none of the teachers identified as a problem financial obstacles to organizing the extracurricular activities, which is explained by more funding opportunities, but also a more active participation of teachers in projects. Almost all of the respondents indicated that they had attended the training organized by REC for CEE – branch Bulgaria for the formation of competencies for writing project proposals, which could explain the activity in their participation in different projects.

Regarding the need for further training and qualification, teachers point to the need for training related to the application of new technologies in school, specific-subject qualification related to biodiversity. Of particular interest to teachers is training related to project proposals writing. In connection with the last recommendation, a seminar could be organized with the teachers related to the projects in the Secondary School. This problem needs to be analyzed in two directions: on the one hand, to provide information to teachers on

how to write projects, organizations that fund similar school initiatives, illustrations of good practice. The second is the implementation of the so-called project-based training with the following accents - methodology, types, stages of its organization. In essence, the concrete practical results of such training are related to creating opportunities for self-financing of the school institution concerned, as well as involving students in such initiatives directly reflecting their learning motivation. As a result of the study, we identified the following three main types of barriers that directly or indirectly affect the conduct of extracurricular activities at school. The essence of each of them will be presented in the following table:

Tabl. 1 Main groups of barriers, related to conducting of extracurricular activities in schools

Type of barriers	***Description***
Administrative	Lack of coordination teacher-principal when it comes to organizing and conducting of extracurricular activities. Teachers often state that there are discrepancies between the extracurricular programs they propose and the programs that are approved by the principal. Lack of good communication between biology teachers in the regions regarding exchange of ideas and experience. Bureaucratic procedures for processing the paperwork is a serious administrative barrier when extracurricular activities are organized. Suggestions for electronic submission of the papers is being articulated. Often teachers face difficulties when designing programs and thematic range for extracurricular activities (especially for teachers with less experience). Not enough biology teachers, willing to organize extracurricular activities not just accidentally but as a practice in schools. As a reason for this teachers state the low pay and the lack of motivation of the students. Not enough time for preparation of extracurricular activities. Less biology lesson hours in the curricula also have negative impact on students' motivation when they have to decide on extracurricular activities.
Organizational	Lack of good facilities in some of the schools requires this to be compensated by teachers with personal finances to purchase the necessary materials for the extracurricular lessons. Lack of modern ICT at school, to allow students organizing different types of activities. Lack of planning and organizational skills to design attractive for students' extracurricular activities. Lack of pre-designed programs for biology extracurricular activities. Insufficient number of students at schools.
Content	Difficulties related to the application of interactive methods in the teaching process and need for further qualification. Difficulties arise from the inability of students to apply their knowledge of interactive methods in teaching specific learning content. Barriers, related to the specific biology teachingn content and the need of additioanl knowledge improvement. Some teachers lack motivation to organize extracurricular activities. Major of the freshly starting young teachers begin their career with lack of confidence, fear and reluctance. Insufficient good command of didactic terminology and the need to plan additional hours for keeping up with the knowledge.

The analysis of the interviews led to the conclusion that the main problems are mainly related to the organization and conduct of the extracurricular activities and the use of modern teaching methods. In addition, the interviewees outline a very important pedagogical practice problem, which we can definitely claim to negatively affect the professional activity of the pedagogues, namely: the inability to combine their own teaching with ensuring of the students involvement. This is related to how active they are applying transmission or direct teaching strategies, which in turn makes students passive observers of their performance in class, respectively, to decrease the interest and discipline and lack of motivation for participation in any extracurricular activities [6].

Conclusion

In order for children to become responsible and active members of society, it is necessary to realize that the rooted values and norms have far more influence on behavior than knowledge in their own right. Such values and norms are formed from an early age and are difficult to change at a later stage of development. For this reason, the inclusion of environmental education in the curriculum of primary schools requires not only additional teaching materials but also a completely new way of teaching [7, 8].

By doing so, students will be able to learn how actions in different spheres of life can affect the environment. This will allow them to address environmental issues in different aspects, as foreseen in the concept of sustainable development.

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CONTENT OF RADIOACTIVE ELEMENTS IN SEDIMENTS OF THE NORTHERN BLACK SEA COAST OF BULGARIA FOR THE PERIOD 2010-2016

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Abstract

The Aim of the study is to investigate the radioactive contamination of the Bulgarian Black sea coast.

In the period between 2010 and 2016 sampling of sediments from Varna bay, Varna Lake, Galata, Emine and other sites at the northern Black sea coast of Bulgaria was done.

Materials and Methods: The samples were analyzed by gamma-spectrometry for determination of ^{137}Cs, ^{234}Th, ^{235}U, ^{226}Ra, ^{228}Ac and ^{40}K.

Results: The values obtained for the specific mass activity of natural radionuclides in sediments are within the values detected in soils of Northeast Bulgaria. The artificial ^{137}Cs is detected in all analyzed samples and is mainly due to the accident at the Chernobyl nuclear power plant in April 1986.

Conclusion: The level of the radionuclides determined in the sediments is within the range of background values and is not hazardous for contamination of sea water.

Keywords: Black sea, radioactivity, marinesediments, caesium - 137, strontium – 90.

Introduction

The Black Sea is the world's largest oxygen-free swimming pool and is therefore an important environment for studying the processes that control the geochemistry of uranium in the liquid and solid sediment phases. Organic-rich sediments in an acid-free water basin such as the Black Sea are enriched in uranium with more than one order of magnitude than ocean basin sediments. According to some authors [1, 2, 3, 4], a major part of the radioactivity accumulates in the sediment. Variations in marine ecosystems have the greatest impact on bottom communities. Accumulated organics, metals, radionuclides and petroleum derivatives in the sediments have a significant effect on the macro-species.

After the Chernobyl accident in 1986 an enormous amount of radioactive elements was thrown out, much of which in the marine environment. According to the data cited in the literature as a result of this disaster radioactive elements with a total activity of about 2400 TBq were deposited in the Black Sea [5,6]. The studies related to the radioactive pollution and behavior of natural and artificial radionuclides in the Black-sea water basin are rather limited and give no complete picture of the radio ecological status of the region [7, 8, 9, 10]. There is a lack of systematic data concerning their physical and chemical peculiarities as well as their concentration levels in bottom sediments, algae, mussel, fish, sea snails and other Black sea marine organisms.

The purpose of this study is to determine the content of these radionuclides in sediments, waters, algae, and other marine organisms, starting with the first step - bottom sediments. The data obtained serves to quantify the processes of sedimentation and distribution in the environment and to assess the potential risk to the biosphere and humans.

Materials and Methods

Sampling of bottom sediments was done in the period 2010-2016, 13 sampling sites from the Varna Bay, 3 of Lake Varna, 12 from Galata, 4 from Emine and 6 from other sites on the northern Black Sea coast of Bulgaria were collected and analyzed. The sampling in Varna bay was performed by the Scientific Research Boat "Prof. Valkanov" owned by the Institute of Fishing Resources", Varna getting up to six miles out to sea.

All samples were analyzed by gamma-spectrometry. The samples were homogenized, dried at 80ºC and sieved through a 2-mm mesh before measurement with a gamma-spectrometer. The samples were stored in air-tight containers for minimum 28 days to allow ^{226}Ra to come into equilibrium with its short-lived progeny. The measurements were done following standard procedures [11]. A Canberra high-purity germanium detector with 20% efficiency an energy resolution of 1.8 keV for ^{60}Co γ-ray energy line at 1332 keV was used. The detector was calibrated with standard reference radionuclide source, type MBSS2, containing ^{241}Am, ^{109}Cd, ^{139}Ce, ^{57}Co, ^{60}Co, ^{137}Cs, ^{113}Sn, ^{85}Sr, ^{88}Y, ^{210}Pb, ^{203}Hg supplied by the Czech. Metrological Institute. The measuring system included a multichannel analyzer DSA 1000 (Canberra, USA). The spectrum was analyzed by GENIE-2000 software with measurement uncertainties less than 10%. Typical counting times were 19–24 h.

The ^{238}U concentration was derived from the weighted mean of the photo peaks of ^{234}Th (63.5 and 92.6 keV), and the ^{226}Ra concentration was derived from ^{214}Bi (609.3 keV) and ^{214}Pb (295.2 and 352.0 keV) in the same way. In addition ^{226}Ra was evaluated at its 186.1 keV line taking into account the contribution of the overlapping line at 185.72 keV of ^{235}U calculating the specific activity of ^{235}U through the specific activity of ^{238}U (ISO 18589-3,2007). For ^{232}Th, the photo peaks of ^{212}Pb (238.6 keV), ^{208}Tl (583.1 keV) and ^{228}Ac (911.1 keV) were used. Activity concentration is expressed as Bq.kg^{-1} dry weight. The experimental errors were less than 10%. The uncertainties of the reported values were evaluated considering counting statistics and calibration errors.

Results and Discussion

The data obtained by gamma-spectrometry of the samples collected in the period 2010 to 2016 is summarized and shown in figures 1-6. In all of the samples the radionuclides studied are detectable within the admissible errors.

Content of technogenic radionuclides in the sediments studied: Figure 1 shows the averaged activity values of ^{137}Cs by studied areas for 2016 . As shown in the figure the ^{137}Cs activity is highest in the Galata samples – 24.1 Bq.kg^{-1} (ranging from 12 to 50 Bq.kg^{1}). The average values for Varna lake are 11.7 Bq.kg^{-1} (ranging from 3 to 20 Bq.kg^{1}), for the Bay of Varna– 7,9 Bq.kg^{-1} (from 1.3 to 18 Bq.kg^{1}), for sediment from the area of

Emine - 16 Bq.kg^{-1} (from 2.2 to 26 Bq.kg^{1}) And for the other sites - 9.5 Bq.kg^{-1} (from 0,9 to 36 Bq.kg^{1}).

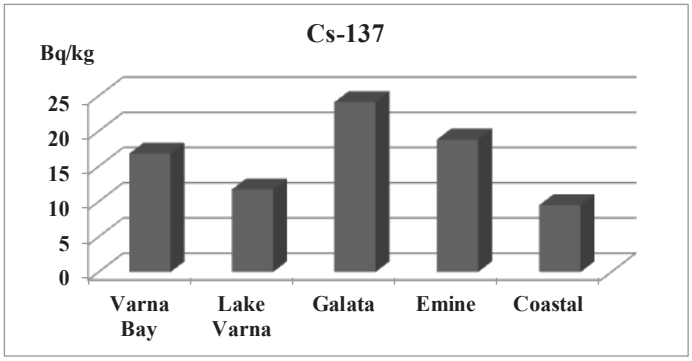

Fig 1. Mean values of ^{137}Cs mass activity in bottom sediments for the different studied areas [in Bq.kg^{-1} dry weight for 2016]

The differences in the ^{137}Cs average activity values for the different regions are not large. Evidence of accumulation in the Galata area is due to a difference in the mechanical composition of sediments. All samples in the Galata area have a high percentage of the silk fraction (silt), while in all other investigated sites the predominant amount of sediment is sandy.

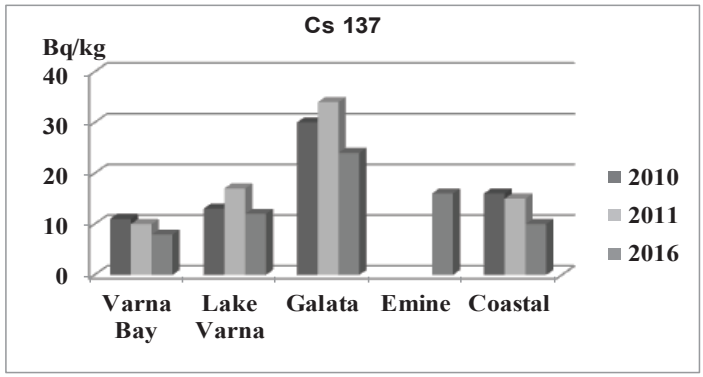

Fig 2. ^{137}Cs mass activity in bottom sediments for the period 2010-2016 [Bq.kg^{-1}d.w.]

In Fig. 2, a comparison of the activities of ^{137}Cs was made for three separate years in the period 2010-2016.

As the results presented on the figure show the content of ^{137}Cs in the sediments tested did not change significantly in the seven-year long period. The values are within the average for this region of Bulgaria and result from the pollution after the accident in the Chernobyl's nuclear power plant in 1986.

Content of natural radionuclides in the sediments studied: The average values for the content of the different radionuclides for three years in the period 2010-2016 in the studied

sediments from the different sites are presented in Figures 3 to 6. The obtained values are within the range detected in the soils of this area. The established differences in the determined activity levels of natural radionuclides in the sediments for the separate sites are due to the difference in composition and structure of sediment - zoo benthos, sand and silt, which naturally leads to a difference in the composition of the elements in them.

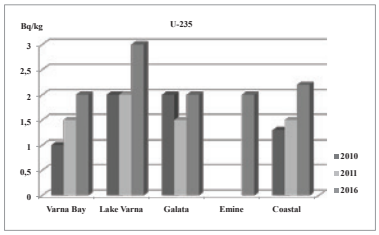

Fig. 3. ^{235}U in bottom sediments [Bq.kg^{-1}d.w.]

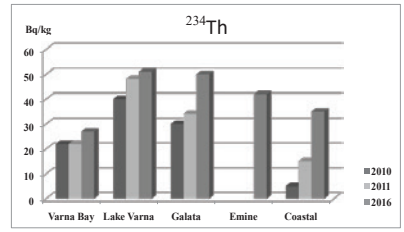

Fig. 4. ^{234}Th in bottom sediments [Bq.kg^{-1}d.w.]

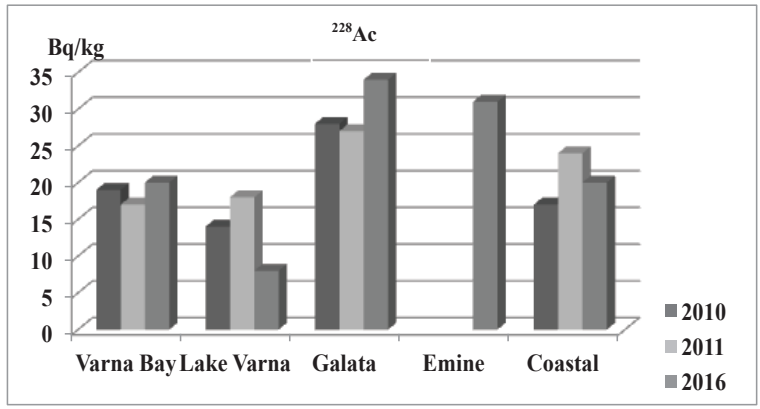

Fig. 5. ^{228}Ac in bottom sediments [Bq.kg^{-1}d.w.]

In the UNSCEAR 1993 Report the average concentrations of ^{238}U and ^{232}Th in the soils of Nordic countries are estimated as 40 Bq.kg^{-1} for each element with quite marked variability, more than 60%. Our results are in good agreement with the cited values. A relatively homogeneous distribution of uranium compared to thorium can be noted which can be explained with the rather conservative and inert behavior of uranium in aquatic systems. The mean residence time for uranium in sea water is considered to be about 5.10^{3}y (Holm and Bojanowski 1989). Compared to uranium thorium is a much more reactive element in sea water. It is removed from the water column rapidly, its residence time being less than 1 y in surface waters and less than 100 years in deep waters. The results for Th^{234} (daughter product of ^{238}U) and Ac^{228}, as a progeny of Th^{228} are indicative for the behavior of thorium.

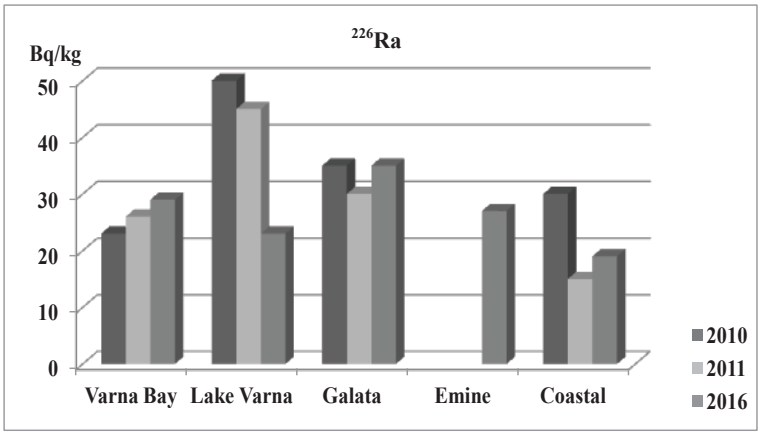

Fig. 6. ^{226}Ra in bottom sediments [Bq.kg^{-1}d.w.]

^{226}Ra is highly radiotoxic and poses a serious risk for the health of humans and living organisms. ^{226}Ra concentrations are similar or higher than those for uranium in the environment. Like thorium it accumulates more effectively in the sediments compared to uranium. The values determined by us in the sediments analyzed are in the range of 5 to 53 Bq.kg^{-1}. The estimated mean value for the content of radionuclides from the ^{226}Ra subseries in the soils of the Nordic countries is also 40 Bq.kg^{-1} [UNSCEAR 1993 Report]

Conclusions

- The established concentration of ^{137}Cs in sediments along the Bulgarian Black sea coast is within the average for this region of Bulgaria and is a result of the pollution after the accident in the Chernobyl's nuclear power plant in 1986.
- ^{238}U, ^{232}Th, ^{226}Ra and their progenies are accumulated in sea bottom sediments, but there are no signs of techno genic contamination with these nuclides in the studied areas.

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REPORTS

Topic:
OTHER RELATED TOPICS

DRY MASS YIELD AND AMOUNT OF FIXED NITROGEN IN SOME FORAGE LEGUME CROPS AFTER TREATMENT WITH ORGANIC FERTILIZER HUMUSTIM

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Abstract

Aim: The study aimed to access some changes in agronomic performance of spring forage pea and vetch after application of the organic fertilizer Humustim.

Materials and Methods: The fertilizer was applied through i) presowing treatment of seeds, ii) during vegetation and iii) combination of both. The field experiment was carried out in three consecutive years.

Results: Treated plants, both peas and vetch were more effective over nontreated with regard to important agronomic characteristics. Dry mass yield increased significantly after treatment of seeds with Humustim at the dose of 1.2 L t^{-1} + one treatment during vegetation; the amount of fixed nitrogen and efficiency of utilization of nutrients of peas were found to be increased; pea's plants accumulated in their dry mass to 42.5 kg ha^{-1} more nitrogen.

Conclusion: Based on the positive results, it is recommended the use of Humustim in the technology of cultivation of peas and vetch in the modern trends of organic farming.

Keywords: organic fertilizer, spring forage pea, vetch, nitrogen fixation

Introduction

Legumes are the key to the construction of sustainable agriculture [1]. With their unique ability to establish symbiotic relationships with Rhizobium bacteria and fix atmospheric nitrogen, they are an important component for ecologically friendly forage systems and allow obtaining of high yields using fewer resources [2].

Pea (*Pisum sativum* L.) and vetch (*Vicia sativa* L.) are valuable forage legumes with multifunctional role. They have a short vegetation period, high dry matter productivity and fixed to 150 kg N ha^{-1} under favorable conditions [3, 4].

The development of sustainable agriculture with preservation the environment is the main vision of the strategy for sustainable development of agriculture. Interest has turned to the use of environmentally friendly products because chemical fertilizers pollute soil and groundwater and selectively accumulated environmentally harmful chemical elements by prolonged use [5, 6].

Humustim is a registered fertilizer for use in organic production in Bulgaria. It is a liquid organic humate fertilizer and growth stimulator. Its application by seeds treatment and/or leaf application promote growth and development of plants and ensure high and qualitative yields from the crops.

The aim of this work was to access some changes in agronomic performance, i. e. dry mass yield, efficiency of utilization of nutrients, accumulation of nitrogen in the dry mass, amount of fixed nitrogen in spring forage pea and vetch after application of organic fertilizer Humustim.

Materials and Methods

The experimental work was conducted on the experimental field of the Institute of Forage Crops, Pleven, Bulgaria on leached chernozem soil subtype without irrigation during three consistent years. Long plots method, 10 m^2 plot size were used. The action of the organic fertilizer Humustim (composition of the liquid formulation is shown at the end of this chapter) was tested on spring forage pea cv. Pleven 4 and vetch cv. Obrazets 666. They were sown at row spacing 15 cm with a sowing rate rated at 110 (for pea) and 200 (for vetch) germinated seeds.m^{-2}. The next variants in 4 replications were studied: Control – nontreated seeds (NS); Nontreated seeds + one treatment during vegetation (TDV) (NS + one TDV); Nontreated seeds + two treatments during vegetation (NS + two TDV); Treated seeds 0.6 L t^{-1} (TS 0.6 L t^{-1}); Treated seeds 0.6 L t^{-1} + one treatment during vegetation (TS 0.6 L t^{-1} + one TDV); Treated seeds 0.6 L t^{-1} + two treatments during vegetation (TS 0.6 L t^{-1} + two TDV); Treated seeds 1.2 L t^{-1} (TS 1.2 L t^{-1}); Treated seeds 1.2 L t^{-1} + one treatment during vegetation (TS 1.2 L t^{-1} + one TDV); Treated seeds 1.2 L t^{-1} + two treatments during vegetation (TS 1.2 L t^{-1} + two TDV). Seeds were treated 24 hours before sowing. Treatment during vegetation was done at the stages of growing up and starting flowering to full flowering with the dose of fertilizer 400 ml ha^{-1}.

The following characteristics were assessed: dry mass yield (from fresh mass yield and% of dry matter) (dried at 60^{O}C); sustainable yield index according to the formulae of

Singh et al. (1990) [7]: SYI (Sustainable yield index)= (Ym-Sd)/Ymax, where: Ym - mean yield, Sd - standard deviation, Ymax - the maximum yield; nitrogen in dry mass yield as productivity of dry mass multiplied by nitrogen content in dry mass (% of dry matter) (for pea); efficiency of utilization of nutrients (a physiological parameter), kg harvested dry mass kg^{-1} total N in plant (a formulae of Bowen and Zapata, 1991) [8] (for pea); amount of fixed nitrogen according to the formula of Carlsson and Huss-Danell (2003) [9] for roughly estimation in the field conditions. Data were averaged and statistically processed using SPSS 2012 [10].

Composition of the liquid formulation of organic humate fertilizer Humustim is as follows: total N – 3.0%; total P – 0.4%; K – 9.7%; humic acids – 32.0%; fulvic acids – 4.0%; macro elements Ca, Mg, Zn, Cu, Co, Mb, B, S, etc.; ash – 18.0%.

Results and Discussion

The results obtained show a higher yield of dry mass in the variants with presowing treatment of the seeds for both, pea and vetch (Table 1). The exceeding in dry mass yield as compared to the control were similar (10.5-12.0%) for the treatment of pea seeds with Humustim at the dose of 0.6 L t^{-1} and one, or two treatments during vegetation did. A higher yield of dry mass was obtained upon treatment with the higher dose (1.2 L t^{-1}) and exceeding as compared to the control reached 19.8% for the treatment of seeds with Humustim at the dose of 1.2 L t^{-1} and one treatment during vegetation. No evident difference between the yields obtained after treatment of the seeds and the combination of treatments during the vegetation.

Table 1. Dry mass yield and sustainable yield index (SYI) from pea and vetch after treatment with organic fertilizer Humustim

Variants	Pea		Vetch	
	dry mass, kg ha^{-1}	SYI	dry mass, kg ha^{-1}	SYI
NS	8344	0.312	7047	0.125
NS + one TDV	8480	0.338	7453	0.132
NS + two TDV	8596	0.341	7540	0.137
TS 0.6 L t^{-1}	9216	0.355	7767	0.142
TS 0.6 L t^{-1} + one TDV	9256	0.358	7900	0.146
TS 0.6 L t^{-1} + two TDV	9348	0.368	7980	0.148
TS 1.2 L t^{-1}	9620	0.378	8220	0.150
TS 1.2 L t^{-1} + one TDV	10000	0.399	8398	0.155
TS1.2 L t^{-1} + two TDV	9800	0.396	8467	0.155
Average	9184	0.361	7863	0.143
LSD 5%	595		491	

The dry mass yield from vetch was lower as compared to pea, but the same trends were found. The highest was the dry mass yield when the treatment of seeds at the dose of 1.2 L t^{-1} + two treatments during vegetation were done and the exceedings of 20.1% as compared to the control was found.

The sustainable yield index increased in proportion to the doses applied for the both crops.

The nitrogen in dry mass yield is an important characteristic and is related to the quality of the forage. In our study, it is defined only in pea and in all variants tested was found higher than that of the control (Table 2). In the variants with the treatment with Humustim only, during the vegetation, nitrogen in dry mass yield was found to be 12.3-13.2% higher as compared to the control – for the lower dose (treatment of seeds at the dose of 0.6 L t^{-1}) – from 19.4 to 21.9%, and for the highest dose (treatment of seeds at the dose of 1.2 L t^{-1}) – from 25.4 to 26.7%. The highest nitrogen content in the dry mass yield was obtained by the presowing treatment of seeds at the dose of 1.2 L t^{-1} + one treatment during vegetation.

The amount of nitrogen accumulated in dry mass from pea was from 19.6 to 42.5 kg ha^{-1} more for treatment with Humustim variants in comparison with the untreated control.

Table 2. Nitrogen in dry mass from pea after treatment with organic fertilizer Humustim

Variants	N in dry mass yield	To the control, +	
	kg ha^{-1}	%	kg ha^{-1}
NS	159.4	-	-
NS + one TDV	179.0	12.3	19.6
NS + two TDV	180.4	13.2	21.0
TS 0.6 L t^{-1}	192.4	20.7	33.0
TS 0.6 L t^{-1} + one TDV	194.3	21.9	34.9
TS 0.6 L t^{-1} + two TDV	190.4	19.4	31.0
TS 1.2 L t^{-1}	201.0	26.1	41.6
TS 1.2 L t^{-1} + one TDV	201.9	26.7	42.5
TS1.2 L t^{-1} + two TDV	199.9	25.4	40.5
Average	188.8		
SE (P=0.05)	4.5		

Physiological efficiency of utilization of nutrients was found significant increased only when the highest dose Humustim was used (Fig. 1).

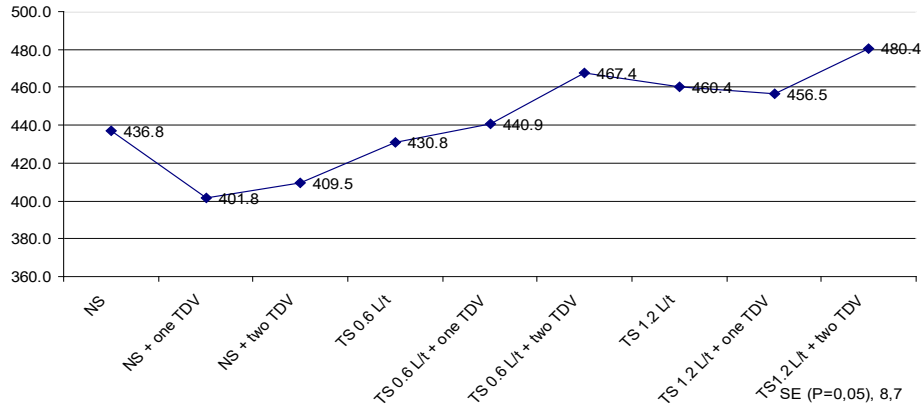

Fig. 1. Physiological efficiency of utilization of nutrients in pea after treatment with organic fertilizer Humustim

Pea and vetch are nitrogen fixing crops. Significant higher amount of fixed nitrogen was found when treatment with Humustim at the dose of 1.2 L t^{-1} and treatments during vegetation were done (Fig. 2). There were no evident differences between the variants for this dose tested. The amount of fixed nitrogen was from 21.7 to 28.2 kg ha^{-1} more than the control.

The amount of fixed nitrogen in vetch increased with increasing the doses of fertilizer. When pea seeds were treated with Humustim at the dose of 1.2 L t^{-1} and treatments during vegetation were done, nitrogen from 19.9 to 24.1 kg ha^{-1} was established, which was more than the amount nitrogen fixed from the control plants. We assume higher amount of fixed nitrogen after treatments with Humustim resulted from the well-developed root system, due to the humic acids included in the composition of Humustim. Humic acids stimulate the growth of the root system of plants, including that of root hairs, where nodules can be located [11]. In our study, with the experimental doses of Humustim we introduced humic acids (%) as follows: with one treatment during vegetation - 12.8; with presowing treatment at the dose of 0.6 L t^{-1} seeds – 0.19, with presowing treatment at the dose of 1.2 L t^{-1} seeds - 0.38, respectively. The presence of molybdenum in the composition of the product also has a positive effect on the nitrogen fixation process.

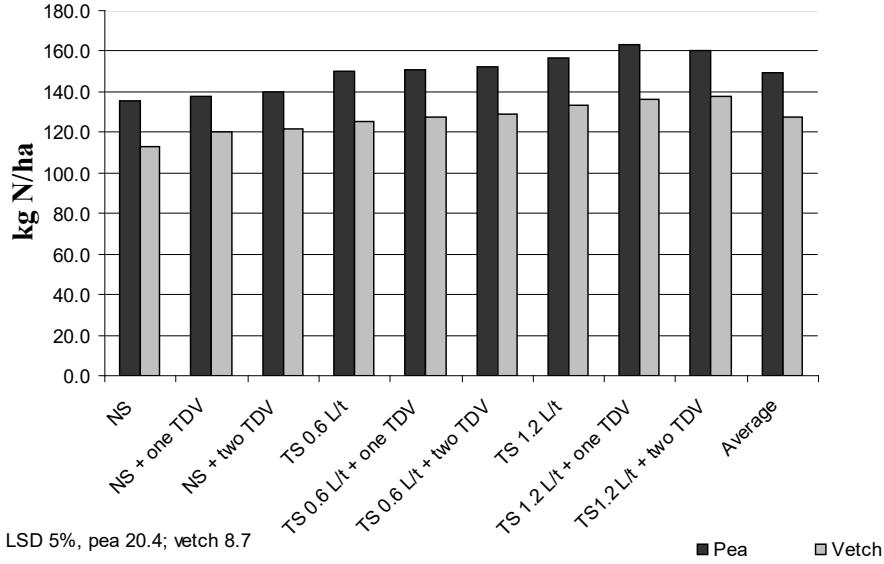

Fig. 2. Amount fixed nitrogen from pea and vetch after treatment with organic fertilizer Humustim

As a whole, plants treated with Humustim, both peas and vetch, were more effective over nontreated in regard to important agronomic characteristics.

Conclusions

Treated with Humustim plants - peas and vetch were with more effective agronomic characteristics comparing with nontrheated controls. Dry mass yield increased significantly

after the presowing treatment of seeds with Humustim at the dose of 1.2 $L.t^{-1}$ + one treatment during vegetation (in vetch with 19.8%, and in pea with 20.1%), under a high sustainable yield index. The amount of fixed nitrogen in pea was found to be increased up to 20.8% (presowing treatment of seeds at the dose of 1.2 $L.t^{-1}$ + one treatment during vegetation), and in vetch up to 21.3% (presowing treatment of seeds at the dose of 1.2 $L.t^{-1}$ + two treatments during vegetation). Treatment with Humustim increased the efficiency of utilization of nutrients by peas. Peas plants acumulated in their dry mass to 4.25 kg/da more nitrogen after presowing treatment of seeds with Humustim at the dose of 1.2 $L.t^{-1}$ + one treatment during vegetation. In the modern trends such as sustainable agriculture it is recommended the use of Humustim in the technology of cultivation of peas and vetch.

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WATER MEMORY DISPLAYED BY THE CONTACT ANGLE DISTRIBUTIONS OF EVAPORATING DROPS

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Abstract

The aim of the paper is to demonstrate the effect of water filtration on the contact angle evaporation distribution of a sample sessile drop.

Materials and Methods: A nuclear filter with holes in diameter of 0.15 micrometers was used to transport a water sample through its holes. The contact angles of the sessile drop are measured during the process of drop evaporation.

Results: The distributions before and after the filtration are shown to be manifestly different which implies a change of water properties due to filtration. Moreover, a difference between the filtered and unfiltered sample's distributions is maintained in time, implying an effect of water "memory".

Conclusions: The water filtration through a nuclear filter causes changes in some water structural properties which tend to be preserved in time.

Keywords: water filtration, nuclear filter, contact angle evaporation distribution, water memory.

Introduction

In this paper we demonstrate the effect of water filtration on the contact angle evaporation distribution of a sample sessile drop. The study of contact angle and evaporation phenomena of sessile drops is useful for the development of surface and environmental sciences as well as for new technologies. In a series of papers it was shown that water properties are sensitive to environmental stimuli, such as chemical content [1, 2, 3], physical influences such as radiation [4], magnetic fields [5] etc., as well as the integral environmental influences [6, 7]. Usually this sensitivity of water is attributed to the so called structure of water [8] – a set of hydrogen bonds forming a statistically stable net of interactions between water molecules. For example the magnetic treatment of water is used in irrigational agriculture to increase the crop results.

Here we discuss differences in the sessile drop contact angles behavior during evaporation caused by a specific treatment of the drop's liquid prior to its contact with the solid. The sample of deionized water, the drops are taken from, was filtered through a folio, called nuclear filter, with small - 0.15 micrometer - holes in it, produced by accelerated heavy ions. In the next Sections, we show that drops taken from filtered and not filtered (control) deionized water samples display manifestly different distributions. Moreover after a single filtration, when repeated measurements of the treated and untreated samples are performed, this difference between distributions continues to persist. In [9] the effect of water filtration

trough a nuclear filter on a transformed contact angle distribution is discussed, but without the impact on the sample demonstrating water memory by repeted measurement as done here. We remark that water filtration trough soil layers is a common phenomenon for an ecosystem.

Materials and Methods

Deionized water was used for the experiments. A filtered sample was prepared with a transport velocity across the folio of W=1,4 mm/sec. The filtration of the deionized water proceeded by forcing a portion of the control sample to move through the holes of a nuclear filter's folio with help of a piston. The folio thickness was 10 micrometer. The holes were produced by bombarding the folio with heavy ions emitted by the accelerator of such particles at the Joint Institute of Nuclear Research at Dubna (Russia). The diameter of the holes was 0,15 micrometer. The number of holes was approximately 200 000 holes/mm^2.

We measured simultaneously the distributions of equal number of three drops taken from each of the samples: from a deionized water sample (the control, unfiltered sample) and from the filtered sample with velocity W at room temperature of 19°C. The contact angles were measured at equal time intervals of 4 minutes with an accuracy of 0.5 degrees. The drops contact areas with the substrate were 4 mm. After the filtration and the simultaneous measurement of the control and the treated samples, they were kept in a refrigerator for 24 hours at a temperature of 4^{O}C. Then a repeated simultaneous distribution measurement of sessile drops taken from the samples was performed.

The method of measurement utilizes evaporating drops whose contact angles are measured during the whole process of drop evaporation [10-14].

The results of measurements are presented graphically. The measured contact angles are plotted on X axis. For each drop the number of contact angle observations with values within each of X intervals is measured during the whole evaporation time and is plotted on Y axis. To compare different distributions we normalize them by dividing them by the total number of measurements. So one obtains graphs of probability distributions.

Results and Discussion

The results of measurements are shown on Figures 1 and 2. On Figure 1 the graph of the control sample (the graph with circles) is compared with the graph of the filtered water sample (the graph with triangles).

Fig. 1 displays the result of water treatment measured immediately after the filtration. The impact of filtration on the distributions is clearly seen. The maximum of the corresponding distribution display a shift to smaller angles, along with a lower value of its maximum compared to the maximum of the control sample graph.

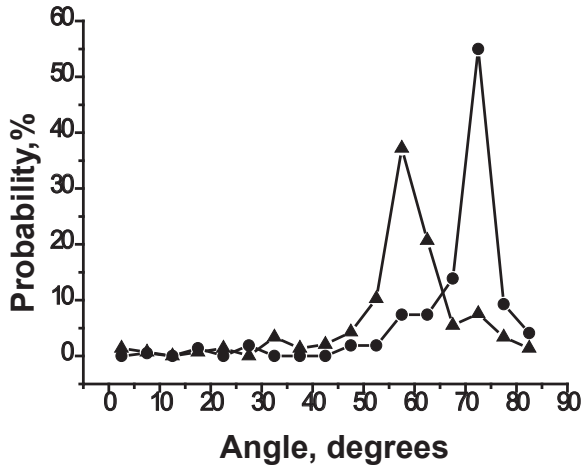

Fig. 1. Probability distributions measured immediately after the filtration: control sample (circles) and sample filtered with velocity of 1.4 mm/sec (triangles)

Fig. 2 displays the filtration impact on the time behavior of the distribution. The control and the filtered samples are simultaneously measured 24 hours after the filtration. One can see a residual filtration impact along with an evolution of the form of the distribution of the treated sample. A possible explanation refers to a difference in water structure between control and filtrated samples as a result of interface interaction between walls of nuclear filter's holes and water flow through the holes.

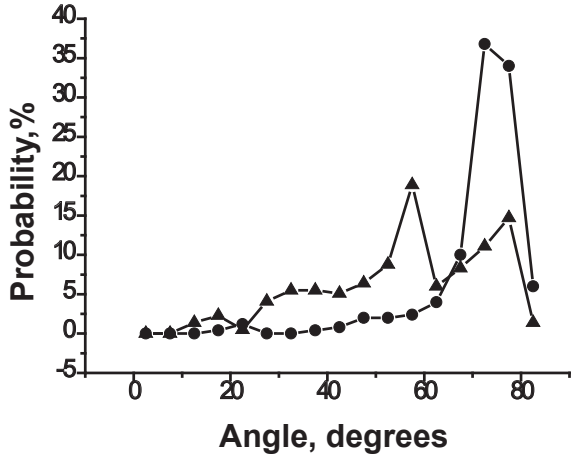

Fig. 2. Probability distributions of the samples measured 24 hours after the single filtration: control sample (circles), and sample filtered with velocity of 1.4 mm/sec (triangles)

Conclusions

The transport of a water sample through the holes of diameter 0.15 micrometer of a nuclear filter changes sample's properties, so that the contact angle evaporation distributions are influenced by filtration. Moreover a residual impact on the distributions after 24 hours is observed. An explanation of these effects could be expected to be given on the basis of the existence of hydrogen bonds forming a statistically stable net of interactions between water molecules.

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