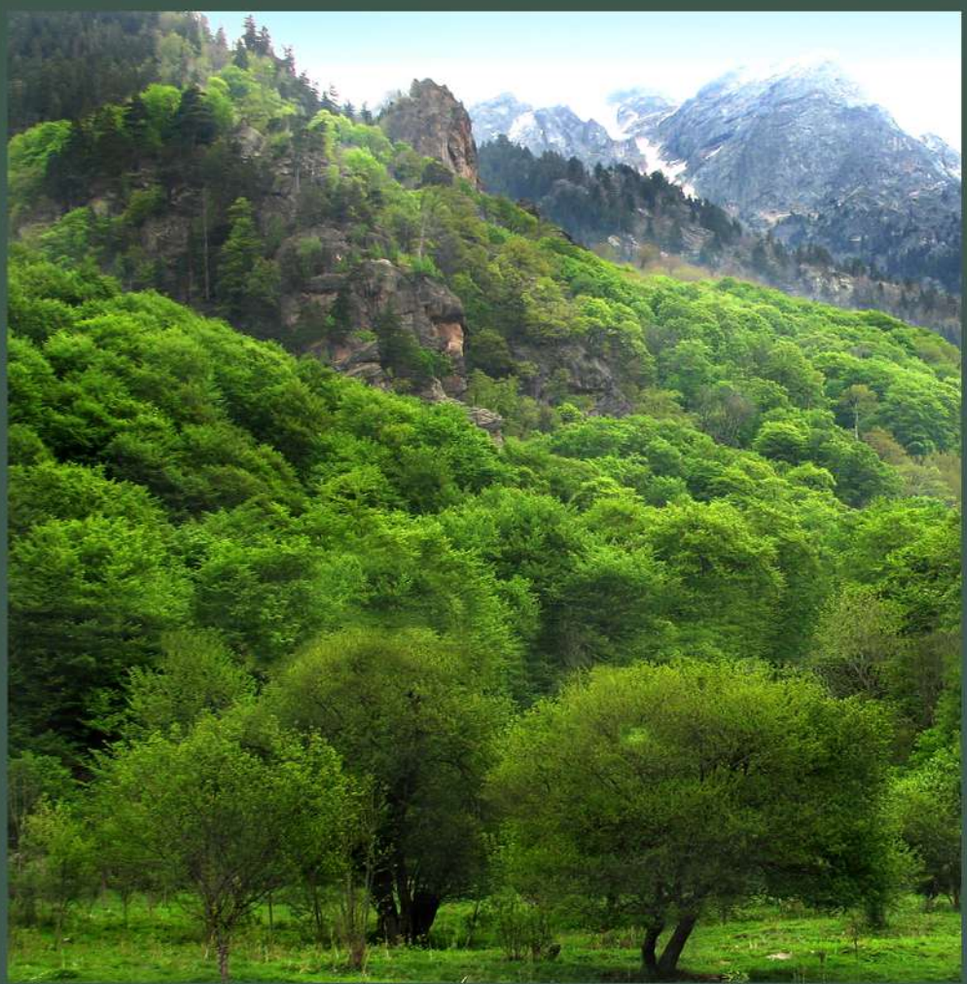
SEMINAR OF ECOLOGY – 2014

WITH INTERNATIONAL PARTICIPATION
DEDICATED TO 70 YEARS USB



Proceedings

24-25 April 2014, Sofia, Bulgaria

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

Seminar of Ecology – 2014 with international participation dedicated to 70 years USB Proceedings

24-25 April
Sofia, Bulgaria

Този сборник съдържа доклади, изнесени на „Семинар по Екология-2014“, с международно участие, проведен на 24-25 април 2014 г. в Институт по биоразнообразие и екосистемни изследвания – БАН, гр. София, България. Семинарът е посветен на 70-годишнината на СУБ. Част от докладите са публикувани в пълен текст, а други като кратки съобщения. Семинарът е организиран от секция „Биология“ към СУБ, Институт по биоразнообразие и екосистемни изследвания – БАН, гр. София, България и с любезната финансова подкрепа на СУБ и на фирмите БУЛГАП ЕООД и Л.К.Б - България ЕООД. Публикуваните в „Сборник по Екология-2014” материали са рецензирани и редактирани.

Съставителите

These proceeding include the reports, presented at the „Seminar of Ecology - 2014“, with international participation, 24-25 April 2014, Institute of Biodiversity and Ecosystem Research – BAS, Sofia, Bulgaria. The seminar was dedicated to 70 years USB. Proceeding includes full text reports and short communications. The seminar was organized by the section „Biology“, Union of Scientists in Bulgarian (USB) and Institute of Biodiversity and Ecosystem Research, BAS, Sofia, Bulgaria with finantial support of USB and companies BULGAP EOOD and LKB Bulgaria EOOD.

All manuscripts have been reviewed and edited.

The editors

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посветен на 70-годишнината на СУБ
Сборник доклади и кратки съобщения
Съставители: проф., д-р Стефка Чанкова, гл. ас., д-р Петя Първанова,
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ДО
УЧАСТНИЦИТЕ В НАУЧНИЯ
„СЕМИНАР ПО ЕКОЛОГИЯ – 2014“
С МЕЖДУНАРОДНО УЧАСТИЕ,
ПОСВЕТЕН НА 70-ГОДИШНИНАТА НА СУБ

УВАЖАЕМИ КОЛЕГИ!

За мен е чест и удоволствие от името на Управителния съвет на Съюза на учените в България и лично от мое име да поздравя участниците в станалия вече традиционен ежегоден Семинар по екология. Още повече, че настоящият семинар е посветен на 70-годишния юбилей от основаването на съюзната ни организация.

Проблематиката на „Семинар по екология – 2014“ е актуална и в съзвучие с националните и европейски приоритети в областта на екологията и опазването на околната среда.

Едва ли може да се оспорва факта, че екологията е интердисциплинарна наука. Екологическите проблеми могат да бъдат решени само на базата на изясняването на сложните взаимоотношения на екосистемите и биосферата. Всеки научен форум в това отношение трябва да се приветства и поддържа. Висока оценка заслужават организаторите на „Семинар по екология – 2014“, тъй като последователно и настойчиво поставят на вниманието на научната общност най-актуалните проблеми на екологията.

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.

Нека да има повече такива изяви и форуми, а добрите идеи и творчески инициативи да не секват!

Адрес: София 1505, бул. Мадрид 39; тел.: председател: 846 33 18; зам.-председатели: 944 11 57; секретар: 946 18 61; "Секции и клонове": 946 18 61; технически секретар: 944 11 57; списание "Наука", фондация "Наука": 943 30 22; "Международна и конгресна дейност": 943 01 26; финансово-счетоводен отдел: 943 19 86; факс: 944 15 90; e-mail: science@usb-bg.org; http://www.usb-bg.org

Уважаеми колежки и колеги,

Убеден съм, че „Семинар по екология – 2014“ ще представлява пореден сериозен принос към родната наука. Приемете моите сърдечни пожелания за успех на семинара и за по-нататъшни творчески успехи във Вашата изследователска работа!

София, 24 април 2014 г.,

Акад. Дамян Дамянов,
председател на СУБ

БЪЛГАРСКА АКАДЕМИЯ НА НАУКИТЕ
ИНСТИТУТ ПО БИОРАЗНООБРАЗИЕ И ЕКОСИСТЕМНИ ИЗСЛЕДВАНИЯ
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До проф. д-р Стефка Чанкова,

Председател на Организационния комитет

на Семинар по Екология 2014

Уважаеми организатори, уважаеми колеги и гости!

От името на Ръководството на ИБЕИ Ви приветствам с поредното издание на станалия вече традиционен Семинар по екология!

Този форум се утвърди като място за среща и нови контакти на специалисти в различни области както от ИБЕИ, така и от други научни институции в София и страната.

Като имам предвид богатата на участници и теми програма на семинара и през тази година, не се съмнявам, че и това му издание ще поднесе много нови научни постижения, ще създаде нови контакти, и ще даде началото на бъдещи съвместни проекти.

24 април 2014 г, София

С най-искрени пожелания за ползотворна работа

и интересни дискусии в рамките на семинара,

проф. д-р Снежана Грозева,
Научен секретар на ИБЕИ

Уважаеми участници в „Семинар по Екология - 2014“, с международно участие, посветен на 70-годишнината на СУБ, драги колеги,

Един от най-значимите проблеми в световен мащаб, пред който е изправен света през 21 век е екологичният. Глобалното затопляне, засушаването, което превръща хектари плодородна земя в пустини, намаляването на озоновия екран, драстичното изсичане на горите, киселинните дъждове, ежегодно увеличаващото се количество радиоактивни и др. токсични отпадъци, развитието и намножаването на много вредители по земеделските и горските култури, силното, а дори и за много региони опасно замърсяване, будят тревога и основателно безпокойство у човечеството за съдбата на планетата ни.

В какво се изразяват някои от по-важните неблагоприятни изменения на околната среда?

- замърсяване на атмосферата и създаване на сериозна опасност за климатичното равновесие;
- замърсяване на повърхностните води и Световния океан;
- силно намаляване на количеството (на глава от населението) и влошаване качеството на питейните води;
- физическо намаление и силно замърсяване на обработваемите земеделски земи като жизненоважен компонент на биосферата на планетата;
- застрашаване на биоразнообразието;
- влошаване условията на средата в големите градове, водещи до нежелателни последствия за здравето на хората;
- засилване противоречията между социално-икономическото развитие и опазването на чистотата на околната среда.

Проблемите, които трябва да се разрешат, произтичат както от бедността, така и от недалновидния начин, по който често се стремим към благосъстояние. Много части от света са впримчени в една порочна спирала – бедните са принудени да използват прекомерно ресурсите на околната среда, за да преживеят ден за ден, ощетявайки природата, което ги прави още по-бедни. Успоредно с това благосъстоянието, постигнато в някои части на света, често е несигурно, тъй като се дължи на методи, прилагани в селското стопанство, горското дело и промишлеността, които носят изгода и прогрес само за кратки периоди.

Антропогенният натиск върху околната среда често се разглежда като резултат от нарастващото търсене на недостатъчни ресурси и от замърсяването, причинено от повишаващите се жизнени стандарти на едно живеещо в сравнително охолство общество.

Как би могло да се противодейства?

Нужна е промяна в мисленето!

Нужна е промяна в поведението на всички групи от населението и особено на политическия, икономическия и управляващия елит.

Нужна е промяна в ценностите и в отношението към околната среда, мобилизация на усилията на всички за преодоляване на екологичните рискове.

И съм убедена, че в рамките на тези два дни чрез проблемите, които нашия Семинар поставя и разглежда ние заедно бихме допринесли за това!

24 април, 2014 г., София

Проф., д-р Стефка Чанкова

Председател на Организационния комитет

ДОКЛАДИ/REPORTS

Тематично направление:
БИОЛОГИЧНО РАЗНООБРАЗИЕ И КОНСЕРВАЦИОННА БИОЛОГИЯ

Topic:
BIOLOGICAL DIVERSITY AND CONSERVATION BIOLOGY

POPULATION STATE AND CONSERVATION OF THE BULGARIAN ENDEMIC *VERBASCUM TZAR-BORISII* (SCROPHULARIACEAE)

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Abstract

Aim: The aim of the study is to investigate the biology, ecology and the population state of the Bulgarian plant endemic *Verbascum tzar-borisii* (Scrophulariaceae) in order to reveals the main treats for the populations of the species and to indicate the measures necessary for their protection.

Material and Methods: The study has been carried out mainly *in situ* following the Protocol for Monitoring of plant species elaborated by Executive Agency of Environment.

Results: Two populations with about 1000 individuals are currently known in the Bulgarian flora: Sivri tepe locality, near to Chernevo village and Probit kamak locality, near to Ravna village, Varna district. The following threats have been identified to directly affect the populations of the target species: forest felling, fires, grazing, succession changes, low germination rate, etc.

Conclusions In order to improve the population state *in situ* and *ex situ* conservation measures have been undertaken and two protected areas have been designated.

Keywords: Bulgaria, Endemic plant, Scrophulariaceae*, Verbascum tzar-borisii*.

Introduction

In the Bulgarian flora genus *Verbascum* (Scrophulariaceae Juss.) is represented by 45 species and it is the second one richest in endemics. One of the rarest taxa is the Bulgarian endemic *Verbascum tzar-borisii* (Dav. ex Stoj.) Stef.-Gat. with very local distribution in the Northeast Bulgaria floristic region – only two populations are currently known – from Sivri tepe locality, near to Chernevo village and Probit kamak locality, near to Ravna village, Varna district (Fig. 1). The species is included in the Bulgarian Biodiversity Act [1], in the Red Book of Bulgaria [2] as 'Critically Endangered' and is one of the target species of the project 'A pilot network of small protected sites for plant species in Bulgaria using the Plant Micro-reserve model' supported by the EU's financial instrument for environmental

and nature conservation LIFE. The aim of the study is to investigate the biology, ecology and the population state of the Bulgarian endemic plant *Verbascum tzar-borisii* in order to reveals the main treats for the populations of the species and to indicate the measures necessary for their protection.

Material and Methods

The study is carried out mainly *in situ* following the Protocol for Monitoring of plant species elaborated by Executive Agency of Environment (http://eea.government.bg/bg/bio/nsmbr).

Results

Morphological description

Verbascum tzar-borisii is a perennial herb with erect, white compressed stems, 40-70 cm high [3]. Basal leaves are 7-15 cm long, 2.5-4 cm wide, entire, dull serrated white compressed, sometimes with glabrescent upper surface, cuneiform at the base, with long petioles, up to 1-5 cm. The flowers are grouped in 2-3 monohaziys and form branched pyramidal panicle. The corolla is pale yellow, 20-25 mm in diameter, with thick branched hairs along the outer surface and the white papillae on the basis. Stamens are 5, with yellowish petioles and white papillae. All anthers are identical, reniform, light yellow, decurrent. The fruits are ovoid, 4-5 mm long. It flowers in May and June, and fruitful in August and September. Entomophilous plant propagated by seeds.

Populations and habitats

Verbascum tzar-borisii grows in very open grassland communities on clayey marls slopes and poor soils. It inhabits a priority for the European Union habitat 62C0* Ponto Sarmatian Steppes (subtype B), represented by unions *Festucion valesiacae, Stipion lessingianae, Pimpinello-Thymion zygioidi,* with significant diversity in the plant communities.

1. Population of *Verbascum tzar-borisii* from "Sivri tepe" locality, Chernevo village, Varna district (N 43.249787°; E 27.601352°).

The area of the occupancy covers 0.84 ha and it characterized by a very poor and stony soil. In 2010, 539 individuals were counted (212 generative и 327 vegetative). The results of the monitoring carried out in 2011 and 2013 show similarities in its population's size and state. The distribution of individuals is highly fragmented among sparse shrub-grassland vegetation. The species is associated with: *Achillea clypeolata, Adonis vernalis, Alyssum borzeanum, Allium flavum, Amygdalus nana, Anthemis tinctoria, Artemisia alba, Asparagus officinalis, Astragalus glaucus, Berberis vulgaris, Bromus sterilis, Campanula sibirica, Caragana frutex, Centaurea caliacrae, C. salonitana, Cephalaria laevigata, Colutea arborescens, Convolvulus cantabrica, Coronilla emerus, Cotinus coggygria, Echinops ritro, Ephedra distachya, Euphorbia nicaeensis, Fraxinus ornus, Goniolimon collinum, Gypsophila glomerata, Haplophyllum suaveolens, Hieracium echioides, H. virosum, Jasminum fruticans, Jurinea tzar-ferdinandii, Lappula barbata, Melampyrum cristatum, Ononis pusilla, Orlaya grandiflora, Plantago lanceolata, Poa bulbosa, Quercus pubescens, Reseda lutea,*

Rhamnus saxatilis, Salvia nutans, Satureja coerulea, Sesili rigidum, Stachys recta, Stipa pulcherrima, Syringa vulgaris, Teucrium polium, Thalictrum minus, Thesium divaricatum, Tragopogon dubius, Veronica austriaca ssp. *jacquinii, V. longifolia, Xeranthemum annuum.* Five of the associated species are rare, endangered or endemic ones: *Alyssum borzeanum, Caragana frutex, Ephedra distachya, Goniolimon collinum Jurinea tzar-ferdinandi.*

2. Population of *Verbascum tzar-borisii* from „Probit kamak" locality, Ravna village, Varna district.

„Probit kamak" locality is one of a series of hills, northern to Provadiyska river, close to Ravna village (N 43.241309°; E 27.341760°). It belongs to the rocky complex Venchanski dyuz, which consists of Lower and Upper Cretaceous limestones (marls, marly clays, calcareous sandstones and limestones (3). The soil is humus-carbonate, very poor, dry, compacted, and highly stony. The vegetation is composed by thermophilous deciduous forests with participation of *Quercus cerris, Q. pubescens, Acer campestre, Carpinus orientalis, Q. frainetto, Carpinus betulus. Verbascum tzar-borisii grows associated with Satureja coerulea, Thymus zygioides, Achillea clypeolata, Alyssum borzeanum, Anthemis tinctoria, Anthericum ramosum, Aster oleifolius, Astragalus vesicarius, Berberis vulgaris, Campanula sibirica, Caragana frutex, Carex humilis, Centaurea marshaliana, Centaurea jankae, Cephalaria laevigata, Chrysopogon gryllus, Convolvulus cantabrica, Coronilla varia, Cotinus coggygria,Dichanthium ischaemum, Dorycnium herbaceum, Echinops ritro, Euphorbia nicaeensis, Festuca valesiaca, Fraxinus ornus, Fumana procumbens, Gypsophila glomerata, Jasminum fruticans, Helianthemum nummularium, Inula ensifolia, Jurinea consanguinea, Jurinea stoechadifolia, Koeleria brevis, Koeleria macrantha, Lappula barbata, Leontodon crispus, Matthiola fruticulosa, Medicago falcata, Melica ciliata, Minuartia setacea, Ononis pusilla, Paliurus spina-christi, Rosa sp., Plantago argentea, Prunus spinosa, Reseda lutea, Quercus pubescens, Rhodax canus, Salvia ringens, Sanguisorba minor, Scorzonera austriaca, Stachys recta, Stipa pulcherrima, Teucrium chamaedrys, Teucrium polium, Tragopogon dubius.* Three of the taxa have high conservation value: the populations of *Alyssum borzeanum, Caragana frutex* and the biggest Bulgarian population of the globally threatened paleoendemic species of Dobrogea, *Centaurea jankae*. In 2010, 515 individuals (272 vegetative and generative 243) were counted in an area of 0.5 ha. Population's structure represents a mosaic of a group located individuals.

Main threats

The following threats have been identified to directly affect the population of the target species: forest felling, fires, grazing, successional changes, low germination rate (about 30%), limited distribution, difficulties of seedling regeneration due to excessive dry and eroded terrain, etc.

Conservation measures taken

1. Action Plan for the Conservation of *Verbascum tzar-borisii* was developed and approved by the National Biodiversity Council at MOEW. 2. Two hundred seeds are deposited in the National Seed Genebank in Sadovo from the two populations. 3. Two protected areas are proclamated. 4. Information meetings in the RIEWs Varna with the local community and experts were conducted. 5. Information boards are placed near the two localities in the villages Ravna and Chernevo.

Conclusions

Entire world population of the species is represented on the territory of Bulgaria and numbering about 1000 individuals. For effective conservation of the species is necessary to strictly follow the provisions of the Action Plan for the Conservation of *Verbascum tzar-borisii.*

Acknowledgements: The study was completed during the implementation of the project 'A pilot network of small protected sites for plant species in Bulgaria using the Plant Micro-reserve model' supported by the EU's financial instrument for environmental and nature conservation LIFE. For determination of some associated species the research received support from the SYNTHESYS Project http://www.synthesys.info/ which is financed by European Community Research Infrastructure Action under the FP7 "Capacities" Programme (ES-TAF-3669, FR-TAF-3913).

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Fig. 1. Location of the studied populations of *Verbascum tzar-borisii* (Dav. ex Stoj.) Stef.-Gat.

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DETERMINATION OF THE ALKALOID GLAUCINE IN FOUR BULGARIAN POPULATIONS OF *GLAUCIUM FLAVUM* (PAPAVERACEAE)

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Abstract

Aim: The study aimed to update the information about the content of the main alkaloid in different Bulgarian populations of the valuable medicinal plant *Glaucium flavum* Crantz. (yellow hornpoppy).

Material and Methods: Plant samples, each consisting of the aboveground part of several plants, were gathered from four Bulgarian populations located near Varvara, Arkutino, Shkorpilovtsi and Sofia. The raw alkaloid mixture was obtained by usual chemical procedure, and the glaucine content was determined by densitometry program Quanti Scan from Biosoft.

Results: The percentage of the glaucine in the raw alkaloid mixture of three of the tested populations (Varvara, Arkutino and Sofia) was high and similar: between 70.2% and 74.2% while that of Shkorpilovtsi was only 31.2%. The glaucine content was highest in Varvara: 35.8 mg/g DW and lowest in Shkorpilovtsi: 4.4 mg/g DW.

Conclusions Plants from Varvara were considered as most appropriate for *in vitro* culture initiation for their highest glaucine content.

Keywords: yellow hornpoppy, glaucine, Bulgarian populations

Introduction

Glaucium flavum Crantz (Papaveraceae) (Fig. 1) is a biennial [2] medicinal plant species under special regime of protection and use according to Art. 10, Paragraph (1), (2), (3) of the Law on Medicinal Plants, its gathering for trade use from all its natural habitats is prohibited in the territory of the entire country by annual order of the Ministry of Environment and Water (order RD-83/3.02.2014). Aerial parts of *G. flavum* are used in pharmaceutical industry as source of the alkaloid glaucine, which has anti-tussive activity. It is used for the production of several trade marks, among them Glauvent®, Glauterpin® and Broncholytin® (Sopharma PLC). Glau-

cine has also anti-inflammatory, analgesic and antipyretic activity with low gastric toxicity [7]. Semi-synthetic structural analogues of glaucine show antioxidant and antivirus activity [11].

The area of distribution of the species is along the entire Mediterranean coast, along the Atlantic coast of Europe, England, Spain, Portugal and up to Ireland and Scandinavian peninsula in North. The species is distributed along the entire Black Sea coast [8]. In Bulgaria populations of *G. flavum* also occur in inland. The plant is confined to well-drained substrate types such as shingle, gravel, sand, cliffs and waste places [10]. The species become a retreat in many of its natural habitats world-wide, it is classified as vulnerable according to IUCN (2001) in all the Scandinavian countries [10] and it is subjected to be rare and endangered in the Egyptian flora due to urban sprawl [5]. It is necessary to make an effort for *ex situ* conservation of the species due to its medicinal properties and conservation significance. That makes the species in target of *in vitro* culture initiation attempts. For culture initiation it is important to use plants with higher alkaloid content, because the alkaloid biosynthesis is expected to be genetically determined. Different chemotypes of the species have been reported differing in their alkaloid profile and the content of the main alkaloids, including glaucine [3, 6, 9]. This made necessary conducting phytochemical studies to establish glaucine content in plants from the Bulgarian populations.

Fig. 1. *Glaucium flavum* Crantz.

Material and Methods

The plant material was collected during the intensive blooming of yellow hornpoppy in July 2013 from four native populations of the species located near Arkutino, Varvara, Schkorpilovtsi and Sofia. Mean population plant samples were gathered consisting of the aboveground part of several plants each. The air-dried and ground plant material (18 g per sample) was exhaustively extracted in Soxhlet apparatus with 96% ethanol. Concentrated ethanol extracts were acidified with 3% HCl and left for 24 hours. The acidified solution was subjected three times to petroleum ether extraction. The purified acidic solution was alkalized with NH_4OH (pH 9-10) and then extracted four times with CH_2Cl_2. The combined CH_2Cl_2 extracts were dried over an-

hydrous Na_2SO_4 and then evaporated. The dried residue constitutes the raw alkaloid mixture. Thin layer chromatography was performed on DC Alufolien Kieselgel 60 F_{254} (Merck) and the sheets were developed with solvent system from petroleum ether:chloroform:acetone:methanol (4:4:1:1). The spots were visualized by spraying with Dragendorf's reagent. Glaucine was quantified using QuantiScan® densitometry program (Biosoft, Cambridge, UK).

Results

In the raw alkaloid mixtures (RAM) obtained from the four populations the highest quantity of alkaloids was measured in the plant material from the population located near Varvara, and the lowest one – from the plant material collected from Schkorpilovtsi (Table 1). Glaucine was the main alkaloid in all four studied populations. Glaucine content in the aerial plant parts was highest in the sample from population near Varvara, while the lowest one was detected in the sample from Shkorpilovtsi (Fig. 2). Although the samples from the populations in Arkutino and Sofia had equal percentage of glaucine, the content of this alkaloid was higher in the population near Arkutino. On Fig. 3 is presented densitogram of alkaloids extracted from the plant material originating from the population near Varvara.

Table 1. Raw alkaloid mixture (RAM) and glaucine in the plant samples from four Bulgarian populations of *Glaucium flavum* (average values of 5 samples)

Population	RAM in the plant sample [%]	Glaucine in the RAM [%]	Glaucine in the plant sample [%]
Arkutino	2.76	70.2 ± 3.6	1.93 ± 0.11
Varvara	4.82	74.2 ± 4.8	3.58 ± 0.02
Sofia	2.12	70.2 ± 5.4	1.49 ± 0.08
Shkorpilovtsi	1.42	31.2 ± 4.1	0.44 ± 0.02

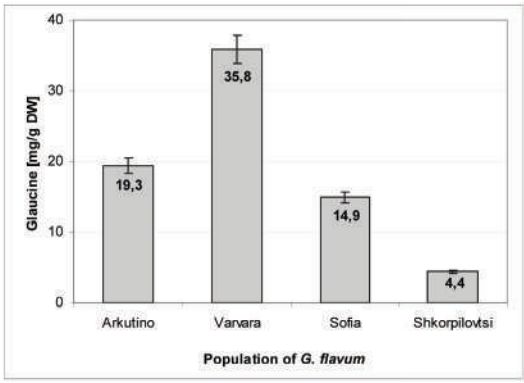

Fig. 2. Glaucine content in the aboveground part of *G. flavum* plants from four Bulgarian populations

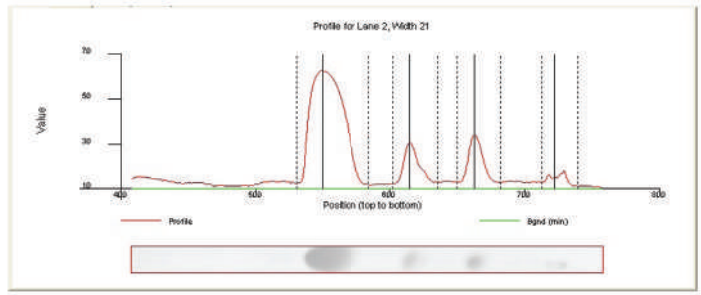

Fig. 3. Densitogram of the raw alkaloid mixture from Varvara population of *G. flavum.*

Discussion

Our studies revealed great differences in the raw alkaloids' and glaucine percentages in the aboveground plant parts of the four studied populations of *G. flavum*. These results are in accordance with previously reported data about other five populations of the species located along southern Bulgarian Black Sea coast [4]. Authors reported variation of glaucine content from 0.9% in the population near Ahtopol to 2.9% in the population near Lozenetz. Our results expanded this range, so the lowest content of glaucine was determined in the sample from Schkorpilovtsi (0.4%), which was 8-time less than that in the sample from Varvara (3.6%). Similar results were published about *G. flavum* populations in Israel where glaucine was from no detectable amounts to 3.6% [6].

The significant differences in glaucine content in the samples from all studied populations of *G. flavum* should be further investigated to find the reasons for such alkaloid variability. One of the reasons could be the complex of the environmental factors: light, water, macro- and micronutrients, pH of the soil, temperature etc., but the available data about their impact is controversial and still insufficiently investigated [13]. Thus, the effect of soil salinity on alkaloids' accumulation studied for plants of *G. flavum* originating from four Israel populations was quite variable according to the different alkaloids in the raw alkaloid mixture and depended mostly on the plant origin at populational and individual level [6]. Authors concluded that the environmental stress though commonly considered as factor stimulating alkaloids' biosynthesis, was useless in the case of this species because glaucine content was highest in the control plants while its amounts in plants exposed to water and salt stress in the natural habitats or irrigated with NaCl solutions in the laboratory, decreased significantly. Salinity didn't affect the content of any of the alkaloids in plants gathered from another one of the tested populations. Similar inter- and intravariability of various populations were reported concerning the content of galanthamine and lycorine which were found to be the main alkaloids of *Leucojum aestivum* plants in most of its Bulgarian populations [12]. The genotypes of *L. aestivum* individuals were proved to be crucial for the alka-

loid patterns and the content of these two alkaloids although some slight fluctuations were observed in relation with the abiotic factors.

On the other hand, as alkaloid biosynthesis needs nitrogen its levels in the soil available to plants are important in the sense that nitrogen lack could limit the alkaloid content. However, some alkaloids are more sensitive to nitrogen availability than others which is in relation with the amount of nitrogen in their precursors [1]. Treatments high in nitrogen led to increase of alkaloid content in tobacco (*Nicotiana*) and lupine (*Lupinus*) [1]. In general, nitrate uptake promotes alkaloid accumulation and is preferred over ammonium uptake. The balance of nitrogen and other nutrients in the soil seemed also to be important.

To clarify the differences in glaucine content noticed for the populations which were object of our study, cultivation of plants originating from Varvara, Shkorpilovtsi and Sofia is in progress. The plants are cultivated in the experimental field of IBER in equal environmental conditions (soil and climatic factors). After a period of adaptation a comparative phytochemical analysis of the cultivated plants will be carried out.

In conclusion, the content of glaucine was highest in plants of the population near village Varvara. This finding gave us the reason to consider the plant material originating from this population as most appropriate for *in vitro* culture initiation.

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PSEUDOMONAS CORRUGATA – CAUSE OF TOMATO PLANTS DIEBACK IN GREENHOUSES IN BULGARIA

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Abstract

Aim: This study aimed to identify the causal agent of tomato plants dieback in greenhouses in different regions in Bulgaria in order to provide relevant advices for growing tomatoes.

Material and Methods: Bacterial strains were isolated from diseased plant parts and subjected to pathogenicity tests. The scheme of Schaad and BiologTM system were used for characterization and identification.

Results: The isolates induced hypersensitivity reaction in tobacco and formation of watery brown spots in tomato stems. The spots later turned to gaps with necrotic rings. The strains were identified as *Pseudomonas corrugata* – a pathogen known worldwide to cause pith necrosis of tomato. The full metabolic profile for the Bulgarian isolates was described.

Conclusion: *P. corrugata* was established as the causal agent of tomato plants dieback ('pith necrosis') in Bulgarian greenhouses. To our knowledge this is the first report of this pathogen in Bulgaria.

Keywords: *Pseudomonas corrugata*, tomato, disease, greenhouses, Bulgaria

Introduction

Tomato stem or pith necrosis is a serious disease causing great losses to tomato production worldwide. It has a 40-year history since it was first observed in England in the early 1970s [13]. The described symptoms included brown discoloration, necrosis and collapse of the pith, vascular browning, external dark stem lesions and adventitious root formation. The pathogen – *Pseudomonas corrugata*, was widespread in glasshouses in England, but it caused severe loss in only a few [13]. However since then, the disease has tended to spread and raise the damages in the infected greenhouse holdings. At the same time the reports of the disease from different countries in the world continue to

count placing pith necrosis in the list of the emerging diseases [2, 10, 11, 12]. Pith necrosis has been observed in field-grown tomatoes but mainly in USA [6, 8]. Since it is favored by high humidity and free water on the plant surfaces [13] different installations like classic greenhouses, high tunnels and hydroponic holdings often ensure optimal conditions for disease development [10]. Compared to traditional outdoor field growing, greenhouse production provides the option of off-season crop production and expansion of markets. The raised incidence of a disease places these productions against a new challenge.

Although *P. corrugata* was initially pointed as a causal agent of tomato pith necrosis later other pathogens were reported to cause similar symptoms. Pith necrosis of tomato caused by *Pseudomonas viridiflava*, and *Pseudomonas cichorii* has been described in commercial greenhouses in many countries [2, 5, 12, 15]. In the beginning of the 21st century *Pseudomonas fluorescens, Pseudomonas mediterranea* and *Pseudomonas marginalis* were identified as the causal agents of the disease [3, 7, 10, 12]. Other soil-borne bacteria isolated from symptomatic tomato plants also induced discolouration of vascular tissues - *Pseudomonas putida, Pseudomonas citronellolis, Pseudomonas straminea* and *Pantoea agglomerans* [1]. A single report described *Xanthomonas perforans* strains able to induce pith necrosis, vascular discolouration, longitudinal splits and external lesions on stems [1].

In Bulgaria pith necrosis was first observed in the early 1990s with causal agent *P. viridiflava* [5]. Since then pith necrosis occasionally developed in different tomato growing greenhouses in the country. This study aimed to identify the causal agent of tomato plants dieback (pith necrosis) in 2012-2013 in greenhouses in different regions in Bulgaria.

Material and Methods

Bacterial strains were recovered from diseased parts of tomato plants – Holland greenhouse varieties, grown in greenhouse conditions in the regions of Samokov and Velingrad, Bulgaria in 2012-2013. Bacteria were isolated as single colonies on King's medium B as described by Klement et al. [9] and pure cultures were obtained after routine purifying procedures.

The pathogenic potential of the isolates was examined by the hypersensitive reaction (HR) test [9] on tobacco cv. Samsun NN and pathogenicity tests on tomato. Tomato plants with 4-5 permanent leaves were inoculated by stem injection and incubated under conditions of high humidity. Bacterial suspensions of 10^4 cfu/ml grown on Potato-Dextrose Agar medium at 28°C, for 24 h were used for infiltrations. Observations for HR were held up to 24th h after inoculations and tissue changes in tomato stems and whole plants were examined periodically between 1st-4th day.

Screening for major differentiating properties for genus *Pseudomonas* and LOPAT test were conducted [14]. Identification of the isolates was carried out by the miniaturized identification system Biolog™ (Biolog™, USA) with GN2 MicroPlate™ test plates. The software program v4.20.05 of MicroLog™ (Biolog™, USA) was used.

Results

The natural symptoms of infection included discoloration followed by necrosis and hollowing of the pith of the stem leading to plant collapse (Fig. 1). The symptoms were similar to those caused by *P. viridiflava*.

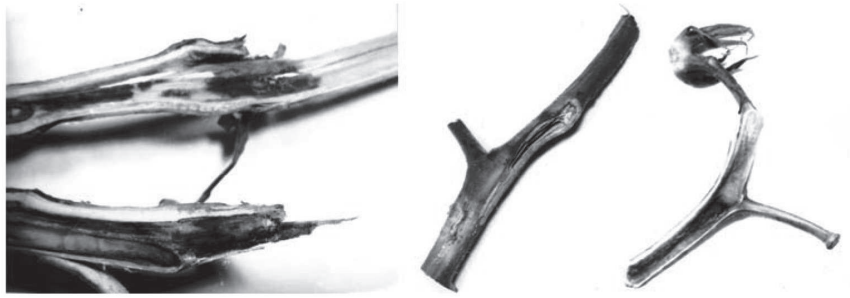

Fig. 1. Symptoms of necrosis and hollowing of stem pith tissues in natural infection of tomato

The isolated bacterial strains induced hypersensitive reaction in tobacco leaves until 24 hours after inoculation. Tomato plants reacted with formation of watery brown lesions on the places of inoculation which enlarged fast. The centers necrotized and turned into gaps. The lower leaves turned yellow and wilted. After cutting of the stems vascular discolouration and beginning of pith necrosis were clearly visible.

The strains were Gram-negative, aerobs, lacking a fluorescent pigment on King's B medium. Their LOPAT test profile (-+--++) was typical for *P. corrugata*.

All strains were identified with BIOLOG™ system as *P. corrugata* with probability 100%, Similarity index 0.845-0.926 and distance index 1.25-1.87.

The isolated strains used as sole carbon sources: Tween 40, Tween 80, L-arabinose, D-arabitol, D-fructose, D-galactose, α-D-glucose, m-inositol, D-mannitol, D-mannose, sucrose, trehalose, pyruvic acid methyl ester, cis-aconitic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, β-hydroxybutiric acid, p-hydroxyphenylacetic acid, α-keto glutaric acid, lactic acid, D-saccharic acid, succinic acid, bromosuccinic acid, glucuronamid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-proline, L-pyroglutamic acid, carnitine, γ-aminobutyric acid, urocanic acid, inosine, and glycerol.

Weak reaction was recorded to dextrin, N-acetyl-D-glucosamine, D-melibiose, monomethyl succinate, acetic acid, formic acid, D-glucosaminic acid, α-ketobutyric acid, malonic acid, propionic acid, quinic acid, glucuronamid, alaninamide, glycil-L-glutamic acid, hydroxy-L-proline, L-ornithine, L-serine, L-threonine, uridine, putrescine, 2,3-butanediol and α-glycerol phosphate.

Bulgarian isolates did not assimilate: α-cyclodextrin, glycogen, N-acetyl-D-galactosamine, adonitol, cellobiose, i-erythritol, L-fucose, gentibiose, α-D-lactose, lactulose, maltose, β-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-

sorbitol, turanose, xylitol, α-hydroxybutiric acid, γ-hydroxybutiric acid, itaconic acid, α-ketovaleric acid, sebacic acid, succinamic acid, glycyl-L-aspartic acid, L-phenylalanine, D-serine, thymidine, phenylethylamine, 2-aminoethanol, D-glucose-1-phosphate and D-glucose-6-phosphate.

The overall abilities of the strains for surviving on different substrates were relatively high as 1/3 out of 95 tested substrates were not utilized (Fig. 2).

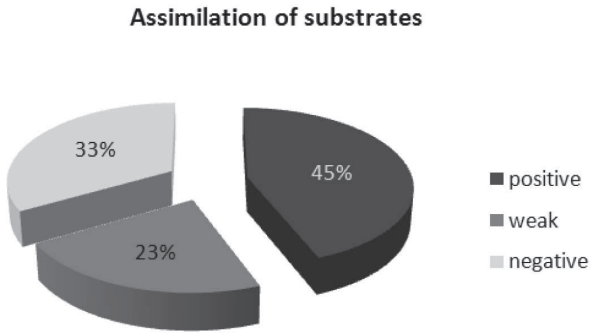

Fig. 2. Ability of *P. corrugata* strains for utilization of the substrates included in Biolog GN2 MicroPlates™

Discussion

P. corrugata was found as a causal agent of pith necrosis of tomatoes for the first time in Bulgaria. Similar symptoms – brown discoloration, necrosis and stem pith collapse have been observed in tomato plants in Bulgarian greenhouses in 1990-1991 but the causal agent was identified as *P. viridiflava* [5]. The disease greatly lowered the quantity of production but the fruits were found to be free of the pathogen [5]. The plants observed in 2012-2013 wilted and collapsed without fruiting. Once introduced in a greenhouse, the disease spread rapidly with pruning, watering and other routine manipulations severely damaging the plants and compromising the production.

Data in the world literature report 11 species responsible for the pith necrosis disease – maybe the largest conglomerate of different bacteria able to cause a syndrome in plant pathology. The pathogens have variable spread and occurrence and it seems that their individual roles in the incidence of the disease are also different. According to the available data, we placed the causal agents of pith necrosis into four groups according to their significance and economical impact (Table 1). The pathogens isolated in Bulgaria attach to the first group of most widely spread and dangerous pathogens which explain the losses in production and the need of strict sanitary measures and a watering regime as means for their control.

Table 1. Bacterial pathogens of pith necrosis reported worldwide

Main	Occassional	Isolated report	Minor/Secondary
P. corrugata [2,6,8,10,11,12,13,15]*	*P. cichorii* [12,15]	*X. perforans* [1]	*P. putida* [1]
P. viridiflava [4,5,12]	*P. fluorescens* [7,12]		*P. citronellolis* [1]
P. mediterranea [3,12]	*P. marginalis* [10]		*P. straminea* [1]
			P. agglomerans [1]

*cited literature is not exhaustive

Conclusion

Pseudomonas corrugata was established as causal agent of tomato plants dieback ('pith necrosis') in Bulgarian greenhouses. This is the first report of this pathogen in Bulgaria.

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CONTRIBUTION TO THE RESEARCH ON MYRIAPODS (CHILOPODA, DIPLOPODA) IN THE MADARA PLATEAU, SHUMEN REGION, NORTH-EASTERN BULGARIA

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Abstract

Aim: This study is the first attempt at deliberate research on the taxonomic structure and the ecological characteristics of the myriapods fauna (Chilopoda, Diplopoda) in the Madara Plateau.

Material and Methods: The material was gathered on 7 sampling sites through pitfall traps recorded on a monthly basis in the period June-October 2012, July-December 2013.

Results: A total of 1127 myriapods were gathered: 975 millipedes and 152 centipedes. Recorded were 10 species of 4 orders: Glomerida, Polydesmida, Chordeumatida, and Julida (class Diplopoda), and 13 species of the orders Scutigeromorpha, Lithobiomorpha, Scolopendromorpha, and Geophilomorpha (class Chilopoda). There are two new species for the region: the centipede *Pleurolithobous patriarchalis* (Berlese, 1894) and the millipede *Glomeris balcanica* Verhoeff, 1906 the latter not been reported in North-eastern Bulgaria so far.

Conclusions Individuals from the order Julida (Diplopoda) and from the order Lithobiomorpha (Chilopoda) were predominant in the region. Eurytopic and polytopic mesothermal, mesophilic, and mesohygrophilic species prevailed. In terms of zoogeographic structure both classes featured highest number of species from the Northern complex followed by the species with Balkan distribution. The Southern complex was represented by only four species of Chilopoda – *Scutigera coleoptrata, Lithobius nigripalpis, P. patriarchalis* and *Scolopendra cingulata*.

Keywords: Madara Plateau, Chilopoda, Diplopoda, BG0000104.

Introduction

The results from taxonomic and ecological research on different groups of organisms at a regional and local level have enormous significance not only for the investigation of the biodiversity of the local flora and fauna but for the preservation of habitats and rare species as well. The specification of the composition and structure of the families of spe-

cies in a given region is not an end in itself and could be used for the clarification of fundamental problems connected with the origin, division, and distribution of species, the dynamics of their areas and the advent of allochthonous and invasive elements. Producing an inventory of the faunistic complexes in a specific geographical region could serve as a sample for research on the role of environment in the biogeographic distribution of species and the formation of a particular biota. A very suitable region for research of the kind are the rudimentary plateaus of the hilly and plateaued plain of Mysia which take up 56% of its total area and have an altitude of 300-500 m. They have a common origin and represent remnants of denudation surfaces and terraces – a result of the constant rise of Mysia plain [1]. The plateaus along the eastern part of Mysia plain are arranged in terraces with the lower plateaus and hills (Ludogorsko, Dobrudzhansko, Madarsko, Frangensko, Momino, Provadiysko, and Royaksko) located in its periphery towards the river Danube and the Black Sea, while the higher ones (the Popovski, Razgradski, and Samuilovski Heights, and the plateaus Lilyaksko, Shumensko, and Stana Plateau) are found in its center. They are fragmented and isolated due to the presence of perpendicular cliff cornices of durable limestone and sandstones on the one side and an intricate network of lowlands and dry valleys on the other [18]. In connection to the latter, plateaus could be considered a type of island habitats with their characteristic and/ or specific flora and fauna. The comparative analysis on the biodiversity in the remnant plateaus can be used to study the distribution of species and the mechanisms for the formation of nature complexes.

This study aims at setting the beginning of extensive and complex research on the taxonomic composition and ecological characteristics of myriapod communities (Diplopoda, Chilopoda) in the Madara Plateau. The idea is that the results of this inventory can be compared to the data on myriapod fauna in the plateau of Shumen which is situated in close proximity, as well as, at a later stage, to compare them to similar research on other plateaus in Northeastern Bulgaria. As a result of research conducted over the span of a few years the faunal list of the myriapods in the region at present includes 22 species millipedes and 37 species centipedes found in the urban and suburban parks of Shumen, in the forest shelter belts close to the town, as well as in the natural habitats in the Shumen Plateau [2, 4]. In the Madara Plateau, which is situated 12 km east of the town of Shumen, up to the present research only two species of Chilopoda – *Scolopendra cingulata* and *Clinopodes flavidus* [9] have been registered (Table 1).

Material and Methods

The research was conducted in the region of the Madara Plateau which rises in the southern part of Mysia Plain and is a wide circum denudial plateau which takes up the platform which is furthest north and the highest in the Royak Plateau. The climate is moderate-continental and the soil is mostly chernozem and grey forest soil. The upper layers of Madara rocks are comprised of thick layers of conglomerates, carbonate sandstones, and sandy limestone [12]. Maximum altitude (429.8 m) is measured at the peak Dvete mogili situated in the north-western part of the plateau (Fig. 1).

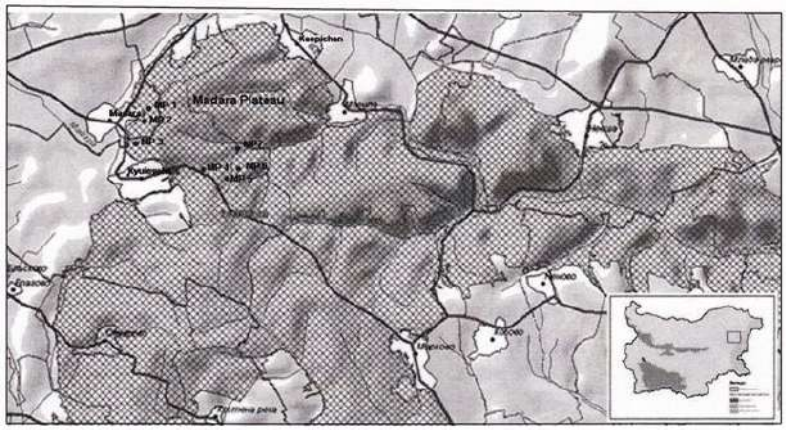


Fig. 1. Map of the study area including location of the sampling plots

The phytocenotic structure of the region is formed mostly by mesoxerophytic oak forests, featuring Hungarian/Italian oak (*Quercus frainetto*) and secondary forests of Oriental Hornbeam (*Carpinus orientalis*). Half of the territory of the Madara Plateau is taken up by mixed deciduous forests comprised predominantly of *Quercus cerris, Q. frainetto, Quercus polycarpa, Carpinus betulus, C. orientalis,* and *Fraxinus ornus* [5]. Characteristic of the more arid parts of the secondary forest ecosystems are the steppe flora species *Stipa lessingiana, Amygdalus nana, Astragalus glaucus, Scutellaria orienthalis, Centaurea marschaliana, Veronica spuria*, etc. Cultivated area (vineyards and fields) take approximately 47% of the territory of the plateau, while about 13% are covered with pastures overgrown with frutescent vegetation.

The material was gathered on 7 sampling plots (Fig. 1) which are representative of the main habitats in the plateau and could be compared to those of the Shumen Plateau – two coniferous forests dominated by *Pinus nigra* (MP 1) and *Pinus sylvestris* (MP 7) respectively, two deciduous forests of *Carpinus orientalis* and *Carpinus betulus* (MP 3 and MP 6), a mixed deciduous-coniferous forest of *Quercus cerris, C. betulus* and *P. sylvestris* (MP 5) and two meadows of steppe and wheatgrass vegetation, separate shrubs, and trees (MP 2 and MP 4). There were 10 pitfall traps in each sampling plot. They were set in a straight line at a distance of 10 m from each other and were reported monthly in the periods June-October 2012 and July-December 2013.

Results

A total of 1127 myriapods – 975 millipedes and 152 centipedes, were gathered at the time of research. Registered were 10 species of Diplopoda of 4 orders – Glomerida, Polydesmida, Chordeumatida, and Julida, represented by one family each. The biggest number of collected individuals for the accounted periods was of *Glomeris hexasticha* (32.5% of the total collected millipedes), followed by representatives of the order Julida – *Cylindroiulus boleti* (22%), *Megaphyllum transsylvanicum* (13.1%), *Leptoiulus trilineatus* (12.5%), and *Megaphyllum bosniense* (12.3%) (Table 1).

Table 1. Species composition, zoogeographic structure and ecological adherence of the species of Myriapoda (Diplopoda, Chilopoda) found in the Madara Plateau.

***Abbreviations**: new species for the fauna of myriapoda in the Madara Plateau; **Chorotypes: CEU** – Central European, **EEU** – East European, **EU** – European, **CPBK** – Carpathian-Balkan, **BK** – Holobalkan, **EMT** – European-Mediterranean-Turanian, **MED** – Holomediterranean, **EME** – East-Mediterranean. **Ecological groups: E** – eurytopic, **P** – polytopic, **O** – oligotopic, **S** – stenotopic; **me-ma** – meso-macro-thermal, **me** – mesothermal, **me-mi** – meso-micro-thermal; **m-hy** – mesohygrophilic, **m** – mesophylic, **m-x** – meso-xero-phylic; **t-x** – trogloxenic, **geo** – geophilic.

№	Species	Author	Zoogeographical structure	Number of specimens	Ecological groups with respect to:			
					Biotopic preferences	Temperature conditions	Humidity	Light conditions
	DIPLOPODA							
	Order Glomerida							
	Family Glomeridae							
1.	*Glomeris hexasticha* Brandt, 1833	*	CEU	317	P	me	m-hy	geo
2.	*Glomeris balcanica* Verhoeff, 1906	*	BK	30	P	me	m-hy	geo
	Order Polydesmida							
	Family Polydesmidae							
3.	*Polydesmus complanatus* (Linnaeus, 1761)	*	CEU	2	P	me	m	geo
4.	*Polydesmus renschi* Shubart, 1934	*	CPBK	2	O	me	m-hy	geo
	Order Chordeumatida							
	Family Anthroleucosomatidae							
5.	*Anamastigona bilselii* (Verhoeff, 1940)	*	BK	32	P	me-mi	m	geo
	Order Julida							
	Family Julidae							
6.	*Leptoiulus trilineatus* (C.L. Koch, 1847)	*	EEU	122	P	me	m	t-x
7.	*Cylindroiulus boleti* (C.L. Koch, 1847)	*	EEU	215	P	me	m	t-x
8.	*Megaphyllum bosniense* (Verhoeff, 1897)	*	BK	120	P	me	m	geo
9.	*Megaphyllum lictor* (Attems,1904)	*	BK	7	P	me-mi	m	geo
10.	*Megaphyllum transsylvanicum* (Verhoeff, 1897)	*	EEU	128	P	me	m	geo
	Total number of collected specimens:			**975**				
	CHILOPODA							
	Order Scutigeromorpha							
	Family Scutigeridae							
1.	*Scutigera coleoptrata* (Linnaeus, 1758)	*	MED	1	P	me-ma	m-hy	t-x
	Order Lithobiomorpha							
	Family Lithobiidae							
2.	*Lithobius (Lithobius) muticus* C.L. Koch, 1847	*	CEU	16	E	me	m-hy	t-x
3.	*Lithobius (Lithobius) nigripalpis* L. Koch, 1867	*	EME	56	E	me-ma	m	t-x
4.	*Lithobius (Lithobius) forficatus* (Linnaeus, 1758)	*	EU	5	E	me-mi	m-hy	geo
5.	*Lithobius (Lithobius) mutabilis* L. Koch, 1862	*	CEU	18	P	me	m	t-x

6.	*Lithobius (Lithobius) lucifugus* L. Koch, 1862	*	CEU	1	E	me	m-hy	t-x
7.	*Lithobius (Sigibius) microps* Meinert, 1868	*	EU	1	E	me	m-hy	t-x
8.	*Pleurolithobous patriarchalis* (Berlese, 1894)	*	EME	28	E	me-ma	m-hy	geo
	Order Scolopendromorpha							
	Family Scolopendridae							
9.	*Scolopendra cingulata Latreille,* 1829	Kaczmarek, 1970	MED	0	P	me-ma	m-x	t-x
	Family Cryptopidae							
10.	*Cryptops anomalans Newport,* 1844	*	EU	17	E	me	m-hy	geo
	Order Geophilomorpha							
	Family Geophilidae							
11.	*Clinopodes flavidus* C.L. Koch, 1847	Kaczmarek, 1970	EMT	4	E	me	m-hy	geo
12.	*Stenotaenia linearis* (C.L. Koch, 1835)	*	EU	2	P	me-ma	m-hy	geo
	Family Dignathodontidae							
13.	Henia illyrica *(Meinert, 1870)*	*	EEU	1	E	me-ma	m	geo
	Family Linotaeniidae							
14.	*Strigamia crassipes* (C.L. Koch, 1835)	*	EU	2	E	me-mi	m-hy	geo
	Total number of collected specimens:			**152**				

Fourteen species of Chilopoda from the orders Scutigeromorpha, Lithobiomorpha, Scolopendromorpha, and Geophilomorpha are registered in the Madara Plateau at present. The species *Scolopendra cingulata*, which was reported in the region of Kaczmarek in 1970 [9] was not found at the time of our study. The biggest variety of species is observed in order Geophilomorpha presented by four species of three families – *Clinopodes flavidus* and *Stenotaenia linearis* (Geophilidae), *Henia illyrica* (Dignathodontidae) and *Strigamia crassipes* (Linotaeniidae). There is a considerable number of species registered with minimal quantity and with separate specimens found in the region – *Lithobius forficatus, C. flavidus, S. linearis, S. crassipes, Scutigera coleoptrata, Lithobius lucifugus, Lithobius microps* and *H. illyrica*, while *Lithobius nigripalpis* features the biggest number of collected centipedes (36.8%) (Table 1).

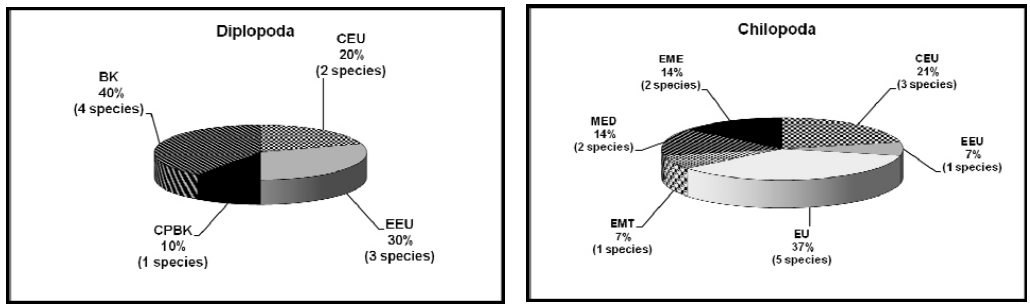
Discussion

As a result from this study the faunal list of myriapods in the Madara Plateau has reached 24 species of the two classes (Chilopoda, Diplopoda). Twenty-two (22) of the species found are new and unrecorded up to now in the region. The number of the species is considerably smaller than the number of the myriapods registered in the region of Shumen and the Shumen Plateau (59 in number), however, we expect that the increase in the span of research and the use of manual collection in other parts, as well as in specific microhabitats (under stones, in rotting wood, soil, and foliage carpet) will lead to an increase in the total number of species in the region. This will allow a thorough comparative analysis on the composition of species and the ecological characteristics of myriapod fauna in the two plateaus to be conducted at a later stage.

At present on the Madara Plateau the "pull" millipede from order Glomerida – G*lomeris hexasticha* is with highest percentage, while in the Shumen Plateau the highest number of millipedes is presented by *Leptoiulus trilineatus* (1474). As a whole the millipede fauna in both plateaus is dominated by species of the order Julida, while the centipede communities both in the Shumen Plateau and in the Madara Plateau are dominated by Lithobiidae [3].

The analysis of the zoogeographic structure of myriapod communities in the region of study used the zoogeographic categories introduced by Stoev [14]. In the millipede community in the Madara Plateau the species with European distribution and the species from the Northern Complex have equal share (Fig. 2-A). Holobalkan species have the highest percentage (based on the number of species) – 40%, followed by species with East-European distribution (30%), and Central-European distribution (20%). European (38%) and Central-European (23%) species predominate in class Chilopoda (Fig. 2-B) and currently there are no registers of species with Balkan distribution. The Southern complex, comprised mostly of thermophilic, stenothermal, and stenotopic species, is not represented in the Diplopoda in the Madara Plateau, while in the faunistic list of Chilopoda in the region it includes a considerable number of species (35%) – with European-Mediterranean-Turanian (1 species), Holomediterranean (2 species), and with East-Mediterranean distribution (2 species).

In both classes of myriapods in the Shumen Plateau, as well as currently on the Madara Plateau, the share of the Northern complex, which is comprised of cryophilic, cold-resistant, eurytopic species with wide distribution in Europe and the Palearctic, predominates over the one of the Southern Complex.



A. B.

Fig. 2. Zoogeographic structure of the myriapod families (A. Diplopoda, B. Chilopoda) in the Madara Plateau

In the current study the ecological groups of myriapods in the Madara Plateau by analogy with those found in the Shumen Plateau are classified according to their biotopic preferences and the degree of their ecological flexibility to the temperature conditions, humidity, and light conditions of their habitat [3] (Table 1).

The millipedes such as detrito- and saprophags are trophically dependent on the type of vegetation which determines the nutritional base of the habitat [15, 16] and this predetermines the lack of eurytopic species among them (Fig. 3). The main part of millipedes in the region, based on the data

from their common distribution in Europe and in Bulgaria, are polytopic. They are typically forest inhabitants but have also been registered in open mountainous habitats with frutescent vegetation, as well as in urban and suburban urbanized habitats. This group is the biggest in number in the region of study and 90% of the registered millipedes can be categorized as such. It is only *Polydesmus renschi* which is oligotopic and inhabits a limited number of habitats with similar ecologic conditions and plant formation. In the Madara Plateau the species was registered in June 2012 only in coniferous forest dominated by *P. nigra* (MP 1) where only two male specimens were found.

Habitat preferences and distribution of the centipedes, which as predators are not trophically bound with the flora of the habitat, are governed mainly by the abundance and accessibility of specific microhabitats as well as by the complex of soil and climate conditions which the different types of biotopes offer [17]. Ten out of the thirteen species of Chilopoda determined in this study are eurytopic (Table 1, Fig. 3) and inhabit various biotopes: caves, forests of different type, open landscapes and urbanized habitats. Four species are polytopic and according to the data from their general distribution are found in different types of habitats – forests and open natural habitats as well as city parks and agro-ecosystems. These are the synanthrope *S. coleoptrata* found only in the pit-fall traps in one of the meadows in the Madara Plateau (MP 4); *Lithobius mutabilis* – in the coniferous (MP 7) and the mixed deciduous-coniferous forest (MP 5), and *S. linearis* – only in the mixed forest (MP 5) and open meadow (MP 2), as well as *S. cingulata*, which has not been registered during this study.

The centipedes are characterized with great ecological flexibility in terms of the temperature regime of the habitat and show activity in a wide temperature range. The main part of the species registered in this study (the lithobiomorphs *L. muticus, L. mutabilis, L. lucifugus* and *L. microps*, as well as the geophilomorphs *Cryptops anomalans* and *C. flavidus*) are mesothermal – they prefer high temperatures of the environment but exhibit terrestrial activity during the cooler fall months as well. These are mostly forest and mountainous species, inhabitants of forest and open mountainous habitats. Among the centipedes in the Madara Plateau the number of meso-macro-thermal species with preference for higher temperatures of the environment registered mostly in the summer months is high. As such can be categorized *L. nigripalpis*, which is the most numerous in the region, *P. patriarchalis, S. coleoptrata, S. cingulata, S. linearis* and *H. illyrica*. They are encountered mostly in xerophytic mesothermal deciduous and coniferous forests and open habitats where they inhabit the wetter regions. *L. forficatus* and *S. crassipes* are meso-microthermal with high ecological tolerance to lower temperatures of the environment. They are mostly mountainous and forest species active during the winter months as well (Fig. 3). There are no representatives of the meso-macrothermal species among the millipedes. The bigger part of the registered species (8) are meso-thermal, and it is only *Anamastigona bilselii* and *Megaphyllum lictor* that are meso-microthermal, resistant and active even in the periods of winter cold (Fig. 3).

Humidity is the factor of the environment with the most significant influence on the distribution and activity of Chilopoda and Diplopoda [8, 10, 11]. At this stage of the study in the millipede fauna in the Madara Plateau predominate mesophilic species with high ecological flexibility in terms of humidity which prefer wet biotopes but can stand periods of drought as well. They are encountered in different types of forests and in shady meadows with frutescent vegetation and sporadic trees. Only three of the species – *Glomeris hexasticha, Glomeris balcanica,* and *P. renschi* are mesohygrophilic. They are sensitive to drought and preferably inhabit biotopes with higher

humidity. In contrast to the millipedes, the bigger part of the centipedes registered in this study are mesohygrophilic and show preference to habitats with constant and relatively high humidity, while only the lithobiomorphs *L. nigripalpis* and *L. mutabilis* and the geophilomorph *H. illyrica* are mesophilic. The only meso-xerophilic species in the region, which is also resistant to longer periods of drought, is *S. cingulata* which is a typical inhabitant of dry and thin xerophitic forests, open meadows, and rocky areas with sparse vegetation (Fig. 3).

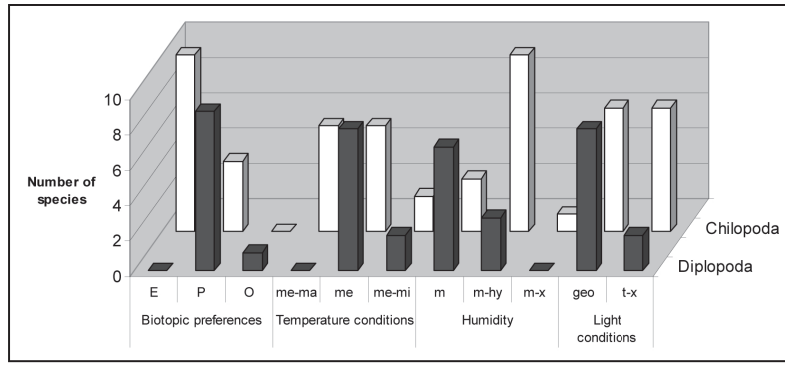


Fig. 3. Ecologic groups of myriapods from the Madara Plateau according to their biotopic preferences and ecological flexibility to the temperature conditions, humidity and light conditions of the habitat

The myriapods as typical inhabitants of caves, upper soil layer, and forest foliage carpet avoid direct sunlight and very few species exhibit terrestrial activity during the light part of the day. The habitats preferred by them as well as their specific environment are characterized by low levels of light, conditions of semi-darkness, or even complete lack of light. That is why geophilic species are predominant in both classes. Only two species of Diplopoda are trogloxenic – *L. trilineatus* and *C. boleti*, while among the centipedes, due to their predator way of life, half of the registered species (7) avoid direct sun light but still, even though rarely, exhibit terrestrial activity during the light hours of the day as well (Table 1).

Among the new species for the region registered during the study, two require special attention – *Pleurolithobous patriarchalis* (Berbese, 1984) and *Glomeris balcanica* Verhoeff, 1906, the latter one being new for the fauna of Northeastern Bulgaria and registered for the first time.

Pleurolithobous patriarchalis is an East-Mediterranean species distributed in Italy, a range of countries in the Balkans, the Near East, and probably introduced in Northern Africa [6, 19]. The most northern point of distribution of the species in Bulgaria is the region of Varna where P. Drensky found only one female specimen in 1931. All other locations of *P. patriarchali*s are south of the Balkan Ridge [13] and it could be assumed that its migration in North-eastern Bulgaria, similar to other Mediterranean faunal elements, has happened along the Black Sea coast. In the region of the Madara Plateau it is registered in bigger part of the studied habitats (MP 3, MP 4, MP 5, MP 6, and MP 7) and up to the moment there have been collected 28 specimens (5♂, 17♀, 6 subad.). The species was registered only during the months of June 2012 and July and December 2013.

Glomeris balcanica is a Balkan species known in the Southeastern Balkans and European Turkey [6]. The species is new for North-eastern Bulgaria [7] and similar to *P. patriarchalis* is not registered in the studies of the myriapods fauna of the Shumen Plateau, Shumen, and the forest shelter belts near the town [2, 4]. In the Madara Plateau *G. balcanica* is registered in three of the studied habitats: coniferous forest (MP 1), open meadow with frutescent vegetation and separate trees (MP 2), and deciduous hornbeam forest (MP 3). A total of 30 specimens (2♂, 11♀, 17 juv.) were gathered only in the period June-September 2012.

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MICROBIOLOGICAL ANALYSIS OF THE WATER OF LUMËBARDHI RIVER (KOSOVO) DURING WINTER SEASON

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Abstract

Aim: The objective of this study is to assess the quality of water, of the river Lumëbardhi during winter season, 2012 year, through the microbiological analysis.

Material and Methods: Samples for microbiological analyses are collected in three localities along the river. Analysed parameters are: Total coliform bacteria, SS (*Salmonella* and *Shigella*), Heterotrophic bacteria, *Streptococcus faecalis* and Fungi.

Results: We determine higher number of microorganism in waters of Lumëbardhi river during the winter season. The locality three is higher polluted with microorganisms, compared with other localities (1 and 2). The number of total coliform bacteria (14.000 cfu/100 ml) is higher at third locality, compared with first locality (5.000 cfu) and second locality (10.000 cfu).

Conclusions According to the bacteriological analysis the river is polluted microbiologicaly. The results from the river section, examined during the investigation, demonstrate that the river water belongs to the fourth class of quality.

Keywords: technique, plate, microbiological, analysis, water

Introduction

The waters of river Lumëbardhi in Prizren city are believed to be strongly contaminated by human waste, because the canalization of the city of Prizren flows in the river.

These reports are related to public and semipublic sources in which contamination or a breakdown in treatment has occurred and to untreated supplies [1, 3, 5, 6]. Whilst most reports are concerned with typhoid, paratyphoid and cholera there have been recent reports of water-borne outbreaks caused by enterotoxigenic *E. coli* [8, 9, 10] and *Campylobacter fetus* [9, 10,15].

Material and Methods

Samples for bacteriological analyses are collected in sterilized bottles (100 ml) at the depth of 10-20 cm under the water surface. The samples are taken at three localities along the river. The media used for the bacteriological analysis of water include nutrient agar (NA) for heterotrophic bacteria, Violet red bile agar for total coliform bacteria (TC), Bile aesculin agar for *Streptococcus faecalis* (FS), SS-agar for *Salmonella* and *Shigella* (FC) and potato dextrose agar for fungi. All the media used were weighed out and prepared according to the manufacture's specifications, with respect to the given instruction and direction. The collected samples were cultivated to selective nutrient agar for each species of bacteria. Cultures were incubated at 37°C except the fungi, incubated at room temperature, 20°C, for 5 days [1]. First locality is before the city, while the second and third localities are after the city Prizren (Fig. 1).

Fig. 1. Sampling localities (1, 2, 3) in the Lumëbardhi River (Kosovo)

Results

The microbiological analysis of the water of the river Lumëbardhi is presented in Table 1.

Total coliform bacteria at first locality were 5000 cfu (colony formed units)/100 ml water, while at third locality were 14.000 cfu/100 ml, which was higher than the recommended value. The higher number of *Streptococcus faecalis* was detected also at third locality (15000 cfu /100), while the lower number of the same bacteria was detected at first locality (6000 cfu/100).

The lower number of *Salmonella* and *Shigella* (1000 cfu/100) was registered at first locality, while the higher number was registered at third locality (6000 cfu/100).

The higher number of fungi is found at third locality (3500 cfu/100 ml), while the lower number is registered at first locality (900 cfu/10).

The higher number of heterotrophic bacteria was registered at third locality (50.000 cfu/100 ml), while at first locality was registered lowest number (20.000 cfu /100 ml).

Table 1. Microbiological analysis of waters of the river Lumëbardhi, during winter season 2012

Group of microorganism	Amount of analysed water	**Locality 1**	**Locality 2**	**Locality 3**
Heterotrophic bacteria	100 ml	20.000 cfu/100 ml	30.000 cfu/100 ml	50.000 cfu/100 ml
Total coliform	100 ml	5.000 cfu/100 ml	10.000 cfu/100 ml	14.000 cfu/100 ml
Streptococcus faecalis	100 ml	6.000 cfu/100 ml	11.000 cfu/100 ml	15.000 cfu/100 ml
Salmonella and *Shigella*	100 ml	1.000 cfu/100 ml	4.000 cfu/100 ml	6.000 cfu/100 ml
Fungi	100 ml	900 cfu/100 ml	2.000 cfu/100 ml	3.500 cfu/100 ml

cfu - colony formed units

Discussion

This study has presented the microbiological analysis of water samples taken from different localities of the river Lumëbardhi.

High counts of FC and FS were obtained in all localities throughout the study period indicating faecal pollution of considerable dimensions. The unequivocal rise in counts of these organisms with the onset of the first measurable rainfall strongly supports the concept of faecal material from the neighbourhood of the wells being washed through the porous laterite or directly round the well shaft itself leading to pollution of the contents [2, 4, 5].

Study results clearly indicated that most of the water of the river at all localities, but especially in third locality, are highly contaminated (IV class) [17], or critical to strongly polluted (III to IV class) [10].

Ideally, drinking water should not contain any microorganisms known to be pathogenic or any bacteria indicative of faecal pollution [11, 13, 16]. Detection of faecal indicator bacteria in drinking water provides a very sensitive method of quality assessment and it is not possible to examine water for every possible pathogen that might be present [7, 12, 14].

A regular monitoring of the water quality for improvement not only prevents diseases and hazards but also checks the water resources from going further polluted [5, 6, 11].

Conclusion

Based on achieving results led us to conclude:

1. The waters of Lumëbardhi River are high polluted by bacteria at all localities.
2. Higher number of all microorganisms are registered at all localities.
3. On the base of coliform bacteria according to Tumpling [17] system the water of Lumëbardhi River belong to fourth class of pollution.

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ALGOCENOSIS OF RIVER VALBONË (ALBANIA), DURING WINTER SEASON 2012

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Abstract:

Aim: The aim of this study is to determine the algocenosis of river Valbona, nearby city Bajram Curri, and the quality of water, through algal bioindicators during winter season of 2012 year.

Material and Methods: Samples for algological, analyses are collected in three locality along the river. Determination done accoding to the algal keys.

Resuslts: During our investigation we determine the different species of algae and bioindicators, through which can evaluate the quality of water and class of bonity.

At the river Valbona, during the winter we determine, 30 species of algae.

Conclusions Dominate the Bacillariophyta with 24 species, following by Chlorophyta with 3 species, Cyanophyta with 2 species, Euglenophyta and Xanthophyta with 1 species.

Keywords: algocenosis, determine, algae, water, river, Valbona, Albania.

Introduction

Algae have long been identified as valuable indicators in the bio-monitoring of stream and river ecosystems [2, 3, 18].

More recently, bio-monitoring has been applied to a variety of water quality problems [2, 7, 17]. Algal communities provide an integrated measure of water quality as experienced by the aquatic biota and have many biological attributes that make them ideal for biological monitoring [8], but their potentials for algal bio-monitoring were not realized so far [4, 5, 6].

Study area. The Valbona valley, with the Valbona stream flowing through it, is situated at north east part of Albania, nearby the border, with Kosovo.

The algae of the river Valbona have attained special significance of algologists in Albania. We examined the phytoplankton and phytobenthos of the river Valbona.

The Valbona river is one of the larger rivers in Albania. Source of the river Valbona are at Ragami village, nearby the border with Montenegro.

It is long about 50 km. In this part absent the industrial and domestic effluents, because no have any factory and do not have too much inhabitants, to discharge the efflunets to the river.

The river Valbona outflow into the rive Drini. It is one of the cleanest river in the country Albania.

Material and Methods

The samples were collected at 3 sampling stations along the river Valbona during the winter of 2012. Water samples were collected in 500 ml glass bottles, 10 cm beneath the water surface, using standard methods.

Fixed in formalin 4%. Epilithon brushed from the stones with toothbrush and the upper layer of epipelon was pipetted off with a vacuum suction system. Epiphyton sampled with the substrate and palced in the plastic bottles. The algae examined using a Leica microscope, with a digital camera Fujifilm, which filmed the algae directly from the sample.

Algal **identification** was done according to the keys: *Cyanoprokaryota*: Komárek, Anagnostidis [6, 7], Elenkin 1938, 1949 [3, 4]; Starmach 1966 [15, 16], *Bacillariophyceae*: Kramer- Lange-Bertalot 1986, 1987, 1991a, b, c, d [10, 11, 12, 13, 14], *Euglenophyta*: Starmach 1983 [15]; Chlorophyta: Starmach 1972 [16].

Results and Discussion

The results are presented in Table 1, the determined algae belonging to the divisions: Cyanoprokaryota, Bacillariophyceae, Euglenophyta, Chlorophyta and Tribophyceae.

The class Bacillariophyceae is dominating compared with other divisions such as Cyanophyta (2 species), Chlorophyta (3 species), Euglenophyta (1 species) and Tribophyceae (1 species).

The Bacillariophyta is represented through its highest number of genus (14) were, Nitzschia and Navicula with 3 species, while 6 genus are represented with 2 species and 6 genus represented with 1 species.

Cyanoprokaryota are represented by 2 taxa: Nodularia spumigena and Oscillatoria mirabilis.

Chlorophyta is represented by 3 species: with dominating gender Cladophora with 3 species: Cladophora praelongum, Desmodesmus perforatus and Microspora floccose

Registered 8 bioindicators species, which belongs to two level of saprobity: oligosaprob (4 species) and oligobetamesosaprob (4 species).

Table 1. List of taxa recorded in Valbone river in winter 2012, with their occurrence in the sampling localities and their saprobity indicator values.

			LOCALITIES		
		Level of Saprobity	1	2	3
30	***Total number of algae***				
	Division Cyanoprokaryota				
1	*Nodularia spumigena Mertens*		1		1
2	*Oscillatoria mirabilis Böcher*		1		
	Division OCHROPHYTA				
	Class Bacillariophyceae				
1	*Achnanthes hungarica (Grunow) Grunow*	o	1		1
2	*A. normani Rabenhorst*	o	1		3
3	*Aneumastus stroesei (Ostrup) Mann*				1
4	*Aneumastus tuscula (Ehrenberg) DGMann & AJStickle*	o-β		1	
5	*Cocconeis pediculus Ehrenberg*	o-β		1	
6	*C. placentula var. lineata (Ehrenberg) Van Heurck*		1	1	1
7	*Cyclotella ocellata Pantocsek*			1	
8	*Cymbella affinis Kützing*	o-β	1		1
9	*C. helvetica Kützing*	o	3	1	
10	*Diatoma ehrenbergii Kützing*		1	1	
11	*D. moniliforme Kützing*		1		
12	*Epithemia adnata (Kützing) Brébisson*				1
13	*Fragilaria capucina Desmazières*	o-β	3	1	
14	*Gomphonema carolinense Hagelstein*				1
15	*Luticola goeppertiana (Bleish) Mann*			1	
16	*Meridion circulare (Grev.) Cleve Agardh*	o	1		1
17	*Navicula capitatoradiata Germain*			1	
18	*N. tripunctata (O.F.Müller) Bory*		3		
19	*Nitzschia acula Hantzsch in Rabenhorst*				1
20	*N. acicularis (Kützing) W. Smith*			1	
21	*N. capitellata Hustedt*		3		
22	*Synedra nana Meister*				1
23	*S.ulna Kützing*		1		
	Division EUGLENOPHYTA				
1	*Euglena oblonga Lemm.*		1		
	Division CHLOROPHYTA				
1	*Cladophora praelongum Brébisson*		1		1
2	*Desmodesmus perforatus (Lemmermann.) Hegewald*			1	
3	*Microspora floccose (Vaucher)Thuret*				1
	Division OCHROPHYTA				
	Class Tribophyceae				
1	*Tribonema vulgare Pascher*		1		1

We recognized 30 species of algae from five taxonomical divisions during winter season 2012.

During the study period, the diatoms (Bacillariophyceae) were accompanied by the Chlorophyta, Cyanoprokaryota, Euglenophyta and Tribophyceae [9, 12].

During winter season 2012, Chlorophyta represented by 3 species, Cyanoprokaryota by 2 species, while the divisions Euglenophyta and Tribophyceae represented by 1 species [10, 11].

From 30 species, 8 are indicators of environmental conditions such as habitats, temperature, streaming and oxygenation, saprobity, halobity, and acidification [1, 2, 3].

Conclusions

Therefore, since the algal species diversity of the Valbona river has been formed under the influence of regional climatic factors, it can be currently recognized as a natural system, with a high buffering and self-purification capacity.

Using comparative floristic methods, we did not reveal any influence of the anthropogenic pollution on the structure of algal communities as a whole.

According to the bioindicators species, investigate waters classified in I class of bonity respectively at oligosaprob level.

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КРАТКИ СЪОБЩЕНИЯ/SHORT COMMUNICATIONS

Тематично направление:
БИОЛОГИЧНО РАЗНООБРАЗИЕ И КОНСЕРВАЦИОННА БИОЛОГИЯ

Topic:
BIOLOGICAL DIVERSITY AND CONSERVATION BIOLOGY

EX SITU CONSERVATION OF THREATENED PLANT SPECIES OF BULGARIAN FLORA ON THE TERRITORY OF UNIVERSITY BOTANIC GARDEN ECOPARK, VARNA

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Aim: Of this study to establish and describe the threatened plant species in the territory of University Botanic Garden Ecopark, Varna.

Material and Methods: This research was conducted on the territory of University Botanic Garden Ecopark, Varna during the vegetation period 2012-2013. Sites for conservation threatened and endangered plant species in University Botanic Garden have been identified and mapped. Made a card with QuantumGIS. Photos taken with the camera Canon EOS 600D.

Results: We have done a map of the location as well as data and number of protected plants. A photo material database of all threatened species has been provided. A research that is the territory of University Botanic Garden shows 45 plants threatened and endangered plant species. The distribution of taxa in categories is as follows: 1 Balkan Endemic [4], 23 species, listed in the Red Data Book of Bulgaria [3, 6], 8 of them are in the category "rare", 14 "endangered" and 1 species is the category "critically endangered"; 34 species are protected by the Law Of the Biodiversity [1]; 1 species is listed in the List of Rare, Endangered and Endemic Plants in Europe [2]; 5 species are subject of the Convention On the International Trade in Endangered Species of Wild Flora and Fauna [5], 2 species are listed in the Black Sea Red Data Book [7], 10 species are relict: 9 of them Tertiary relict and 1 Glacial relict. Most of the protected species are introduced and naturalized to the territory of the University Botanic Garden. There are naturally preserved habitats in the University Botanic Garden, Varna which favours the autohtonic plants with conservation significance.

Conclusion:

1. University Botanic Garden, Varna has value of Ex Situ of conservation significant plant species of Bulgarian flora (45 species). Appears to be an enriched and used for scientific, educational and exhibition purposes;

2. University Botanic Garden can be a source of genetic material of successfully introduced and adapted species.

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GENETIC POLYMORPHISMS OF BULGARIAN AND PORTUGUESE COMMON BEAN (*PHASEOLUS VULGARIS*) GERMPLASM

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Introduction: Researches into genetic polymorphism are directly related to biodiversity, genetic variability and adaptation of organisms to changing abiotic and biotic factors.

One of the most sensitive and precise tool for measuring genetic relationship are the DNA markers [1]. Amplified Fragment Length Polymorphism (AFLP) analysis is commonly used to study genetic variability and genotype identification [2]. AFLP technique is based on the principle of amplification of short genomic DNA fragments. Another very important tool for analyzing genetic diversity are SSRs (Simple sequence repeats) or microsatellites [3].

Common bean (*Phaseolus vulgaris* L.) the most important grain legume all over the world is a traditional crop of great economic relevance. The bean seeds are rich in carbohydrates, proteins, minerals, vitamins and fibers. Common bean is a diploid (2n=22) and has a relatively small genome.

Aim: to study the genetic variability of Bulgarian and Portuguese common bean (*Phaesolus vulgaris*) germplasm.

Material and Methods:

DNA extraction: Total genomic DNA was isolated from 100 mg of young leaves of 5 individual seedlings from 22 different accessions. The innu PREP Plant DNA Kit (Analytikjena) was applied in accordance with the procedure provided by the company producer. DNA quantity was measured with NanoDrop, at a wavelength of 260 nm. DNA was visualized by UV light in an 1% agarose gel.

ALFP analysis: This analysis was performed with the following 17 accessions: 9 Portuguese landraces, 7 Bulgarian accessions (2 landraces, 2 commercial varieties -"Tipeau 1 and Tipeau 2", 1 genotype resistant to lodging –"Dobrudjanski ran", 2 medium early genotypes – "Dobrudjanski 2 and Dobrudjanski 7") and 1 CIAT line with high nitrogen fixation ability (BAT 477). Genomic DNA (50 ng/µL) was digested with 5 U of *Eco*RI and 1 U of *Mse*I at 37^0C for 2 h. *Eco*RI and *Mse*I adapters were ligated to digested DNA fragments in 5x rapid ligation buffer and 5 unit T4 DNA ligase were added and incubated at room temperature. Taq polymerase enzyme was used in all PCR reactions. Selective amplification was performed using eight different AFLP primer combinations. Three primer combinations were found to be inappropriate. Preamplification of the diluted (10-fold) ligated DNA was carried out with primers complementary to the *Eco*RI and *Mse*I adapters, with one selective nucleotide adenine and cytosine,

respectively, in a thermal cycler (Techne, TC-512). The second amplification was carried out with five selective primer combinations of *Eco*RI and *Mse*I with three selective nucleotides. PCR products were electrophoresed on 1% agarose gels for product amplification checking

SSR analysis: This analysis was performed with the same 17 accessions referred in the AFLP analysis plus 1 Bulgarian landrace and 1 Bulgarian commercial variety, 2 Portuguese landraces and 1 Angolan accession. Seven microsatellites were used to perform the genetic diversity analysis of the selected accessions. The SSR loci amplification was performed using forward primers (F) fluorescently labeled at the 5´end and unlabelled reverse primers (R). PCR reactions consisted in a 50 ng/µL template DNA, 10x PCR buffer, 2 mM dNTP, 25 mM $MgCl_2$, primer F/R and 1 unit Taq DNA polymerase in a total reaction volume of 25 µL. Amplification was performed in a different thermal cyclers. PCR products were checked on 1% agarose gels.

For both ALFP and SSR analyses PCR products were analyzed by capillary electrophoresis - ABI310 Genetic Analyser (Applied Biosystem) and CEQ 8000 Genetic Analysis System (Beckman Coulter) respectively. Genetic diversity parameters and clustering (PCA analysis) were estimated using GenAlEX software.

Results: A total of 506 polymorphic bands were detected with the ALFP analysis. Percentage of polymorphic loci was 51.98% (74.31% for Portuguese genotypes, 81.62% for the Bulgarian and 0% for the Mexican). According to the SSRs results it was observed that the loci are highly homozygotic, what is expected considering that the common bean is a self-pollinating plant. Nevertheless, all microsatellite loci used were found to be polymorphic.

Conclusions Preliminary results from both analyses showed:

- the ALFP analysis revealed two main groups – Portuguese and Bulgarian. Mexican genotype clustered with the Portuguese.
- the SSR analysis revealed a few differences between the Portuguese and Bulgarian germplasm.

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SPATIAL NICHE PARTITION AMONG FIVE SYMPATRIC LIZARDS IN NORTH-WEST BULGARIA

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Lacertid lizards (family of Lacertidae Oppel, 1811) are very important and characteristic part of the European reptile communities. In Bulgaria, the family is represented with nine species. In spite of the dominating role of the lacertid lizards a sympatric occurrence of more than five species is very rare. Arnold [1] describes sympatry of seven species near Gacko in Bosnia and Herzegovina. For Bulgaria, Tzankov [2] describes sympatry of six lacertids in North-East Rhodopes Mountains. The Snake-eyed skink is the only representative of its family (Scincidae Gray, 1825) in Bulgaria and co-occurs in many of the lacertids' habitats.

Aim: The aim of the present study is to give more data about the habitat use, share of spatial niche and ecological requirement of species in a small community in North-West Bulgaria.

Material and Methods: The study area is located in Montana district near Ogosta Dam in North-West Bulgaria. We describe seven general types of habitats – stony shore of the dam, sandy shore with low vegetation, oak forest, meadows and grasslands, ecotone between the forest and the meadow, pine forest and road sides covered with shrubs.

The studied species are the Green lizard (*Lacerta viridis* (Laurenti, 1768)), the Wall lizard (*Podarcis muralis* (Laurenti, 1768)), the Balkan Wall lizard (*Podarcis tauricus* (Pallas, 1814)), the Meadow lizard (*Darevskia praticola* (Eversmann, 1834)) which belong to the family of Lacertidae and the Snake-eyed skink (*Ablepharus kitaibelii* Bibron & Bory de Saint-Vincent, 1833) from the family of Scincidae.

A total of 620 individual locations were documented, which fall among in the seven general types of habitats. To express the diversity of the species by habitats, and also the diversity of used habitats by the species, Shannon diversity index was used.

Results: The Green lizard is the species with the widest niche. It was found in all of the habitats but was most common in the grasslands with shrubs, which provide cover, places for sunbathe and observation. The rest of the species, in view of the competition with the dominant *L. viridis*, have narrowed spatial niches. The Wall lizard was found in

all of the habitats but is predominate in the stony shore of the dam. The Balkan wall lizard is presented with highest number of individuals in the sandy shore of the dam and in the grasslands, and the Meadow lizard is the species with the narrowest niche, being confined mainly to oak forests. The Snake-eyed skink is most common in the grasslands with shrubs and in the ecotone – niche overlap with the Green lizard and the Balkan wall lizard.

The highest number of species was found in the grasslands – all of the five species because of the intermediate position between the other habitats.

Conclusion: All of the species display specific habitat preferences and in this way avoided the competition among them.

Keywords: habitat use, lizards, ecology, Lacertidae, Scincidae

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NEW DATA ON AQUATIC OLIGOCHETS (OLIGOCHAETA LIMICOLA) IN THE BURGAS LAKE (VAYA)

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The Burgas Lake (Vaya) is a shallow wetland on the Black Sea coast, west of the city of Burgas. This natural lake is an important bird area along the Via Pontica flyway - the largest air highway for migratory birds from/to SE Europe. During the last 70 years however, the Vaya Lake has gone through essential changes, as shallowing, combined with flattening of the bot-

tom, decreasing of the surface area and decreasing of salinity. The influence of various anthropogenic factors contributes to the alteration of the general picture of the lake. Some of these factors are the discharge of communal and industrial waste waters, the construction of a depot for technology wastes from oil refinery "LUKOIL-Neftohim Burgas" AD, the clogging of the channel, connecting the lake to the Black Sea and the construction of a drainage system. These lead to drying up of parts of the wetland area and disturbing the water balance of the lake by pumping out water for irrigation. Important role for the lake's state plays the input of nutrients by the communal and industrial waste waters, the irregular storage of stock-breeding wastes on the lake shore, the overloading the communal WWTP = wastewater treatment plant for the main city and suburbs. Having in mind all the anthropogenic impacts could be expected low diversity and numbers of the macroinvertebrate complex.

Research aim: Excluding some single studies of the lake [1, 3, etc.], complete and systematic hydrobiological studies have not been performed so the aim of this study was to present actual data on species diversity and abundance of the aquatic oligochets in Vaya Lake (South-Eastern Bulgaria) - a Ramsar site and an Important Bird Area.

Material and Methods: The survey was conducted in 8 sampling points; samples were taken in every season during the period 2004-2007 from the exact lake bottom, outside the area of the fringing communities. Qualitative samples of benthic macroinvertebrates were taken according to the standard method ISO 9391:1995. Field and cameral work was done in accordance with the standard method EN/ISO 5667-3:2003/AC:2007. The species diversity, density and biomass of the oligochets were measured.

Results: In the past the salinity of the lake varied widely (from 0.76 ‰ to 18.14 ‰) according to [3]. In those conditions euryhaline species formed the lake flora and fauna. Nowadays the channel, connecting the lake with the sea is nearly covered with reed, limiting the flow of salt water and forming the lake as a freshwater wetland. The changes in the salinity drove changes in the fauna and macrozoobenthos community in particular. Physiographic and hydrological conditions of the lake along its entire area do not vary so the oligochets composition is limited in variety of species and numbers.

The macrozoobenthos of the Vaya Lake is presented mainly by species from fam. Chironomidae (Diptera) and Oligochaeta Limicola [1]. Within the previous studies [3] only one naidid species (*Nais eliguis* Műller, 1773) was registered. In the current research this species did not present at all, and the oligochete complex was dominated by three species: *Limnodrilus hoffmeisteri* (Claparede, 1862), *Limnodrilus claparedeanus* (Ratzel, 1868) and *Potamothrix hammoniensis* (Michaelsen, 1901). Findings of oligochets from fam. Naididae (single specimens from genera *Nais* and *Dero*) were sporadic during the whole studied period. Within the oligochete complex three tubificid species had the highest density. The dominant was *L. hoffmeisteri*, followed by *L. claparedeanus* and the juvenile (immature) forms of the *Limnodrilus* sp. The highest density of *L. hoffmeiteri* and *L. claparedeanus* determined the highest biomass of the same species. *L. hoffmeisteri* is one of the most common oligochete species of the inland water bodies, occuring in mass under poly- and α-mesosaprobic conditions together with other representatives of fam. Tubificidae, especially with *Limnodrilus udekemianus* (Claparede, 1862) and *Tubifex tubifex* (Műller 1774) [2]. The euribiotic species *T. tubifex* registered once only in 2004 in the following three year period seemed to be replaced by the ponto-caspian relict species *P. hammoniensis*.

The co-dominant *L. claparedeanus* is widely distributed component of the zoobenthos in deeper lakes and reservoirs and in larger rivers under organic loading [2]

The second co-dominant *P. hammoniensis* is very common in Danube and Black Sea coastal water bodies, where it may have high population density. It used to be a basic component of oligochete fauna in native waterbodies, classified being under eutrophic, even hypereutrophic conditions [2].

In the station, situated near the inflow of the three west rivers (Aitoska R., Sanar-dere R. and Chakarliyska R.), supplying the lake with fresh water, the inflow can explain the high oligochets' density and biomass during all seasons and years. The highest density and biomass of the oligochete fauna was established in the middle area of the lake that can be considered as the less influenced by anthropogenic impacts.

Some specific attention deserve two stations where no macroinvertebrates were registered. One of them was next to the urban WWTP, which discharges treated communal and industrial wastewater into the lake and under certain conditions it may lead to their degradation and complete depletion of oxygen in the bottom layer. The other station was situated in the channel once used to connect the lake and the sea. Currently, the natural connection is interrupted by large amounts of waste materials and fringing reeds.

Conclusion: Consequently, because of the shallow flat and almost uniform in composition bottom of the Vaya Lake, the number of species in the oligochete complex was limited. When comparing to earlier studies, the community structure showed lasting changes where the euribiotic species *T. tubifex*– tolerant to high levels of organic pollution, was dominated while in nowadays it was replaced by ponto-caspian relict species *P. hammoniensis* – a native tubificid in coastal water bodies along the Black-Sea coast and the Danube River.

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ДОКЛАДИ/REPORTS

Тематично направление:
АНТРОПОГЕННИ ВЪЗДЕЙСТВИЯ ВЪРХУ ЖИВАТА ПРИРОДА

Topic:
ANTROPOGENIC IMPACT ON THE LIVING NATURE

PESTICIDES IN THE ENVIRONMENT-SOURCES OF POLLUTION AND EFFECTS ON ECOSYSTEMS /A Review/

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Abstract

Application of plant protection products is essential in the modern agricultural practice, the yield and food production would be reduced significantly, without their usage. But there are also some clear evidences that the widespread treatment with these substances leads to the pollution of environment and cause irreversible damages to ecosystems and their inhabitants. There are many pathways for pesticides to penetrate to the environment and their residues may remain there for a long time after the treatment. From the environment pesticides can easily enter into the food and drinking water and to cause a lasting negative effect on the people and human health. Although pesticides are used to control the pests, they also harm other living organisms in the environment, which are necessary for the maintenance of the ecological balance. Pesticides from the group of neonicotinoids are considered as a particularly dangerous, because of their high persistence in the environment and more pronounced toxic effects. Pesticides from the group of POPs (persistent organic pollutants) are also very harmful for the inhabitants of the ecosystem, because of their long-term stay in the soil and bioaccumulation. One of the most negative consequence of availability of pesticide residues in the soil is the formation of highly toxic metabolites. Another harmful effect from improper application of this substances is the provoking a resistance in the pests, which requires creation of new, more powerful tools to combat them. As a consequence, an increase in the amount and frequency of pesticide treatment in agricultural practice is necessary after that. Timely measures are of a great importance, aimed to reduce the global pesticide use and also, the application of alternative approaches to protect crops from pests.

Keywords: pesticides, pollution, environment, ecosystem, pest

Modern agricultural practice is impossible without usage of plant protection products. Pesticides are chemical substances, applied for treatment of various plant pests – diseases,

weeds and pests, including prevention, destruction, attracting, repelling or control. Currently there are about 1200 – 1400 active substances of pesticides, belonging to different chemical classes and are known under different trade names and formulations.

A great part of the pesticides belongs to the so-called **persistent organic pollutants (POPs)**, which are toxic organic substances and are particularly dangerous, because of their accumulation in the environment, where they remain for an extended period of time. Active substances accumulate in the biosphere; it is possible to be transported via atmospheric and deposited at a long distance; they can cause significant adverse effects on human health or the environment, regardless of the distance of the sources. As a result of human activity in recent decades, these pollutants have become widespread in major regions around the world (including those, where POPs have never been used). Extensive contamination of the environment and living organisms cause a significant exposure to several species, including humans, resulting in acute and chronic toxic effects. POPs concentrate in living organisms through a process called bioaccumulation, which means that POPs are gradually bioconcentrated through the food chain in fish, birds and mammals, and ultimately in humans also. No matter of the fact, that POPs are not soluble in water, they easily accumulate and concentrate in the fatty tissue of humans and animals, and due to their migration can be found in regions where they have never been applied. The specific effects of POPs include the development of cancer, allergies, impairment of the central and peripheral nervous system, immune system disorders also. Some POPs are considered as endocrine disrupters, which through changes in the hormonal system can damage the reproductive and immune systems of exposed organisms. This dangerous combination of toxicity, persistence, mobility and possibility for transmission over long distances, contributes for the distribution of POPs all around the world (http://www.moew.government.bg/?show=top&cid=244, https://obsoletepesticides.net/site/).

The modern pesticides from the group of **neonicotinoids** are considered particularly harmful to pollinators. These synthetic analogues of nicotine were produced in the late twentieth century and differ from this natural pesticide by a higher persistence in the environment and more pronounced toxic effect [8]. They are systemic insecticides, which penetrate into the plant tissues and act through them by attacking and suppressing neural impulses in insects. They affect the central nervous system, causing paralysis and death and therefore, these insecticides are thought to be more effective than others [12]. They also destroy the digestive, immune, and especially the nervous system of almost all insects, mites and arachnids, the majority of fish and amphibians, many birds and bats. Studies, conducted in Europe in the 90s proved that neonicotinoids residues can accumulate in the pollen and nectar of treated plants and pose a potential risk to insects – pollinators, but the effect that these residues might have on bees is not yet completely established [6]. The data from recent studies conducted in Europe, maintain the thesis for the persistence of neonicotinoids. They easily contaminate ground and surface water and remain there for an extended period of time.

Benefit from the application of plant protection products is undeniable, cultures and their yield will be drastically decreased without their participation. On the other hand, there are clear evidences that the widespread use of pesticides leads to the pollution of the environment and food and causes irreversible damages to ecosystems and their inhabitants.

There is a big number of useful species, which play an important role in the proper functioning of the ecosystems, which are affected by the harmful effects of pesticides.

I. Main factors for pesticide environmental pollution

Of particular importance for the presence of pesticide residues in the environment is their incorrect use: non-compliance with the specified dose and treatment time; the failure of some specific techniques and rules of treatment is a substantial factor also. Consequently, the active substances fall within the soil and in groundwater and surface water, which ultimately affects all residents of the ecosystems.

Storage in unsuitable premises contributes to their penetration into the soil and groundwater as a result of the rains and destroying the integrity of the packaging. Subsequently pesticides are spread in the environment, reaching water sources and arable land.

Substances fall within the soil not only through their direct contribution, but also through the dead leaves of treated plants, especially when the substances are persistent. Their application for seed treatment prior to sowing is the source of distribution also.

The use of insecticides in households also leads to their presence in the environment. Residues of active substances can remain in the environment long after application and have a durable negative effect on the people and ecosystems [15].

II. Behavior and effects of pesticides in the environment

1. Pesticides in the atmosphere.

Pesticides fall into the atmosphere mainly in two ways: through the dispersion of substances in the air, or through evaporation from treated areas. Presence of pesticide substances in the atmosphere represents a tremendous risk to the environment, because of their dispersion from the wind – they can fall at unwanted places where can contaminate other areas and affect ecosystem`s inhabitants.

2. Pesticides in the hydrosphere and their impact on aquatic organisms.

Water pollution by pesticides is a widespread phenomenon and their presence in the groundwater is also a serious problem. They can reach surface waters by leaching from treated plants and soil; pesticides may fall in the basins directly from the air and soil or through the products emitted by humans and animals; also as a result of passing them by the wind after treatment of cultivated areas. Hydrosphere is an environmental part, inhabited by many species of animals and other organisms, including microorganisms, invertebrates, various types of aquatic plants etc., which may be affected by the presence of substances in the area.

3. Pesticides in the soil and the impact on soil microflora.

Soil is an important component of the biosphere, due to the huge amount of organisms and their products in it. Some of accumulated chemicals in the soil display high biological activity and influence the life of soil organisms, causing damages to the soil microflora. It was found that arthropods and earthworms living in the soil, accumulate persistent pesticides in their bodies, which lead to their destruction.

All types of pesticides have a significant impact on the biological activity of the soil. This influence is determined by their chemical composition, the dose, the proper time of introduction of substances, the duration of treatment, physico-chemical properties of soil, climatic conditions, the applied agricultural activities, etc. Some pesticides are absorbed by the colloidal particles in the soil and retain their activity.

Detoxification of active substances in the soil is carried out by physicochemical, chemical and biological means. After falling into the soil, they are subjected to a transformation, advanced by soil microorganisms, some of which are able to decompose the imported pesticides into the soil; they are crucial for the activation of detoxification of these substances. For the decomposition of the pesticide in the soil, the type of the soil and its humidity, ambient temperature and the content of organic substances in it, are of a great importance.

The presence of pesticide residues in the soil also affect soil fertility. At the normal amount of substances the growth of microorganisms and their activity is not inhibited and proceeds normally. Intensive treatment with them may cause a reduction of beneficial soil microflora. After treatment with herbicides for example, the development of the various groups of soil microorganisms in the treated areas is inhibited for a period of typically 10-20 days. But the use of high doses can lead to the suppression of the soil microflora for a longer period, like two, three, even more than six months. According to soil researchers, the loss of bacteria and fungi causes a soil degradation. **The excessive use of chemical fertilizers and pesticides has an effect on soil organisms that resembles the overuse of antibiotics in humans.**

Indiscriminate application of chemicals in the agricultural practice can work for several years, but after a while there is not enough soil beneficial organisms, to retain nutrients in it. For example, plants development depends upon a variety of soil microorganisms, which convert natural nitrogen into nitrates, which the plants can use. Mycorrhizal fungi grow in the roots of many plant species and contribute to the absorption of nutritional substances. These fungi can also be damaged by the herbicides in the soil; the growth of many species of mycorrhizal fungi is suppressed by pesticides.

Another negative consequence from pesticide presence in the soil is that after their partial destruction, the formation of metabolites, that are no less toxic than the initial substances is possible. Derivative compounds, obtained from pesticide degradation in the soil are connected with the humic substances in it and form a durable, poorly degradable complex compounds, which remain in the soil for a long period. There are known a large number of transformation products (metabolites) of pesticides. Persistence and movement of these substances and their transformation products are defined by their solubility in the soil, the soil sorption constant, half-life time [1]. They can be removed from the soil through the process of runoff and leaching and thus cause contamination of the water sources for the population.

Pesticides and their metabolites are retained by the soil to a varying degree, depending on the interaction between the soil and the properties of the substances. The most significant feature of the soil is the content of organic matter in it. As the content of organic matter in the soil is higher, there is a stronger adsorption of pesticides and their metabolites. The important factor for this process is the pH value of the soil. The adsorption of a big number

of pesticides (e.g. herbicide 2,4-D ,2,4,5-T, picloram, atrazine) increases with decreasing pH value of the soil.

4. Effects on non-target organisms (birds, mammals, bees, etc.).

Pesticides are designed to control pests, killing harmful insects, but their toxicity affects a number of useful species, that are essential for the normal functioning of ecosystems and human life. Active substances also harm other living organisms in the environment, necessary for the maintenance of ecological balance, such as insects, like bees, which are first in importance, birds, earthworms, fish and other aquatic organisms, and non-target organisms also [4, 15]. Pesticides have adverse effects on the behavior of the earth bees (*Bombus terrestris* L.) and wood bees stingless bees [10]; cause high mortality in larvae and adults of tree species ladybugs [9]. All these kinds of organisms are essential for nature and humanity, their participation in vital processes such as pollination, soil formation, etc. are necessary for the proper functioning of ecosystems. Generally, insecticides are considered the most acute toxic class of pesticides, but herbicides may also pose a risk to non-target organisms [1]. Various pesticides have varying ability to accumulate in the environment, depending on their chemical nature; individual plant species also differ in their ability to accumulate and retain pesticide residues.

Population of honey bees and other pollinators declined in the recent years all around the world. Several stressors are suspected as a potential cause, and last but not least is the use of pesticides in agriculture. The routes of exposure of bees from pesticides are several: by direct spraying during treatment of the crop; flying bees through clouds of dust, released during the sowing of treated seeds; feeding with contaminated pollen and nectar from sprayed areas and taking drops of water from guttation of treated plants.

According to several authors, the so-called Colony Collapse Disorder (CCD) that appeared in the last decades and caused the global reduction and disappearance of bees worldwide, is largely due to the increasing application of pesticides in the agriculture and household [13, 14]. Destruction and reducing of bee population concerns not only honey bees; a reduction of wild pollinators – bumblebees, etc., is reported worldwide [3, 11]. This problem is of great concern, because the role of bees is not only limited to the production of honey and other bee products; they are also the main pollinators of many plants, with great economic importance, as well as wild species and field plants, which is extremely important for the biodiversity of ecosystems.

Pesticides have not only a direct toxic effect on bees. Particularly dangerous is the sublethal effect of their use, which is manifested as changes of certain features of the bees behavior, disorders in physiology and immune system also; this effect can make the colony more sensitive (e.g., more susceptible to disease and parasite), which may lead to its collapse [2].

Several studies confirm the idea that even in very small concentrations, pesticides can make honey bees vulnerable to parasites and diseases by weakening of their immunity and subsequent unlocking of microbiological and parasitic diseases [5, 7].

Honey bees are not the only inhabitants of ecosystems, that are directly or indirectly affected by the use of pesticides. As already mentioned, treatment with insecticides can affect other beneficial insects such as bumble bees, insects, biological agents, etc. [9, 10].

Another important and very dangerous consequence of improper pesticide application is the **emergence of resistance in pests**, which requires the creation of new, more powerful tools to combat them and also, an increase of the amount and frequency of pesticide applications in agriculture. A pesticide resistance develops when substances are used frequently or when the same or similar active substances are used repeatedly. A pesticide resistance means that the pesticide do not controls for a long time pests, diseases and weeds, for which it is intended. The number of pests that are resistant to pesticides is increased, but the availability of pesticide products which can be used against pests is decreased.

Conclusion

The use of plant protection products is of substantial economic importance, in order to obtain high quantity and better quality from the crops production. But their implementation should be done with concern for the environment and preservation of ecological balance. There are lots of heavy consequences from the application of pesticides in the modern economy, but the most dangerous is their overall negative impact on ecosystems and their inhabitants. This could lead to their total destruction and be detrimental to the life of the planet, especially taking into account the fact that with the development of chemical industry, these weapons become more powerful and has a wider spectrum of destructive force. Timely measures are necessary to be taken to reduce the global application of pesticides, and the use of alternative approaches to protect crops from pests.

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ESTIMATION OF GENOTOXIC EFFECT OF INSECTICIDE MOSPILAN AT GOLDEN FISH

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Abstract

Aim: The aim of this study is to determine the genotoxic effect of insecticide Mospilan in peripheral erythrocytes of fish at golden fish (*Carassius aureatus*).

Material and Methods: Concentration of insecticide Mospilan (Chemical Abstracts name 1-[2-(2,4-dichlorophenyl) pentyl]-1H-1,2,4-triazole), it was in first aquarium, 15 ml insecticide Mospilan/40 liter water, in second aquarium 12 ml insecticide Mospilan/40 liter water, in third aquarium 10 ml insecticide Mospilan/40 liter water, in fourth aquarium 8 ml insecticide Mospilan/40 liter water. Fifth aquarium use as control, without insecticide Mospilan, contains only drinking water. The fish we treated for two days.

Results: We determine higher number of micronucleus in peripheral erythrocytes of fish treated with insecticide Monspilan (for each treated group with insecticide), compared with control group 4 micronucleus /2000 erythrocytes).

Conclusions Based on an investigation we can conclude that insecticide Monspilan damage the chromosome of erythrocytes of fish *C. aureatus*.

Keywords: erythrocyte, fish, insecticide, Monspilan induced, micronucleus.

Introduction

The erythrocyte micronucleus bioassay has been used with different fish species to monitor aquatic pollutants displaying mutagenic features [1, 2, 6], demonstrated that fishes inhabiting polluted waters have greater frequencies of micronuclei compared to those raised in clean pond. The micronuclei frequencies may vary according to the season, the kind of pollution involved, and the species of fish. These structures are easy to visualize in erythrocytes and are therefore often used as a measure of chromosomal aberrations [5, 9].

Because counting of micronuclei is much faster and less technically demanding, the micronucleus assay has been widely used to screen for chemicals that cause these types of damage [2] and the damage resulting from it [3, 4, 8].

Material and Methods

The waters of the lake, were captured the golden fish, is characterized by a low level of industrial and agricultural activity.

We used one species of fish, *Carasius aureatus*. After the capture, they placed in aquariums with aerated tap water and taken to the laboratory. After acclimation to reduce the stress of capture and transport, fish treated in aquarium with insecticide for five days.

Slides stained with Giemsa. The frequency of erythroblasts, micronuclei and nuclear abnormalities were estimated by counting 2000 cells in extensions. At each aquarium put 10 fish.

Golden fish *C. aureatus* chosen for this study because it is very adapt for investigation, also due to proven sensivity to genotoxic chemicals. In each aquarium put ten (10) fish, total number of fish is 50. Concentration of insecticide, it was in first aquarium 15 ml insecticide Mospilan/40 liters water, in second aquarium 12 ml insecticide Mospilan/40 liters water, in third aquarium 10 ml insecticide Mospilan/40 liters water, in fourth aquarium 8 ml insecticide Mospilan/40 liters water. Fifth aquarium use as control, without insecticide Mospilan, contain only drinking water.

Experimental design: Fish *C. aureatus* placed in five different aquaria, each one containing tap water (negative control) and four different aquaria containing different dilution of insecticide Mospilan. The fish was cut in caudal region and smears of peripheral blood were made on clean slides.

Slide preparation and staining: For each fish, prepare three slides. Slides were coded, for each fish. The smears are air-dried and fixed in absolute ethanol for 25 minute. The fish treated for 5 day. After fixation, the slides were stained in aqueous Giemsa (diluted in distilled water ratio 1:3) for 45 minute.

Results and Discussion

The insecticide Mospilan induced high frequencies of micronuclei in peripheral blood erythrocytes of golden fish. The frequencies of micro nucleated (MN) erythrocytes were estimated for each fish, in each aquaria are presented in Table 1. At first aquaria, we detect the 59 micronuclei (MN), which is higher compared with other aquaria and with control group. At second aquaria, we determine 47 MN, while the third has 41 and fourth aquaria have 32 MN, at 2000 erythrocyte. The average number of micronucleus (MN) at all groups treated with insecticide mospilan are 46 MN/2000 erythrocytes, statistically are significantly higher compared with control group ($P<0.001$).

Table 1. Average number (per aquarium) of micronuclei (MN) in 2000 erythrocytes of peripheral blood of fish *C. aureatus* after 2 days treatment in different concentration of insecticide Mospilan

Aquariums	Average number of MN/2000 erythrocytes per aquarium	Significance*
Aquarium 1 (15 ml insecticide/40 l water):Aquarium control	59	P<0.001
Aquarium 2 (12 ml insecticide/40 l water):Aquarium control	47	P<0.001
Aquarium 3 (10 ml insecticide/40 l water):Aquarium control	41	P<0.001
Aquarium 4 (8 ml insecticide/40 l water):Aquarium control	32	P<0.001
Aquarium control - without insecticide	5	
Average number of MN at treated fish, without control group	184:4= 46 MN	

*Statistical processing is done with *Statistical* software *SigmaStat* 3.1®, 2004

The MN frequencies observed in the present study (Fig. 1) are in agreement with the literature. Fish serve as useful genetic models for the evaluation of pollution in aquatic ecosystems. Micronucleus bioassay offers several types of unique information as a bioindicator for chromosomal aberrations not available from other methods: (1) the integrated effect of a variety of environmental stresses on the health of an organism and the population, community, and ecosystem; (2) early warning of potential harm to human health based on the responses of wildlife to pollution; and (3) the effectiveness of remediation efforts in decontaminating waterways [13].

MN assay was used for field water quality monitoring [10]. The MN frequencies measured are consistent with the results observed in *C. auratus* by Cavas [3] and Cavas and Konen [2] and in the range of variability of 1–2 orders of magnitude reported by Bolognesi et al. [1].

Our findings also confirmed that the alkaline comet assay and nuclear deformations in addition to micronucleus test on fish erythrocytes in vivo are useful tools in determining the potential genotoxicity of commercial herbicides [2]. The average of erythrocytes in our study was similar to those observed by Ranzani-Paiva et al. [12], in *Prochilodus lineatus* from the Paraná river [7, 11].

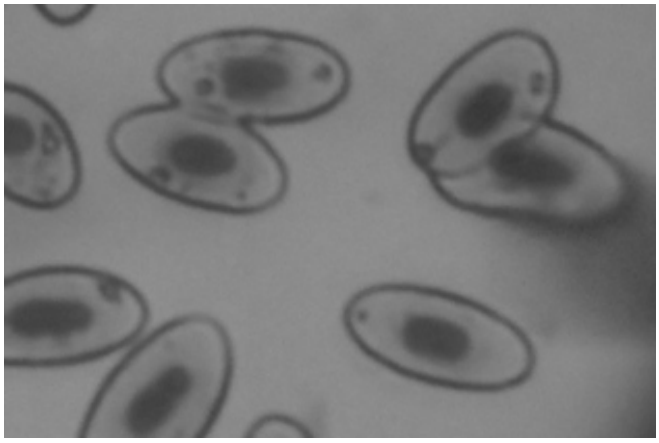

Fig. 1. Erythrocytes with one and two micronuclei

Conclusion

The frequency of micronuclei from first till fourth aquaria, are significantly higher ($P<0.001$) at treated fish, compared with control group of fish. Based on this investigation we can conclude that insecticide Mospilan has highly genotoxic effect in genetic material (DNA) of erythrocytes of fish *C. aureatus.*

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WATER BUGS (HETEROPTERA: NEPOMORPHA AND GERROMORPHA) AS BIOINDICATORS IN ECOLOGICAL STUDIES

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Abstract

Aim: To review the possible application of water bugs (Heteroptera: Nepomorpha and Gerromorpha) as bioindicators in ecological studies.

Material and Methods: Papers from "Web of science" and the search engine "Google scholar" have been selected by key words "indicator" and "Heteroptera".

Results: Only the articles most relevant to the topic of the current study were selected. The studies show that Gerridae and Notonectidae species can be used as heavy metal indicators. Micronectidae and Aphelocheiridae could be good indicators of high oxygen content of waters. The species of aquatic Heteroptera (Nepomorpha and Gerromorpha) are suitable as indicator groups for terrestrialisation of floodplain habitats. The positive relationship between gerromorphan richness and the Habitat Integrity Index could be applied in ecological studies.

Conclusion: The observed data point at the suitability of some water bugs as bioindicators for organic and heavy metal pollution, terrestrialisation and habitat integrity in certain conditions.

Keywords: indicator, Heteroptera, bioindicator, pollution

Introduction

Because of the pollution, habitat destruction, flow modification and invasion by alien species, fresh waters are experiencing a decline in biodiversity for the past decades [10]. Obtaining and evaluating reliable water quality information plays a key role in environmental protection and understanding of these processes of decline. Besides the instrumental measuring techniques, bioindicative systems are used to provide integrated information permitting prophylactic care of the environment [7]. In this report, the term "bioindicator"

is used for "an organism (or part of an organism or a community of organisms) that contains information on the quality of the environment (or a part of the environment)" [7]. Macroinvertebrates are widely used as bioindicators and this review focuses on one of the insect suborders - Heteroptera. Species in this diverse suborder occupy a broad spectrum of aquatic habitats and have a variety of shapes. Water bugs include two infraorders: Nepomorpha and Gerromorpha [15].

Our aim is to review the possible application of water bugs (Heteroptera: Nepomorpha and Gerromorpha) as bioindicators in ecological studies.

Material and Methods

Papers from "Web of science" and the search engine "Google scholar" have been selected by key words "indicator" and "Heteroptera".

Results

Only the articles most relevant to the topic of the current report were selected. A study on the effect of water quality on water bugs shows that *Aphelocheirus aestivalis* (Fabricius, 1794) is restricted to waters with high oxygen content and low nutrient concentrations [6]. The mentioned above species forms an air film on the body surface called plastron which enables these insects to be virtually independent of contact with atmospheric air. Therefore they can stay permanently below the surface as long as the water is sufficiently well aerated [14]. Some species of *Micronecta* are sensitive to pollution and cannot live in oxygen-poor waters [3]. Worsening in oxygen conditions is connected to gradual elimination of Micronecta sp. in the following order: *M. poweri* (Douglas & Scott, 1869), *M. griseola* (Horváth, 1899) and *M. minutissima* (Linnaeus, 1758), that is why Micronectidae are good indicators of high oxygen content of waters [5].

Most of the other water bugs are less sensitive to low oxygen concentration, which can be explained by the fact that all adults (except *Aphelocheirus aestivalis*) do not depend for breathing on oxygen dissolved in water since they mostly collect air at the water surface. When compared water bugs with stoneflies (Plecoptera), the latter are much more sensitive to low oxygen concentrations. Most mayflies (Ephemeroptera) and caddisflies (Trichoptera) are also more sensitive than water bugs [6].

Corixidae are the most speciose and also the most widely distributed family of aquatic bugs. They occupy a relatively wide range of habitats [12]. Some of the Corixidae species occur in water bodies with diverse chemical conditions. Therefore it could be better instead of the presence/absence of a single species as indicator of water quality to be used the percentage species composition of a corixids, which has shown a relationship with conductivity of lake waters. The conductivity is positively correlated with the concentrations of most major ions, and reflects chemical conditions in fresh water and water quality in general [11]. Savage [11] has developed a model, designed to test whether a given species composition of Corixidae from a lake of known conductivity corresponds to the expected pattern in natural

unpolluted waters. If there is agreement in both the corixid communities and conductivity between sample and model then it is believed that the lake is in a normal state. If there is a lack of agreement, further investigations on aspects of water quality are needed [11].

Insects are not often used as bioindicators of heavy metal pollution because the individuals of many species are not easy to collect large enough sample or there is short seasonal supply. Often their origin is unknown due to their flying ability. In many cases a taxonomic expert is needed to determine the species. Using Notonectidae and Gerridae seems to overcome many of the abovementioned problems. These insects are predators [12] and thus inclined to bioaccumulate heavy metals. They are often easy to collect in large number, species identification is easy on species level [1, 8 and 9] and even adults do not disperse for long distance [1, 8 and 9]. Species of genus *Gerris* (*G. argantatus* Schummel, 1832, *G. odontogoster* (Zetterstedt, 1828), *G. lateral*is Schummel, 1832 and *G. thoracicus* Schummel, 1832) have been tested as bioindicators for heavy metal pollution [8, 9]. According to these studies, gerrids are good at detecting differences in iron and manganese concentrations in polluted vs control site. There is only one study which confirms that *Anisops sardeus sardeus* Herrich-Schäffer, 1849 (Notonectidae) is an excellent accumulator of Zn, Co and Pb (1).

Aquatic Heteroptera (Nepomorpha and Gerromorpha) are suitable as indicator groups for terrestrialisation of floodplain habitats [13]. The mentioned study was carried out in the Alluvial Forest National Park in Austria, where engineering measures led to a loss of connectivity between the main course of the river and its backwater systems, and also to a reduced connectivity within the backwater systems. Connectivity in the paper is used as the average annual duration (days per year) of the upstream surface connection of the water bodies with the main stem of the Danube River. When connectivity is low, the entry of the biogenic material and soft sediment leads to terrestrialisation of these backwaters and, in general, to the loss of biodiversity. In this study, species diversity patterns of aquatic Heteroptera (Nepomorpha: Corixidae; Gerromorpha: Mesoveliidae, Hydrometridae, Veliidae, Gerridae) with respect to the type of water bodies and local habitat quality parameters are investigated. The results display that heteropterans have potential significance as indicator group for aquatic habitats with strong terrestrialisation processes [13].

A study on Habitat Integrity Index at Rio das Mortes River in western Brazil has shown that the index is positively correlated with Gerromorphan's richness but there is no such correlation with the Nepomorpha [2]. Habitat integrity of a river is defined as "the maintenance of a balanced, integrated composition of physical, chemical and habitat characteristics on a temporal and spatial scale that are comparable to the characteristics of the natural habitats of the region" [4].

Discussion

We can summarize, that water bugs are suitable as bioindicators for organic and heavy metal pollution, terrestrialisation and habitat integrity in certain conditions.

Nevertheless, further study is needed for sufficient clarity which case would benefit from the application of the shown relationships. The possible use of other predacious water bugs (not only gerrids and notonectids) as heavy metal bioindicators should be tested. The

value of water bugs as indicators in ecological studies would be strongly enhanced by additional data about their habitat requirements, diversity and distribution.

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SPECTRAL REFLECTANCE SIGNATURES IN SOIL SALINITY STUDIES

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Abstract

Aim: Salinity occurs through natural or human-induced processes that result in the accumulation of dissolved salts in the soil water to an extent that inhibits plant growth. Soil salinization has become a serious global-scale problem. Numerous studies and efforts in controlling soil salinity have been made. Nearly 60 percent of the salt-affected soils around the world are in irrigated farmlands, and this trend is increasing. Salinization is a major reason for degradation of soil resources and decline of soil fertility. From an ecological and economic point of view it is extremely important to establish both the occurrence and distribution of soil salinization and the intensity of the process. The goal of this study is to examine soil spectral reflectance properties as detectors and quantitative indicators of soil salinity.

Material and Methods: Remote sensing techniques are widely used in soil surveys for land assessment and monitoring the spatial distribution and temporal change detection of salt-affected soils. In this paper, some results are presented from spectrometry studies of saline soils. Ground-based and airborne reflectance measurements in the visible and near infrared ranges of the electromagnetic spectrum were performed over soils with varying degree of salinity. Different data processing methods were applied to relate soil spectral response to salinity.

Results: The specific information carried by the spectral reflectance features was used to identify saline soils and assess the degree of salinization.

Conclusions The practical application of the research results requires a greater number of *in situ* and remote sensing measurements of soils with different types of salinization, and especially of soils with lower salinity.

Keywords: remote sensing, spectral reflectance, soil salinization

Introduction

At present, the concepts of precision and sustainable agriculture are turning into operational practices [3, 12]. These concepts are often characterized as an evolutionary step in agriculture. They include strategic issues related to crop species, soil properties, fertilization, growth monitoring, and yield prediction. Precision agriculture is a knowledge-based agricultural management system to improve crop production efficiency by adjusting farming inputs to specific conditions within each area of a field. In other words, precision farming is the use of information technologies to achieve site-specific management of crops. Site-specific management accounts for the within-field variability of factors that influence yield, identifies the causes of this variability and effectively modifies crop farming practices. Relevant to soil salinity problem is as well the world-wide concern of natural resources preservation. Soil cover is an essential component of the natural environment. It resembles the relationships between the other environmental components (rocks, water, climate, vegetation, human activities) and

is an indicator of the ecological status of the landscape. Assessment of soil condition is important in detecting land degradation and monitoring the results of remediation and revitalization measures. More precise characterization of the variable soil properties is needed for sustainable agriculture running. One of the fundamentals of precision farming is the implementation of the new informational capabilities provided by remote sensing technologies. Soil studies are undoubtedly among the priorities of remote sensing observations [2, 6, 8, 9].

One of the main processes leading to soil resources degradation is salinization. It depends on environmental and anthropogenic factors such as high temperatures, poor drainage, increased mineralization of groundwater, intensive irrigation and over-fertilization. Soil salinity is one of the biggest problems worldwide related to "wearing out" of soil resources and loss of biodiversity in both terrestrial and aquatic ecosystems. Secondary salinity due to human activities has significantly increased the extent of the problem. Besides being unsuitable for agricultural use saline soils are pollutants for the surrounding areas. According to global statistics salinization affects nearly 10% of the soil resources and 50% of the irrigated land in the world. In Bulgaria salinization accompanies the most fertile soils used for intensive agriculture (Sliven, Burgas, Plovdiv and Veliko Tarnovo regions). From a practical point of view, it is very important to establish both the presence and distribution of salinization as well as the intensity of the process, i.e. to quantify the extent and rate of salinity. A dramatic example of soil salinization as a serious environmental problem, especially when resulting from human activity, is the disappearance of the great basin of the Aral Sea [15] and the expansion of large areas of salt-affected soils (Fig. 1).

Remote sensing techniques are widely used in soil surveys for detection and monitoring of the spatial- temporal distribution of salt-affected soils. Numerous publications have been devoted to the use of spectral data from multiple remote sensing sensors to detect and describe soil salinization [1, 4, 5, 7, 10, 11, 13, 14]. They point out difficulties associated with the complexity of the process, its dynamic nature and the interrelated factors on which salinization depends (inherent soil properties, climat conditions, and agricultural activities).

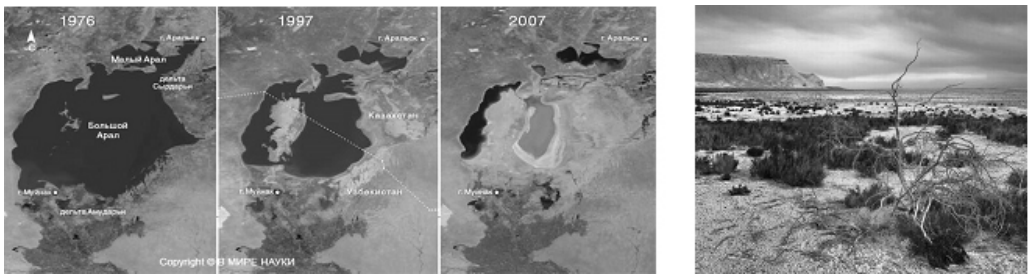


Fig. 1. Progressive disappearance of the Aral Sea and spreading of salt-affected soils over large areas

In remote sensing assessment of soil salinization, a frequently utilized approach is to use plant indicators since plant species have different tolerances to soil and water salinity [4, 7]. Usually, attempts are made to identify and distinguish between various salt-tolerant

and salt-sensitive species on the basis of multispectral image data. Salt-sensitive species exhibit inhibited growth preluding their eventual death. Salinity-induced stress depends on the salt levels and the particular species tolerance level. As a consequence, vegetation becomes more dominated by more salt-tolerant plant species. These are commonly observed in saline areas and can be considered salinity indicator species. Since plant species vary in their sensitivity to salts, some species will be affected by low concentrations, while others will tolerate high salt concentrations. As so, an indicator of slightly saline soils will be plant inhibited growth, while for high salt-containing soils such an indicator will be the presence of halophytes. But a number of plant species grow vigorously in both non-saline and saline environments. Besides, salinity indicator species vary from region to region. Their presence at a particular location is influenced by seasonality, climate conditions, and other factors. As a result, it is not uncommon to find a mosaic of plant species of varying salt-tolerance across a site. Further investigation should always be carried out to confirm if the vegetation is really indicating salt-affected soil. All this not only requires region-specific local knowledge but it is the reason of ambiguity and unreliability of salinity assessment. The use of such secondary indicators for monitoring and mapping salinized areas by remote sensing gives in some cases good results but is limited to medium and low salinity where tolerant plants grow fairly well.

In other publications remotely sensed data are used to detect and characterize saline soils by applying empirical relationships between spectral features and *in situ* measurements of soil electrical conductivity. The electrical conductivity is a soil properties associated with the degree of salinization [1, 4]. A number of works indicate the ability of remote sensing data to differentiate between highly saline and unsalted soils and no success in distinguishing low and moderate salinity levels [1, 4, 5, 11]. It is worth mentioning the synergetic use of measurements in different spectral ranges (visible, infrared, thermal, microwave) for soil salinity assessment [11, 14].

In our paper some results are presented from spectrometry studies of saline soils. The main goal of this study was to empirically investigate the relationship between soil spectral reflectance signatures and salinity level, and on the basis of experimental evidence to show the applicability of soil reflectance response for identification of salinization and assessment of the salinity degree.

Material and Methods

Ground-based and airborne reflectance measurements in the visible and near infrared ranges of the electromagnetic spectrum were performed over soils with varying salinity. Different data processing methods were applied to identify salt-affected soils and to examine the performance of soil multispectral response as a quantitative indicator of the degree of salinization.

Due to the dry climate and poorly drained conditions salinization is observed in the surrounding areas of many saline lakes (endorheic basins) such as the Adjinour lake (20 000 hectares of salt-affected soils) on the territory of Azerbaijan where a part of our research encompassing ground-based and airborne experiments has been carried out (Fig. 2). Soil salinity degree in the region varies from 6-7% to

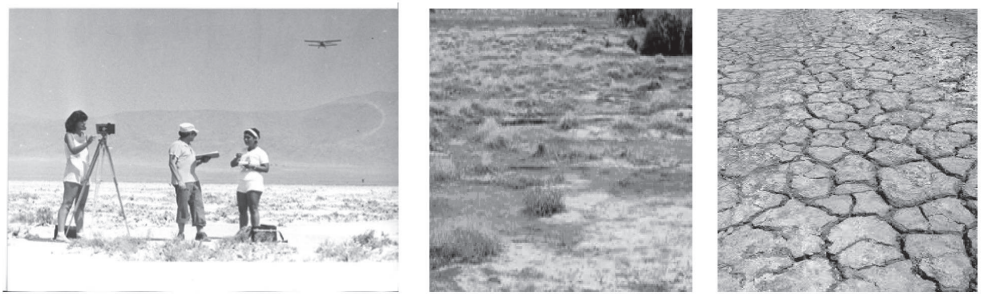


Fig. 2. Ground-based and airborne spectral measurements of saline soils in the area of Adjinour Lake, Azerbaijan

15-20% depending on the type of the solonchak. Multispectral data has been acquired as well over less saline soils (1-4%) in Vietnam (on ancient wave-cut platform), Mongolia (natural solonetz, meadow solonchak) and Bulgaria, Belozem (meadow solonetz, solonchak-solonetz).

Soil spectral reflectance signatures in the visible and near infrared band (400-850 nm) have been measured with multichannel spectrometric systems from airborne platforms (aircraft, helicopter) and in the field. Ground-based and remotely sensed data have been analyzed in respect to its suitability to serve as an indicator of soil salinity.

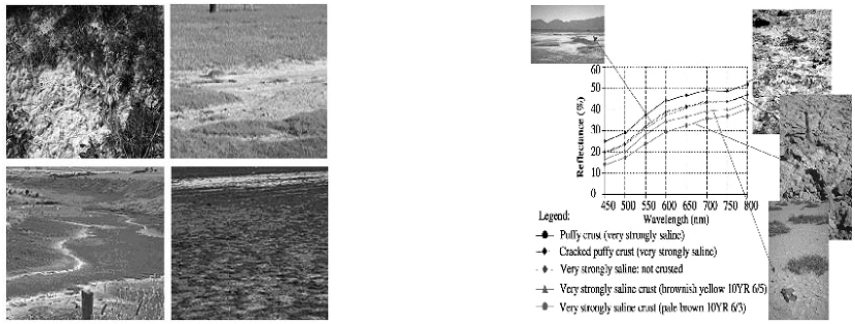
The interpretation of multispectral remote sensing data relies upon the specifics of the spectral reflectance patterns of different objects. The application of remote sensing data for recognition and characterization of saline soils requires detailed knowledge of their spectral response as a function of salinity and other physical and chemical properties. Soil salinization is a multifactor phenomenon whose heterogeneity, spatial dynamics and seasonal dependence complicate their survey both with conventional field methods and remote sensing techniques. Soil texture, surface roughness, color, mineral composition, organic and moisture content also are causes for significant variations of soil spectral reflectance properties. Analyzing the datasets we use different spectral indicators to resemble peculiarities and distinctive feature of saline soils reflectance. These are reflectance factors for selected wavelengths, reflectance ratio indices, slopes of the spectral reflectance curves at different portions of the spectral range 0.4-0.8 μm, clustering by a two-dimensional space representation, multiple regression, and others. Herein some of the data processing results are presented.

Results and Discussion

Soils tend to accumulate salts in the surface layer with local concentration and deposits of light-colored crust, whitish and whitish-grey salt patches, stripes or crystals (Fig. 3a). This leads to the formation of spectral mixtures and causes difficulties for the interpretation of the acquired remote sensing data. Besides, saline soils differ in their structure varying smooth to rougher as well as in color from white to light grey and darker. Color is a dominant factor affecting optical properties of soils. It is greatly determined by the humus content which could be higher in solonetz soils and alkaline saline soils. The concentration

of organic matter leads to pronounced variations of soil reflectance patterns. For example, the reduction of two color-difference units of the Munsell Scale causes reduction of soil reflection in the whole spectral range [11]. This is illustrated by Fig. 3b which presents the spectral reflectance signatures of highly saline soils with different color.

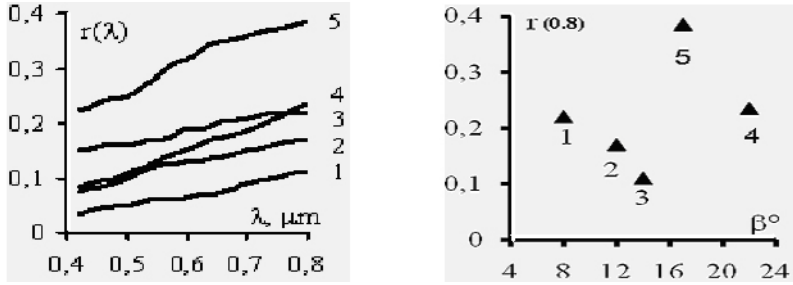
All soils are characterized by increasing reflectance in the wavelength range under consideration. In Fig. 4a the spectral characteristics of various soil types are plotted. The values of the reflectance factors and the slope



(a) (b)

Fig. 3. Different salinity manifestation on the soil surface - salt crust, patches, strips and dark alkali solnetz (a); reflectance spectra of highly saline soils with different color (b)

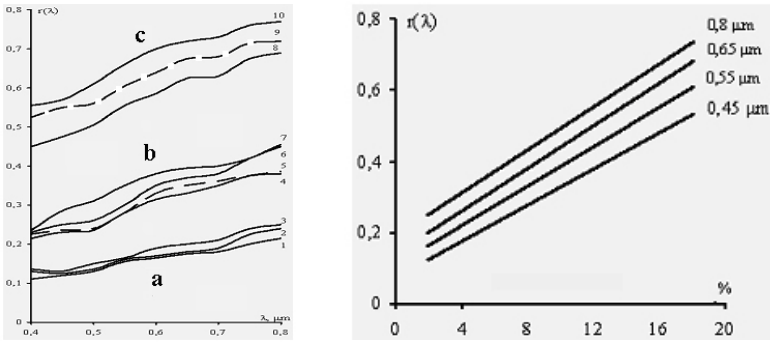
of the spectral curves can serve as classification features. Salt-affected soils (4 and 5) are distinguished by their higher brightness and steeper slope. Using these two features saline soils are confidently identified and separated from non-saline soils. This is illustrated in Fig. 4b where the soils from Fig. 4a are presented in the two-dimensional space of the spectral curves slope (the angle β^o) and the reflectance values at 0.8 μm.



(a) (b)

Fig. 4. Spectral reflectance signatures of different soil types (a): 1 - leached black soil, 2 - brown forest soil, 3 - alluvial-meadow soil, 4 - meadow solonetz, 5 - solnochak; soils location in the two-dimensional space of the slope of the spectral curves and the values of the reflectance factors at 0.8 μm (b)

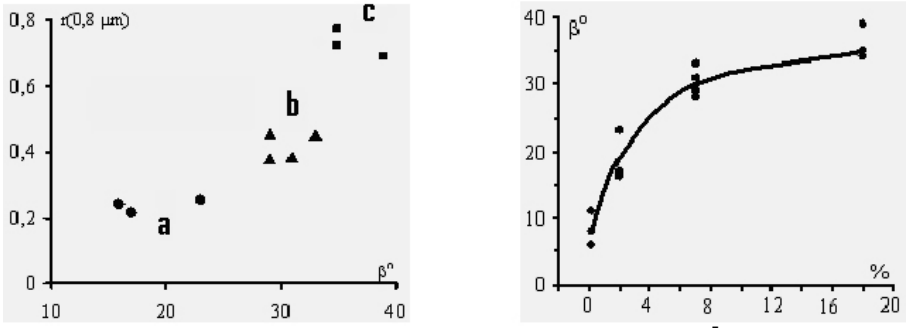
The same is confirmed by Fig. 5 where the spectral reflectance characteristics of differently salinized soils are plotted. The ground-based reflectance measurements are shown with solid lines and the dotted lines refer to airborne data. As it can be seen, according to their reflectance values the soils are distinctly grouped by the salinity degree into low (a), medium (b) and highly (c) saline. Increased reflectance associated with higher salinity is observe in the whole wavelength range. This is illustrated also by the established dependence of the reflectance factors at different wavelengths on the salinity level (concentration).



(a) (b)

Fig. 5. Spectral reflectance characteristics of saline soils with different salt content (a): a – 1-2% (meadow solonetz), b – 6-8% (solonchak), c – 15-20% (solonchak); dependence of the reflectance factors at different wavelengths on the salinity level (b)

In Figure 6a the three groups of salt-affected soils are presented in the two-dimensional space of the gradient β^o and the reflectance factor r (λ) at 0.8 μm. The reflectance goes up and steeper with the increased salt content.



(a) (b)

Fig. 6. Representation of saline soils (from Fig. 5) in the two-dimensional space of the spectral curves slope (angle β^o) and the reflectance values at 0.8 μm (a); dependence of the slope of soil spectral curves on the salt content (b)

The soils form three non-overlapping clusters which means that they can be reliably distinguished by these two classification features. Data analysis in a two-dimensional space allows better detection and differentiation of saline soils especially in cases when this is not possible using only one feature. In Figure 6b the empirical relationship between the gradient β^o of the reflectance curves and the salt content in soils is presented. It shows the steeper-slope trend with increasing salinity. The practical use of such dependencies for identification and characterization of saline soils requires extensive and more detailed experiments particularly over low-salinity soils.

Conclusions

Recognition and classification of soil salinity is the first step to combat against salinization. Recent advances in the application of remote sensing technologies for monitoring degraded lands and specifically salt-affected soils, have shown promising abilities. The results presented in the paper illustrate the potential of multispectral data to identify soil salinization. The implementation of different data processing methods significantly improves this potential. It has to be pointed out, however, that the classification and the spatial distribution assessment of salt-affected soils faces serious difficulties typical for both, remote sensing and traditional ground surveys. These difficulties are associated with the spatial and temporal heterogeneity of soil salinity, and the climate-dependent dynamics of salinization processes. Along with the salinity level, soil type, color, surface roughness organic, mineral and water content strongly influence soil spectral reflectance data and often are causes of ambiguity of data interpretation. An efficient approach to remote sensing application in saline soils surveys could be the synergetic use of data from multiple sensors and wavelength bands together with GIS databases and detailed field inventory.

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ELECTROKINETIC STUDY OF ULTRASOUND-INDUCED PERMEABILIZATION OF BARLEY THYLAKOID MEMBRANES AND CHLORINA BARLEY MUTANTS UNDER LOW AND HIGH LIGHT

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Abstract

Aim: The effects of ultrasound (22 kHz) on barley thylakoid membranes (*Hordeum vulgare* L.) of wild type (*WT*) were investigated. The barley plants of *WT* and two *chlorina* mutants, *chlorina 126* and *chlorina f2*, were subjected to light intensities of 100 (LL, low light) and 1000 μmol photons m^{-2} s^{-1} (HL, high light), respectively in order to specify the effect of light exposure on the electrokinetic potentials at the membrane surfaces.

Material and Methods: Chloroplasts were isolated from leaves of barley (*Hordeum vulgare* L.) according to [7]. The electrophoretic mobility (EPM) measurements were performed using the particle electrophoresis technique (OPTON Cytophotometer). Ultrasound treatment (22 kHz) was applied and the lipid peroxidation was analyzed.

Results: We determined the electrophoretic mobility, zeta potential and surface charge density of thylakoids under ultrasound (22 kHz) treatment and low and high light exposure. Lectin-membrane interaction was

tested on the EPM of thylakoid membranes from wild type after ultrasound (US) treatment at intensity of 15 W cm^{-1} in dependence of energy supplied by the system. The lectin binding may provide information about changes in glycoprotein complexes of the membrane. We studied the effect of *Phaseolus vulgaris* agglutinin (Phytohemagglutinin PHA-P) on the surface electric charge of thylakoids in low ionic strength media, pH 7.80. PHA-P induced a decrease in EPM of thlakoids at doses of 3.3 – 13.3 μg/ml after US exposure (0.820 kJ). There was an enhancement in zeta potential of barley thylakoids (*WT*) upon pre-treatment with the 3.3 μg PHA-P/ml after US treatment (1.820 kJ).

Conclusion: Comparison of the electrokinetic properties of thylakoids from wild type plants and *chlorina* mutants revealed that zeta potential in *chlorina f2* possess less negatively charged outer surface of the thylakoid membrane under low light during barley plant growing than in wild type and *chlorina 126*.

A decreased lipid peroxidation by US (22 kHz, 15 W cm^{-1}) exposure before and after lectin pre-treatment of thylakoids (*WT*) was observed. It could be due to a protection of lectin molecules to a recombination of free radical products as a consequence of an indirect action of ultrasound permeabilization on thylakoids from wild type.

Keywords: ultrasound, thylakoid membrane, zeta potential, barley, lipid peroxidation

Introduction

The electric charge distributed over the surface of biological membranes plays an important role in the regulation of the molecular membrane processes, but also in the processes of permeabilization of thylakoid membranes with external influences such as ultrasound (22 kHz). We used the barley thylakoids as a model for ultrasound-induced permeabilization of the membrane. US treatment (15 W cm^{-1}) could cause oxidative stress resulting from iron-dependent reactions that produce excessive amounts of highly reactive free radicals in thylakoid lumen as hydroxyl and alkoxy radicals, as well as H_2O_2, which in turn initiate various oxidation reactions of cellular components notably including lipid peroxidation [5].

In this study we report for the first time: 1. the reduction of EPM, zeta potential and surface electric charge of *chlorina f2* thylakoids because of strong complex aggregations of barley thylakoids upon low light treatment; 2. using US (15 W cm^{-1}) exposure, as well as additional lectin PHA-P pre-treatment to decrease the lipid peroxidation products generated which depends on US energy supplied (820 kJ or 1820 kJ) to the membrane vesicles.

Material and Methods

Plant material, culture and preparation of thylakoids

Experiments were carried out with 3-week-old barley (*Hordeum vulgare* L.) plants grown in a climatic chamber at 25°C, light intensity of 100 μmol photons m^{-2} s^{-1} (provided by white fluorescent lamps) and a photoperiod of 12 h light/12 h dark. Knop nutrient solution was included at watering twice per week. Whole plants were then exposed at a low light (LL) intensity of 100 μmol photons m^{-2} s^{-1} or a high light (HL) intensity of 1000 μmol

photons m^{-2} s^{-1} for 5 h. Plants kept at 25°C for 5 h at 100 or 1000 μmol photons m^{-2} s^{-1} were used as LL and HL controls respectively.

Chloroplasts were isolated from the leaves of 3-week-old seedlings (*Hordeum vulgare* L., wild type and *chlorina* barley mutants) according to Takeda et al. [7] with some modifications. The final pellet was resuspended to a concentration of 2.1-2.8 mg chlorophyll mL^{-1} [6]. For experiments the following buffer containing 10 mM Tricine (KOH), 5 mM $MgCl_2$, pH 7.80 or 10 mM Tricine (KOH), 5 mM $MgCl_2$, 10 mM NaCl, pH 7.80 were used.

Microscopic (visual) microelectrophoresis

The electrophoretic mobility (EPM) measurements were performed using the particle electrophoresis technique with OPTON Cytophotometer according to [4]. Data are means of three independent experiments. The zeta potential (ζ, mV) and surface charge density (σ, C m^{-2}) were calculated by [1, 3].

Ultrasound treatments

The preparations were treated with an ultrasound (22 kHz) with varying ultrasound exposure in the range 0.820 kJ or 1.820 kJ energy supplied by the system. We used the intensity of 15 W cm^{-1}. The experiments are carried out at a room temperature of 25°C in a Sonopuls (Bandelin). The US treatment was applied to an Eppendorf cups containing 0.5 ml of chloroplasts. At the end of exposure, the final thylakoid suspension (exposed and unexposed to US) was stored in ice at 0°C before measurements.

Detection of malondialdehyde (MDA)

Following the ultrasound treatment the samples were subjected to the analysis of thiobarbituric acid-reacting substances (TBARS) products according to the method described by [5] with slight modifications.

Statistical analysis

The experiments were performed in triplicate. The significant differences between means were determined by use of ANOVA. One-way analysis of variance was performed with Holm-Sidak method for comparison the significance of the treatment.

Results

Effects of US (22 kHz) on the electrophoretic mobility of thylakoids

Phytohemagglutinin treatment (3.3 – 13.3 μg PHA-P/ml) resulted in the significant changes in the EPM of *WT* thylakoids up to 18%. Ultrasound exposure caused a reduction (13.9%) of the EPM of *WT* thylakoid membranes. US treatment of PHA-P pre-treated thylakoids (3.3 – 13.3 μg PHA-P/ml) didn't cause a statistically significant influence on the EPM of thylakoid membranes. There was an effect of protection in EPM values of US exposure (Fig. 1).

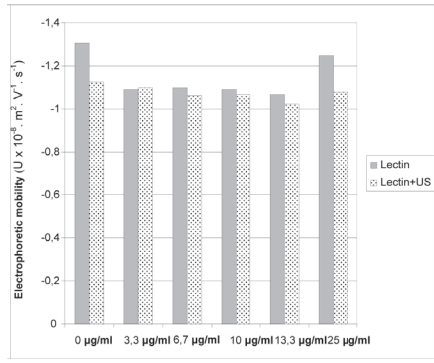

Fig. 1. Effect of ultrasound (22 kHz, 15 W cm^{-1}, 0.820 kJ) exposure on EPM of thylakoid membranes (*WT*). The medium contained 10 mM Tricine (KOH), 5 mM $MgCl_2$, 10 mM NaCl, pH 7.80. Electrophoretic mobility of thylakoids (*WT*) treated by phytohemagglutinin (PHA-P)

There was an effect of increase in EPM values of US-exposed (1.820 kJ) thylakoids (WT) with 13%. The EPM of WT thylakoids increased after treatment of 3.3 µg PHA-P/ml (with 16.7%), while that of thylakoids (WT) remained stable after lectin pre-treatment at doses of 6.7 – 13.3 µg/ml upon US exposure. The variation of the effect of ultrasound treatment (1.820 kJ) resulted in the insignificant changes in the EPM of WT thylakoids without US exposure of lectin pre-treated thylakoid membranes (Fig. 2).

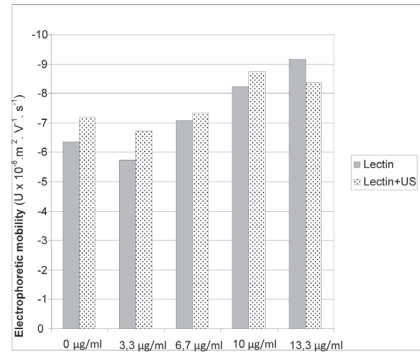

Fig. 2. Effect of ultrasound (22 kHz, 15 W cm^{-1}, 1.820 kJ) exposure on EPM of thylakoid membranes (*WT*). The medium contained 10 mM Tricine (KOH), 5 mM $MgCl_2$, 10 mM NaCl, pH 7.80. Electrophoretic mobility of thylakoids (*WT*) treated by phytohemagglutinin (PHA-P)

Evaluation of lipid peroxidation in the US exposed thylakoids

US treatment (0.820 kJ) of *WT* thylakoids did not influence the lipid peroxidation of the

membranes. The TBARS of lectin pre-treated thylakoid membranes in the presence of 3.3 – 10 μg PHA-P/ml concentrations of lectin was significantly modified after US exposure of the samples by a reduction of 34.6% in comparison to TBARS of the same thylakoids before US treatment (Fig. 3).

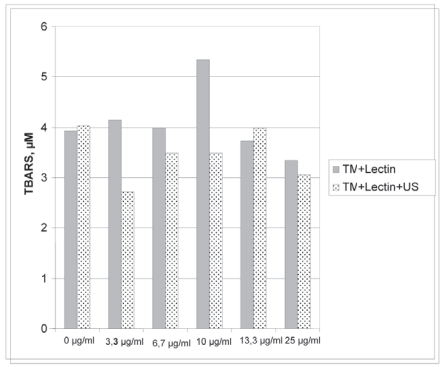

Fig. 3. Effect of ultrasound (22 kHz, 15 W cm^{-1}, 0.820 kJ) on lipid peroxidation of control (untreated) and phytohemagglutinin (PHA-P) pre-treated thylakoids (*WT*). Thiobarbituric acid reactivity substances in thylakoids, suspended in 10 mM Tricine (KOH), 5 mM $MgCl_2$, 10 mM NaCl, pH 7.80 before and after exposure to US

A statistically significant increase in lipid peroxidation of WT thylakoids upon US treatment with 45% versus preparation without US exposure was observed. Lectin pre-treatment of *WT* thylakoids in the presence of (3.3 or 10-25 μg/ml) doses of PHA-P decreased strongly TBARS upon US exposure (1.820 kJ) comparing the values of lectin pre-treated thylakoids (*WT*) before US exposure (Fig. 4).

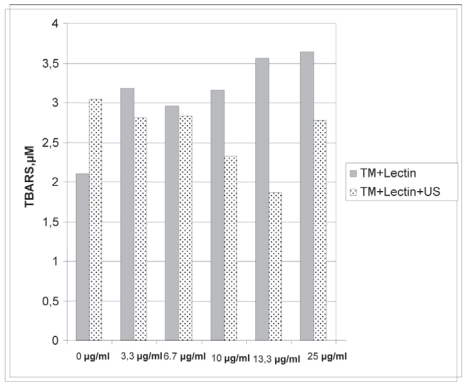

Fig. 4. Effect of ultrasound (22 kHz, 15 W cm^{-1}, 1.820 kJ) on lipid peroxidation of control (untreated) and phytohemagglutinin (PHA-P) pre-treatedthylakoids (*WT*). Thiobarbituric acid reactivity substances in thylakoids, suspended in 10 mM Tricine (KOH), 5 mM $MgCl_2$, 10 mM NaCl, pH 7.80 before and after exposure to US

Zeta potential of thylakoid membranes in wild type and chlorine barley mutants under low and high light

The changes in the zeta potential of barley thylakoids upon light treatment of wild type and chlorina barley mutants were studied. *Chlorina f2* is devoid of chlorophyll (Chl)b, causing a complete loss of LHCIIb [8]. *Chlorina f2* is a Chl-b-deficient mutant. It lacks the 25-kDa polypeptide of LHCIIb and has a strongly reduced amount of the 28-kDa polypeptide of LHCIIb, as well as a reduced amount of CP24 [2].

Treating the plants with low light showed that EPM, ζ and σ of *chlorina f2* started to decrease in comparison to the same parameter of wild type (Fig. 5).

The high light treatment of *WT* and *chlorina* barley mutants was not accompanied by significant changes in EPM, ζ and σ of thylakoids compared to the same parameters of the wild type thylakoid membranes (Fig. 6).

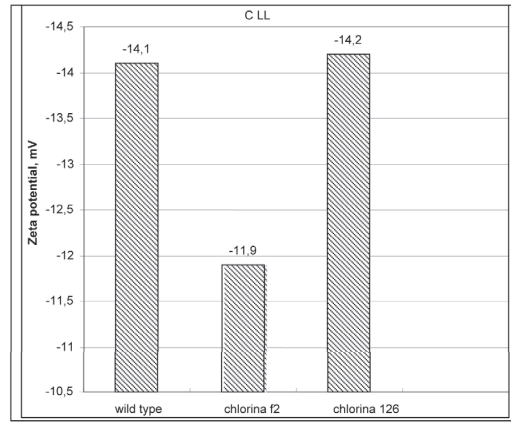

Fig. 5. The influence of 'low light' on the zeta potential of thylakoids isolated from wild type barley plants and *chlorina 12*6 and *chlorina f2* mutants. The solution contained 10 mM Tricine (KOH), 5 mM $MgCl_2$, pH 7.80

The high light treatment of *WT* and *chlorina* barley mutants was not accompanied by significant changes in EPM, ζ and σ of thylakoids compared to the same parameters of the wild type thylakoid membranes (Fig. 6).

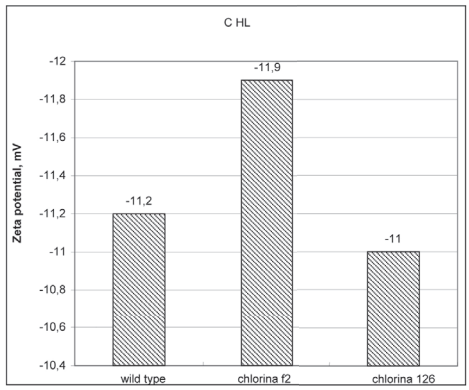

Fig. 6. The influence of 'high light' on the zeta potential of thylakoids isolated from wild type barley plants and *chlorina 126* and *chlorina f2* mutants. The solution contained 10 mM Tricine (KOH), 5 mM $MgCl_2$, pH 7.80

Discussion

Low light exposure of wild type and chlorina barley mutants decreased electrokinetic potential of c*hlorina f2* without significant changes in zeta potential and surface electric charge of thylakoids in wild type and *chlorina 126*.

Ultrasound exposure of wild type barley thylakoids after pre-treatment with phytohemagglutinin (PHA-P) lectin affected strongly TBARS products comparing the values of thylakoids before ultrasound treatment. PHA-P lectin molecules could protect the thylakoid membranes by a US (15 W cm^{-1}, 0.820 kJ) exposure. Lectin pre-treatment even led to a decrease in free radical products upon US (15 W cm^{-1}, 1.820 kJ) exposure.

Moreover it has been found that wild type thylakoid membranes were more resistant to US treatment (0.820 kJ) than wild type thylakoids when the US exposure (1.820 kJ) was applied.

Experiments with isolated chloroplasts are in progress to clarify the possible reasons for the observed stabilizing effect of lectin molecules on the electrokinetic properties of thylakoids under ultrasound-induced permeabilization of the membranes.

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КРАТКИ СЪОБЩЕНИЯ/SHORT COMMUNICATIONS

Тематично направление:
АНТРОПОГЕННИ ВЪЗДЕЙСТВИЯ ВЪРХУ ЖИВАТА ПРИРОДА

Topic:
ANTROPOGENIC IMPACT ON THE LIVING NATURE

MACROPHYTE-BASED ECOLOGICAL STATUS ASSESSMENT OF SUB-MEDITERRANEAN RIVERS IN BULGARIA

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Aim: The Water Framework Directive (Directive 2000/60/EC) requires ecological assessment of water bodies with biological communities. In this paper, an over view was given on the Sub-Mediterranean Rivers in Bulgaria and their ecological assessment based on macrophytes as a biological quality element.

Material and Methods: Data for thirteen rivers (Azmaka, Biserska, Byala, Dereorman, Krumovitsa, Luda reka, Manastirska, Melnishka, Popovska, Sokolitsa, Varbitsa and Stara) in South Bulgaria studied in 2009 were presented together with site descrip-

tions (flow velocity, shading, mean depth, substrate type, and altitude) and environmental variables (acidity, electrical conductivity, dissolved oxygen and oxygen saturation, NH_4, NO_3, PO_4, total nitrogen and phosphorus, COD and BOD5). The abundance of each species was estimated according to a five-degree scale according Kohler [2]. Macrophyte relative abundances were quantified based on percent frequency of occurrence in 9 sampling sites.

Reference Index (RI) which defines type-specific reference and non-specific disturbance indicating taxa, and transformation into ecological quality ratio (EQR) was calculated after Schaumburg et al. [3] and Gecheva et al. [1].

Results: The majority of studied rivers were slow-moving, lighted, with sandy or stony bottom and average depth up to 1 m. The altitude varied between 50 (Azmaka River) and 510 (Varbitsa River) m a.s.l. The pH values were slightly in the alkaline range. Highest values of electrical conductivity, $N\text{-}NO_3$, $P\text{-}PO_4$, BOD and COD were deremined at Dereorman River. Two river sites were characterized by absence of macrophytes, while additional two rivers were dried out. At the remaining nine sites 40 taxa were identified, among them 7 bryophytes and 1 charophyte. The 32 vascular plants registered, included 11 hydrophytes and 21 helophytes. The most frequently distributed hydrophyte was *Potamogetonnatans* L. (registered at 5 sites, relative abundance 56%), while the most common species of the group of helophytes was *Sparganiumerectum* L. (registered at 6 sites; relative abundance 67%).

EQR indicating good and high ecological status was achieved in 8 of the 9 river sites where macrophytes were registered. Only Dereorman River was evaluated in poor ecological status based on macrophytes as BQE.

Conclusion: First attempt was presented to describe macrophyte development in Sub-Mediterranean Rivers in Bulgaria and to reveal the variation in community structure.

The assessment of the river sites according to the adopted Reference Index resulted predominantly in high and good ecological status.

Acknowledgments: The research was a part of the project: “Developing classification system to wards ecological status and potential assessment of the defined surface water types (rivers and lakes) on Bulgarian territory (in compliance with System B)” executed by Consortium for Biomonitoring, financed by Operational Programme Environment 2007-2013.

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POTENTIAL THREATS FOR AIR POLLUTION IN WESTERN RHODOPES: A REVIEW

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Aim: Western Rhodopes were identified as an air quality study area since background monitoring station 'Rozhen' is situated within this relatively free of local industrial emissions region. Nevertheless, traditional sources of atmospheric pollution together with cross-border pollution have a serious potential impact. A review of existing assessment and ecological monitoring programs dealing with air quality issues in the territory of Western Rhodopes has been performed. It allows the identification of the most dangerous sites for the air health damages which should be used as relevant sites for biological monitoring. Biomonitors can be further selected in order to facilitate the monitoring operation, its development and harmonization in the Western Rhodopes region.

Material and Methods: The review process has taken into account 12 literature sources, which could be classified into 3 broad categories: reports on the state of environment of the Regional Inspectorate of Environment and Water - Smolyan, municipal programs and reports under the Conservation of Globally Significant Biodiversity in the Landscape of Bulgaria's Rhodope Mountains Project (Rhodope Project).

Results: Four pollutants exceeded air quality standards: particulate matter, SO_2, NO_x and O_3 [1].

Measured critical levels for ozone at background station 'Rozhen' suggest serious effects on vegetation and high mountain tourism in summer.

The threats for air pollution could be summarized as follows:

- Mass-consumption of solid fuels for domestic heating;
- Delay in environmentally friendly technologies implementing caused by economic stagnation;
- Overbuilding with mutually exclusive activities (e.g. hotels and wood-processing) in Municipalities of Chepelare, Satovcha, etc.;
- Steam generators installing with inadequate diameter and height of the chimney-stack.

No significant industrial pollutants were registered at the selected territory [2]. Eleven dangerous sites (in the area of Asenovgrad, Laki, Batak, Velingrad, Barutin, Smolyan, Rudozem, Madan, Zlatograd, Banite, Ardino) for the air health damages were selected and they are recommended to be used in biomonitoring.

Conclusion: This review registered 3 potential threat sources (solid fuels for domestic heating, vehicle pollution and combustion plants) and a large number of point and diffuse sources of pollution. This preliminary work will pave the way for further biomonitoring with mosses in selected 11 sites within the Western Rhodopes region.

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BIO-MONITORING OF SOIL SAMPLES FROM THE AREA OF KCM PLOVDIV

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Introduction: The toxic impact of a number of substances in plant bio-systems is an important factor for environmental pollution. Of particular interest is the contamination of soils and waters with heavy metals and their impact on physiological status, adaptive ability and agricultural productivity. The mutagenic effect of heavy metals requires special attention and research. Heavy metals bioavailability, accumulation and toxicity depends on many environmental factors. Metal ions present in excessive amounts become toxic. The critical concentration for one metal may be different at different phases of development,

and depends on the chemical and physical conditions. Two or more metals at toxic levels can be either synergistic or antagonistic.

Two main approaches are commonly used to monitor environmental pollutants. The first one is based on physical and chemical analysis. It could be:

Very sensitive;

With good resolution;

Very cost-effective.

Special equipment and special laboratories are required.

The second one is based on bioassays - different test-systems are applied at different levels - from living organism to cellular and/or sub-cellular.

The aim of this study was the application of a complex of fast, highly sensitive to low doses of contaminants methods for monitoring of soil samples from the area of KCM Plovdiv.

Material and Methods: The unicellular green algae are a robust model system for plants and have, therefore, been used for the development of various test-systems for screening of the effect of physical and chemical mutagenic/promutagenic factors upon plants.

The wild type *Chlamydomonas reinhardtii* 137C, cultivated in our lab for a long time, was used because it is a widely spread, photosynthetic eukaryote, a single cell organism, which has a typical plant cell structure, a haploid genome, a short life cycle and is sensitive to different pollutants and especially to heavy metals [3].

Five soil samples from different plots near KCM Plovdiv were studied. Measurement of heavy metals content in soil was done by ICP-AES. Extraction was performed with 0.01M $CaCl_2$ solution for 48h.The cell suspension was treated for 72h under continuous light on a shaker. The toxic and genotoxic potential of the soil extracts was observed by several methods: Spot-test – rapid test for toxicity; Growth rate – for testing the inhibitory effect of test samples (according to ISO 8690:2004). The toxic and genotoxic potential of the soil samples was assessed by the colony forming ability ("clonal" assay). Cell survival was determined by counting colonies visible to naked eye [1] after 7-10 days growth in the light in TAP solid nutrition media. The mutagenic potential of the soil samples was assessed by the "visible" mutants - assay is based on changes in size, morphology and pigmentation of surviving colonies.

Oxidative stress markers - malondialdehyde (MDA) as a biomarker for lipid peroxidation, and intracellular hydrogen peroxide – were measured in quantity by formula:

$$\mathbf{MDA} = \frac{\mathbf{(A535\text{-}A600).a.b}}{\mathbf{s.FW}}$$

HSP70B was also used as an early warning marker for oxidative stress. The level of heat shock proteins (HSP70B) was determined by gel–electrophoresis and Western blotting analysis [2].

The results were determined by one-way ANOVA test.

Results: Soil samples were analyzed in different ways: cell survival, growth and mutation rate of algae culture. Data for cell survival are represented by the survival fraction (SF). The fraction of surviving colonies was calculated by the method of

Bryant [1] shows a slight stimulatory effect (SF = 1.1) in the treatment of the cell suspension with soil extracts №2 and 3. Weak genotoxic effect (SF = 0.8) and weak mutagenic effect (MI = 4.46) was observed after treatment with soil extract №4.

This assay is informative for the type of induced and realized genetic damage - low-size mutations, which are smaller than 1/3 of the average colony size (impaired cell division), pigment mutations (gene point mutations), morphological mutations (altered cell wall structure and composition).

The higher values of induction of HSP70B after treatment with the soil extracts №1 and №5 corresponds to the results of the Spot –test and the growth rate. The lower value of HSP70B for soil extract №3 corresponds to the results of the survival test.

Conclusions

• Our results also confirmed that in gene toxicological studies a battery of test systems with different resolution must be used.

• Data from spot-test and growth rate showed that soil samples have different effect. Genotoxic effect had soil samples 1 and 5.

• Treatment with soil extract № 4 showed low survival rate and week mutagenic effect.

• Some inhibition of HSP70B was observed after the treatment with soil extract 3.

• The lower value of HSP70B for soil extract 3 corresponds to the results of the survival.

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TRACE ELEMENTS IN SOILS AND HERBS IN THE VICINITY OF SMELTER "KCM 2000"-PLOVDIV (BULGARIA)

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Abstract

Area of smelter "KCM 2000"-Plovdiv, is one of the "hot spots" in Bulgaria in terms of contamination with trace elements. For the purposes of this study four test sites were chosen at various distances from the smelter, according to the wind rose. In each one both soil and herb samples were collected simultaneously. Concentrations of As, Cd, Cu, Hg, Ni and Pb in all samples were analyzed by ICP-MS. Coefficient of soil technogenic pollution and the coefficient of bioaccumulation of the studied trace elements in herbs were calculated. Our results showed that the soils in the vicinity of "KCM" are priority contaminated with Pb, Cd, Ni and As. *Plantago lanceolata* and *Carex divisa* were found as accumulators of Pb, Cd and Cu, and *Papaver rhoeas* - of Cd, Hg and As.

Introduction: The area of smelter "KCM 2000", Plovdiv, is one of the "hot spots" in Bulgaria in terms of contamination with trace elements (National State of the Environment Report, 2010). As a result of many years of production, all components of the environment are considerably contaminated - air, water and soil. Trace elements cause one of the most dangerous pollution of the soil, because they could be sorbed firmly in the root layer and most of them are toxic to many plants. This requires continuous monitoring of the trace elements technogenic accumulation in soils and ecosystems in order to consider measures for their remediation.

The aim of the present study was to establish the content of the main pollutants (As, Cd, Cu, Hg, Ni and Pb) in the soils and naturally growing herbs in the vicinity of the smelter "KCM 2000".

Material and Methods: For the purposes of the study four test sites were chosen at various distances from the smelter, according to the wind rose. Each site was subdivided to 5 sampling squares, and both soil and plant samples were collected simultaneously (BDS 17.4.5.01-85). Sample preparation and mineralization were performed according to standard ISO 11466.

Content of As, Cd, Cu, Hg, Ni and Pb was determined by inductively coupled plasma mass spectrometry (ICP-MS) using instrument Agilent 7700 ICP-MS, DF 1000. Concentrations of studied elements in soil samples were compared with the Bulgarian hygiene norms (Regulation Norm 3, 2008). Coefficient of soil technogenic pollution (C_t) and the coefficient of bioaccumulation (C_b) of the studied trace elements in plants were calculated. Raw data were processed using statistical software package Statistica 7.0 (StatSoft Inc., 2004).

Results: We found that the concentrations of studied trace elements were higher in sampling sites situated in South direction from the smelter (Site 2, 3 and 4) in comparison with those in North direction (Site 1). Distance from the emitter also had a considerable effect to the contamination level which is in agreement with other authors [1, 2, 3, 4,

8]. Coefficient of soil technogenic pollution was found as follows: As – from 0.1 (Site 1) to 16 (Site 4); Cd – from 1.7 (Site 1) to 30 (Site 2); Cu – from 0.35 (Site 1) to 1.4 (Site 2); Hg – from 0.06 (Site 1) to 0.94 (Site 2); Ni – from 1.3 (Site 1) to 2.3 (Site 3); Pb – from 2.9 (Site 1) to 63.8 (Site 3). Values of the coefficient of bioaccumulation of the studied trace elements confirmed the poor ability of Cu, Ni and Pb to move from soil to plants. *Plantago lanceolata* and *Carex divisa* showed similar bioaccumulation levels for Cu (C_b = 0.1-0.14), Ni (C_b = 0.1-0.12) and Pb (C_b = 0.04-0.08). *Plantago lanceolata* was found as better accumulator of Cd (C_b = 1.3), followed by *Papaver rhoeas* (C_b = 1.1). From the studied plant species, only *Papaver rhoeas* showed good bioaccumulation of As (C_b = 2.6) and Hg (C_b = 2.0).

Conclusion: Soils in the studied area are contaminated with Pb, Cd, Ni and As. Of the studied plant species, *Plantago lanceolata* and *Carex divisa* were found as bio accumulators of Pb, Cd and Cu, and *Papaver rhoeas* – of Cd, Hg and As.

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HYPOGLYCAEMIC EFFECTS OF GLYPHOSATE BASED HERBICIDE ON COMMON CARP (*CYPRINUS CARPIO* L.) AND BIGHEAD CARP (*ARISTICHTHYS NOBILIS* RICH.) LIVER

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Abstract: In the present work the main objective was to study the impact of a glyphosate based herbicide on glycogen storage in common carp (*C. carpio*) and bighead carp (*A. nobilis*) liver using PAS-reaction on cryosections. We used different concentrations of the test herbicide in laboratory conditions for 96 hours. Results showed glycogen storage depletion in the liver of both fish species with increasing the herbicide concentration. We observed a clearer tendency towards glycogen depletion in the bighead carp liver which indicated a higher sensitivity to glyphosate.

Introduction: Agricultural chemicals may enter the aquatic environment through atmospheric deposition, surface run-off and they frequently affect non-target aquatic organisms. Glyphosate is a non-selective, systemic herbicide that can control most annual and perennial plants. Fish are an important aquatic indicators and are frequently exposed to pesticides. In the liver, which is the main detoxification and metabolic organ, toxicants may alter carbohydrate metabolism and indirectly affect the function of other organs. Data from studies carried out on the effects of glyphosate based herbicide on fish liver glycogen storage are relatively scarce and on bighead carp are absent in available literature.

The aim of the present study was to investigate the effects of glyphosate based herbicide and the degree of expression on liver glycogen in two freshwater fish species.

Material and Methods: Histochemical analysis was performed according standard histochemical procedure described by Pearse [2] at the Department of Anatomy, Histology and Embryology at Medical University of Plovdiv, Bulgaria. Positive PAS-reaction was presented in purple-magenta staining and the degree of intensity of histochemical staining of all specimens, including control fish livers were appraised semi-quantitatively by using the grading system of Peebua et al. [3], which we slightly modified: (-) – negative reaction of histochemical staining; (+/-) – weak positive reaction of histochemical staining (weak purple staining); (+) – moderate positive reaction of histochemical staining (purple-magenta staining); (++) – strong positive reaction of histochemical staining (intense purple-magenta); (+++) – strong positive reaction of histochemical staining in the hepatocytes (intense magenta).

Results: Liver is the main store of carbohydrates. We found positive PAS-reaction in all test groups, including control. In the fish group exposed to 20 mg/l and the control we observed a similar degree of expression of PAS-reaction in both fish species. Degree of expression of histochemical staining in the common carp liver was moderate and in

the bighead carp liver was strong positive. At the higher herbicide concentrations of 40 mg/l and 72 mg/l we found a weak positive PAS-reaction which indicates depletion of glycogen storage in the liver of both fish species.

Conclusions Changes in liver glycogen indicate that carbohydrate metabolism in fish might be impaired under the effect of this herbicide [1]. Positive PAS-reaction which we observed in common and bighead carp showed a similar degree of expression of histochemical staining. Tendency was towards glycogen depletion with increasing the herbicide concentrations. We consider that the reduced intensity of PAS-reaction is probably a result of metabolic changes due to glycogen depletion. This trend was more pronounced in the liver of bighead carp which indicates that this fish species is more sensitive to the herbicide effects. We also think that this chemical may impact the whole organism, not just the liver. Therefore, we suggest that further investigations need to be carried out.

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ADAPTATION OF CFGE FOR ASSESSMENT OF DNA SUSCEPTIBILITY OF HUMAN LYMPHOCYTES TO ZEOCIN

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Introduction: A lot of environmental genotoxins could seriously damage DNA inducing DSBs which if not repaired can cause serious diseases in organisms including cancer. Development of sensitive biomarkers for detection of such injuries is of main interest in many studies. A variety of methods for detection of DSBs induction in numerous test-

systems are used in recent years. Constant field gel electrophoresis (CFGE) is one of them [1, 2]. The method is very sensitive, rapid and economical than other methods and allows the use of whole cells for measurement of DNA damage. The cell insertion in agarose plugs prevented their further damage during laboratory processing.

Human peripheral blood lymphocytes are used as a sensitive biological indicator of exposure to various genotoxins including mutagens with DNA-damaging potential. This test-system has a number of advantages such as circulation through all the tissues, easy obtaining of a large number of cells with a possibility of multiple repetitions, easy for cultivation, stable karyotype.

Aim: The aim of this research is to adapt constant field gel electrophoresis (CFGE) in our lab on human lymphocyte test-system and to use this method to study and compare the susceptibility of human lymphocytes from smokers and nonsmokers to the radiomimetic Zeocin.

Material and Methods:

- Human lymphocytes *in vitro*

Lymphocyte cultures from peripheral venous blood of clinically healthy donors (smokers and nonsmokers) age between 27 and 45 years were prepared. To separate lymphocytes from erythrocytes and granulocytes Ficoll-Paque gradient centrifugation was used [3]. Each lymphocyte culture containing RPMI 1640 medium (Sigma), 12% calf serum (Sigma), 40 μg/ml gentamycin (Pharmacia) and 0.1% phytohemagglutinin PHA (Sigma) was incubated at 37°C and then was treated with Zeocin (InvivoGen) in an appropriate manner indicated below.

- Compound

Zeocin (Cas No.: 181494-14-4) - standard radiomimetic, belonging to bleomycin's antibiotic group was described in our former studies [1].

- Experimental design

Constant field gel electrophoresis is applied to obtain DSBs induced by radiomimetic Zeocin. The first step was to adapt this method in our lab to human lymphocytes. In our study we followed the same steps as for *Chlamydomonas reinhardtii* with minor modifications [1].

Lymphocyte cultures ($5x10^5$ cell/ml) from smokers and nonsmokers are treated on ice with 100, 150, 200 μg/ml Zeocin for 15 min in a dark at the 18^{th} hour (G1) after PHA stimulation. Untreated cultures are used as a control. After the treatment cells are washed in serum-free medium and 80 μl of each cell suspension is pippeted with equal quantity of low melting agarose (LMA) solution (0.8%) into plug molds and left to solidify on ice. After then the agarose cell plugs are transferred in lysis buffer containing proteinase - K (1 mg/ml). The lysis procedure started on ice for 30 minutes and continued for 18 hours at 37^oC. The volume of lysis solution was increased from 0.75 to 1.25 ml compared to that described for *Chlamydomonas reinhardtii* [1]. The plugs are washed three times in Tris-EDTA buffer and left at room temperature for 30 minutes. After the washing plugs are inserted into the wells on 0.8% agarose gel (prepared in 0.5 TBE buffer, pH 7.9–8.1) containing ethidium bromide (1.1μg/ml) for electrophoresis run. The wells are covered with LMA. The running conditions were - 20 V, 10 mA for 40 hours in an electrophoresis chamber (HE 99X, Amersham Biosciences).

The movement of DNA out of the starting wells into the electrophoresis gel has been measured using UV-gel scan and computer analysis of DNA - ethidium bromide fluorescence (Syngene software; GeneTools). This gives information about the quantity of DSBs induced in cells by radiomimetic.

Fraction of DNA released into the gel (FDR) was calculated according to the following formula:

$$\mathbf{FDR} = \frac{\mathbf{DR}}{\mathbf{DR+DW}}$$

Where DR is the quantity of DNA released outside the starting well, DW - DNA in the well. This enables us to compare the susceptibility of human lymphocytes from smokers and nonsmokers to DSBs inducer Zeocin, which was the second step of our study.

- **Statistics**

Student's t-test and chi-square method were used for statistical analysis.

Results

1) Our CFGE data show an enhancement of DSBs after Zeocin treatment in smokers' and nonsmokers' lymphocytes compared to the negative control.

2) The effectiveness of action of the radiomimetic depends on the concentration applied in our study. The higher concentrations of Zeocin - 150 and 200 μg/ml increase two-fold the FDR compared to the negative control ($P<0.001$). No statistically significant difference is calculated between the negative control and the lowest concentration Zeocin (100 μg/ml).

3) The more pronounced susceptibility to Zeocin is obtained for smokers' DNA than that of nonsmokers'. Statistically significant difference is found for the higher concentration - 150 μg/ml ($P<0.05$).

Conclusions For the first time we adapt this method for human lymphocytes much better than Dahm-Daphi et al., [2]. The optimized by us running conditions appear to be essential for the migration of the largest heavy fraction of DNA from the agarose plugs. This allows measuring the extent of DNA damage. Include the ethidium bromide in the agarose gel makes the method rapid and safety.

This study indicates that CFGE is successfully adapted and used in our lab on human lymphocyte test-system. CFGE is effective, sensitive and appropriate for DSBs detection induced by radiomimetic Zeocin in human lymphocytes from various donors.

Our study shows that lymphocytes of smokers are more susceptible to DSBs inducers than the lymphocytes of nonsmokers.

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КРАТКИ СЪОБЩЕНИЯ/SHORT COMMUNICATIONS

Тематично направление:
МЕХАНИЗМИ НА АДАПТАЦИЯ НА ЖИВИТЕ СИСТЕМИ

Topic:
MECHANISMS OF ADAPTATION OF THE LIVING SYSTEMS

BIOCHEMICAL AND MOLECULAR RESPONSES OF *PHASEOLUS VULGARIS* L. CULTIVAR AND THREE MUTANT LINES TO DROUGHT STRESS

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Introduction: Drought is one of the main environmental stress that could affect agricultural systems, leading to the decrease of crops production. Drought also leads to overproduction of reactive oxygen species (ROS) resulting in oxidative damage at

cellular level. This overproduction of ROS could cause serious damages in proteins, lipids, carbohydrates and DNA and etc. *Phaseolus vulgaris* L. (Common bean) is an important food source, economically important product, and model organism for legumes. Common bean is sensitive to water deficit comparing with other crops.

Aim: The aim of this work is to compare stress response of *Phaseolus vulgaris* L. cultivar and three mutant lines to drought stress, induced by PEG treatment.

Material and Methods: Cultivar Dobrudjanski 2 (D2) and three mutant lines (D_2-0,0031 M NEU – Line №2; D_2-0,0062 M EMS – Line №3 and D_2-0,0125 M EMS – Line №6) derived by the methods of chemical mutagenesis were studied. Plants at the third leaf phase were split into three groups: untreated control group and two others treated with different concentrations of polyethylene glycol (PEG - MW 10 000) - 8% and 16% for 24h. Four biochemical and molecular markers for oxidative stress were analyzed - malondialdehyde (MDA), [3]; total hydrogen peroxides (H_2O_2), [4]; proline (Pro), [1]; and heat shock protein content (HSP70B), [2]. The significance of differences in the test responses has been assessed using Two-way ANOVA (GraphPad Prism, version 5.00).

Results and Discussion:

1. *Total hydrogen peroxides (H_2O_2) content.* Total hydrogen peroxides was used as a biochemical marker for oxidative stress. Constitutive levels of H_2O_2 of mutant lines were compared with those of cultivar D2. Our results demonstrate that variation depending on the genotype existed. All mutant lines were with higher constitutive contents of H_2O_2 - about 80% for mutant lines 2 and 6, and about 40% for mutant line 3. PEG treatment reflected to an increased content of total hydrogen peroxides in a dose –dependent manner for D2 and mutant lines 3 and 6. No effect of concentrations was registered for mutant line 2, where the higher accumulation of H_2O_2 was observed. Based on the induced H_2O_2 levels genotypes could be ranked as follows: L2 > L6 > D2 ≥ L3.

2. *Malondialdehyde (MDA) content.* Malondialdehyde content is commonly used as an indicator for lipid peroxidation. Similar constitutive levels of MDA were measured for all investigated genotypes. After the treatment with both concentrations of PEG an increasing of the MDA with 15 - 50% was established. Nevertheless the fact that constitutive levels of MDA were similar for all genotypes differences among induced MDA levels were found. The best pronounced effect of PEG was observed in mutant line 6 (about 50% higher content of MDA comparing with the control). In terms of the measured MDA values after the treatment with 8% and 16% PEG genotypes could be arranged as follows: L6 > L2 ~ L3 > D2.

Slight increasing of MDA and H_2O_2 found after the treatment with PEG could be considered as an indication that experimental conditions applied by us were not very stressful for cultivar D2 and mutant lines L2, L6 and L3.

3. Proline (Pro) content. Pro is commonly used as a biochemical marker for stress. At the same time Pro is well known an osmoprotectant, involved in defense mechanisms, protecting cellular membranes, stabilizing proteins/protein complex and etc. Based on the variation of constitutive levels of Pro, genotypes could be split into two groups. The first one includes cultivar D2 and mutant line 3 with approximately simi-

lar Pro content (100 and 100.126% respectively). The second group includes mutant lines 2 and 6 with 3-4 fold higher Pro content comparing with genotypes from the first group. After the treatment with PEG, the content of Pro was significantly increased – 2-5 fold for all genotypes. The effect of both concentrations and the genotype was established. The most well expressed effect of the concentration was manifested for cultivar D2 (118% difference between 8% and 16% PEG) followed by mutant line 2. No correlation between the level of constitutive Pro contents and induced after PEG treatment was found.

4. *Molecular marker-heat shock protein content (HSP70B).* Heat shock proteins (HSP70B) could be used as an early warning marker of stress and their induction could be related to the non-specific cell defense mechanisms [2]. Based on their constitutive contents of HSP70B genotypes could be split into 2 groups. The first one includes genotypes with higher levels of HSP70B in comparison with the parental cultivar D2- mutant lines 6 and 2 (86% and 56%). Mutant line 3 belongs to the second one - constitutive level of HSP70B is about 60% lower than that in D2. Increasing of PEG concentrations from 8% to 16% leads to an enhancement of HSP70B accumulation in D2 with 84% and 124% respectively. The lowest constitutive level and the most significant HSP70B induction after PEG treatment is established for mutant line 3. The HSP70B content measured in this mutant line after the treatment with 8% PEG is 477%, and after 16% PEG – 293% in comparison with the control. Our results demonstrate the abcense of some correlation between constitutive and induced levels of HSP70B. Typical for the genotypes with high constitutive levels of HSP70B (mutant lines 6 and 2) is slight overproduction of HSP70B and contrary – typical for genotypes with low constitutive levels of these proteins (like mutant line 3) is well expressed overproduction.

Based on our results here we confirm our previous finding in *Chlorella* species, isolated from contrasting habitats that HSP70B could be used as an reliable early warning stress marker. Our results could be interpreted from another point of view – overexpression of HSP70B could be considered as cells compensatory mechanism.

Conclusions

1. Slight increasing of MDA and H_2O_2 found after the treatment with PEG could be considered as an indication that experimental conditions applied by us are not very stressful for parental cultivar and mutant lines. Even that genotypes differ in their response in some extend.

2. HSP70B could be specified as a very reliable marker for environmentally induced stress. The abcense of correlation between constitutive and induced levels of HSP70B outline an interesting dependence - high constitutive levels of HSP70B - slight overproduction HSP70B and contrary – low constitutive levels of these proteins - well expressed overproduction.

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INTERDISCIPLINARY INTERACTION FOR THE BIOTECHNOLOGICAL DEVELOPMENT OF BALKAN MEDICINAL PLANT SPECIES

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Aims: Within the scientific PhytoBalk project in the framework of the *Bulgarian-Swiss Research Programme* an *in vitro* collection of medicinal and aromatic plants characteristic for the Balkan region is being set up at the facilities of the Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences. The aim of the work is to develop biotechnological approaches for the controlled delivery of biologically active compounds of medicinal and aromatic plants characteristic for the Balkan region.

Material and Methods: Investigated plant species were chosen on an ethnobotanical principle. Plant material of species of the *Hypericum* and *Pulsatilla genera*, *Sideritis scardica* Sofia 2 cultivar, *Inula britannica*, *Artemisia alba* were collected from Bulgaria.

Polyphenolic contents, enzymes, molecular markers of oxidative stress in the *in vitro* culture plant material were measured spectrophotometrically. Structural and functional alterations of photosynthetic membranes were characterized by 77 K fluorescent spectroscopy, electrophoretic profile by 10% SDS-PAGE. Essential oils were prepared by micro-steam distillation and extracts for isolation of non-volatile chemical constituents by exhaustive ultrasonic extraction with solvents with increasing polarity – hexane, chloroform and methanol. Extracts were further subjected to successive Sephadex LH 20 column chromatography as well as preparative thin layer chromatography leading to purification and isolation of individual compounds.

Results and Discussion

Table 1 presents a summary of the conditions for long-term maintenance of the species under investigation in the PhytoBalk project.

Table 1. Obtained conditions for long-term maintenance of stock shoots of the investigated species

Plant species	Medium formulation	Sub-culture period
Sideritis scardica Sofia 2 cultivar	Macro- and microelements after Murashige and Skoog (MS), Gamborg vitamins, supplementation of 0.2 mg/l benzyladenine (BA) + 0.02 mg/l naphthyl acetic acid (NAA), 30 g/l sucrose, 6.5 g/l agar	1.5 months
Hypericum richeri	The basic macro- microsalts and vitamins of MS medium + 0.2 mg/l BA + 0.1 mg/l indole-3-butyric acid (IBA), 6.5 g/l agar.	3 months
Hypericum rumeliacum	The basic MS formula, 30 g/l sucrose, 6.5 g/l agar	3 months
Hypericum calycinum	The basic MS formula, 30 g/l sucrose, 6.5 g/l agar	7 months
Hypericum tetrapterum	The basic MS formula, 30 g/l sucrose, 6.5 g/l agar	7 months
Hypericum perforatum	The basic MS formula, 30 g/l sucrose, 6.5 g/l agar	7 months
Pulsatilla montana ssp. *balcana*	The basic MS formula, 30 g/l sucrose, 6.5 g/l agar	1.5 months
Pulsatilla halleri ssp. *rhodopaea*	The basic MS formula, 30 g/l sucrose, 6.5 g/l agar	1.5 months
Pulsatilla slaviankae	The basic MS formula, 30 g/l sucrose, 6.5 g/l agar	1.5 months
Clinopodium vulgare	The basic MS formula, 30 g/l sucrose, 6.5 g/l agar	7 months
Inula britannica	The basic MS formula, 30 g/l sucrose, 6.5 g/l agar	4 months
Artemisia alba Turra	The basic MS macro- microsalts and vitamins, Gamborg vitamins, glycine after MS formula,	4 months

Modification of plant growth regulators (PGR) supplementation in *A. alba* led to obtaining of two distinctive essential oil profiles in the aerial parts of the plant: one with monoterpenoid and another with sesquiterpenoid domination. Electrophoretic profile of the samples indicated differences in the protein bands in relation to the two terpenoid profiles of the oils. Studies on thylakoid membranes revealed that domination of the monoterpenoids was associated with a higher degree of aggregation of LHCII. On the contrary, sesquiterpenoid domination was associated with a decrease in LHCII aggregation. Thus, a relation between the macromolecular organization of photosystem

II and the factors affecting the terpenoid biosynthesis in *A. alba* were demonstrated. Phytochemical investigation on the non-volatile components of the extracts of field collected material of the plant led to the isolation of 7 flavonoids (quercetin-3,4'-dimethylether, centaureidin, axillarin, quercertin 3-methyl ether, kaempferol, quercetin and luteolin) and 3 coumarins (scopoletin, umbeliferone and fraxidin-8-O-glucoside). Their structures were elucidated by NMR, MS and UV [1]. Qualitative similarity by thin layer chromatography was established with *in vitro* derived plant material of the plant. Through modification of plant growth regulators supplementations and vitamin content, optimizations were achieved affording stimulation of polyphenolic content in the *in vitro* cultured plants. PGR supplementation also suppressed antioxidant enzymatic activity, but in the same time stimulation of polyphenolic production was observed, which contributed to the preservation of comparable levels of the markers of oxidative stress (levels of endogenous hydrogen peroxide) and lipid peroxidation (malondialdehyde) indicating of interrelations between the enzymatic and non-enzymatic defense of the plant *in vitro*.

Phytochemical investigation of the extracts of the aerial parts of wild collected *Inula britannica* led to the isolation and identification of sesquiterpene lactones pulchellin C, gailiardin, britannin, ivalin and 11,13-dihydro-inuchinanolide. Their structures were determined by spectral methods [2]. Qualitative similarity of the sesquiterpene lactone profiles of the intact plant and *in vitro* derived material was shown by TLC using sulfuric acid spray reagent for visualization. High performance thin layer chromatographic analytic technique was developed for the rapid qualitative characterization of samples of the different *Hypericum* species showing similar concentration levels of hypericins in *in vitro* and *ex situ* biomass, indicating the potential of Balkan *Hypericum* species as biotechnological producers of hypericins. Optimization of PGR and vitamin supplementation as well as initiation of callus, suspension and conventional genetically non-transformed are in process in order to develop approaches for the controlled delivery of plant biomass with desired properties for the further scale-up of obtained processes in bioreactor system.

Conclusion: Obtained results will be used for scientifically based targeted delivery of plant material with defined secondary metabolite profile. Further research is in progress to evaluate the potential biological activity of extracts, fractions and individual compounds of the studied *in vitro* culture systems.

Keywords: *in vitro* culture optimization, secondary metabolite production, Balkan medicinal and aromatic plants

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PROTECTIVE ACTIVITY OF DIFFERENT EXTRACTS OF *CLINOPODIUM VULGARE* L.

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Plants of Lamiaceae are highly regarded by some ethnicities for their medicinal properties, including anti-inflammatory and antioxidant activities. Their beneficial properties are attributed mainly to compounds belonging to the classes of terpenoids and phenolics [1]. A member of the family, the wild basil *Clinopodium vulgare* L., is a common perennial herbaceous plant that grows throughout Europe, Western Asia, North Africa and North America [1]. Its leaves are traditionally used as a spice. In traditional medicine, particularly in Bulgaria, this herb is used as a medicine for skin irritation and swelling, and relieving the symptoms associated with mastitis and prostatitis [1].

Strong *in vitro* antitumour activity on A2058 (human metastatic melanoma), HEp-2 (epidermoid carcinoma larynx, human) and L5178Y (mouse lymphoma) cell lines as well as good antioxidant activity were shown in previous studies on the aqueous extract

of *C. vulgare* L. Antibacterial activity [1] was obtained for ethanol, ethyl acetate and acetone extract.

The aim of this study was to test the potential of *Clinopodium vulgare* L. (Wild basil) to scavenge free radicals and to protect plasmid DNA.

Material and Methods: Aerial parts of *Clinopodium vulgare* L. were collected at the beginning of July 2013 (period of flowering) from approximately 18 km southeast from Sofia, Bulgaria in Lozenska mountain (an environmentally clean region) - altitude of 600-700 m over the sea. The plants were air-dried in clean, dark and airy room and stored in ventilated paper boxes before preparation of materials for further investigation. Plant organs (leaves, flowers and stems) were gently separated and treated individually. Randomly collected parts from these plant organs and from the whole plants were milled/homogenized in a grinder-mill before water extraction, lyophilization and preparation of butanol extracts.

Phytochemical analysis of *Clinopodium vulgare* L. was done with GC/MS of ethyl acetate extracts from a water infusion of the aerial parts of the herb. An aliquot of the lyophilizate was dissolved in 0.1N HCl and the solution extracted with ethylacetate. The extract was dried with a portion of anhydrous Magnesium sulphate and then introduced for GC/MS analysis.

GC/MS experiments were performed using a *Thermo Scientific Trace GC* equipped with a HP-35 30 m x 0.25 mm capillary column and quadrupole mass spectrometric detector.

Five *Clinopodium vulgare* L. extracts (4 aqueous - total, leaves, flowers, stems and 1 buthanolic total extract) were studied using DPPH assay and DNA topology assay.

The DPPH assay, based on a color reduction of 1,1 - diphenyl-2-picrylhydrazyl hydrate from purple to yellow, was applied according to Sharma and Bhat, [2] with slight modifications. The color change was monitored at 517 nm wavelength after 20 minutes of incubation in the dark at 4^0C. Scavenging activity was calculated by the following equation:

Percentage inhibition = $[(A_A-A_B) /A_A] \times 100$, where A_A is absorbance of DPPH solution and A_B is absorbance of tested extract solution. Ascorbic acid was used as a standard.

DNA topology assay was applied according to Čipák et al. [3]. As a positive control 0.08 mM Fe^{2+} was used. The transformation of supercoiled pBR322 DNA to relaxed circular form was visually detected using agarose gel electrophoresis. Briefly, the reaction mixture with total volume 10 μL containing 300 ng supercoiled pBR322 DNA, extracts in a concentration range 10 – 1000 μg/ml, 0.08 mM Fe^{2+}, and phosphate buffer (10 mM, pH 7.0) was subjected to electrophoresis in 1% agarose gel. After 1 h the structural modifications were photographed with UV transillumination using G: BOX (Syngene).

The DNA was made visible by staining with ethidium bromide. Percentage of supercoiled and relaxed DNA forms was densitometrically determined by a computer program (ImageJ v1. 48v).

Results: Because the application forms of the herb are aqueous extracts, the organic extracts derived from a water infusion of the aerial plant parts– both in acidic and alkaline medium. The chromatograms obtained show an intensive peak of an acidic compound in the chromatogram of the acidic extract. This compound is mass spectrally recognized as decarboxilated form of Caffeic acid, which could originate from caffeic acid and/or its con-

jugated forms in acidic medium and high GC-inlet temperature; the confirmation of these hypotheses is a subject of our further investigations.

The antioxidant activity of different *Clinopodium vulgare* L. extracts measured by the DPPH assay was relatively strong in comparison with standard - ascorbic acid. The calculated inhibitory concentration 50 (IC50) for each extract showed that the leaf extract was the strongest radical scavenger (239.48 μg/ml ± 2.68). Based on the activities of the different extracts, they could be arranged in the following way: leaves, total (244.52 μg/ml ± 11.13), flowers (281.42 μg/ml ± 5.36), stem (429.90 ± 7.85), total buthanolic (459.00 μg/ml ± 11.32).

DNA topology assay results show no DNA damaging activity of the extracts in the tested concentrations. All of them are able to protect plasmid DNA against the damaging action of Fe^{3+}. The most pronounced protective effect is established for the aqueous total and leaf extracts where all the tested concentrations protect the plasmid DNA. On the other hand, the aqueous flowers and stems extracts, as well as the total buthanolic extract are able to protect pBR322 DNA only at concentrations 500 and 1000 μg/ml.

Discussion: Here it was shown that aqueous extracts of different *Clinopodium vulgare* L. parts possess strong scavenging and DNA protective activities. Even though, the most pronounced scavenging and DNA protective activities were observed for leaf aqueous extract. This could be related to the high quantity of water soluble antioxidant phytochemicals such as phenolics and glycosilated forms of flavonoids, polyphenols and terpenoids. The total aqueous extract also possesses high antioxidant and DNA protective activity. It could be speculated that these activities are mainly due to the presence of high quantity of water soluble antioxidant compounds as saponines, because it contains water extractable components of all the aerial parts of the plant including leaves and flowers.

Conclusion: Our preliminary results provide information for potential protective properties of different *Clinopodium vulgare* L. extracts. The further investigation of their antimutagenic and anticarcinogenic activities is in a progress.

Keywords: *Clinopodium vulgare* L., DPPH, DNA topology assay, gas chromatography–mass spectrometry

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IN VITRO DETERMINATION OF ANTIOXIDANT CAPACITY OF PEA (*PISUM SATIVUM* L. CV. RAN) PLANTS

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Introduction: Reactive oxygen species (ROS) are oxygen forms with higher reactivity than molecular oxygen that are normally present in living tissues at low steady-state levels. The unfavorable environment enhances the ROS production that can lead to oxidative stress. To cope with ROS the plants possess enzymatic and non-enzymatic antioxidant mechanisms commonly termed antioxidant system which is underlying the antioxidant capacity (AOC). Methods characterizing the AOC of non-enzymatic antioxidants of foods or biological systems are extensively investigated [1-3]. The AOC was recommended as screening criterion for the plant tolerance to unfavorable environment [1]. The variation between plant species and genotypes to tolerate the environmental stresses was linked to leaf AOC and metabolism. Cultivar Ran 1 is Bulgarian early spring-grown pea possessing tolerant photosynthetic apparatus and male gametophyte viability that can be due to features in its antioxidant system.

The purpose of the study was to examine the AOC along the pea plant during its vegetative growth. We used three assays which reflect different features of the non-enzymatic antioxidant system in order to obtain a picture of the organ distribution of non-enzymatic antioxidants and their protective efficacy during pea vegetative growth.

Material and Methods: Pea plants were grown hydroponically in climatic room (20^0C; light intensity 100 $\mu mol.m^{-2}.s^{-1}$; day/night photoperiod: 12/12h). The fresh and dry weights of roots, stem, leaves, and shoot apex were measured twice: once, in three true leaf stages plants, and 10 days later, in five leaf stages plants. At the same time the same organs samples (in duplicate) for AOC assays were collected, fixed with liquid nitrogen and lyophilized. Each plant sample was extracted (acetone/water/acetic acid=70/29.5/0.5 v/v) at room temperature under stirring. The mixture was centrifuged at cooling and the clear supernatant was used for AOC assays.

The AOC was determined by HORAC (hydroxyl radical averting capacity), ORAC (oxygen radical absorbance capacity) and F-C (Folin-Ciocalteau) assays which assess

different functions of certain non-enzymatic antioxidants [1-3]. The **HORAC assay** [3] is based on the generation of free hydroxyl radicals due to catalytic decomposition of H_2O_2 in the presence of divalent cobalt (CoF_2) at 37^0C and pH 7.4. Fluorescein was used to monitor the generation of free hydroxyl radicals and their scavenging by pea antioxidants. The reaction was performed in 10 mm light path quartz fluorescence cells on Perkin Elmer LS-5 fluorimeter equipped with thermostated cell holder. Gallic acid (GA) was used as a standard. The **ORAC assay** [1, 3] was carried out on the same equipment and applied also fluorescein as a fluorescent probe. The peroxyl radical generation was achieved by decomposition of AAPH at 37^0C and pH 7.4. Trolox (a water soluble vitamin E analogue) was used as standard. For HORAC and ORAC assays sigmoidal fluorescence decay curves were obtained for blank (sodium phosphate buffer), standards and pea samples, and their net areas under curves (AUC) were calculated. The AOC of each pea sample was evaluated by formulae [1, 3] using blank, standard and sample AUC. The **F–C assay** is based on the reaction of phenolic compounds with the Folin-Ciocalteau reagent [1, 2]. Gallic acid was used for the standard curve construction. The assays results were expressed as μmole standard (GA or Trolox) equivalents per gram sample DW.

Results: During the studied growth period the pea plants developed all leaf stages before flower formation. Root and shoot biomasses enhanced and it was accompanied by AOC increase. Highest AOC was localized in the green organs such as leaves, especially those in the shoot apex; stem and root AOC were lower. The AOC increase during the organ growth was expressed in different magnitude by the three assays because the assays estimated the antioxidant function of different compounds. Their identification is needed.

The **HORAC** values reflect the prevention capacity of the pea organs against hydroxyl radical generation. Hydroxyl radicals can be generated by the catalytic decomposition of H_2O_2 in the presence of transition metal ions (Fe^{+2}, Cu^{+2}, Zn^{+2}, etc.) known as non-enzymatic reactions of Fenton and Haber-Weiss [3]. These radicals are short lived with a high rate constant and the biological antioxidants are not able to scavenge them at physiological conditions. However antioxidants, acting as metal chelators, prevent the hydroxyl radical formation, thus acting as preventive antioxidants. The HORAC assay measures the capacity of the antioxidants present in pea organ to chelate Co(II) prior to the Fenton reaction occurring. The assay was sensitive to the type of pea organ and its age as the HORAC values differed between roots and shoot ones, and changed with the organ growing. Among the natural antioxidants phenolic compounds chelating metals, showed HORAC values dependent on their chelating ability. Flavonoids with catechol, 4-oxo, and 50–OH arrangements strongly inhibited Fenton-induced oxidation; phenolic acids demonstrated lower HORAC; glucose and rutinose groups influenced positively HORAC as their hydroxyl groups increased the chelating ability [3]. Strong antioxidants such as Trolox, vitamin C, melatonin did not react significantly [3]. Flavonoids accumulate mostly in surface organs at sunlight. Pea leaf guard and epidermal cells are predominant sites for accumulation of flavonol glycosides and anthocyanins; mature pea leaf contains mainly quercetin glucoside. Similarly, catechins, proanthocyanidins, leukoanthocyanidins are found in pea roots.

The **ORAC** assay provides a direct measure of the hydrophilic chain-breaking AOC against the peroxyl radicals. There were obvious differences in ORAC of pea root, stem and leaf; as well ORAC changed with the progress of organ growth. This assay indicates the AOC against peroxyl radical of chain-breaking antioxidants such as flavonoids, vitamin E, ascorbate (vitamin C), beta-carotene, glutathione [1-3]. Flavonoids with catechol structure in the B-ring strongly inhibit the lipid peroxidation. Tocopherols and tocotrienols (vitamin E) donate phenolic hydrogen to lipid free radical that inhibits the chain propagation during the lipid oxidation. Cooperated with carotenoids the tocopherols react with other ROS such as singlet molecular oxygen that also prevents the lipid peroxidation. Tocopherol composition and content differed along the plant; the pea plant contained only alpha-tocopherol mainly in shoot apex. The tocopherol protection against lipid peroxidation is enhanced by ascorbate-glutathione cycle due to tocopherol recycling. The leaves maintain ascorbate and glutathione in high reduction state at optimal conditions; the pea leaves possessed large ascorbate pool, also pea embryonic axes synthesized and accumulated ascorbate rapidly; almost all root cells contained ascorbate as well. Glutathione function is to regenerate the ascorbate via the ascorbate-glutathione cycle. Besides, glutathione can directly scavenge the hydrogen peroxide (thus acting as preventive oxidant); as well it can react non-enzymatically with other ROS such as singlet oxygen, superoxide and hydroxyl radicals.

The **F–C assay** (known as **total phenolics assay**) characterized another part of pea antioxidant profile. It measures the reducing capacity of total phenols and other oxidation substrates [1, 2]. There were differences between the reducing capacities of pea root, stem and leaf. Generally, the green organs possessed higher level of reducing compounds compared to root. There was no large change in reducing capacities of pea organs during studied growth period besides an increase in shoot apex at the 10^{th} day. Presumably, the compounds with reducing ability did not alter fast. This slower change can be due to the complex phenolic metabolism [1]. Actually, the phenolic compounds contribute essentially to the antioxidant defense, and their efficiency is considered being higher than that of tocopherol or ascorbate [1-3].

Conclusions By means of three different assays we demonstrated that the green pea organs and mostly the youngest one (shoot apex), possessed high AOC, respectively these were antioxidant-rich source. The antioxidant properties of pea non-enzymatic antioxidants were dynamic, and had potential to change in response to growth conditions. The assays estimated the antioxidant functions of different compounds belonging to several molecular families. Therefore, the pea organs did not have the same HORAC, ORAC and F-C values. It is recommendable to use several methods to obtain a more detailed picture of the type and distribution of antioxidants and their protective efficacy in the plant. The quantification of AOC by plant organ and developmental stage is important for the food industry too, especially for the production and preservation of its nutritional value.

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ДОКЛАДИ/REPORTS

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PLANT EXTRACTS WITH POTENTIAL TO CONTROL *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* - CAUSAL AGENT OF BACTERIAL CANKER OF TOMATO

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Abstract

Aim: The aim of this study was to test the effect of different plant extracts against the causal agent of bacterial canker of tomato - *Clavibacter michiganensis* subsp. *michiganensis* as potential means for biocontrol.

Matherial and Methods: Plants materials from A*rtemisia absinthium, Ambrosia atemisifolia, Conium maculatum, Clematis vitalba, Datura stramonium, Hedera helix, Melilotus officinalis, Melilotus albus, Echium vulgare, Tanacetum vulgare* and *Hypericum perforatum* were used for methanol and *n*-hexane extractions. *In vitro* tests were conducted by the agar diffusion method. Pilot seed treatment test was conducted.

Results: Extracts from *Melilotus officinalis* and *Hypericum perforatum*, alone and in combinations, showed a promising potential to be used for control of *Clavibacter michiganensis* subsp. *michiganensis*. Extract from *H. perforatum* showed a very good effect for tomato seed disinfection from this pathogen.

Keywords: *Clavibacter michiganensis, Hypericum perforatum, Melilotus officinalis,* plant extracts, antibacterial effect

Introduction

Bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis (Cmm)* is a serious disease of tomato that can occur in greenhouses and in the field. Since its first report in 1910 in Michigan (USA), bacterial canker has spread worldwide and causes serious losses. The main host is tomato but natural infection has also been reported on pepper and several wild members of family *Solanaceae. Cmm* is one of the few plant pathogenic bacteria which induce systemic infection. The pathogen invades the plant through natural openings and wounds, and can survive with seeds, plant debris, on stakes and equipment [9].

Tomato plants of all ages are susceptible to bacterial canker. The symptoms are variable and depending on age, cultivar, way of introduction of the pathogen and environmental factors but in most cases generally leading to plant death. *Cmm* can be present in low levels on symptomless plants, multiplying rapidly in favorable weather conditions. The pathogen is spread by rains and by human activity and may survive in infected plant debris or equipment for long enough to establish a resident population on newly planted tomato seedlings in the following season [9].

Control of disease is extremely difficult once the pathogen has been introduced into a greenhouse or a field. Measures include use of pathogen-free planting material, cultural practices such as destruction of crop residues, deep ploughing, crop rotation, and general sanitation measures like disinfection of tools, structures and equipment [9]. There is no known effective treatment for bacterial canker. Copper-based chemicals are only capable of reducing epiphytic populations of *Cmm* and generally have negligible effect for disease control [9]. Treatments with the plant defense activator acibenzolar-S-methyl ester (ASM, Bion™) give partial effect against *Cmm* infection [13]. Sources of resistance are available [9] but have not yet been introduced into commercial cultivars.

Infected seeds are the most important source of inoculum - symptomless, with normal germination but carrying bacteria on the surface or within the inner tissues [8]. Infection levels in production fields grown with *Cmm* infected seeds varies greatly and is correlated to the environmental conditions [5, 8, 9], however, seed contamination rates as low as 0.01-0.05% could be enough to initiate an epidemic [5]. That's why disease management is mainly directed towards production of pathogen-free seeds and disinfection of seeds. Traditional methods include fermentation for extraction of seeds, hot-treatment and soaking of seeds in solutions of disinfectants (bleach, hydrochloric acid, hypochlorites, o-phenylphenol, etc.); however, none of them has proved to be completely safe for *Cmm* eradication. Other disadvantages include time requirements for fermentation and necessity of accurate temperatures for hot water treatment in order not to significantly influence germination [2, 3, 8, 9]. Limitations of chemical treatments are mainly connected with the levels of disinfection and germination [2]. On the other side, handling with synthetic chemicals has caused various problems such as toxicity to users [4] for which reason many of them has been forbidden for use in Europe. A comparative study of traditional treatment methods re-

vealed that disinfection of the seeds from *Cmm* can be achieved only by a thermo-chemical method involving the application of mercury-containing organic substance [2], which can no longer be used. Investigations on a substance synthesized by the Bulgarian actynomycete strain *Streptomyces hydroscopicus* 155 (bactericin) showed comparative results with the thermochemical method [3]. However, in field experiments conducted from treated seeds grown until fruit stage, the plants still gave 3.7-18.6% infected fruits [3]. The active substance of bactericin is an elaiophylin type macrolide antibiotic [3] which currently places its potential use in doubt.

Yearly crop losses and limitations of known measures for control require elaboration of alternative techniques especially for use in organic farming. An economical and efficient alternative for disease control is the use of natural products derived from plants, since they do not affect environment and their residues are easy to degrade.

Effects of plant extracts and essential oils against pathogens have been extensively studied recently. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, flavonoids and essential oils. In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores and some have been found to have antimicrobial properties [6]. Nevertheless, from all known plant species, estimated at 250'000- 500'000 [6], only a small percent have been investigated in these aspects.

Different plant products have shown to be effective against certain plant pathogens but only few have been tested against *Cmm*. A good effect was shown by cinnamon, basil, fenchel, thyme, oregano, dictamnus and marjoram essential oils [7, 14, 15] but as general rule oils greatly decrease germination ability [15]. In Mexico an extract from a specific plant species - pecan (*Carya illinoensis*) showed efficacy [4]. *Allium sativum* and *Ficus carica* extracts showed only partial effect [1]. Other data consider extracts from fungi [12], compost (based on soil antagonists) [10] or vinegars [15].

The aim of this study was to test the effect of different plant extracts against the causal agent of bacterial canker of tomato in aspect of possible disease control.

Material and Methods

Plant materials

Fresh plant parts (leaves and flowers) from different species (Table 1) were collected from the region of Sofia field, Bulgaria. Plant materials were oven-dried (22-60^{0}C according to the leaves' turgor and thickness) to absolute dry weight, ground and stored in air-tight brown bottles.

Extractions

Two solvents with different polarity – methanol and n-hexane were used. Extractions were prepared in Soxhlet extractor at water bath (80^{0}C) for 4-5 hours.

Methanol extracts were concentrated in vacuum evaporator at 55^{0}C, 300 mbar. After the evaporation of the solvent the concentrates were divided into liquid and soft fractions at 70^{0}C, 72 mbar. N-hexane extracts were concentrated in vacuum evaporator at 40^{0}C,

325 mbar until single solid fractions were obtained. The liquid, soft and solid fractions were stored at 16^0C in air tight brown bottles.

The liquid fractions were diluted in water (%, v/v) up to 24 h before the assay. The soft and the solid fractions were diluted in water (%, w/v) using dimethylsulfoxide (DMSO) up to 24 h before the assay.

Antibacterial assay

The *in vitro* test for antibacterial activity was completed by the agar diffusion method on Nutrient agar with 0.2% glucose. Bacterial suspension of *Cmm*, 100 µl, 1x108 cfu/ml was used for inoculum. The wells were filled with 2x35µl of each substance (extract or combination of extracts) and left for 2h prior to incubation. Incubation was held at 30ºC for 48h. The antibacterial activity was determined by measuring the inhibition zones in millimeter (diameter) on the 24^{th} and 48^{th} hour.

Seed treatment tests

Seed treatment tests were carried out with artificially infected seeds from tomato cv. Yana and methanol extract from *H. perforatum* in three repeats of 100 seeds each. Healthy seeds were infiltrated with bacterial suspension of a 48h culture $1x10^6$ cfu/ml under vacuum (1 atm.) for 60 min [2]. The seeds were left to dry for 2h and treated with the extract under vacuum (1 bar) for 60 min. Healthy seeds treated with sterile distilled water, with DMSO, with the extract and artificially inoculated but not treated seeds were used as controls. All the seeds were divided into two equal parts. The first part was tested for infection and the second part was tested for germination. TTC and Nutrient agar media were used for infection tests from the seed surface and inner tissues (endosperm and embryo). Major differentiation tests were conducted if needed [11]. Germination was observed in laboratory conditions in wet chambers, at 24^0C in dark.

Table 1. Plant species tested for antibacterial activity

Plant name	Family	Bayer Code	Common name	Solvents
Ambrosia artemisiifolia L.	Asteraceae	AMBEL	common ragweed, bitterweed, carrotweed, hogweed, Roman wormwood	methanol (m) *n*-hexane (h)
Artemisia absinthium L.	Asteraceae	ARTAB	absinthium, absinthe wormwood, wormwood, green ginger	methanol (m) *n*-hexane (h)
Conium maculatum L.	Apiaceae	COIMA	hemlock	methanol (m) *n*-hexane (h)
Clematis vitalba L.	Ranunculaceae	CLVVT	old man's beard, traveller's joy, graybeard	methanol (m) *n*-hexane (h)
Datura stramonium L.	Solanaceae	DATSL	Jimson weed, purple thorn apple, datura	methanol (m) *n*-hexane (h)
Hedera helix L.	Araliaceae	HEEHE	common ivy, ivy	methanol (m) *n*-hexane (h)
Melilotus officinalis (L.) Pall.	Fabaceae	MEUOF	yellow sweetclover, yellow melilot, ribbed melilot, common melilot	methanol (m) *n*-hexane (h)
Melilotus albus Medik.	Fabaceae	MEUAL	bokhara clover, honey clover, tree clover, white sweetclover, white melilot	*n*-hexane (h)
Echium vulgare L.	Boraginaceae	EHIVU	viper's bugloss, blueweed	*n*-hexane (h)
Tanacetum vulgare L.	Asteraceae	CHYVU	tansy	*n*-hexane (h)
Hypericum perforatum L.	Hypericaceae	HYPPE	St John's wort, goatweed	methanol (m)

Results and Discussion

Water solutions of soft and liquid fractions of methanol extracts of four of the tested plants (*A. absinthium, A. atemisifolia, C. maculatum* and *C. vitalba*) showed no effect on the growth of *Cmm* (Table 2).

Satisfactory results (≥10 mm inhibition zones) were observed by using water solutions of *n*-hexane extracts from *A. absinthium* (5%), *H. helix* (5%) and *E. vulgare* (0.8 – 2%), as well as of soft fractions of methanol extract of *D. stramonium* (5%) which suppressed bacterial growth. Both methanol and *n*-hexane extracts of *M. officinalis* (2-5%) were effective against the pathogen. Best results were obtained from applications of water solutions of soft fractions of methanol extracts of *H. perforatum* (Table 2).

Table 2. Antibacterial activity of leaf and flower extracts of the tested plants against *Clavibacter michiganensis* subsp. *michiganensis* (inhibition zone, mm)

Plant species	methanol extracts		*n*-hexane extracts	
	5% (w/v)	2% (w/v)	5% (w/v)	2% (w/v)
AMBEL	0	0	0	0
ARTAB	0	0	8	11
COIMA	0	0	7	7
CLVVT	0	0	7	7
DATSL	11 + 4pg*	8	25pg	9pg
HEEHE	7	7	10	7
MEUOF	9	11 + 8pg	12	13
MEUAL	nd**	nd	15pg	7pg
EHIVU	nd	nd	nd	10
CHYVU	nd	nd	7pg	7pg
HYPPE	15 + 12pg	13 + 4pg	nd	nd

*pg – poor growth of *Cmm* (normal after 48h)

**nd – not determined

On the basis of the results obtained from the single application of the extracts, an assay testing different combinations was conducted. The results varied greatly from completely no effect to a good inhibition zone of 14 mm (Table 3). Reduction of the inhibition effect compared to the individual extract applications was observed for combinations numbered 11, 14-17 (Table 3). Slightly greater effect was achieved by combinations numbered 2 and 13. A clearly synergic effect was observed for combinations numbered 7, 10 and 20 (Table 3). Best antibacterial activity against *Cmm* was achieved for combinations numbered 2, 13

and 20 made from methanol and *n*-hexane extracts of *D. stramonium, M. officinalis, M. alba* and *H. perforatum*.

Table 3. Antibacterial activity of plant extracts combinations against *Clavibacter michiganensis* subsp. *michiganensis* (inhibition zone, mm)

No.	Plant extract in combination*	Proportion	Inhibition zone, mm
1	MEUOF(m) 5%/DATSL(m)5%	1:1	11
2	MEUOF(m) 5%/DATSL(m)5%	2:1	12
3	MEUOF(m) 5%/DATSL(m)5%	1:2	11
4	MEUOF(m) 2%/DATSL(m)2%	1:1	8
5	MEUOF(h) 5%/DATSL(h)5%	1:1	9
6	MEUOF(h) 2%/DATSL(h)2%	1:1	7 + 2pg**
7	MEUAL(h) 2%/DATSL(h)2%	1:1	10
8	MEUOF(h) 2%/MEUAL(h) 2%	1:1	11
9	MEUAL(h) 5%/DATSL(m) 5%	1:1	9 + 6pg
10	MEUAL(h) 5%/DATSL(h) 5%	1:1	9 + 8pg
11	MEUOF(m) 5%/DATSL(h) 5%	1:1	8
12	MEUOF(m) 2%/DATSL(h) 2%	1:1	8
13	MEUOF(h) 5%/DATSL(m) 5%	1:1	13
14	MEUOF(h) 2%/DATSL(m) 2%	1:1	7
15	MEUAL(h) 2%/DATSL(m) 2%	1:1	0
16	MEUOF(m) 2%/DATSL(m) 2%/ARTAB(h) 2%	1:1:1	7
17	MEUOF(m) 2%/DATSL(m) 2%/ARTAB(h) 2%/EHIVU(h)0.8%	1:1:1:1	7
18	MEUOF(h) 2%/DATSL(m) 2%/EHIVU(h) 0.8%	1:1:1	11
19	MEUOF(h) 2%/DATSL(m) 2%/MEUAL(h) 2%	1:1:1	9
20	MEUOF(h) 2%/DATSL(h) 2%/MEUAL(h) 2%/HYPPE(m) 5%	1:1:1:1	14

*Bayer codes of plant species (first letter of solvent) % of extract, w/v

**pg – poor inhibition effect of the growth (negative after 48h)

A pilot seed treatment test for the effect of *H. perforatum* extract for decontamination of tomato seeds was conducted. Periodical observations for germination were carried out. The laboratory tests proved that complete disinfection of the tomato seeds from the pathogen *Cmm* was obtained. The germination of the treated seeds was delayed for 24-48h compared to the water infiltrated control seeds. However, on the 7th day after placing in wet chambers the germs of treated seeds are equal in size than these of the same control. According to the resulted percents, the treatment with *H. perforatum* methanol extract was favorable for the germination of the tomato seeds (Table 4).

Table 4. The effect of *H. perforatum* methanol extract for decontamination of tomato seeds cv. Yana

Seeds	Germination (%, average of three determinations)
Water infiltrated control	81.4
DMSO infiltrated control	80.5
Healthy, extract treated control	83.8
Infected, non-treated control	83.3
Infected, treated	91.3

Conclusion

Methanol and *n*-hexane extracts from *M. officinalis* and methanol extract from *H. perforatum*, as well as a combination of extracts of *M. officinalis* and *D. stramonium* and a combination of extracts of *M. officinalis, M. alba, D. stramonium* and *H. perforatum*, showed a promising potential to be used for control of *Clavibacter michiganensis* subsp. *michiganensis* or as templates for new, more effective control bioproducts.

Extract from *H. perforatum* showed a very good effect for tomato seed disinfection from this pathogen.

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VIRUS DISEASES ON MEDICINAL PLANT *SILYBUM MARIANUM* L. GAERTH

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Abstract

The objective of the research was the establishment and identification of widespread plant viruses on *Silybum marianum* - milk thistle, caused diseases on this medicinal plant species.

Material and Methods: Leaf samples with virus disease-like symptoms were collected from the plantations of the Institute of Roses, Essential and Medicinal Cultures near Kazanlak, Bulgaria in the period 2009 - 2011. The samples from individual milk thistle plants were tested by the serological ELISA method with kits for the following viruses *Cucumber mosaic virus* (CMV), *Alfalfa mosaic virus* (AMV) and *Tomato spotted wilt virus* (TSWV) and by the indicator method, using test plant for one of the viruses - AMV.

Results: Three important plant viruses on milk thistle were established: CMV in 37% of the analyzed plants; AMV in 20% and TSWV in 19%. The viruses caused chlorotic spots, turning to necrotic spotting, wilting and perishing of leaves and entire plants had poor seed yield for the pharmaceutical industry.

Conclusions The results of the ELISA and indicator methods testify that the yellowing of some milk thistle plants in the field could be caused by virus diseases.

Keywords: *Silybum marianum*, virus pathogens - CMV, AMV, TSWV.

Introduction

Silybum marianum (L.) Gaerth.- white thorn or thistle - milk thistle, Marian thistle, Mediterranean milk thistle and Scottish thistle, synonym *Carduus marianus* L. is cultivated for seed production as a raw material for the pharmaceutical industry of large areas in some European countries (Austria, Bulgaria, Germany, Hungary, Poland);

American (Argentina) and Asian (China) countries. The medicine, prepared from thistle seed extracts, is used against the diseases of liver, gallbladder, different kinds of cancer and Alzheimer's disease. The seed extract contains 65-80% silymarin (a complex mixture of polyphenolic molecules) and 20-35% fatty acids. Except for the treatment of liver diseases (cirrhosis, jaundice and hepatitis), gallbladder diseases and diabetes type II, silymarin is also used to protect liver against poisoning, e.g. with poison fungi as *Amanita phalloides*. The thistle seed extract is known as cancer inhibitor, reducing the growth of cancer cells in breast cancer and other cancers and for lowering of cholesterol levels.

S. marianum is an annual or biennial plant of the *Asteraceae* family, originally native to Southern Europe but became wide spread throughout the world via Asia.

The areas cultivated with *S. marianum* increased (from 1385.4 hectares in 2009 to 1596.3 hectares in 2012) in Bulgaria (Ministry of Agriculture and Foods, Department of Agrostatistics, Investigation of Yields of farming cultures-crops for 2009 and 2012). This fact shows the exceptional interest to the seed production of this medicinal culture. It is necessary to know the plant viruses and diseases caused on this perspective medicinal plant.

There are scarce data in the phytopathological literature about virus diseases on *S. marianum*. *Tomato spotted wilt virus* (TSWV) was artificially transmitted to milk thistle by Gognalons et al. [6] and naturally proven by Antignus et al. [3].

The objective of the research was the establishment and identification of widespread plant viruses on *Silybum marianum* – milk thistle, caused diseases on this medicinal plant species.

Material and Methods

Samples from leaves with symptoms of virus diseases were collected from the trial fields of the Institute of Roses, Essential and Medicinal Cultures (IREMC) near Kazanlak, Bulgaria in the period of 2009 - 2011. The samples were tested by the serological ELISA method – DAS-ELISA [4] and in the case with AMV and by using the indicator method [7] with test plant species *Nicotiana glutinosa*. Kits purchased from the German company LOEWE, Biochemica were used for the following viruses: *Cucumber mosaic virus* (CMV), *Alfalfa mosaic virus* (AMV) and *Tomato spotted wilt virus* (TSWV). The extinction values were measured using a spectrophotometer SUMAL PE, Karl Zeiss, Jena, Germany. All samples with extinction values two and a half times higher than the negative controls were assumed as virus positive. Negative controls were samples of symptomless healthy plants and positive controls – indicator plants infected with the respective viruses. Optical density (OD) or extinction means the logarithm of the ratio between intensity (If) of fallen and passed (Ie) light via the solutions: OD = lg (If/Ie). The optical density was shown by the measured extinction values for the samples (Fig. 1, Fig. 2 and Fig. 3). Cut off was the boundary value that served as a threshold of all extinction values over two and a half times higher than the negative controls, i.e. a threshold for all milk thistle plants samples with positive reaction to the viruses.

Results

Cucumber mosaic virus (CMV) was established in 10 samples from 27 analyzed milk thistle plants that, i.e. 37% (Fig. 1, Table 1).

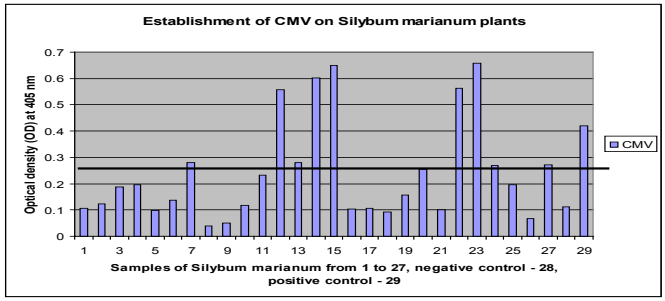

Fig. 1. Establishment of *Cucumber mosaic virus* (CMV) on *Silybum marianum*

Table 1. Established viruses on *Silybum marianum*

Name of the virus	Total number of analyzed plants	Plants with virus infection	Percentage in relation to the total number of analyzed plants
Alfalfa mosaic virus (AMV)	5	1	20
Cucumber mosaic virus (CMV)	27	10	37
Tomato spotted wilt virus (TSWV)	26	5	19

Alfalfa mosaic virus (AMV) was proven in 1 from 5 tested milk thistle plants or 20% (Fig. 2 and Table 1). One from 5 light yellow colored seedling plants was infected with AMV. This plant had severe chlorotic spots and almost entire yellowing leaf laminas. The attendance of AMV in one plant milk thistle was confirmed by the symptoms on the test plant *Nicotiana glutinosa*.The test (indicator) plant species *N. glutinosa*, infected with material from the AMV positive by ELISA yellowing seedling plant of *Silybum marianum*, showed systemic reaction such as large chlorotic areas on the leaves (Fig. 2 and Fig. 7).

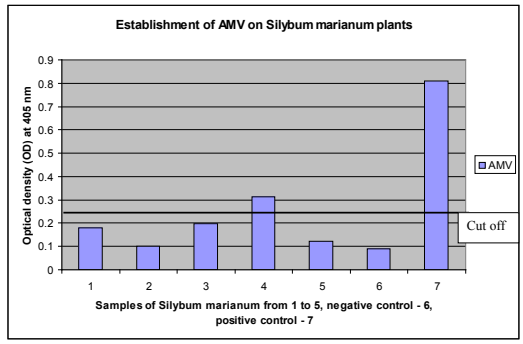

Fig. 2. Establishment of *Alfalfa mosaic virus* (AMV) on *Silybum marianum*

Tomato spotted wilt virus (TSWV) was found in 5 of 26 analyzed milk thistle plants, i.e. 19% (Fig. 3 and Table 1). AMV, CMV and TSWV were established in significant viral concentration, defined by the optical density (OD).

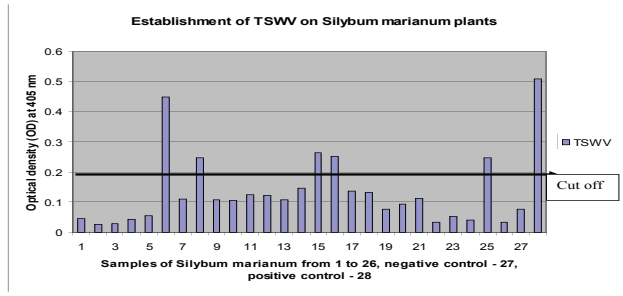

Fig. 3. Establishment of *Tomato spotted wilt virus* (TSWV) on *Silybum marianum*

CMV was established in 5 *S. marianum* plants in comparatively low concentration over 0.25 OD and in 5 plants in almost moderate concentration over 0.55 OD (Fig. 1). Only 5 plants were analyzed for AMV and in 1 of them the virus was proven in nearly moderate concentration over 0.3 OD (Fig. 2). TSWV was present in 4 milk thistle plants in comparatively low concentration over 0.25 OD and in 1 plant nearly moderate concentration – over 0.45 OD (Fig. 3). The typical symptoms for the respective virus such as ring spots, caused as a rule by TSWV or mosaic, often caused by CMV, have not been established until now, but we expect to find milk thistle plants with such symptoms in other observations of the crops. Antignus et al. [3] observed ring spots and tip necroses caused by TSWV on *S. marianum* in Israel, probably in high viral concentration in the crop.

Board 1. Symptoms of viral diseases on *Silybum marianum*

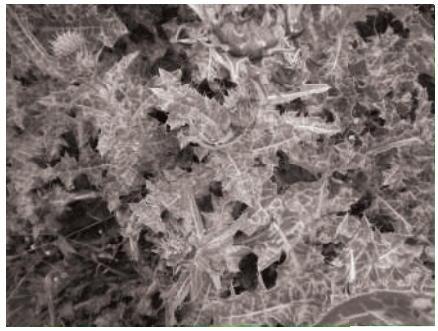

Fig. 4. Part of *Silybum marianum* L. with flowered plants

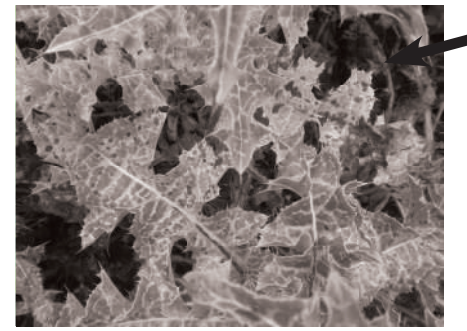

Fig. 5. Part of *Silybum marianum* L. plantation
On the right up – chlorotic leaf with large necrotic area, caused by virus pathogens

Fig. 6. *Silybum marianum* L., on the left – symptomless leaf; on the right – chlorotic leaf, in which TSWV and CMV were established.

Fig. 7. Chlorotic spotting, caused by *Alfalfa mosaic virus* (AMV), originating from milk thistle seedling plant on *Nicotiana glutinosa* – infection after artificial inoculation

Discussion

The results shown on Fig. 1, Fig 2 and Fig. 3 testified positive of virus infections that caused chlorotic spotting or complete yellowing of leaves (Fig. 5), followed by necrotic spotting (Fig. 6) and dying of some leaves and entire plants. These damages probably affect the normal seed yield. The established CMV, AMV and TSWV are large wide spread on a number of cultural and wild plant hosts. These viruses cause serious diseases on some of the hosts such as vegetables and flowers. The transmission of infections is carried out by virus transmitters (vectors). Aphids are vectors for CMV and AMV and thripses – for TSWV. CMV was established in 1/3 of the tested milk thistle plants in our study. This shows that in spite of the protective mechanisms of the leaves (thorns), they are attractive as food for the aphids.

Conclusion

Large spread in the nature plant viruses caused disease, expressed as yellowing of the *Silybum marianum* leaves, followed to necrotic spotting, wilting and sometimes dying of parts or entire plants. This viruses were: *Cucumber mosaic virus* (CMV) in 10 from 27 tested plants (37%); *Alfalfa mosaic virus* (AMV) in 1 from 5 tested plants (20%) and *Tomato spotted wilt virus* (TSWV) in 5 from 26 tested plants (19%). The results showed that viruses are responsible for some diseases in *S. marianum* crops.

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STUDY OF THE RADIUM-226 AND THORIUM-234 ACCUMULATION IN COTTON PLANTS FROM CONTAMINATED SOILS OF BUHOVO AREA

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Abstract

The preservation of soil in its natural state is practically impossible nowadays as the whole global surface is influenced to some extend by anthropogenic factors. The development of methods for reduction of the transfer of pollutants like radionuclides from contaminated soils to the agricultural production is of particular importance in order to guarantee the purity and quality of the farm products. The aim of the work is to study the effect of application of modified and natural zeolites for the assimilation of radioactive elements in agricultural products. We present and interpret the results of a vegetation experiment carried out in vessels with cotton of variety "Chirpan-603" on soil from the tailing pond of Buhovo, contaminated with natural radioactive isotopes. Two kinds of zeolites were folded in the soil – natural zeolite from the Beli Bair deposit and modified organic zeolite based on the natural calcite. The plants were air-dried and analyzed by gamma-spectrometry with multichannel analyzer CANBERRA DSA 1000.

The transfer coefficients for the different plant organs are determined. The results obtained show for both isotopes least accumulation in the plant seeds. The considerable accumulation in the cotton wadding should be remarked. The highest accumulation for both isotopes was observed in the plant stems. The application of zeolites in the soil has not decreased significantly the accumulation of isotopes in the plants.

Keywords: plants, soil, radioactivity, transfer coefficient, Radium-226, Thorium-234

Introduction

The protection of soil in its natural state is practically impossible nowadays as the whole global surface is influenced by anthropogenic factors. It is therefore necessary to study the sources of pollution and the territories affected. This will enable the finding of effective ways and means for their restoration, protection and sustainable use.

The development of methods for reduction of the transfer contaminants like radionuclides from polluted soils to the agricultural production is of particular importance in order to guarantee the purity and quality of the farm products.

The aim of the work is to study the accumulation of some natural radioactive elements in plants grown on soil contaminated by uranium mining activities as well as testing reduction of the accumulation in plants by addition of zeolites to the soil.

Material and Methods

For realization of the aims of the study a pot vegetation experiment with cotton of variety "Chirpan-603" on soil polluted with natural radioactive elements was carried out. The soil was collected from the tailing pond of Buhovo. For the experiment cotton was chosen as a technical crop suitable for growing on polluted soils as in this case the plant production obtained will not be used as food which would have caused additional dose accumulation for the population.

The content of the radioactive isotopes ^{234}Th and ^{226}Ra in the soil is shown in Table 1.

Table 1. Activity concentration of ^{234}Th and ^{226}Ra in soil

	Th-234	Ra-226
Activity	89 ± 8 Bq.kg^{-1}	46 ± 5 Bq.kg^{-1}

The two zeolites available for us in the moment: natural zeolite from the "Beli Bair" field and modified zeolite based on natural calcite were folded in the soil as follows:

Control - 5 kg soil
Variant 1 - 5 kg soil + 250g natural zeolit
Variant 2 – 5 kg soil + 500g natural zeolit
Variant 3 – 5 kg soil + 250g/modified zeolit
Variant 4 – 5 kg soil + 500g modified zeolit

The zeolites are aqueous alumosilicates of alkaline and alkaline earth elements with the following general formula:

$$M_{x/n}Al_xSi_yO_{2(x+y)}\cdot pH_2O, \quad (1)$$

where: *M* is a metal with alkaline properties, most often Na, Ca and/or K and more rarely Mg, Ba, Sr;
n is the valence of the cation M;
the x/y ratio varies between 1:1 and 1:6, and p/x is between 1 and 4.

The zeolites have well expressed ion exchange properties which determines their exchange capacity, ion exchange selectivity and kinetic parameters in different systems. The ion exchange selectivity reflects their ability to extract ingredients from diluted solutions in the presence of other ions. The high-silicic types are usually selective towards the big univalent and bivalent cations (K, Rb, Cs, NH_4, Ba), and the low- silicic – towards the small cations like Na, Ca.

In our experiment we have used zeolite Clinoptilolite which is industrialy obtained and has wide application in our country. These are volcanic tufts at oligocene age formed by acid (rhyolitic), glassy, igneous rocks.

The second product used is a modified zeolite which is Clinoptilolite modified with the purpose of changing its alumosilicate composition. This is done by treatment with high acidity solutions thus extracting a significant part of the aluminum without breaking the crystal mineral structure. During treatment the Si/Al ratio changes from 5-6 to 15-20. As a result the exchange capacity and the selectivity towards polar molecules decrease but selectivity towards non polar molecules increases. Thus the sorption capacity for benzol of the Clinoptilolite with partially extracted aluminum grows 4 times and for nitrogen it doubles [1, 2, 3].

The plants were air-dried and analyzed by gamma-spectrometry with multichannel analyzer CANBERRA DSA 1000 and high-purity germanium detector with 20% efficiency and energy resolution of 1.3 keV for ^{60}Co γ-ray energy line at 1332 keV. The spectrum was analyzed by GENIE-2000 software with measurement uncertainties less then 10%. Typical counting times were 19–24 h. ^{234}Th concentration was derived from the weighted mean of the photopeaks at 63.5 keV and 92.6 keV and ^{226}Ra was evaluated at its 186.1 keV photopeak taking into account the contribution of the overlapping line at 185.72 keV of ^{235}U.

Results and Discussion

The percentage allocation of ^{234}Th and ^{226}Ra activities in cotton plant organs is shown in Fig. 1 and Fig. 2.

It is evident from the graphs that the least accumulation for both isotopes is in the plants seeds – 9% of the ^{234}Th activity and 6% of the activity of ^{226}Ra. It should be noticed that there is a considerable accumulation in cotton wool – 30% of ^{234}Th and 32% of ^{226}Ra. The highest accumulation for both isotopes is determined in the stems.

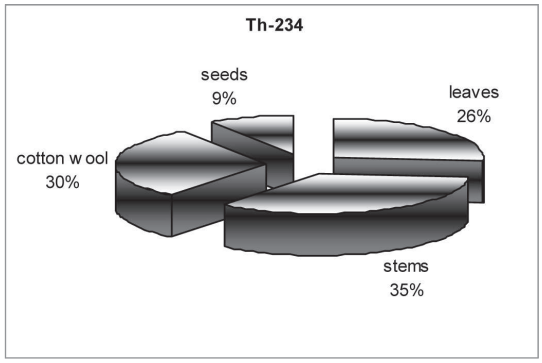


Fig.1. Allocation of ^{234}Th in the plant organs

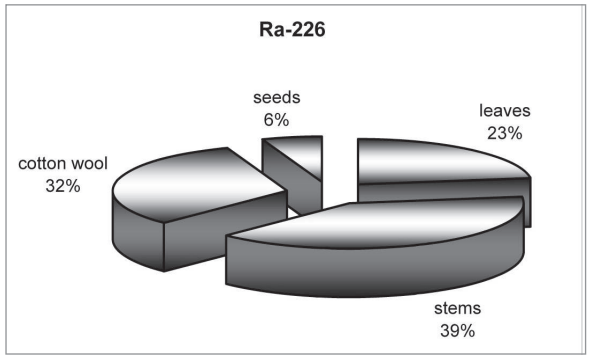
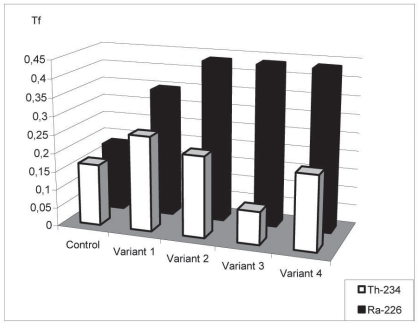


Fig. 2. Allocation of ^{226}Ra in the plant organs

In order to value the degree of radioactive elements accumulation the so called transfer coefficients are defined – the ratio 'activity per 1 gram air-dry plants' to 'activity per 1 gram air-dry soil.

The transfer coefficients for the investigated isotopes in the different plant organs are shown in Fig. 3, Fig. 4, Fig. 5 and Fig. 6.



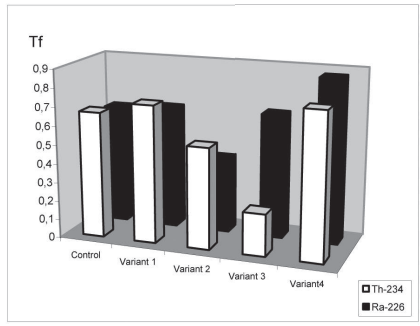
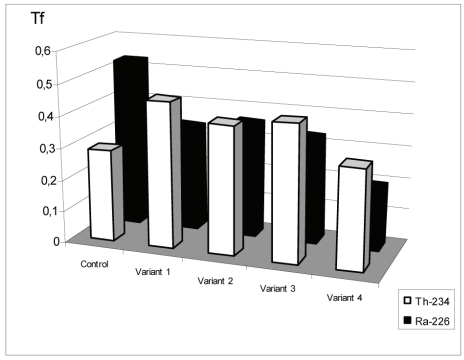


Fig. 3. Transfer coefficients in leaves

Fig. 4. Transfer coefficients in stems

The results show that the transfer coefficients of ^{226}Ra are highest in cotton stems and lowest in cotton seeds. It should be noticed that the accumulation in cotton wool which is the usable part is with relatively high transfer coefficients.

The results presented in the figures show that the application of zeolites in the soil did not resulted in considerable reduction of ^{226}Ra accumulation. Some decrease in the transfer coefficients is observed in cotton wool and the seeds – down to 2.7 in variant 4 which is about 1.2 times lessening– in the leaves and stems at the same time the accumulation of radium even increases.



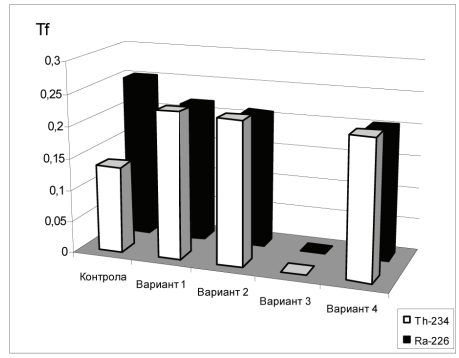


Fig. 5. Transfer coefficients in cotton wool **Fig. 6. Transfer coefficients in seeds**

As for ^{234}Th the transfer coefficients are highest in the plant stems and cotton wool.

The application of zeolites did non result in decrease of thorium accumulation by plants. The only exception observed is in variant 3 where the application of 50 mg modified zeolite per 1 kg soil decreased the transfer coefficients in the leaves and the stems twice. But in cotton wool the thorium accumulation increases.

The accumulation of both elements is with similar transfer coefficients. There is some difference in the plant leaves only.

Conclusions

- The accumulation in the cotton seeds is less then that in the leaves, stems and cotton wool,
- The transfer coefficients for both elements are highest in cotton wool and plant stems,
- The results obtained show that the application of both zeolites in the soil did not influence noticeably the accumulation of the observed radioactive elements in the plants.

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INFLUENCE OF DOUBLE MICROBIAL ASSOCIATIONS WITH AM-FUNGI AND *RHIZOBIUM* ON THE GROWTH OF ALFALFA AND RED CLOVER AND ON THE SOIL STRUCTURE

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[2]Academician Metodi Popov Institute of Plant Physiology and Genetics, BAS, Sofia, Bulgaria

Abstract

The aim of the study was to determine the effect of inoculation with mycorrhizal biofertilizer "Mycosym TRI-TON" applied single or in combination with N-fixing Rhizobial bacteria on the growth of alfalfa and red clover, the N and P content of plant biomass and on the soil structure.

Material and Methods: A pot and a field experiments with the two forage grasses on slightly eroded Leached Cinnamonic soil were carried out. The following parameters were determined: dry weight and N and P content of shoots and roots, percentage of root mycorrhization, acid phosphatase activity and percentage of water-stable aggregates in the soil.

Results: Positive effect of the inoculation on the studied plant parameters and on the content of water-stable aggregates in the soil was obtained. It was established specificity in the phosphorus translocation and its distribution in the plant parts for two grasses tested.

Conclusions The results obtained outline the possibility for application of forage grasses treatments with "Mycosym TRI-TON" and symbiotic N-fixing bacteria in contemporary systems for soil erosion protection.

Keywords: Arbuscular myccorrhizal fungi, Rhizobium inoculation, legume grass, plant parameters, water-stable soil aggregates

Introduction

Present-day intensive agriculture causes decrease of the soil fertility of natural pastures and meadows and the species diversity of the forage grasses in grass cover. This determines the increasing interest on the studies, connected with factors, bringing about these negative processes and with the contemporary biotechnologies, applied for their overcoming. A great part of these technologies are based on the utilization of biofertilizers, consisted of AM fungi and N-fixing bacteria [2, 4, 6, 9, 14]. Both groups of mi-

croorganisms, applied alone or in combination improve plant nutrition and contribute to soil pollution protection. Besides that, many authors report that extracellular mycorhizal hyphaes, along with excreted from them and soil microorganisms mucopolysaccharides exert positive effect on water-stable soil aggregates formation [6, 10, 12].

The aim of the study is to examine the effect of the inoculation with the mycorrhizal fertilizer "Mycosym TRI-TON", applied single or in combination with symbiotic N-fixers from the genera Rhizobium on the growth of alfalfa and red clover, N and P content in plant biomass and on the changes of soil structure in pot and small-plots field experiments.

Material and Methods

The first experiment was carried out with alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.), grown on slightly eroded Leached Cinnamonic soil from the area of Suhodol (Sofia district). Pots containing 1 kg of soil were used in the experiment. The agrochemical properties of the soil were as follows: humus – 2.07%; NH_4-N – 6.4 mg/100 g; NO_3- N – 6.6 mg/100 g; $P_{(P205)}$ – 1.4 mg/100 g; $K_{(K2O)}$ – 22 mg/100 g; pH $_{(H2O)}$ – 5.3. The experimental scheme included inoculation of the plants with mycorrhizal granular biofertilizer "Mycosym TRI-TON", applied single and in combination with N-fixing bacteria. The biofertilizer contained mycorrhizal fungus *Glomus intraradices* consisted of more than 50 spores/g, more than 200 IMP (Infective Mycorrhizal Propagules). It was added to the soil (at 2 cm depth under soil surface) before sowing of the seeds in quantity 0.5 g/pot. The seeds were previously treated with suspension of N-fixing microorganisms (1.108 cells/ml) as follows: red clover - *Rh. trifolii* 294 and lucerne – *Rh. meliloti* 116. The strains tested are from the collection of Soil Microbiology Department of "Nikola Pushkarov" Institute of Soil Science, Agrotechnology and Plant Protection. During the experimental period the dry shoot biomass weight was determined in two cuts (first – budding and beginning of flowering and second – three weeks later) and the root biomass dry weight - at the end of the study. The N content of the roots and shoots (by the method of Kieldal), their P content - by molybdenum-vanadate method and the percentage of roots mycorrhization - by the method of Philips and Hayman [13] were also analysed. The activity of the acid phosphatase activity in both rhizospere soil and roots of control variants and of those, treated with "Mycosym TRI-TON" was analyzed using the method of Tabatabai and Bremner [15].

The second experiment for erosion control was carried out in the experimental field in Suhodol, Sofia area on the same type of soil. The experimental design included plots of 2 m^2 and hay yield area of 0.25 m^2 in 5 replications. The seeds of red clover and alfalfa were treated before sowing with the suspension of the tested Rhizobium strains (1.108 cells/ml). The inoculum of "Mycosym TRI-TON" was applied during the sowing time in amount of 15 g/m^2. At the end of the experiment, the air dry shoot yield, shoot N and P content and the root mycorhization were determined. After finishing the active vegetation of the plants, in order to study the influence of the mycorrhizal preparation on the soil structure, samples were taken in

depth 0-10 and 10-20 cm. The percentage of water-stable aggregates was determined by the method of Katschinski.

The statistical treatment of the data on plant biomass included determination of the least significant differences (LSD) ($P \leq 0.05$) among the treatments (STATGRAPHICS. Plus 2.1).

Results

The data, presented in Table 1 showed that the application of the biofertilizer "Mycosym TRI-TON" exerted positive effect on the quantity of the biomass of both tested plants. The shoot weight in the treated variants was higher than the control for both cuts. These results were probably due to the better plant nutrition after the biofertilizer addition and they corresponded to higher percentage of root mycorrhization. The highest and statistically proven increase of shoot biomass was established in the treatments with double inoculation. The same tendency was observed also for root weight. The increase of the percentage of root mycorrhization in inoculated treatments as compared to the control was about 15% for alfalfa and more than 20% for red clover. These data confirmed the results, reported by other authors, which established that double inoculation of the legume grasses (included AM fungi and nodulating bacteria) was more efficient than single one [1, 16]. They are also in accordance with our previous investigations with other pasture grasses [7]. The study included treatments of esparsette (*Onobrichis simplex* L.), bird,s foot trefoil (*Lotus corniculatus*) and white clover (*Trifolium repens*) with "Mycosym TRI-TON" and nodulating bacteria in pot and field conditions.

It was established an increase in the N and P content of plant biomass in the treated variants in comparison with the controls for both legume grasses (Fig. 1). Again the increase was the highest for the treatments with double inoculation, especially for the shoots P content. Specificity in N and P distribution in the plant biomass was observed. At the end of the experiment, the N content of shoots was higher than those of the roots for both plant species tested while the P shoot content of alfalfa was less than those of the roots. In red clover these quantities was equalized. The P content was the highest for symbiotrophic association alfalfa – AM fungi - *Rh. meliloti* 116 and red clover – AM fungi - *Rh. trifolii* 294. The effect of double inoculation on P content of plant biomass in alfalfa was higher as compared to the red clover.

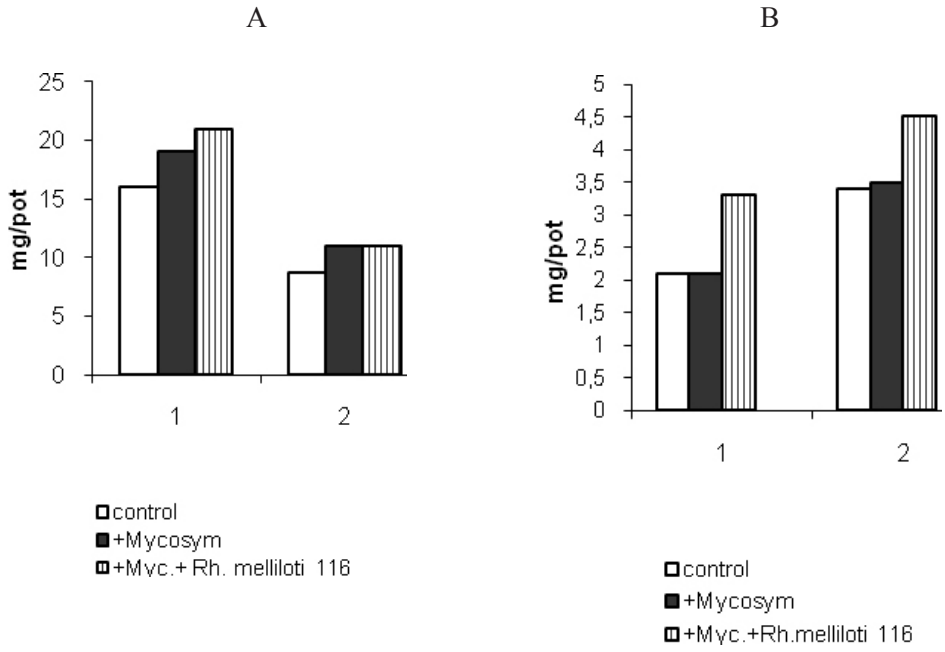
The data on acid phosphatase activity are presented in Fig. 2. For both legume grasses this parameter was higher in treated with biofertilizer variants compared to the control. The increase in soil acid phosphatase activity was probably due to the stimulation of phosphate-decomposing bacteria in the rhizosphere zone. These data corresponded to the higher increase of the plant biomass and its higher N and P content due to the improved plant nutrition. Higher values of acid phosphatase activity were obtained for red clover. The symbiotrophic associations studied in the pot experiment were tested also in field conditions. The data represented in Table 2 show an increase in the hay yield and

Table 1. Effect of inoculation of pasture grasses on the yield of dry plant biomass and root mycorrhization in pot experiment

Treatments	Shoots (g/pot)		Roots (g/pot)	Mycorrhization (%)
	I cut	II cut		
Lucerne				
Control	*0.53[a]	0.63[a]	1.30[a]	21.11
+ Mycosym	0.62[ab]	0.75[ab]	1.60[ab]	28.15
+ Myc.+*Rh. meliloti* 116	0.88[b]	0.90[b]	1.90[c]	31.70
Red clover				
Control	0.47[a]	0.90[a]	1.20[a]	20.55
+ Mycosym	0.62[ab]	1.40[ab]	1.43[ab]	29.22
+ Myc.+*Rh. trifolii* 294	0.88[b]	1.61[b]	1.60[b]	33.44

*Values in the same column, which are followed by different letters are significantly different at $P \leq 0.05$

Alfalfa



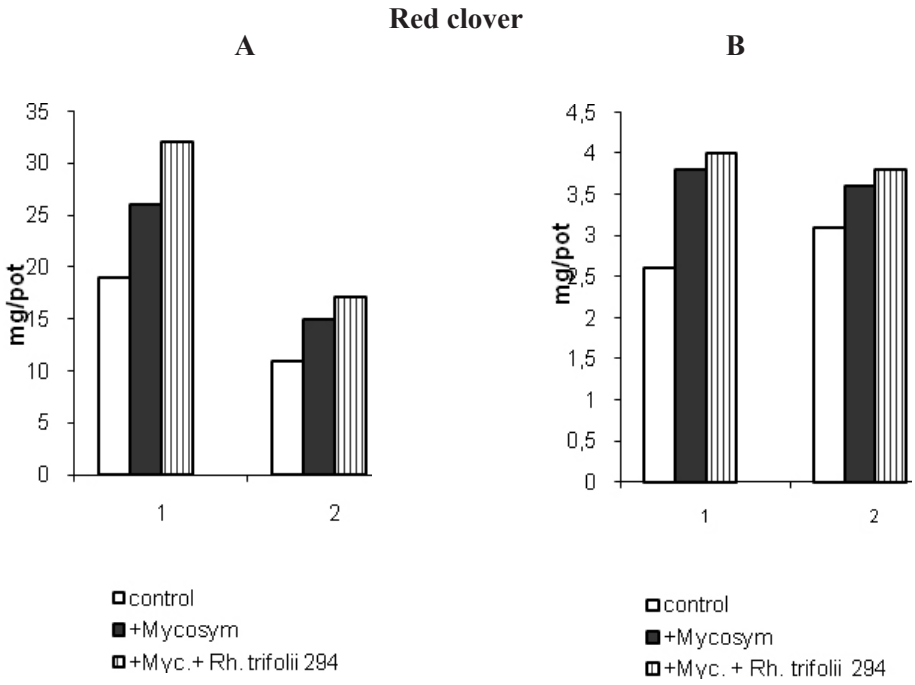


Fig. 1. (A) Nitrogen content of shoots (1) and roots (2), (B) Phosphorus content of shoots (1) and roots (2)

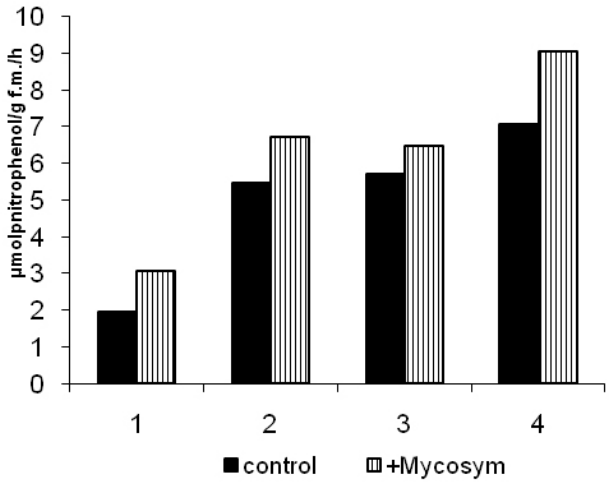


Fig. 2. Effect of treatment with „Mycosym TRI – TON”on acid phosphatase activity in alfalfa (1-soil; 2-roots) and red clover (3-soil; 4-roots)

Table 2. Effect of inoculation on the hay yield of legume grasses in field conditions

Treatments	Dry hay (kg/2)	P (%)	N (%)	Roots mycorrhization (%)
Alfalfa				
Control	0.782[a]	0.170	1.9	31.23
+ Myc.+ *Rh. meliloti* 116	0.875[a]	0.205	2.1	46.19
Red clover				
Control	0.430[a]	0.135	1.8	25.50
+ Myc.+ *Rh. trifolii* 294	0.590[a]	0.146	1.9	49.32

*Values in the same column, which are followed by different letters are significantly different at $P \leq 0.05$

Table 3. Effect of treatment with „Mycosym TRI - TON“on the soil structure

Treatments	Depth (cm)	Water-stable aggregates (%)		
		< 0.25mm	> 0.25mm	> 1mm
Alfalfa				
Control	0 - 10	22.3	79.6	50.2
Control	10 - 20	17.2	85.2	58.4
+ Mycosym.+ *Rh. meliloti* 116	0 - 10	19.1	80.1	61.2
+ Mycosym + *Rh. meliloti* 116	10 - 20	14.2	84.3	70.3
Red clover				
Control	0 - 10	27.2	70.2	39.6
Control	10 - 20	16.3	81.5	47.1
+ Mycosym. + *Rh. trifolii* 294	0 - 10	20.3	75.2	49.1
+ Mycosym. + *Rh. trifolii* 294	10 - 20	19.6	80.9	55.7

in the N and P content of the shoots compared to the control. These data were in accordance with higher percentage of root mycorrhization (Table 2) and higher content of water-stable aggregates (Table 3). The percentage of soil particles ≥ 1 mm increased with about 10 - 20% by the inoculated treatments of alfalfa and red clover at both soil depths. The increase in the part of water-stable aggregates, which improve soil structure confirmed the positive effect of extracellular micellium of AM fungi for conglutinating of soil particles, was established by many authors [3, 8, 11]. These results outline the possibility for application of forage grasses treatments with “Mycosym TRI-TON” and symbiotic N-fixing bacteria in contemporary systems for soil erosion protection.

Discussion

Rhizobial bacteria and AM fungi are plant beneficial microorganisms having the potencial to improve biomass production. Their utilization for plant inoculation contributes to soil fertility conservation, reduction of mineral fertilization and ecological safe crop production. The combined inoculation with AM fungi and N-fixing bacteria could have positive or detrimental effect on plant growth depending on the type of mycrosymbionts interaction. Mycorrhizal colonization can affect root exudation and hense the composition and function of rhizobacterial community. Some authors reported for competition between AM fungi and Rhizobial bacreria for sites of root penetration [1, 8]. Catford et al. [5] showed that an established symbiosis in alfalfa could systematically exert an inhibition on the second one. This inhibition could be explained by the common regulating signals between plant and both AM-fungi and Rhizobial bacteria. It has been established that root flavonoids are the molecular signals involved in the general recognizing process between plant and microorganisms [9].

On the other hand there is much information confirming that AM-fungi improve growth, nodulation and N-fixation in legume-Rhizobium symbiosis [1, 13, 15]. This effect is due to the fact that N_2-fixation depends on steady adequate supply of phosphates to roots and nodules. AM fungi are able to take up phosphates from soil solutions with low phosphate concentrations more efficiently than simple roots. They take up, accumulate and transfer large amounts of phosphates to the plant by releasing the nutrients in root cells containing arbuscules. From them phosphates are transferred to cortical root cells, ready to be used by plants. In this way, providing phosphates, AM-fungi assist nodule formation and their functioning. The interactions in symbiotic system AM fungi – *Rhizobium* – leguminous plant are complex and that is why empirical studies should be carried out for each concrete symbiotrophic association.

In our study the increase of root and shoot biomass and their N and P content demonstrated synergistic interaction between tested AM fungi and Rhizobial strains. Obviously, plants recognized inoculants as beneficial partners.

The obtained results, concerning quality and quantity of plant biomass and soil structure changes indicated that the studied mycrosymbionts could be utilized for forage grasses treatments with "Mycosym TRI-TON" and symbiotic N-fixing bacteria in contemporary systems for soil erosion protection.

Conclusions

The mycorrhizal biofertilizer "Mycosym TRI-TON", tested in pot and small-plots field experiments increase the yield and the N and P content in plant biomass of alfalfa and red clover. The highest effect was obtained by the dual inoculation with AM fungi and Rhizobial bacteria.

The AM fungi influence the phosphate distribution in the plant biomass and activate acid phosphatase activity.

Extracellular mycelium of AM fungi exerts favorable effect on soil structure, increasing the part of water-stable aggregates.

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REDUCTION OF THE INFECTION WITH TOMATO MOSAIC VIRUS AND CUCUMBER MOSAIC VIRUS IN TOMATOES THROUGH INDUCERS OF RESISTANCE

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Abstract

Aim: Our aim is to induce resistance to ToMV and CMV in tomatoes with the elicitors EXIN and BION.

Material and Methods: In our study we used two substances to induce Systemic Acquired Resistance (SAR): EXIN and BION. They were tested on three tomato cultivars – Buffalo heart, Giant and Nazareth. Confirmation of the virus infection in the treated plants was done by DAS-ELISA.

Results: We received different percentage of reduction DAS ELISA values for ToMV and CMV. Treating the plants with these substances expressed good results against ToMV for the cultivars Giant and Nazareth and better for Buffalo heart. DAS ELISA values for CMV in the treated plants was slightly reduced.

Conclusion: Best results according to DAS ELISA values for ToMV and CMV were achieved by combination treatment EXIN+BION which induced resistance to CMV in 92.4% (non infected plants) and ToMV in 72% (non infected plants) of the experimental plants cv. Buffalo heart.

Keywords: ToMV, CMV, SAR, tomato resistance

Introduction

Tomatoes are damaged by more than a hundred viruses worldwide [6]. In Bulgaria Tomato mosaic virus (ToMV), *Cucumber mosaic virus* (CMV), *Tomato Spotted Wilt Virus* (TSWV) and *Potato Virus* Y (PVY) are of the greatest importance for tomato production.

ToMV belongs to the *Tobamovirus* genus and infects tomato and tobacco plants systemically. Symptoms are mosaic and characterized by intermingled light and dark green regions [11]. CMV was first described as a disease of cucurbits in in Michigan [3]. The virus can infect a large number of indicator plant species and has been isolated from over 500 naturally infected species. Isolates of CMV are heterogeneous in symptoms, host range, transmission, serology, physicochemical properties, and sequence of genomic RNAs [4]. The host range of the collective isolates of CMV is over 1300 species in more than 500 genera of over 100 families, with new hosts reported each year. CMV infects most of the major horticultural crops as well as many weed species; the latter act as reservoirs for the virus [4].

The symptoms induced by CMV are not generally specific to CMV, but rather reflect sets of host responses to viral pathogens. Therefore, symptoms such as light green–dark green mosaics, generalized chlorosis, stunting, leaf filiformism, and local chlorotic or necrotic lesions associated with various strains of CMV are not specific to CMV, but can also

be elicited by other viruses in the same plant species. Some strains of CMV can induce a bright yellow chlorosis in some *Nicotiana* species [4].

Systemic acquired resistance (SAR) refers to a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens. After the formation of a necrotic lesion, either as a part of the hypersensitive response (HR) or as a symptom of disease, the SAR pathway is activated. SAR activation results in the development of a broad-spectrum, systemic resistance [8, 13]. The SAR signal transduction pathway appears to function as a potentiator or modulator of other disease resistance mechanisms. When SAR is activated, a normally compatible plant-pathogen interaction can be converted into an incompatible one [10]. Conversely, when the SAR pathway is incapacitated, a normally incompatible interaction becomes compatible [2]. The mechanism by which this modulation occurs is not understood; however, at least part of the resistance response could be due to expression of the SAR genes.

BION is known as an elicitor of SAR in plants. BION successfully protected wheat against powdery mildew by affecting multiple steps in the life cycle of the pathogen [5] and induced resistance against different pathogens on tomato crops like the fungi *Fusarium oxysporum* f.sp. *radicis-lycopersici* [12] and *Xanthomonas* spp. bacteria [7]. BION was also used to induce resistance against Tobamoviruses (TMV and ToMV) in *Nicotiana glutinosa* [9].

Our aim is to reduce the infection with ToMV and CMV in tomatoes by the elicitors of induced resistance EXIN and BION.

Material and Methods

We used the two elicitors (EXIN and BION) to induce SAR in three tomato cultivars (Buffalo heart, Giant and Nazareth), all of them sensitive to ToMV (necrotic tomato strain) and sensitive to CMV (necrotic pepper strain).

The standard virus inoculums was prepared by using the leaves showing necrotic symptoms harvested from the pre-maintained ToMV-infected tomato (cv. Ideal) and CMV infected pepper (cv. Sivria) plants. Homogenization of the infected leaf tissue (1g) was with 5 ml potassium sodium phosphate buffer pH 7 (1:10, w/v) in a pre-chilled pestle and mortar. The virus inoculums were swabbed over carborundum pre-dusted leaves. After 10 min, inoculated leaves were washed with distilled water. Infection and treatment of the plants were in a phase 5-7th leaf of the tomato cultivar.

CMV and ToMV symptoms observation was done every week and confirmation with DAS-ELISA [1] test was performed. Plants were treated once three days before artificial infection with the viruses. BION (benzo [1, 2, 3] thiadiazole-7-carbothioic acid-S-methyl ester or benzothiadiazole, SYNGENTA) was used at a concentration of 3mM and BION 1.75 mM in combination with EXIN (Phytoxin VS, with the active ingredient of 4.5% salicylic acid in at a concentration of 1 packet (10 ml) in 1 L of water). The plants were divided into groups of 15 plants each and were treated in several schemes: 1) with an aqueous solution of 3mM BION, pH = 7.; 2) EXIN and 3) EXIN+BION. For each treatment were used controls for comparison: K (healthy plant treated with water only), K (infected plant not treated with chemicals, only with water), K-BION (healthy plant treated with BION only, measuring for toxicity), K-EXIN, K-BION+EXIN (healthy plants treated with this

combination). About 21 days after inoculation, samples were taken for DAS-ELISA for CMV or ToMV virus infection after the treatments with BION and EXIN before artificial virus inoculation. Homogenization of tomato leaves was 1:10 w/v (0.5 g) in 5 ml buffer potassium sodium phosphate buffer pH 7. Plants showing DAS-ELISA values under the cut-off (twice the negative control value) are considered as non infected according to the manufacturer's instructions. Reading of the reaction was made with Multifunctional detector type DTX 880, at a wavelength of 405 nm. The results are represented in percent non infected plants.

Results

We observed different symptoms on tomato plants and fruits (Fig. 1 and Fig. 2). Most of them were mosaic, chlorotic and necrotic spots on tomato leaves and necrotic lesions on tomato fruits.

Fig. 1. Necrotic fruit symptoms caused by ToMV

Fig. 2. Tomato leaf symptoms caused by CMV

Our results indicated relatively good protection of tomato cultivars against ToMV and CMV. We received different percentage of non infected plants after treating with the substances and artificial virus inoculation. Treating the plants with these substances expressed good results against ToMV for the cultivars Giant and Nazareth and better for Buffalo heart (Fig. 3). Percentage of non infected with CMV plants (non infected plants) after treatment with 3mM BION alone was less (45%) compared with ToMV – 65.8% (Fig. 3). Treatment of the plants with EXIN alone resulted with almost no effect against CMV and ToMV virus infection (Fig. 3). Treatment of the tomato cultivars with the combination EXIN + BION resulted with significantly high percentage of non infected plants with CMV and ToMV compared with mono treatments (Fig. 3). Better protection of this combination was achieved against CMV – 92.4% non infected plants from the cv. Buffalo heart, 73% for the cv. Giant and 52.8% for the cv. Nazareth. We received lesser percentage of non infected tomato plants treated with this combination and inoculated with ToMV – 36% to 72% (Fig. 3).

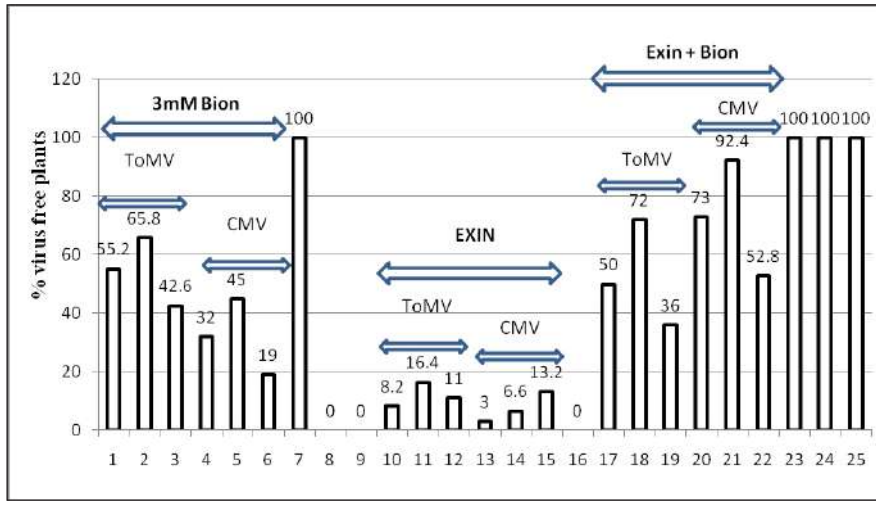

Fig. 3. Percentage of virus free plants received by DAS ELISA results after treatments with BION and EXIN once 3 days before artificial virus inoculation

Legend:

1, 4, 10, 13, 17, 20 - tomato cultivar Giant
2, 5, 11, 14, 18, 21 - tomato cultivar Buffalo heart
3, 6, 12, 15, 19, 22 - tomato cultivar Nazareth
7 – tomato plants from the three cultivars not treated and non inoculated (K-)
8 – tomato plants inoculated with ToMV only (K+ToMV)
9 - tomato plants inoculated with CMV only (K+CMV)
16 - tomato plants treated with EXIN only for toxicity
23- tomato plants treated with BION only for toxicity
1-3/ 10-12/ 17-19 – artificial inoculation of plants with ToMV
4-6/ 13-15/ 20-22 - artificial inoculation of plants with CMV
24 – tomato plants treated with EXIN + BION only for toxicity
25 – tomato plants treated with H_2O only

Discussion

Different tomato cultivars (Giant, Buffalo heart, and Nazareth) showed different DAS-ELISA values for ToMV and CMV when treated with BION and EXIN. Best results were achieved by combination treatment EXIN+BION which induced resistance to CMV in 92.4% (non infected plants) and ToMV in 72% (non infected plants) of the experimental plants cv. Buffalo heart.

The effects of pre-treatment of tomato plants with BION have been evaluated against ToMV in tomato seedlings [9]. Pre-treatment of the indicator plant *Nicotiana glutinosa* with BION followed by challenge inoculation with tobamoviruses produced a reduced number and size of local lesions (79% protection over control to ToMV

inoculation). The following quantification of viral concentration provided evidence that BION pre-treatment reduced the viral movement to upper leaves [9].These results confirm our findings that pretreatment of tomato plants with BION can induce resistance against ToMV.

Systemic acquired resistance was induced in tomato cultivars – cv. Ideal (79.2% non infected plants), cv. Roma (85.8%) and cv. Balkan F1 (72.6%) when treated once 3 days before PVY inoculation with 3mM BION [14]. When treated with EXIN 3 days before PVY inoculation the percentage of uninfected plants was 46.2% for the cv. Ideal, 26.4% for the cv. Roma and 0% for the cv. Balkan F1. Three days after viral inoculation the percentage of uninfected plants was 0% in cultivar Ideal, 6.6% in cultivar Roma and Balkan 13.2% F1. [14] The percentage of non infected plant received against PVY compared with the percentage of non infected plants against ToMV and CMV differ which is probably due to the different cultivars and viruses.

In the present study we proved that BION and EXIN can be successfully used prior to CMV and ToMV inoculation of tomato plants to induce SAR against these economically important viruses in Bulgaria.

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INDUCTION OF RESISTANCE IN PEPPER TO POTATO VIRUS Y BY ACTIVATION OF DEFENSE MECHANISMS OF THE HOST PLANT

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Abstract

Aim: Our aim is to induce Systemic acquired resistance to Potato virus Y (PVY) in pepper plants using BION and EXIN.

Material and Methods: Plants were treated once with EXIN and BION in two variants: 3 days before and 1 day after artificial infection with PVY. Confirmation of the results was made with DAS-ELISA.

Results: Treatment of the pepper plants with the two elicitors of Systemic acquired resistance (SAR) induced resistance against PVY resulting in reduction of the DAS ELISA values for PVY. We received different results when treating the plants before and after virus inoculation.

Conclusion: The combination treatments with EXIN+BION once 3 days before PVY inoculation induced resistance to PVY in 86% (13 non infected plants from 15) of the experimental plants cv. Kurtovska kapia.

Keywords: PVY, SAR, pepper

Introduction

PVY was first recognized in 1931 as an aphid-transmitted member within a group of viruses associated with potato degeneration, a disorder known since the eighteenth century [15]. PVY is the type species of the genus *Potyvirus*, one of the six genera in the family Potyviridae [14]. PVY is naturally spread by vegetatively propagated material and by aphids in numerous species in a nonpersistent manner [7]. Transmission by contact has also been reported. PVY has a wide host range and is highly variable with some host specificity [14]. PVY is one of the most damaging plant pathogens causing significant losses in four main crops around the world: potato, pepper, tomato, and tobacco. In surveys of viruses with worldwide economic

importance, PVY was listed in the top-five viruses affecting field-grown vegetables [17]. PVY was also found responsible for damages in petunias in Europe and in eggplant crops in India.

PVY is the causal agent of major diseases and production losses in pepper crops. In some situations it can affect 100% of the plants and can be the most important disease [10]. Efficient control strategies depending on the crop have been developed. However, none of them seems capable to take into account PVY evolution and to suppress risks of new epidemics.

SAR refers to a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens. SAR has been recognized as a plant response to pathogen infection for almost 100 years [4]. However, most of the early studies were mainly descriptive and lacked quantitative tools to analyze the response. Thus, considerable effort has been devoted to identifying and isolating biochemical markers for SAR that could be used to distinguish it from other inducible plant resistance responses. A large body of evidence suggests that salicylic acid (SA) plays a key role in both SAR signaling and disease resistance. Initially, the leve1 of SA was found to increase by several hundred-fold in tobacco or cucumber after pathogen infection, and this increase was shown to correlate with SAR [13]. Since these reports, a considerable amount of data has established a correlation between the concentration of SA and the establishment of enhanced disease resistance not only in tobacco and cucumber but in other plants as well [3, 9]. These data, coupled with the finding that exogenous SA can induce SAR and SAR gene expression, led to the suggestion that SA was involved in SAR signaling [18].

The aim of this study is to induce SAR to PVY in pepper plants using the elicitors BION and EXIN.

Material and Methods

Pepper plants of the cultivar Kurtovska kapia (sensitive to PVY) were treated in two variants: once, 3 days before artificial inoculation [11] with PVY^N strain or once, 1 day after virus inoculation. The plants of each variant were divided into groups of 15 plants each and treated in three schemes:

I scheme - with Exin 4.5 HP (Phytoxin VS, with the active ingredient of 4.5% salicylic acid) at a concentration of 1 packet (10 ml) in 1 L of water,

II scheme – with BION (benzo [1, 2, 3] thiadiazole-7-carbothioic acid-S-methyl ester or benzothiadiazole, SYNGENTA) at a concentration of 3mM,

III scheme – with combination with EXIN and 2.35 mM BION.

Spraying was carried out in a greenhouse with temperatures 21-24°C and a air humidity of 45% at a dose of 5-15 ml solution (depending on the size of the plant) of the compounds.

Inoculation and treatment were in phase 4-5^{th} leaf of the pepper plants. The standard virus inoculums were prepared by using the leaves showing mosaic symptoms harvested from the pre-maintained PVY-infected pepper (cv. Sivria) plants. The leaves (1 g) were homogenized in 5 ml of the potassium sodium phosphate buffer (pH 7.2, 0.1 M) in a pre-chilled pestle and mortar. The virus inoculums were swabbed over carborundum pre-dusted leaves. After 10 min, inoculated leaves were washed with distilled water.

Twenty-one days after inoculation, samples were taken for DAS-ELISA [6] for PVY virus infection after the treatments with BION and EXIN before artificial virus inoculation. Homogenization of pepper leaves was 1:10 w/v (0.5 g) in 5 ml buffer potassium sodium phosphate buffer

pH 7. Plants showing DAS-ELISA values under the cut-off (twice the negative control value) are considered as non infected according to the manufacturer's instructions. Reading of the reaction was made with Multifunctional detector type DTX 880, at a wavelength of 405 nm.

Results

Symptoms of the infection with PVY only of pepper plants (without any treatment) are mostly visible on the vegetative parts but rarely on fruits. They consist of chlorotic or necrotic symptoms on leaves (Fig. 1 and Fig. 2) and later the fruits become dry (Fig. 3).

Fig. 1. Necrotic leaf symptoms caused by PVY

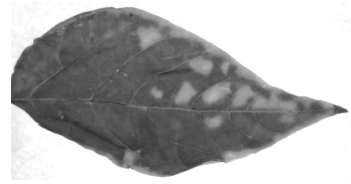

Fig. 2. Chlorotic leaf symptoms caused by PVY

Fig. 3. Dry pepper fruit caused by PVY infection

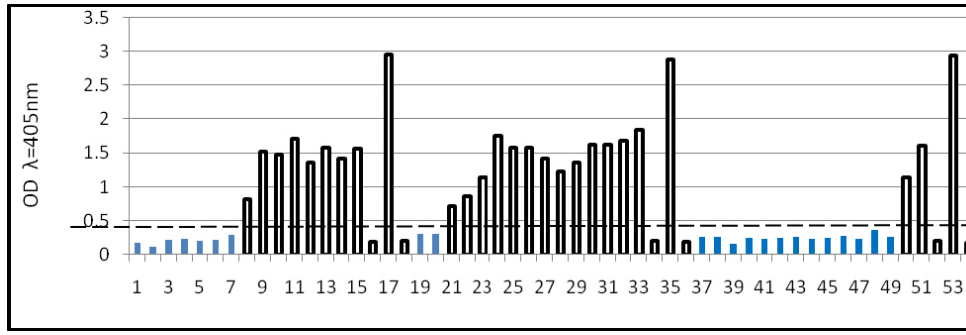

Fig. 4. DAS ELISA results after treatments with BION and EXIN 3 days before artificial virus inoculation with PVY (PVY inoculation of plants 3 days after treatments with elicitors)

Legend:

1-15 – plants treated with BION and inoculated with PVY
16- plants treated only with BION (for toxicity)
17- plants infected with PVY only (K+)
18- non treated and non inoculated plants (K-)
19-33 - plants treated with EXIN and inoculated with PVY
34 - plants treated only with EXIN (for toxicity)
35 - plants infected with PVY only (K+)
36 - non treated and non inoculated plants (K-)
37-51 - plants treated with BION + EXIN and inoculated with PVY

52 - plants treated only with BION + EXIN (for toxicity)
53 - plants infected with PVY only (K+)
54 - non treated and non inoculated plants (K-)

Treatment of the pepper plants with the two elicitors of SAR induced resistance against PVY resulting in reduction of the virus symptoms in all schemes. Near about half of the pepper plants treated with BION remained negative for PVY infection after virus inoculation (Fig. 4). The situation of the pepper plants treated with EXIN was completely different. We received only around 13% (2 plants from 15) non infected plants after virus inoculation (Fig. 4).

The best result was received after treatment of plants with the combination BION+EXIN. The healthy plants were 86% (13 from 15 plants) 21 days after the infection with PVY (Fig. 4).

All the pepper plants treated 1 day after virus inoculation were infected with PVY with the exception of only one plant treated with BION which was virus free (Fig. 5).

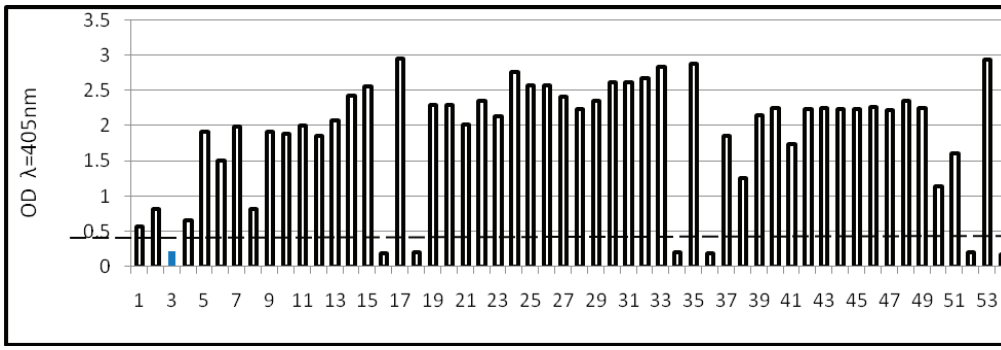

Fig. 5. DAS ELISA results after treatments with BION and EXIN 1 day after artificial virus inoculation with PVY (Inoculation of plants 1 day before treatments with elicitors)

Legend:

1-15 – plants treated with BION and inoculated with PVY
16- plants treated only with BION (for toxicity)
17- plants infected with PVY only (K+)
18- non treated and non inoculated plants (K-)
19-33 - plants treated with EXIN and inoculated with PVY
34 - plants treated only with EXIN (for toxicity)
35 - plants infected with PVY only (K+)
36 - non treated and non inoculated plants (K-)
37-51 - plants treated with BION + EXIN and inoculated with PVY
52 - plants treated only with BION + EXIN (for toxicity)
53 - plants infected with PVY only (K+)
54 - non treated and non inoculated plants (K-)

Discussion

From the different treatment schemes the combination treatments with EXIN+BION was successfully used to prevent infection of PVY in 86% of the pepper plants cv. Kurtovska kapia applied once, 3 days before virus inoculation. The treated plants in this scheme were without any symptoms. The treatments made 1 day after virus inoculation proved to be inffective for control of PVY infection.

BION was evaluated in greenhouse and field tests to control different phytopathogenic fungi and some bacteria. Its appliance prior to artificial inoculations induced resistance against fire blight and bacterial canker of tomato with a reduction of disease severity [1, 16]. Very little data is available concerning induction of resistance against viruses. Positive results were obtained in experiments with sugar beet for resistance to *Tobacco necrosis* virus [2] and it was established that pretreatments of tomato and pepper seedlings reduced the concentration of ToMV and TMV, respectively [8]. EXIN was developed by a Vietnamese company and it was established that pretreatments reduced damages by phytopathogenic bacteria and fungi (*Pseudomonas solanacearum, Erwinia carotovora* and *Sclervtium rolfsii*) of tomato, potato and cabbage [5].

In this study we confirm that pretreatments with BION and EXIN induce resistance against plant viruses. Our results also confirm that treatments with EXIN of infected plants caused no significant effect on the development of the disease as previously reported [5] and we established that treatments with BION of infected plants had also no significant effect for control of PVY infection.

The combined pretreatment with BION and EXIN was applied in our previous study to control PVY infection in tomato cultivar Ideal where 14 of 15 treated plants remained virus free [12]. Similar results were achieved in this study with pepper plants where 13 of 15 treated plants remained virus free in confirmation that this combination scheme is successful for control of PVY infection of vegetable crops.

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MONITORING OF FUNGICIDES USED AGAINST POWDERY MILDEW AND APPLE SCAB IN NON-COMMERCIAL ORCHARDS IN THE SOFIA FIELD

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Abstract

Aim: A monitoring of plant protection aimed at the treatment by fungicides against apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*, most important fungal diseases reducing the yield from apple orchards and aggravating its quality.

Material and Methods: The monitoring encompassed four locations in the Sofia field. It includes mainly inquiry concerning fungicides used for spraying and cultivars grown in the orchards. Field observations were realized during the vegetation period for manifestation of symptoms of apple scab and powdery mildew.

Results: The number of chemical treatments is minimized significantly in comparison with those recommended for plant protection of industrial plantations. Only fungicides approved in Bulgaria were used.

Conclusion: The main conditions for minimizing of fungicide treatments are: application in the appropriate phenological phase, planting of cultivars resistant to pathogens and prevention against the initial infection.

Introduction

The most important diseases of apple that reduce yield and deteriorate produce quality are apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*) [1, 2, 10]. There are many fungicides on the market with different effect and active ingredient. Each company promotes its product as the solution to the problem but the use of fungicides should comply with certain requirements and one of them is not to create resistant forms in the population of the pathogen [6]. This imposes limitations as to the number of treatments with one and the same fungicide during vegetation, when the active ingredient of the preparation accounts for a systemic effect on the pathogen [3, 8, 10, 13]. That is why the label of every fungicide on the local market should contain information about the maximum number of treatments per vegetation.

In Bulgaria, a lot of pest control products for cultivated plants can be purchased and used by people, who are not plant protection experts but they own or handle farms with small noncommercial orchards – an old and stable tradition in our country.

Plant protection on such farms has its peculiarities, mostly related to the safety of people and farm animals because people's houses, animal housing and water sources, etc., are often in close proximity to the cultivated plants that are subject to chemical treatment. This imposes the careful use of purchased chemicals to protect people and animals from poisoning and keep the environment clean. The control of plant protection safety and efficiency is in the prerogatives of the specialized division of the BAFS[1]. The scientific institutes of the Agricultural Academy also render assistance to these farms. Whenever necessary to use fungicides, the farmers can seek advice in the regional divisions of the agency and the scientific institutes. To be able to give a competent advice, the experts should have preliminary information about the local phytosanitary conditions, which depend not only on the given environment but also on the preferences of the farmers to the cultivated plants and cultivars, the size and structure of the farm, etc.

The present article reports data of the monitoring realized during the period October 2012 – September 2013. It was aimed at the fungicide treatment and its effi-

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cacy against apple scab and powdery mildew on different apple cultivars grown in orchards in four locations in the field of Sofia.

The results of the monitoring showed that the control of both diseases on small non-commercial farms can be efficient with a limited number of fungicide treatments conformed to the diseases' cycle.

The other important prerequisite for efficiency is the choice of cultivars.

Material and Methods

The monitoring encompassed four locations in the Sofia Field, namely Sofia City (Capital municipality), the municipalities of Bozhurishte, Kostinbrod and Slivnitsa. It was targeted at the apple cultivars, grown on small non-commercial farms (Table 1) and the plant protection activities against apple diseases during the period October 2012 – September 2013. It was fully supported by the private farmers, who had asked the advice of the Regional Plant Protection Division of BAFS as well as the Department of Phytopathology of the Nikola Pushkarov Institute of Soil Science, Agrotechnologies and Plant Protection (ISSAPP) in Sofia.

The choice of locations was finalized after a survey among the farmers, inquiring into information about the fruit cultivars in the orchards, owners' education and expertise in the field of apple diseases, how the fruit trees were taken care of motivation for cooperation, consent and cooperation to access the farm.

The chosen locations were examined for powdery mildew and apple scab infection in early summer (June 5, 2013 – July 17, 2013). Leaf samples were collected for additional laboratory analysis. The presence of infection from both pathogens was detected microscopically. The spread of both diseases was recorded as percentage of leaf infection (one sample - thousand leaves per cultivar). The damage index (DI) on hundred fruits per cultivar was calculated by McKiney`s formula [7, 12].

There were two orchards in the area of Kostinbrod. The first one was on the territory of ISSAPP, where the inter row space was maintained as fallow and weeds in the rows were treated with herbicides. Apple trees of cv. Florina were planted in a neighboring private plot in the autumn of 2012. In this case, the soil was cultivated only around the tree trunks and the remaining area was covered with grass.

The orchard in the area of Slivnitsa was also created in the fall of 2012 on a preliminarily prepared plot. This plot was kept free of weeds during the vegetation in 2013 by means of shallow soil cultivation, trenching around the tree trunks and spraying with herbicides against perennial weeds.

In Stolichna municipality, the observations were carried out in the yard of a private summer house. The yard was covered with grass. The trees were old, with high trunks and large canopies. The soil under them had not been culti-

vated, except for shallow tillage prior to fertilization. The grass between the rows was mowed and that under the trees - treated with herbicides.

In the municipality of Bozhurishte, the observations were done in the yard of an amateur pomologist. He used to trench every year, making the so called "watering cup". The unwanted grass on the rest of the area was destroyed mechanically or by herbicide treatment during the preparation of the soil for vegetable or flower growing.

Results and Discussion

According to preliminary data, the specified 12 cultivars had a different level of resistance to the pathogens of apple scab and powdery mildew [4, 5, 6, 9]. Moreover, the apple trees in the different locations were grown in different conditions with regard to the agrotechnical and plant protection activities (Tables 1 and 2).

Table 1. Characteristic of apple variety

№	Cultivars	powdery mildew	apple scab
1	Florina	weak susceptible	resistant
2	Golden delicious	susceptible	susceptible
3	Granny Smith	moderate susceptible	weak susceptible
4	Idared	severe susceptible	resistant
5	Jonathan	severe susceptible	moderate suscepible
6	Kantarka	weak susceptible	severe susceptible
7	Melrose	susceptible	severe susceptible
8	Mutsu	resistant	resistant
9	Prima	weak susceptible	resistant
10	Red delicious	resistant	susceptible
11	Starksimson delicious	weak susceptible	susceptible
12	Yellow bellflower	susceptible	severe susceptible

Table 2. Description of the objects and fungicide use

Location (features)		**cultivars**
Sofia, Capital municipality (yard of summer villa)		Jonathan, Kantarka, Red delicious, Yellow bellflower
fungicides/active substances	**BBCH***	**Phenological phase**
urea	95 - 97	50% of leaves are yellow/end of fall of the leaf
Strobi (kresoxim - methyl)	10 - 54	„mouse ears”
Strobi (kresoxim - methyl)	57	pink (blossom) button
Score (difenoconazole)	73	after the fall of young fruits in June
Location (features)		**cultivar**
Bozhurishte municipality (garden out of the town)		Florina, Golden delicious, Melrose, Prima, Starksimson delicious
fungicides/active substances	**BBCH**	**Phenological phase**
urea	95 - 97	50% of leaves are yellow/end of fall of the leaf
Champion (copper hydroxide)	0	repose
Tiram (dimethyldithiocarbamate)	10 - 54	„mouse ears”
Horus (cyprodinil)	57 - 59	pink (blossom) button – white ball
Horus (cyprodinil)	69	end of florescence
Tiram (dimethyldithiocarbamate	71	fruit size up to 10 mm
Shavit (triadimenol)	73	after the fall of young fruits in June
yard of summer villa		Mutsu
fungicides/active substances	**BBCH**	**Phenological phase**
Urea	0	repose
Score (difenoconazole)	10 - 54	„mouse ears”
Score (difenoconazole)	10 - 54	„mouse ears”
Location (features)		**cultivar**
Kostinbrod municipality (garden out of the town)		Florina, Golden delicious, Granny Smith, Idared, Red delicious
fungicides/active substances	**BBCH**	**Phenological phase**
Strobi (kresoxim - methyl)	10 - 54	„mouse ears”
Strobi (kresoxim - methyl)	57 - 59	pink (blossom) button – white ball
Score (difenoconazole)	73	after the fall of young fruits in June
Location/ features		**cultivars**
Kostinbrod municipality (garden out of the town)		Florina
fungicides/active substances	**BBCH**	**Phenological phase**
Urea	95 - 97	50% of leaves are yellow/end of fall of the leaf
Urea	0	repose
1% Bordeaux mixture	1	start of leaf sprout
Champion (copper hydroxide)	57	pink button
Champion (copper hydroxide)	69	end of florescence
Champion (copper hydroxide)	73	after the fall of young fruits in June
Champion (copper hydroxide)	39	about 90% of shoots are developed to the final extent
Location (features)		**cultivars**
Slivnitsa municipality (garden out of the town)		Florina, Golden delicious, Red delicious
fungicides/active substances	**BBCH**	**Phenological phase**
Score (difenoconazole)	11 - 54	the leaf blades do not reach final extent
Score (difenoconazole)	31 - 32	about 20-30% of shoots are developed to the final extent
* Key for phenological growth stages according to Meier et al. [10]		

The percentage of healthy leaves in the vicinity of Kostinbrod was between 90.3% (Golden Delicious) and 98.4% (Florima) (Table 3).

Apple scab was found in Golden Delicious – 7.3%, Florina – 3.8% and Idared – 3.3%. Powdery mildew was found in all cultivars but the leaves were only slightly infected – 5.5% in Red Delicious, 4.9% in Idared and 1-1.6% in Florina.

The trees of cv. Florina on the first plot were not treated. Scab control was probably additionally enhanced by the autumn ploughing into the soil of the fallen leaves as a measurement against pathogen's development and creation of infectious potential for the following year.

The application of fungicides with copper content in the newly planted orchard with cv. Florina served as prevention against the fire blight bacteriosis that had started emerging in the area (Table 3).

Table 3. Kostinbrod municipality

Date of observation 05.06.2013	healthy %	sick - %	
cultivars		apple scab	powdery mildew
Golden delicious	90.3	7.3	2.4
Red delicious	94.5	0.0	5.5
Idared	91.8	3.3	4.9
Granny Smith	96.6	0.0	3.4
Florina	98.4	0.0	1.6
new garden in lawn			
Date of observation 05.06.2013	healthy %	sick - %	
cultivars		apple scab	powdery mildew
Florina	95.2	3.8	1.0

Only scab infection was observed in the area of Slivnitsa (Table 4). The lack of powdery mildew infection could be explained with the lack of primary infection as well as the fungicide treatment at the beginning of the vegetation. The existence of scab infection indicates the necessity for additional plant protection activities against the disease. Such activities should include not only spraying but also prevention measures such as autumn ploughing of the fallen leaves into the soil.

Table 4. Slivnitsa municipality

Date of observation 05.06.2013 cultivars	healthy %	sick - %	
		apple scab	powdery mildew
Golden delicious	95.0	5.0	0.0
Red delicious	98.0	2.0	0.0
Florina	96.8	3.2	0.0

The scab infection in the orchard of Stolichna municipality was between 2-4.3% in all cultivars (Table 5). This was possibly related to the preventive carbamide spraying in the fall of 2012 that reduced the accumulation of infectious potential in the orchard as well as the use of efficient fungicides for scab control at the start of vegetation.

Powdery mildew was found only in cv. Jonathan – 2.9% and Kantarka – 3.8%. This showed the efficiency of pruning as a prevention measure against the disease as well as the efficiency of the fungicides used (Table 5).

Table 5. Capital municipality

Date of observation 09.07.2013. cultivars	healthy %	sick - %	
		apple scab	powdery mildew
Red delicious	97.3	2.7	0.0
Jonathan	94.2	2.9	2.9
Yellow bellflower	95.7	4.3	0.0
Kantarka	94.2	2.0	3.8

The data for Bozhurishte municipality showed a comparatively severe scab infection, found in all cultivars and ranging from 6.6% in Melrose to 12.3% in Golden Delicious (Table 6), while powdery mildew infection was minor and only in two varieties – Prima and Melrose.

The comparatively higher percentage of scab infection could be explained with the lower efficiency of the fungicide sprayings (Table 6).

Table 6. Bozhurishte municipality

Date of observation 17.07.2013 cultivars	healthy %	sick - %	
		apple scab	powdery mildew
Golden delicious	87.7	12.3	0.0
Florina	90.7	9.3	0.0
Starksimson	88.0	12.0	0.0
Melrose	90.1	6.6	3.3
Prima	87.3	10.9	1.8
Mutsu	100.0	0.0	0.0

The trees of cv. Golden Delicious, Granny Smith, Idared, Jonathan, Melrose, Mutsu, Red Delicious and Yellow Belle Fleur that gave fruit during the current vegetation were infected by scab up to 1.2–3.2% (Table 7) cv. Florina proved to be resistant.

Table 7. Manifestation of apple scab on the fruits

LOCATION (municipality)	CULTIVAR	DI %
KOSTINBROD	Florina	no fruits
	Florina	0.0
	Idared	2.5
	Golden delicious	2.8
	Granny Smith	2.3
	Granny Smith	1.5
	Red delicious	2.1
BOZHURISHTE	Florina	0.0
	Golden delicious	2.5
	Melrose	1.4
	Mutsu	1.2
	Prima	no fruits
	Starksimson	2.5
CAPITAL	Jonathan	1.7
	Red delicious	1.8
	Yellow bellflower	3.2
SLIVNITSA	newly planted trees	no fruits

Conclusions

The results of the monitoring show that the apple scab disease was frequent in the small non-commercial orchards with minimized fungicide application. Powdery mildew infection was detected more scarcely in the monitored cultivars.

Cultivar Florina confirmed its resistance to apple scab infection on fruits. The presence of the pathogen in leaves even if a small degree is important for the infectious cycle during the summer and for the accumulation of infectious potential in the orchard.

The use of fungicides can be minimized if applied in the critical phases of the trees' development, when initial infections take place.

The number of fungicide treatments in the small farms is limited for objective reasons. Other activities for prevention such as removal of fallen leaves, pruning, etc., must be done or required in order to avoid the accumulation of infection for the next vegetation.

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ДОКЛАДИ/REPORTS

Тематично направление:
ИНВАЗИВНИ ВИДОВЕ

Topic:
INVASIVE SPECIES

INVASIVE PLANT SPECIES ALONG THE MAJOR RIVERS IN STRANDZHA NATURAL PARK

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Aim: We investigate the spread of invasive plant species along the major rivers in Strandja Natural Park: Rezovska, Veleka and Mladezhka.

Material and Methods: Object of the study are five invasive plant species: *Ailanthus altissima* (Mill.) Swinge, *Amorpha fruticosa* L., *Bidens frondosa* L., *Erigeron annuus* (L.) Desf. and *Robinia pseudoacacia* L. The research was conducted by the route method in 2013.

Results: We have mapped the spread of the invasive plant species along the rivers Rezovska, Veleka and Mladezhka. We have determined the number of locations and the number of invasive plant species. We have identified risk areas of occurrence and distribution of the invasive species.

Conclusion: It is taking measures to limit the spread of the invasive plant species and a gradual recovery of the affected habitats.

Keywords: Invasive plants, rivers, Strandzha Natural Park

Introduction

The invasive alien species are the second most important cause of biodiversity loss in the world, after the loss and destruction of the habitats. For the scale of the process is revealing the fact, that in Europe as foreign identify 6658 species of terrestrial plants. Approximately 10-15% of these species are considered as invasive [12].

In Bulgaria, according to DAISIE (Delivering Alien Invasive Species Inventories for Europe), were distributed 734 alien species of vascular plants (17.89% of the vascular flora of Bulgaria). Of these, 60 species are described as being invasive [9], but their number is greater. Most of the invasive plants in Bulgaria are carried with a different purpose: to decorate, erosion control, medical uses, food, wood and others. Only a few plants have infiltrated the country accidentally, for example by importing other types of plants. All alien species once come in natural or semi-natural habitats and ecosystems interact with native species and affect local biodiversity in the following ways: Competition with the native

species for water, nutrients and light; displacement of native species; hybridization with native species (genetic pollution); changing of the habitats.

Besides biodiversity, invasive alien species threaten human health (some of them cause allergies) or cause serious economic damage (weeds or pests in agricultural and forestry crops). In Europe, most of the worst invasive species cause loss of biodiversity and lead to changes in the structure of the communities, while a small number cause direct damage to species of conservation status.

We investigate the spread of the invasive plant species along the major rivers in Strandzha Natural Park: Rezovska, Veleka and Mladezhka.

Strandzha Natural Park is the largest protected area in Bulgaria. It was declared a national park by Order NoRD-30 from 24 January 1995, State Gazette number 15/1995. The goal is long-term conservation of the unique nature of Veleka River and Rezovska River watersheds and ensuring sustainable socio-economic development in the region. The category of the park is changed in the natural park by Order NoRD-350 from 14 July 2000, State Gazette number 66/2000. Within its boundaries are included 21 settlements with nearly 8000 people. In the park areas present their own status of nature conservation: 5 reserves, 19 protected areas and 7 landmarks. After twice reducing the area of the park in 2001 and 2013, its total area is 116054.21 ha.

Rezovska River rises east of the town Kovchas, Turkey. After Paspala village, Turkey, is a border river between Bulgaria and Turkey. The river flows mostly eastward in a deep valley and empties into the Black Sea through the estuary. In its middle and lower course have formed a number of meanders. The length of the river on Bulgarian territory is 70 km with a catchment area of 183.4 km^2.

Veleka River stems from many karst springs located 3 km southwest of Ahlatla village, Turkey and about 5 km southeast of Belevren village, Bulgaria. The length of the river in Bulgarian territory is 123 km, and its catchment area is 994.8 km^2. In the upstream the river valley is narrow and deep and almost entirely forested. Near the mouth of the river valley is wide and a typical estuary. The width of the riverbed is also modified: from 20 m upstream to 80 m at the confluence of Mladezhka River. At the mouth of Veleka River its width can reach up to 150 m with an average high water. Changes, and the depth of the river, which at the upper reaches is only 0.8-1.0 m, and estuary reaches 7-8 m. The river is navigable for more than 9 km.

Mladezhka River has a length of 30.4 km and a catchment area of 232.2 km^2. Its karst springs are located about 1 km west of Mladezhko village, in the eponymous landmark. The river flows in a wooded valley south of the Bosnian ridge and flows into Veleka River in the area Thracian camp located south of Zabernovo village. In large part it dries droughts in downstream [4].

Material and Methods

Object of the study are five invasive plant species: *Ailanthus altissima* (Mill.) Swinge, *Amorpha fruticosa* L., *Bidens frondosa* L., *Erigeron annuus* (L.) Desf. and *Robinia pseudoacacia* L. The survey covers bands with an average width of 20 m on both sides of the

major rivers in Strandja Natural Park: Rezovska (only Bulgarian bank of the river), Veleka and Mladezhka. The research was conducted on the route method in 2013. The identification of the invasive species is carried out with the Handbook for Plants in Bulgaria [3]. The coordinates of the boundaries of the polygons, occupied by habitats, were detected with GPS Garmin Oregon 450. The used coordinate system was WGS 84 UTM 35N. The file conversion from gpx to shp format is done with free software DNR GPS. To determine the area, occupied by polygons and length of transects, mapping of terrain, as well as mapping, we used software product Quantum GIS ver.1.7.3.

Results and Discussion

We established five invasive plant species along the major rivers in Strandzha Natural Park. Two of these are trees, one is a shrub and two are herbaceous plants. Two of species: *Ailanthus altissima* and *Robinia pseudoacacia* are included in the List of the worst invasive species in Europe. Four of species: *Ailanthus altissima, Amorpha fruticosa, Bidens frondosa* and *Robinia pseudoacacia,* are among the ten most dangerous invasive species in Bulgaria [9]. *Erigeron annuus* is a new species of floristic region of Strandzha. Here is a brief description of each of the species:

***Ailanthus altissima* (Mill.) Swinge (Simaroubaceae)**

Global distribution: The origin is from East Asia (China and North Vietnam). It is naturalized in all continents except Antarctica. It was brought from China to Europe for the first time in 1740.

Distribution in Bulgaria: It is found throughout the country, from sea level to 1800 m asl. It was brought to the country in the period 1888-1900, and early in 20th century is considered the second most widespread alien species after *Robinia pseudoacacia*. The first evidence of its mass spread spontaneously in the country since 1928 [9].

Morphology: Tree 20-30 m high with a diameter of 80 cm, gray-brown bark, smooth, in older trees slightly longitudinally cracked, young twigs yellowish or blackish, slightly fibrous. Leaves imparipinnate, 45-100 cm long, located at the tips of young shoots. Leaflets 11-25 (42) in number, 4-12 mm long and 2.5-4.0 mm wide, oval lanceolate or ovate lanceolate, edge ciliate, hairy young, old almost naked, grinding with an unpleasant odour. Flowers greenish yellow, gathered in 10-20 cm long racemes. The fruit is a nut with wing, 3-4 cm long, rhombic wrong. Flowering: June – July. Fruiting: October [8].

Biology: It grows very quickly and is able to reach a height of 15 m in just 25 years. Never reaches more than 50 years of age, but are reported some individuals of 70 years of age. Insect pollinated plant. Seeds productivity is remarkable: an individual can form up to 325.000 seeds per year. Fruits and seeds are spread by wind. After formation, they may remain on the branches until spring and this strongly prolongs the spread. Water and cars can also help spread. The germination of the seeds is very high: 90%, and in the laboratory to reach 96%. The plant is propagated very successfully by vegetative. The destruction or damage to the main stem stimulates stem and root cuttings. Unlike most wood species *Ailanthus altissima* form root cuttings even when the main shaft is normally developed. Even the seedlings form a horizontal roots, able to form new shoots. In one year the average

growth of stem sucker is 180 cm, a root sucker: 80 cm and 40 cm of stalk of seed origin. New shoots can be formed at a distance of 26.5 m from the main stem [1].

Ecology: The species tolerates a wide range of temperature, soil moisture and atmospheric humidity. One of the most drought-resistant tree species of the temperate zone [9]. In terms of lighting regime it prefers well-lit areas, but successfully compete with other tree species even in the presence of only 2% to 15% of the normal amount of sunlight. The plant inhabits successfully extremely varied soils (including poor, acidic and salty) and is markedly resistant to impacts, leading to the destruction of habitats. It is highly tolerant to most industrial air pollutants (including high doses of dust and sulfur dioxide). The plant is susceptible to ozone [1].

The plant releases allelopathic substances that inhibit the development of competing species. Such inhibitors are the most abundant in the bark and the roots, but also occur in the leaves, seeds, and wood. In young plants their quantity is higher than in older [6].

The plant has a high resistance against herbivores. The seeds are also not used as food of animals. In its native range it has established relationships with 32 species of arthropods and 13 species of fungi. Of these three insect species have the potential to achieve the biological control: *Eucryptorrhynchus chinensis*, *Eucryptorrhynchus brandt* and *Orthopagus lunulifer* [15]. Besides them in its country larvae of several species of the Lepidoptera, as *Actias selene* and *Eurema hecabe*, use the leaves for food. In North America larvae of *Atteva aurea* also feed on the leaves of the plant, although the butterfly comes from Central and South America.

***Robinia pseudoacacia* L. (Fabaceae)**

Global distribution: The origin is from North America. It is naturalized in all continents without South America and Antarctica. It was brought to Europe for the first time in 1601 as an ornamental plant in France.

Distribution in Bulgaria: It is found throughout the country, from sea level to 1100 m asl. [7]. It was introduced in culture in our country in the middle of 19th century in private gardens. The first evidence of spontaneous distribution in natural habitats in the country was since 1903 [9].

Morphology: Tree 15-25 m high, dark-gray bark, longitudinally cracked, young twigs olive to dark-gray, smooth. Leaves imparipinnate, 5-30 cm long, the base with two 15-40 mm long prilistnitsi converted into spines. Leaflets 7-21 in number, 25-45 mm long and 12-25 mm wide, elliptic or ovate, entire, green above, gray-green beneath. Flowers white, gathered in 10-20 cm long racemes. The fruit is oblong beans, strongly laterally flattened, 5-10 cm long and 1 cm wide, dark brown to black, naked, with 4-10 seeds. Seeds 5-6 (8) mm long, 3-4 mm wide, reniform, dark brown to black, smooth. Flowering: May – June. Fruiting: September – October [7].

Biology: Life expectancy reaches 90 years. It has an extensive root system: as 6-8 m deep, and 50 m width. There are a variety of data about the rate of increase: according to some authors average 0.75 m per year, while others 12.8 m to 10 years, 20.7 m in 25 years, 25.6 m for 40 years. Insect pollinated plant. It starts to form fruits and seeds of 6 years, reaches a maximum between 15 to 40 years of age and continued until age 60. The plant forms fruit each year, but in the 1-2 or 2-3 years (data from different authors) forms a large

number of fruits. Fruits and seeds are spread by wind. After formation, they may remain on the branches until spring and this strongly prolongs the spread. The plant forms a large number of seeds, but germination is low (7-8%). The seeds remain viable for more than 10 years. It was found that 15% of them retain their germination about 40 years, and individual seeds germinate even after 88 years. The seedlings have rapid growth of open well-lit areas. They occupy new territories side by root cuttings. This leads to a dense mono-dominant communities completely inhibit the growth of natural vegetation.

The plant is propagated vegetatively very successfully by the formation of stem and root cuttings. The formation of root cuttings started when the plants are 4-5 years old. It is readily induced in the destruction of above-ground parts. The process is stimulated naturally in the presence of open areas, good lighting, especially in sandy soils. The shoots need enough light to survive [12].

Ecology: This is one of the most easygoing to soil and climate tree species. It can borrow and disrupted habitats. Roots come in symbiosis with nitrogen-fixing bacteria of the genus *Rhizobium*, which allows species to rapidly colonize acidic and contaminated soil. The symbiosis enhances fertility of the colonized habitats, which affects their floristic composition, reducing the number of species that prefer nitrogen-poor soils. The plant does not tolerate strong shading [9, 12]. The tree holds allelopathic effect comparable to that of *Ailanthus altissima* [2].

***Amorpha fruticosa* L. (Fabaceae)**

Global distribution: The origin is from southeastern parts of North America [9]. It is naturalized in Europe and temperate parts of Asia. In Europe it was brought for the first time as an ornamental plant in England in 1724.

Distribution in Bulgaria: It is found throughout the country, from sea level to 1200 (1500) m asl. It was introduced in culture in the early 19th century decorative and anti-erosion purposes. The first evidence of spontaneous distribution in natural habitats in the country was since 1898 [9].

Morphology: Shrub 1-3 (4-6) m high, bark black-gray or brown, smooth. Leaves imparipinnate, 10-20 cm long, in the base with 4-5 mm long, lance styliform, falling stipules. Leaflets 11-25 in number, 15-40 mm long and 8-20 mm broad, ovate or elliptic, entire, dark green above, gray-green beneath. Flowers blue or purple, gathered in 7-15 cm long racemes. The fruit is oblate beans, 0.7-0.9 cm long and 3 mm wide, brown, nude, with 1 (2) seeds. Seeds 5.0 mm long, 1.8 mm wide, oblong-ovate, brown, smooth. Flowering: May – July. Fruiting: August – October [7].

Biology: Insect pollinated plant. Fruits and seeds are spread by wind, water currents or animals. After formation, they may remain on the branches until spring and this strongly prolongs the spread [9]. The germination of the seeds is 45-50%. The plant is propagated vegetatively very successfully: by stem and root cuttings, green cuttings and layers.

Ecology: The plant prefers a well-drained and nutrient-rich soil. It tolerates prolonged flooding and even salt water. It occurs on both the acid and on the neutral and alkaline soils. Easily adapts to and barren, dry and sandy soils [13]. This is due to the nitrogen-fixing bacteria that live in symbiosis with the roots of the plant. It prefers well-lit areas, but bears

little shading. It withstand low temperatures, and if frost, quickly recovering from the stem and root cuttings [9, 12].

It has a high allelopathic potential compared with a large number of invasive species, and including *Ailanthus altissima* and *Robinia pseudoacacia* [2]. All parts of the plant contain substances repellent and highly toxic effect on insects [9].

***Bidens frondosa* L. (Asteraceae)**

Global distribution: The origin is from North America. It is naturalized in Europe, Asia and New Zealand. In Europe, it was first established in 1834 on the island of Sicily, Italy [10].

Distribution in Bulgaria: It is found in many parts of the country, from sea level to 1000 m asl. [14]. For the first time the plant has been established in the country in 2001 in the vicinity of Pazardzhik [10]. The wide distribution of the species in the country testified that he penetrated much earlier, but remained unnoticed due to its similarity with the local species *Bidens tripartitus*. It was reported as new to the flora of Bulgaria in 2004 [9].

Morphology: Annual herbaceous plant with single stem, upright, 20-100 (180) cm high, with four corners to round, longitudinally furrowed, almost naked, often purple at the bottom, branched, branches opposite. Leaves imparipinnate, leaflets 1.5-8.0 (10) cm long and 0.5-2.5 (3.0) cm wide, oblong lanceolate to ovate lanceolate, dark green, sometimes purple, absently short fibrous bare. Flowers in basket inflorescence. Basket 10-15 (20) mm in diameter, multiflowers, located at the tips of the branches and in the axils of the upper leaves and forming complex inflorescences, rarely single. Phyllaries in 2 rows, the outer phyllaries, green or purple; internal phyllaries elongated ovoid, dark brown to almost black. Inflorescences bed flakes long as fruits other. The flowers are tan, tubular, about 5 mm long, tongues colors mostly undeveloped. Fruit is achenes with 2 awn, 5-8 (10) mm long (excluding awn), 2.0-3.5 mm wide, wedge-shaped, flattened, brown, fibrous diluted to almost naked. Flowering: June – October. Fruiting: July – November [14].

Biology: Insect pollinated plant. It forms a very large number of seeds – up to 7000. Fruits and seeds are spread by wind or animals. The fruits are of two types: external and internal. The external fruits are wider adapted to spread short distances, with a short rest period. The internal fruits are narrow and long, with a longer beard adapted to distribute a greater distance and with a long period of rest [9].

Ecology: The plant prefers moist sites. It endured somewhat of a drought, so there is a greater ecological plasticity than local species *Bidens tripartitus*. Often occurs with both him and with *Bidens vulgatus*, forming mixed groups. Compete for space and nutrients with native species and especially *Bidens tripartitus* and *Bidens cernuus*. Compared with them grow considerably faster and reaches two times larger [9].

***Erigeron annuus* (L.) Desf. (Asteraceae)**

Global distribution: The origin is from North America (Canada and the Northern U.S.). It is naturalized in Europe, Asia, Central America and Australia. It was brought to Europe as an ornamental plant in the late 17th century [9].

Distribution in Bulgaria: It is found in many parts of the country, from sea level to 1000 m asl. [14]. Plant species is not listed in floristic region of Strandzha. In Bulgaria it considered unintentionally introduced species, although it may have been introduced as an

ornamental plant. It was first reported in Bulgaria in 1974, by material collected in artificial plantation from *Populus deltoides* in northeastern Bulgaria in 1971 [9].

Morphology: Annual herbaceous plant, rarely biennial or perennial herbaceous plant. Stem erect 35-100 (150) cm high at the bottom of unbranched, short-fitting to spread hard-fibrous long, branched at the top, just short fiber to bone. Leaves at the base of the stem ovate or elliptic, 5-10 (17) cm long and 2.5-4.0 (7.0) cm wide, coarsely toothed, hairy absently, declining during flowering. Lower stem leaves ovate lanceolate or elliptic, 6-10 cm long and 1.5-2.5 cm broad, jagged. Stem upper leaves elliptic lanceolate. Flowers in basket inflorescence. Multiflowers baskets forming paniculiform or corymbiform synflorescences. Involucral leaflets linear lanceolate, almost identical, green or brown. Flowers two types: tongue and tubular. Ray florets female, numerous (80-120), white or pale blue, 4-6 mm long, up to 2 times longer than involucral leaflets. Disk florets bisexual, yellow, 2-2.5 mm long. Fruit is achenes, 1.25 mm long, shortly pubescent. Pappus 2-seriate, outer of scales or setae, inner absent in ray florets, in disk florets of 10-15 long bristles. Flowering: June – September. Fruiting: July – October [14].

Biology: Insect pollinated plant. It forms a huge number of seeds: up to 10000-12000. Fruits and seeds are spread by wind. Seeds not need a period of rest in order to germinate. However, they may retain their vitality for a long time – over 20 years. Under the right conditions the plant can give more than one generation per year. Some of the seeds germinate in the fall, wintering young plants form leaf rosettes, and the next year formed flowering stems [9].

Ecology: The plant prefers a well-lit areas, but tolerates partial shade. It occurs primarily on moist to moderately moist soils. It is not pretentious to soil conditions and tolerates clay or rocky soils. It grows well on very acid soils. Aggressively spreads to areas with disturbed vegetation cover. According Szabó [11] it has a high allelopathic potential – several times higher than that of invasive tree species. According Fabbro & al. [5] in real field conditions *Erigeron annuus* did not show greater allelopathic activity of native species.

We mapped the spread of the invasive plant species along the rivers: Rezovska, Veleka and Mladezhka (Fig. 1). We found that Rezovska River is the least affected by the invasion of alien species. The reasons for this are complex. First is the location of the river on the border between Bulgaria and Turkey. The strengthen border controls during the Cold War and the establishment of permanent boundary fencing at a great distance north of the border led to the almost complete absence of business near the river. Exceptions are two villages, located on Bulgarian territory near the river – Slivarovo and Rezovo villages. It is in the vicinity river is most affected by the introduction of invasive species. Other important factors are: the mountainous nature of the terrain and the relatively low population of the area on both sides of the border. Strandzha Mountain is the most sparsely populated areas in Bulgaria. The state of the area, however, is changing. On July 2, 2006 due to heavy rains a flood destroyed much of the coastal vegetation along Rezovska River and its tributaries and changes the appearance of the river valley. We have found that it has a beneficial effect on the development of invasive species in two ways: 1. Accumulation of sediments suitable as a substrate for colonization by invasive species; 2. Destruction of natural vegetation and release niche for settlement of pioneer species. Another important reason for the presence

of invasive species is the planting of belts of *Amorpha fruticosa* and *Robinia pseudoacacia* on the Bulgarian coast near the mouth of the river in order to strengthen it.

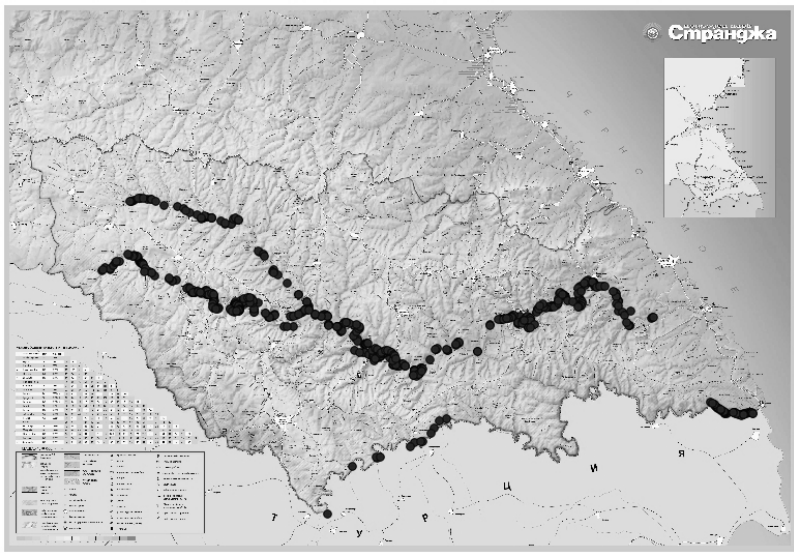

Fig. 1. Spread of the invasive plant species along the rivers Rezovska, Veleka and Mladezhka

Veleka River is most affected by invasion of alien species. The reasons for this are complex again. Near to the river are 6 villages (Zvezdets, Stoilovo, Gramatikovo, Kosti, Brodilovo and Sinemorets) and 3 holiday areas (in Kachul locality, south of the Gramatikovo village and south of the Brodilovo village). One of the arable land in the villages is located along the river. The reason for this is the more fertile soil and the possibility of irrigation. After reforms in agriculture, the land division and the depopulation of the area, most of the arable land were abandoned. Many of them accommodated invasive plant species. Another important reason for the increase of the invasion was the flood of 2006. In conversations with the locals is clear that after the flood their lands appear hitherto unknown *Ailanthus altissima* and *Amorpha fruticosa*, and *Robinia pseudoacacia* strongly expands its distribution. The third reason favoring the entry of invasive species is the presence of road infrastructure on one or both sides of the river on providing a link between the villages, villa areas and farmlands. It is located in a great part of its length: from the confluence of Mladezhka River to the mouth of Veleka River in the Black Sea, near to Sinemorets village.

We determined the number of locations, the area and the number of invasive plant species (Table 1). The distribution of identified specimens and localities for the three rivers is as follows: *Ailanthus altissima* – 1011 individuals in 125 localities, *Amorpha fruticosa* – 14556 individuals in 161 localities, *Bidens frondosa* – 137348 individuals in 97 localities, *Erigeron annuus* – 233 individuals in 12 localities and *Robinia pseudoacacia* – 10618 individuals in 264 localities.

We found the habitats along the major rivers in Strandzha Natural Park, that have invaded invasive species: 91E0 *Alluvial forests with *Alnus glutinosa* and *Fraxinus excel-*

sior (*Alno-Padion, Alnion incanae, Salicion albae*); 91G0*Pannonic woods with *Quercus petraea* and *Carpinus betulus*; 6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels; degraded habitat 6210 Semi-natural dry grasslands and scrubland facies: on calcareous substrates (Festuco-Brometalia); riparian galleries with *Fraxinus oxycarpa*; mixed deciduous forests; plantations of *Populus nigra*; shrublands; riparian strips of sand, gravel or rocks; hay meadows; abandoned farmland; road infrastructure (asphalt, stone covering, soil roads).

Table 1. Summary data for populations of invasive plant species along the major rivers in Strandzha Natural Park

River	Invasive plant species	Number of locations	Area (m^2)	Numbers
Rezovska	*Robinia pseudoacacia*	14	5547	334
	Amorpha fruticosa	20	4349	5994
	Bidens frondosa	8	1446	338
	Erigeron annuus	11	100	187
Veleka	*Ailanthus altissima*	114	44160	945
	Robinia pseudoacacia	207	142969	8894
	Amorpha fruticosa	137	55662	8013
	Bidens frondosa	89	28191	137010
	Erigeron annuus	1	20	46
Mladezhka	*Ailanthus altissima*	11	1902	66
	Robinia pseudoacacia	43	26416	1390
	Amorpha fruticosa	4	1240	549

We have identified risk areas of occurrence and distribution of the invasive species: sediments (sand and gravel), the mouth of the tributaries of the rivers, river branches that dry up in summer, riverbanks that were deforested during the flood in 2006, fishing grounds, roads, crossing rivers and edge of forest roads located near rivers. The number of the species was highest near the villages.

Conclusion

Along the major rivers in Strandzha Natural Park we found five invasive plant species: *Ailanthus altissima*, *Amorpha fruticosa*, *Bidens frondosa*, *Erigeron annuus* and *Robinia pseudoacacia*. As a result of deliberate human activity in the park are imported only two of them: *Robinia pseudoacacia* (cultivated for logging and erosion control purposes) and *Amorpha fruticosa* (cultivated for erosion control purposes). Today the majority of individuals of these species are spontaneously spread over a great distance from the places of culture and of considerably larger size. The other three species are caught in the park without deliberate human intervention.

The number of invasive plant species and their individuals are directly related to the length of the rivers and the number of settlements located nearby. We found that the main

reasons for the increasing spread of invasive species belong to two groups: anthropogenic impacts and catastrophic natural events.

The high reproductive potential of the invasive species and dense groups that they form are some of the most serious threats to populations of native species inhabiting the valleys of large rivers in the park.

All results are provided to the Directorate of Strandzha Natural Park in order to take measures be taken to limit the spread of invasive plant species and a gradual recovery of the affected habitats.

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ДОКЛАДИ/REPORTS

Тематично направление:
ЕКОСИСТЕМНИ ИЗСЛЕДВАНИЯ И УСЛУГИ

Topic:
ECOSYSTEM RESEARCH AND SERVICES

THE NEED TO INTRODUCE AN ENTRANCE FEE WHEN VISITING THE ECO-TRAILS IN BULGARIA (THE CASE OF THE "CANYON FALLS" TRAIL - SMOLYAN)

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Abstract

Aim: The main aim of the paper is to discuss the future development of "Canyon Falls" eco-trail and the need to introduce an admission fee to generate funds for maintenance.

Material and Methods: A survey of visitors to the eco-trail is conducted. In a single day 25 respondents are interviewed. A comparison is made with other similar sites in Bulgaria and Europe, where admission fee is collected.

Results: The survey results show that only 16% of the respondents believe that a fee should not be collected. The average value of the admission fee determined by visitors that replied with "yes" to the question about the need of introducing a fee is 3.58 BGN.

Conclusions Maintenance of eco-trails in Bulgaria requires generating of financial resources to ensure the quality and quantity of ecosystem goods and services provided to people by these natural sites.

Keywords: biodiversity, ecosystem goods and services, eco-trail, admission/entrance fee.

Introduction

Hiking and cognitive ecotourism in Bulgaria in the 90s of last century marked continuous development, showing increasing visitor interest, motivated mainly by the desire of

ordinary people to get in contact with nature and the existence of gradually improving infrastructure. A core element of hiking infrastructure is the availability of numerous trails developed throughout the country. There exist more than 100 trails in the country, which are actively promoted and are subject to targeted visit. The majority of eco-trails is located in mountainous areas and is included in the territorial scope of protected areas under the Protected Areas Act (PAA) or necessarily fall within NATURA 2000 sites. Spatial location of eco-trails is associated with sites that create high quality and quantity of ecosystem/landscape services. Valuation and preservation of ecosystem /landscape goods and services is a priority in the sustainable development of the EU Member States, as in our country. Similar findings are well known, as well as the fact that the development of eco-trails evolves chaotically, without clarification of the term "eco-trail", the criteria and standards for their construction and the responsibilities for their maintenance.

The development of a national program “Bulgarian eco-trails” is an initiative of the Bulgarian Association for Rural and Ecotourism (BARET) and the idea emerges with the construction of the first eco-trail in Bulgaria (Emenska trail) in 1997. The theoretical basis of the initiative are formulated in a paper presented by Petrov and Kisselkova [7] at the international scientific conference dedicated to the 110th anniversary of Sofia University “St. Kliment Ohridski” in 1998. The same authors [7] offer two restrictive benchmarks for the “eco-trail” category, namely attractiveness and biocentricity and twenty criteria to be met by this category. The idea of Bulgarian eco-trails is further developed by Kisselkova and Petrov [4] in the context of natural potential and sustainable development of mountain areas. Requirements for establishing tourist itineraries and trails are published by Todorov [9], which describe three development stages of each route and trail (preliminary, during period and after period). The boom which occurred in the organization and construction of trails since 2000 provoked a discussion at a special roundtable on the mountain trails in Bulgaria (2006). It was organized by the “Mountains and People” Association in cooperation with the project “Conservation of Globally Significant Biodiversity in the Landscape of Rhodope Mountains” of United Nations Development Programme (UNDP). The roundtable discussed three main groups of issues, related to eco-trails [6]:

• Which trails and facilities in Bulgaria could be named “eco-trails” and is there a way to regulate their construction and naming?;

• Who manages and who is responsible for the security of “eco-trails”?;

• How could the security and sustainability of the established trails be guaranteed?

Discussed issues show the main problems faced by the existing eco-trails and we can ascertain that these issues are not resolved till nowadays. Regardless of the existing problems, the construction of trails and improving the theoretical foundation still continues. Development of eco-routes, respectively eco-trails in the area of Middle Rhodopes is traced by Aleksova [1], with particular attention given to the need of their monitoring. The “Three Smolyan Lakes” eco-trail is analyzed as a resource for the development of mountain tourism by Todorov and Gavrilov [10]. The conservation status of “Soskovcheto” reserve in integrated tourism developement is studied by Grigorova [2].

The form of establishment and functioning of eco-trails in the country is not known in European countries or more precisely their development abroad is based on other grounds. Some comparative information is presented in Table 1. For example, in Sicily there are organized

paid tours to Mount Etna using three itineraries with different length and difficulty and entirely with sports character including a competitive element [12]. They are organized episodically and the fee includes refreshments, meals, pasta festival and medical insurance [11].

Eco-trails or routes in different countries are located mostly in national parks, where there is usually an entrance fee. For example, entrance fees are introduced for Torres del Paine National Park located in the Chilean Patagonia, Sagarmatha National Park in Nepal, Sequoia National Park located in Sierra Nevada (California), Tasman National Park (Australia) and Plitvice Lakes National Park in Croatia.

In Bulgaria entrance fees for visiting natural sites are collected only for electrified caves and exceptionally in some other cases as was the entry fee for the visit of "Water Lilies" reserve along the Ropotamo river and the current charges for visiting "Krushunski Waterfalls" trail, "Shipka" National Park – Museum, "Samuilova Fortress" Museum, etc. Meanwhile attempt is made by Kirilov to develop a management model for the development of ecotourism in protected areas [3], but analysis and argumentation for introducing entrance fees are missing. The presented situation about the development of eco-trails in Bulgaria and the willingness of visitors to donate money for their maintenance stimulated the intention of the authors practically to explore the issues.

Table 1. Comparative information on entrance fee in Bulgaria and other countries

Fees for trails in Europe	Entrance fees for natural sites in different parts of the world	Entrance fees for natural sites in Bulgaria.
Tour of Mount Etna on 01.08.2014, with three itineraries; Taxes until 30.04.2014 or 1.08.2014, respectively: I-64 km - 40 € or 100 € II-24 km -15 € or 25 € III-16 km - 8 € or 12 €	Torres del Paine National Park -30 $ CAD Sagarmatha National Park 3000 NPR = 30 $ USD Sequoia National Park - 20 $ USD per car and 5 $ USD per pedestrian Tasman National Park - 10 $ AUD per entry Plitvice Lakes National Park - 8 €,12 € and 14 € (depending on the season)	"Krushunski Waterfalls" trail – 2 BGN Devetashka cave – 2 BGN "Shipka" National Park-Museum- 2 BGN "Samuilova Fortress" Museum – 2 BGN and 1 BGN for children, students and seniors

The involvement of the authors' team in the subproject "Sustainable development of mountain areas in Bulgaria" of the above-mentioned program, related to the evaluation of ecosystem/landscape goods and services in three mountain areas of the country, one of which is the municipality of Smolyan, is the reason to fulfill the idea of researching the attitude of visitors of the studied trail towards the potential payment of an entrance fee for visiting the specified nature site. Despite the lack of legal regulation in this respect, or rather the prohibition to collect an entrance fee at natural sites (Art. 60 of the Regulation .... MEW, 2005) [8], the authors were encouraged by some exception provided by Art. 58, paragraph (2) of the same Regulation and by the desire of visitors to donate a certain amount of money to be used for the maintenance of the eco-trail.

Material and Methods

The main objective of this study is to justify the need for introducing entrance fees when visiting the eco-trails in Bulgaria in order to generate financial resources to ensure their future maintenance.

The selected object of study in the research is the "Canyon Falls" trail spatially situated in the Rhodopean Mursalitsa Ridge and administratively falling within the municipality of

Smolyan. The authors visited the site several times and along with accumulated information about other trails in the country, the idea of interpreting the opportunities for long-term preservation and development of eco-trails in Bulgaria emerged.

Methodological tool for gathering the necessary empirical information is a specially developed questionnaire. The use of the survey approach appears as a variant of the method of contingent valuation (conditional method) applied in the valuation of ecosystem /landscape goods and services. It is based on the declared preferences of people living in a given area, expressed by their willingness to pay and their intentions for action. The practical work in recruiting respondents for the survey took place during a weekend day (Saturday, November 2nd, 2013) featuring significant visits to the eco-trail probably because of the last sunny days of autumn. A rough estimate of the authors shows that during that day more than 300 visitors passed through different sections of the "Canyon Falls" trail. Within the time frame (3 hours) of hiking along the eco-trail 25 visitors were surveyed and it should be noted that there was no formal or informal refusal to fill in the questionnaire.

The questionnaire includes 12 questions. The first five of them are related to determining the profile of the respondents. The next three questions are intended to clarify the relationship between the ecosystems/landscape goods and services provided at the site and the existence of environmental problems in the municipality identified by the respondents. One of them aims at identifying the origin of environmental problems and has a semi-open character with five possible answers, allowing respondents to indicate more than one answer. Question № 9 aims to find out what is the annual number of visits to the eco-trail by each respondent and the seasonal distribution of visits. Question № 10 requires respondents to answer whether Smolyan municipality is able to maintain the infrastructure in the area of the eco-trail with own funds. The last two questions of the questionnaire have a direct focus on the main purpose of this study, namely whether respondents are inclined to pay an entrance fee when visiting the eco-trail and if the answer is "yes" they are requested to indicate a certain amount. The authors have set as a pre-requisite the questions to be clear, unambiguous, understandable and to allow the resulting data to be analyzed depending on the profile of the respondents.

Results and Discussion

The distribution of respondents by gender demonstrates significant prevalence of women over men – 72% to 28% (Fig. 1a). Such a contrast in the sex distribution is not deliberately sought, but some of the respondents were children and most often fathers took care of the children while mothers were completing the questionnaire. There were situations of men carrying a small child on the shoulders and approaching such a respondent is inappropriate. In terms of age structure the profile of respondents shows dominant presence of the most active part of the population. The age group between 31 and 50 years of age has a share of 68%, followed by the younger age group (between 19 and 30 years) with a share of 28% and the youngest group of up to 18 years has only a few representatives with a share of 4% (Fig. 1b). Data discussed allows making the conclusion that the eco-trail is most frequently visited by young people in the most active working age.

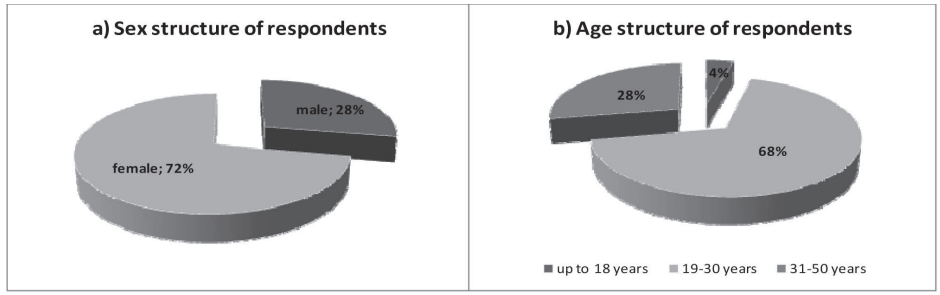

Fig. 1. Sex and age structure of respondents

The educational profile of respondents shows that more than half of the respondents are with higher education – 60%. The group with special secondary and college education represents only 4% of total, and the second largest is the group of respondents with secondary education - 36% (Fig. 2a). The profile of respondents reflects the fact that eco-trails are demanded mainly by people with higher education and in general by people with at least secondary education. Based on the monthly income the profile of respondents illustrates representation of almost all categories provided in the questionnaire (Fig. 2b). There are no representatives of the category with the lowest income and the unemployed. Answers to the question about income show that 52% of respondents have a monthly income of more than 400 BGN, and 24% are of monthly income of more than 800 BGN (Fig. 2b). An important feature in the profile of the respondents is the presence of 36% students.

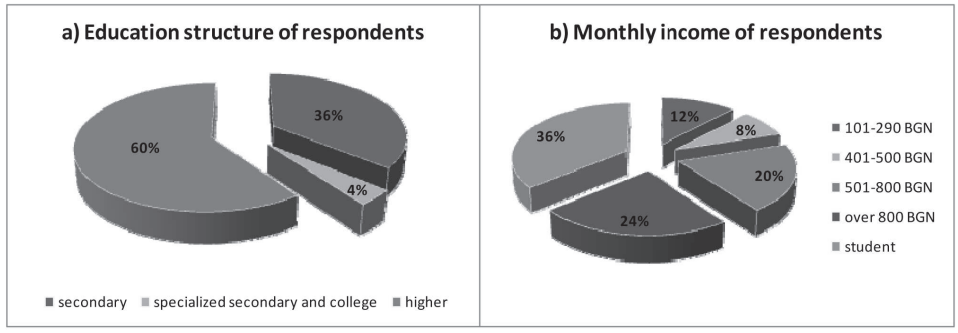

Fig. 2. Education structure and monthly income of respondents

All men representing 28% of respondents indicate that environmental problems do exist in Smolyan municipality and 60% of women respond with "yes" as well (Fig. 3a). However, 12% of the women do not consider any environmental problems in the municipality. The distribution of answers on the origin of environmental problems shows upward increase from global to local. The highest degree of responsibility is set at the municipal level indicated by 31% of surveyed (Fig. 3b). The ranking of the origin of environmental problems is determined in the same order as would the authors do.

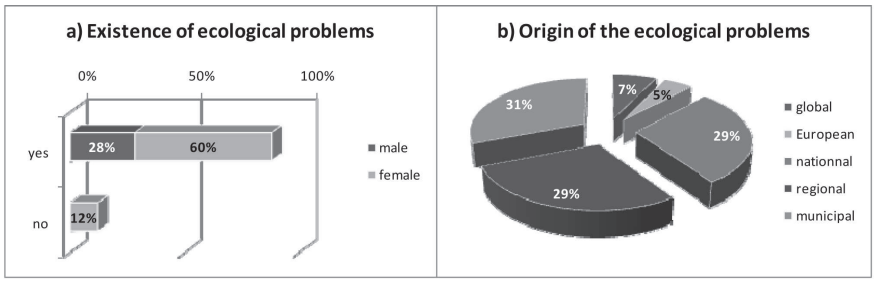

Fig. 3. Existence and origin of ecological problems

Question № 8 is expected to reflect the understanding of the surveyed whether the eco-trail ensures the preservation of ecosystem services provided by nature. Positive responses are given by 20% of the men and 44% of the women, while 8% of the men and 24% of the women responded negatively (Fig. 4a). The displayed distribution of negative answers seems logical as it is expected that people providing negative answer most probably do not understand the concept of ecosystem services. Question № 10 of the questionnaire intends to identify the perceptions of visitors whether the municipality of Smolyan is able to support the maintenance of the eco-trail's infrastructure by own funding. Positive response is given by 8% of the men and 24% of the women but the majority comprising 20% of the men and 48% of the women provided negative answer (Fig. 4b). The prevalence of negative responses (68%) is an illustration of a relatively strong opinion about the inability of Smolyan municipality, the center of which is also a regional center, to maintain the trail using own financial resources. If the poll is held at an eco-trails managed by smaller and financially weaker municipality, the answer probably will be even more emphatic.

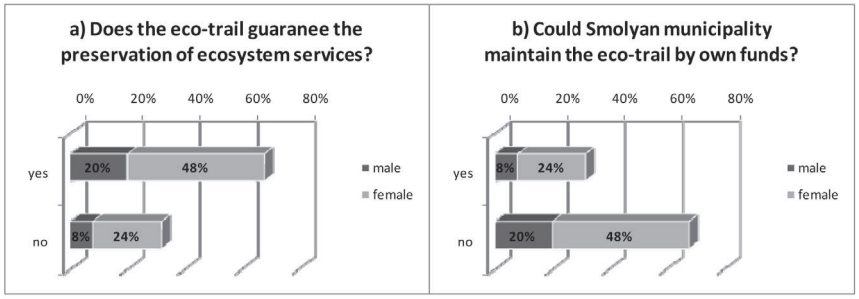

Fig. 4. Preservation of ecosystem services and maintenance of the eco-trail

The visit of the ecotrail by seasons is reflected in the answers to question № 9 (Fig. 5a). The majority of surveyed visits the trail in autumn (66%), but the trail is attractive also in summer (21%) and spring (13%). Nearly half of the respondents (44%) come to the eco-trail twice a year and one of the respondents visits the trail three times a year. It is important also to identify the regional origin of visitors. Those who completed the questionnaire are mainly citizens of two of the major cities in Bulgaria - Sofia (56%) and Varna (24%) or

80% in total (Fig. 5b). Respondents coming from Chepelare (12%) can be considered as local visitors, but small towns in the country as Svoge and Chirpan are also presented with 4% each (Fig. 5b).

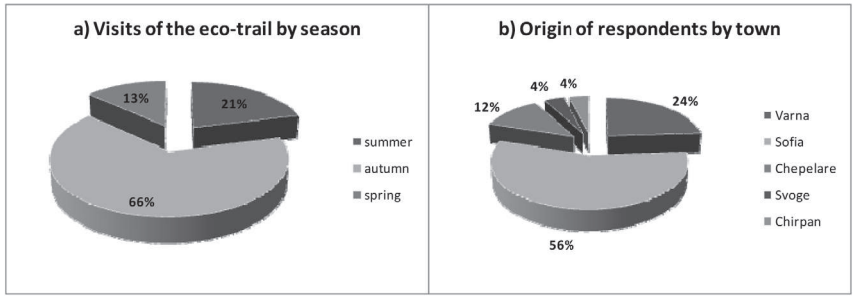

Fig. 5. Visits by season and origin of visitors by town

Next question of the questionnaire (№ 11) is whether to collect entrance fees for visits to eco-trails. Positive answer is given by 76% of the respondents (16% of the men and 60% of the women) and a negative response is indicated by 24% of the surveyed (12% of the men and 12% of the women) (Fig. 6a). That categorical positive response (76%) predetermines the same share of responses to the last question (№ 12) regarding the amount of the potential entrance fee. The suggested amount varies from 2 to 5 BGN and more, respectively 2 BGN -32%, 3 BGN – 8%, 4 BGN – 20%, 5 BGN - 12% and over 5 BGN - 12%. Another 16% of those that provided an answer believe that there should be no entrance fee (Fig. 6b). The average value of the amount of the entrance fee based on the answers of respondents is approximately 3.60 BGN.

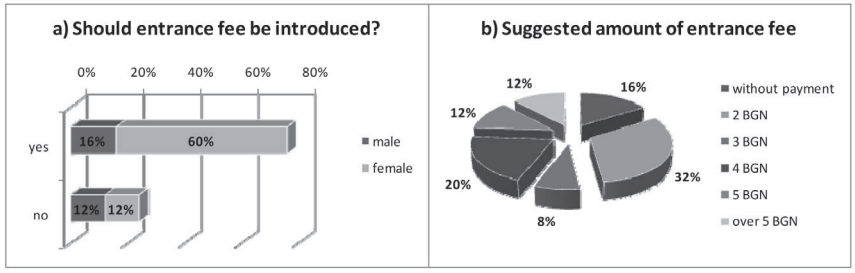

Fig. 6. Introducing and amount of entrance fee

The subject of the present research considers the principle issue of the future development of eco-trails in Bulgaria, their management and maintenance. The assumption of the authors that there should be an entrance fee for eco-trails visits needed to be justified through consultation with visitors. The existence of eco-trails is associated with the raising of own financial resources that can be generated by visitor fees. In the case studied the renovation of the "Canyon Falls" eco-trail and the construction of the visitor center at its starting point is carried out under a project funded by the "European Territorial Cooperation Programme Greece - Bulgaria 2007-2013" in partnership

between the Municipality of Smolyan as a leading partner and Avdira Municipality in Greece. The funding of the project is respectively 841 492 EUR for the Municipality of Smolyan and 317 550 EUR for the Municipality of Avdira. Immediately after the survey media information was spread about the potential bankruptcy of Smolyan Municipality. It stated that "Smolyan municipality is threatened with seizure of accounts and bankruptcy. Municipal administration owes money totaling 110 000 BGN based on three lawsuits brought by a transport company. If within 15 days the required amount is not paid, there will be seizure of accounts and bankruptcy of the municipality". The facts provided strengthen the arguments of the authors for introducing entrance fee for visitors of the studied trail and in general for all trails in the country, because if a municipality in a regional center is threatened with bankruptcy similar threat for smaller and poorer municipalities is even more realistic.

Conclusion

Development of national peripheral space is a priority for each country, as is the case with Smolyan Municipality for Bulgaria and Avdira Municipality for Greece. The "European Territorial Cooperation Programme Greece - Bulgaria 2007-2013" is a typical example of eliminating the effect of "overlapping periphery", discussed by Koulov [5], which in the European Union is very expressive. Development of tourism and especially of eco-trails in the specified mountain area is a tool for sustainable development of the region on both sides of the border line. All issues relating to ownership, management and security of eco-trails still remain open but the authors have made an attempted to suggest an option for the maintenance of eco-trails in consultation with their users. The following conclusions can be made as a result of the survey:

1. The convincing answers of respondents in favor of introducing entrance/visitor fee indicate the right direction to generate the financial resources necessary for the maintenance of the eco-trails;
2. This type of study suggests consultation on the introduction of an entrance fee for other categories of protected sites and areas under the Protected Areas Act in Bulgaria as well;
3. The amount of entrance fee proposed by respondents of about 4 BGN is fully comparable to the remuneration of Bulgarian citizens;
4. The existence of entrance fee for eco-trail visits will have a stimulating effect on environmental awareness and behavior of citizens;
5. The clarification of the terminological concept of an eco-trail is an urgent imperative as is the fulfillment of the "Bulgarian eco-trails" national programme.

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КРАТКИ СЪОБЩЕНИЯ/SHORT COMMUNICATIONS

Тематично направление:
ЕКОСИСТЕМНИ ИЗСЛЕДВАНИЯ И УСЛУГИ

Topic:
ECOSYSTEM RESEARCH AND SERVICES

ASSESSMENT OF OAK DENDROCHRONOLOGICAL SERIES FOR EUSTRESS IDENTIFICATION IN PROTECTED ZONE

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Aim: Stress syndrome is considered as all nonspecific reactions or deviations from the functional norms under the influence of complex environmental impact [2]. In contrast to the destructive stress (distress), eustress is reversible, recurring state of reduced radial growth for a set of years caused by adverse environmental factors. Application of the holistic ecological approach to research eustress (study the final total result of stress situations - impaired growth and production) rather than a metrological one gives the possibility for fast assessment of forest ecosystems.

The main aim of investigation is to present results of *Quercus cerris* L. and *Q. frainetto* Ten. eustress in *SCI "Zapadna Stara Planina i Predbalkan" following a new holistic approach and using an original software application.

Material and Methods: The object of this research is *Q. cerris* L. and *Q. frainetto* Ten., dominated in xerothermic oak ecosystems in SCI "Zapadna Stara Planina and Predbalkan", Bulgaria. Occupying altitudinal belt of rolling plains and foothills, i.e. near settlements, the oak forests are subjected to intensive exploitation that continues to this days. Parts of the still preserved xerothermic oak forests in Bulgaria are included in the protected zones of "Natura 2000" to ensure their preservation and improvement of the future perspective. The national evaluation of the habitat area 91M0 Pannonian-Balkanic turkey oak-sessile oak forests in the protected areas is 75.104 ha. Analyses were conducted with 48 and 60 series, respectively of *Q. cerris* L. (age from 42 to 108 years) and *Q. frainetto* Ten. (age from 42 to 77 years) from 5 locations per each species. SPPAM, version 1.2 (http://www.e-ecology.org) is applied for the analysis of data [3]. Sequences with $R^2 > 0.45$ and locations with EPS (Expressed Population Signal) > 80% are included in the analysis. The used indicator for assessment of stem growth is the average growth index (It = MW/AW, where MW is measured width and AW – computed only for sequences with trusted approximations) sequences. This

*Site of Community Importance (protected zone) announced in European countries

approach allows the elimination of the influence of trees' age on the growth [1]. The calculated eustress characteristics are: duration (D), frequency (F) for 100 years and negative deviation (A), "K" – coefficient, "Ct"- coefficient, coverage (Cov.) and cardinality (Card.) [3].

The climatic database CRU – **TS was used as a source of data on temperature and precipitation for the period 1901-2009. The climatic type of year (CT) is described by the deviation of average annual temperature (dT) and annual sum of precipitation (dP) of year from the respective climatic norm [3]. The used climatic types of years are: hot (H) – $dT > \mu_{ti}$, cold (C) – $dT < -\mu_{ti}$, wet (W) – $dP > \mu_{pi}$ and dry (D) – $dP < -\mu_{pi}$. The year is with normal (N) average temperature, when $-\mu_{ti} \leq dT \leq \mu_{ti}$ and with normal sum of precipitation, when $-\mu_{pi} \leq dP \leq \mu_{pi}$ [3].

Results: The correlation dependences between It of *Q. cerris* L. and *Q. frainetto* Ten. and $T_{averg.}$, DT, $P_{averg.}$ and dP are weak according to the scale [3], although the use of dP and dT leads to a certain increase of the correlation coefficient (r), which was twice as pronounced for *Q. frainetto* Ten. series. For *Q. cerris* L. series of indexes calculated "r" are positive, while for *Q. frainetto* Ten. series of indexes the negative correlation between growth index and temperature parameters used is established.

The correlation coefficient between It and $T_{averg.}$ is larger than "r" between It and $P_{averg.}$ for the *Q. cerris* L. series and an inverse relationship was observed for *Q. frainetto* Ten. series. In the analyzed period the climatic background was determined by the dry and cold years for both studied oaks. The percentage contribution of climate types HD and CW - warm and dry and cold and wet predominates. The biggest part of the total number of stress years for both oak species is occupied by dry years (CD + HD + ND), 43% and 51% respectively for *Q. cerris* L. and *Q. frainetto* Ten. The number of identified stress years, average and maximum set depth, the frequency and duration of eustresa in *Q. frainetto* Ten. are with higher values of the same indices for *Q. cerris* L. (on average 30 to 25, 0.305 to 0.259, 9 to 43 and 8 to 41). There were 11 common eustress years for *Q. cerris* L. locations studied, two of them - 1983 and 1986 were stress years in 71.43% of locations. The dry years - HD, CD and ND climatic type (78.26%) dominated among the common eustress years. The cold and wet climate type also initiated eustress for some *Q. cerris* L. locations as there has been sudden change of warm to cold or wet to dry year in the two previous years. There were 12 common eustress years for the *Q. frainetto* Ten. locations and five of them - 1967, 1969, 1983, 1985 and 2001 were stressful for 80% of the locations. Among the common stressful years dominated climatic types of dry years (HD, CD and ND) - 58.54%. Were also identified climate types HW and CW, CN and NN as adverse years. Following climatic types occurred as eustress years due to sudden change in CT in previous two years. For three of the *Q. cerris* L. locations studied are obtained types of eustress manifestation that would have a negative, but not critical significance for the forest existence (F1D1A4, F3D3A4 and F2D2A4 – very rarely to rarely, very short to short and deep eustress). The established types of eustress manifestation for two of *Q. frainetto* Ten. locations carry some risk for the forest existence (F2D1A5 and F4D3A4 – rarely to often, very short and deep to very deep

**http://www.cgiar-csi.org/data/item/55-cru-ts-30-climate-database

eustress) as will lead to depletion of trees and reducing of their immunity to diseases and pests, and reducing of their productive and reproductive potential.

Conclusion: The weak correlation between growth index and climatic parameters is established for series of indexes of other tree species [3]. *Q. cerris* L. and *Q. frainetto* Ten. form mixed forests in the protected zone, but have some differences in their environmental characteristics. The *Q. frainetto* Ten. growth index indicates a higher value of "r" with precipitation parameters studied and lower negative correlation with temperature parameters studied unlike the *Q. cerris* L. growth index. The *Q. frainetto* Ten. series show greater sensitivity to deviations in climatic parameters from climatic norms and has larger eustress values compared to the *Q. cerris* L. series. A more clear dependence between climate and It is obtained by analysis of the climate types impact on the growth and identification of years with low growth – adverse or eustress years. The study conducted showed the influence of mainly dry years on the stem growth of both oak species. Over 40% and 50% of the dry years caused the appearance of eustress at *Q. cerris* L. and *Q. frainetto* Ten. trees. Stressful influence of other climatic types is often due to an abrupt change in ecological regimes in the previous two years. For the *Q. cerris* L. locations 11 common eustress years was established and for those of *Q. frainetto* Ten. they were 12. Five common eustress years have been identified for both oak species: 1968, 1969, 1983, 1985 and 1986, although mentioned ecological differences between the two species. The type of eustress is not yet risky for the forest existence at about 50% of the locations studied. The data from the conducted survey can be useful to fill the database, also for the evaluation and monitoring of oak forests.

Acknowledgements: Co-funded project "Application of information technologies for modelling of FE as an approach for developing of DGVMs" to COST Action FP1106 and project "Dendrochronological studies of oak forests in SCI "Zapadna Stara Planina i Predbalkan" at the Faculty of Biology, 2014.

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SIZE-AGE AND DIET COMPOSITION OF SOME OF THE MOST IMPORTANT COMMERCIAL FISH SPECIES IN THE BLACK SEA BASIN

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Introduction: Fish productivity in the Black Sea is assessed to be higher than in the other seas of the Mediterranean basin. However, when in the early 1970s industrial fishing started, the stocks of pelagic predators (bonito, mackerel, bluefish, dolphins) quickly became severely depleted [1]. Some species, such as the Black Sea mackerel and the large horse mackerel, disappeared entirely from the Black Sea area. After this reduction of apex predators, the fisheries started targeting mainly small pelagic species like sprat, anchovy and the small variety of horse mackerel [2]. Because of their position and linkage within the food webs, these small pelagic fish species play essential roles in the functioning and dynamics of the modern Black Sea ecosystems.

Aim: The aim of this study is to determine the current size-age structure of the most important commercial fish species in the Black Sea. Besides being targets of the commercial fisheries, these small pelagic fishes (sprat, anchovy) are under the predatory press of horse mackerel and bluefish. Therefore, the knowledge of the food spectrum of these predator species and the proper assessment of the predator–prey relationships will allow us to estimate the amounts of consumed fish and fish mortality due to predation. The data from this study will be implemented in the detailed Black Sea ecosystem model Ecopath, which will serve as a major instrument for the implementation of the ecosystem approach to the management of marine bioresources.

Material and Methods: Samples of several pelagic fish species were collected weekly from commercial fishing vessels trawling along the southern Black Sea fishing area, for the following periods:

- sprat (*Sprattus sprattus* L.) – May 2012-December 2013;
- anchovy (*Engraulis encrasicolus* L.) – May-June 2012 and June 2013;
- horse mackerel (*Trachurus mediterraneus ponticus* A.) – May-June 2013;
- bluefish (*Pomatomus saltatrix* L.) – October-November 2013.

The size-age structure of the populations was investigated on the basis of the following parameters: total length, weight, age, and condition factor (a standard measure of the relationship between weight and length). The age was determined through otolith reading, and the total length and weight of fish examined were measured to the nearest 0.1 cm and to the nearest 0.1 g, respectively. For bluefish and horse mackerel, stomach content analysis was also performed. In order to determine the importance of each food item in the stomach contents, an Index of Relative Importance (%IRI) was calculated.

Results and Discussion:

Size-age structure

The percentage of the 2nd-year-class sprat specimens increased in the autumn months, reaching 61.77% in December 2013. The share of the 3-year-old specimens was very small (on average 0.28% in 2012 and 1.16% in 2013), reaching a maximum again in December 2013. Age class 4 was registered only for the winter period of 2013 and its share constituted on average 1.11% of the total sprat samples.

The same increasing trends in the autumn months were observed for the condition factor which reached a maximum value of 0.62 for both 2012 and 2013, and for the mean total body length (TL) whose highest values were 7.83 cm (2012) and 8.19 cm (2013).

Our results indicate that the sprat population in the coastal zone is dominated by 1st age-class individuals. As the breeding season of sprat approaches and the temperature of the seawater decreases in October-December, the number of larger, sexually mature specimens increases. This phenomenon of migration and distribution of the sprat shoals over the coastal waters, which are characterized by optimal temperatures for the sprat spawning process, is an important mechanism for the successful reproduction.

During the spring season the dominant age classes in the Black Sea anchovy population were the 2nd and 3rd. The other age groups were also well represented. The mean TLs for the same period were 10.63 cm (2012) and 10.72 cm (2013).

In the spring (May-June) the anchovy migration route passes through Bulgarian coastal waters. Here the anchovy schools feed intensively [3], and this is also the breeding season for this species. This is likely the reason that in the sampling period, the anchovy population in the coastal areas of the Bulgarian Black Sea showed a balanced age structure where older, sexually mature individuals were also present.

Stomach contents

In the spring months the Black Sea horse mackerel was found to feed mainly on benthic invertebrates. Of the 273 horse mackerel stomachs analyzed, 158 were full (57.9%). A total of 18 prey species belonging to the taxonomic groups Amphipoda, Cumacea, Decapoda, Isopoda, Mysidacea, Mollusca, Polychaeta, Tanaidacea, and Pisces were identified from stomach contents. The polychaete *Neanthes fucata* was the preferable prey item (%IRI = 75.6). The other important ingested preys were isopod *Idotea balthica* (%IRI = 12.19), and amphipods *Gammarus insensibilis* (%IRI = 7.16) and *Microprotopus maculatus* (%IRI = 1.95). There were no sprat and anchovy specimens found in the horse mackerel stomachs. The only registered representatives of Pisces among the prey items were from the Gobiidae family, with a contribution to the %IRI less than 0.1.

Food items were found in only 23.08% of the analyzed bluefish stomachs. The registered prey species were horse mackerel (*Trachurus mediterraneus ponticus*), whiting (*Merlangius merlangus euxinus*), and red mullet (*Mullus barbatus*). The most important food item in the *Pomatomus saltatrix* diet was found to be the horse mackerel (%IRI > 99). Therefore, we conclude that the bluefish population had a

high predatory impact on the *T. mediterraneus* ponticus population in the Black Sea in the study period. The other registered victims, red mullet and whiting, were present but of limited significance, with values of %IRI = 0.11 and %IRI = 0.14, respectively.

Conclusion: The investigation of the size-age structure of the fish stocks is essential, as it is indicative of the general condition of the populations of the commercially valuable fish species.

The trends in the seasonal dynamics of the food spectrum of the horse mackerel and bluefish, their intensity of feeding, as well as the reasons associated with those trends also need to be further examined and clarified.

The preliminary results presented here are part of a larger, ongoing study, whose aim is to contribute to ecosystem trophic models Ecopath and Ecosim. These models will provide an overview of the trophic flows, the feeding interactions, the effects of changes in fishing pressure, and an evaluation of the impact of fisheries and environment on the Black Sea ecosystems. These prognoses will play a crucial role for the sustainable management of Black Sea marine bioresources.

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ДОКЛАДИ/REPORTS

Тематично направление:
ЛАНДШАФТНА ЕКОЛОГИЯ

Topic:
LANDSCAPE ECOLOGY

A NEW APPROACH FOR LANDSCAPE ECOLOGICAL RESEARCH IN MOUNTAIN AREAS

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[2]Space Research and Technology Institute, BAS, Sofia, Bulgaria

Abstract

Aim: To propose an approach for landscape ecological research in mountain areas.

Material and Methods: Digital landscape maps are developed by using methods of spatial analysis and mapping in GIS environment. The study uses watersheds as natural systems that have functional integrity and naturally determined borders to generalize the information and create a new perspective on landscape functions' analysis. The hemeroby index is integrated in the evaluation of landscape degree of anthropogenization.

Results: Analytical information bases for evaluation of landscape structure and functions for adaptive management of mountain areas are developed.

Keywords: landscape ecology, landscape classification, GIS

Introduction

This study attempts to implement an interdisciplinary approach to the study of Bulgarian mountains, in response to the modern needs of integrated and sustainable planning and management of mountain areas. On this basis, the investigation applies the systems approach and uses landscapes, understood as complete natural and anthropogenic systems, as object of analysis.

Researchers' attention focuses on landscapes functions interpreted from the perspective of the natural capital concept. This theory is essential in modern geospatial research, especially in the context of the debates about 'sustainability' and their current role in solving the problems associated with environmental degradation, land use conflicts, demographic problems and increasing pressure on natural resources. The study follows the concept of multifunctionality of landscapes. It reveals the presence of landscape features and func-

tions that can pose practical interest for various fields, such as economy, culture, ecology, but their effective real life usage is possible only in conditions of integrated management.

This report presents the theoretical justification of this complex landscape-ecological research, applied to mountainous regions.

Theoretical rationale of the study: Argumentation of the expansion of the investigation within landscape boundaries

The present can be seen as a period of steady increase in problems with complex character, the solution of which requires joint efforts in the political sphere, coordination of research from different scientific fields, application of interdisciplinary approaches, formation of broad and precise information databases. The success of science-based policy decisions in practice depends on the systematization of the knowledge, as well as the approaches to problem solutions, and, above all, the deployment of systems thinking [2].

Our research aims to develop a rich database for the current status, structure, and functions of mountain landscapes and formulation of advanced solutions in support of sustainability in their planning and management. On this basis, the study adheres to the theory and practice of modern inter-and transdisciplinary landscape ecology. "Landscape" in the study is interpreted as a real object with independent spatial characteristics - structural and functional unified system of interacting components in certain geographic conditions. Most importantly, modern landscape theories present it as a holistic entity - a space where economic, environmental and social goals must be balanced in support of sustainable development. Landscape as a result of human interactions with nature is situated today "at the heart of sustainability" [10:7].

All this motivates our study to organize the research process within the boundaries of the complex systems – the landscape.

Our adherence to the concept of landscape is based on the following important arguments associated with the objectives and expected results of the study:

1) Using a "landscape scale" [1] or "landscape context" [6] allows for the disclosure of that study scale of the complex spatial systems, which in the highest degree identify them as separate objects with certain properties and functions. A great advantage of this analysis is the provision of an integrated and consistent information on landscape structure, functions, internal and inter-dependencies, which is important for the planning and management of natural and anthropogenic systemic interactions.

2) Landscape functions can be objectively and fully analyzed and evaluated only if they be investigated as single (complete) objects. They cannot be judged only by observation and research into their constituent parts [12]. The use of "land cover" is an established practice, which in a small-scale studies is the perfect basis for conducting economic evaluations and analyses of land use, while the fact that it reflects the spatial correlation of "landscape elements" [4] which are "structural parts of the landscape" [4: 36] should not be overlooked. This should be a guiding factor in any planned form of anthropogenic interference.

3) Modern landscape ecological theory expands its influence on spatial planning. It helps planning as a process to be associated with a new understanding of space. "Space"

planning replaced the old styles that excessively focus on controlling anthropogenic environment and provides a new perspective on this well-established field of activity being reoriented towards integration of sectoral policies in the context of localities and regions [9]. The implementation of this integration is highly dependent on the correct identification of geographical localities in which to direct anthropogenic efforts to form the desired spatial results. The new potential of spatial planning is determined by the capabilities of the landscape concept to propose a scale for spatial interventions and formulate spatial units for optimal planning.

4) Systems analysis, realized on the basis of landscapes, suggests involvement of local people in the process of spatial planning. It adequately reflects their needs and supports their identification with local landscapes which motivates public involvement in the decisions (set in the strategies and development programs). This approach is particularly appropriate in the presence of a strong dependence of the population on local resources as seen in mountainous regions.

Material and Methods

Area of study

Mountain regions, representative for the Bulgarian conditions - Central Balkan Mountains and the Western Rhodopes - are selected for the purposes of the study. The sites' selection is consistent with a number of criteria, among which stand out the following:

• Mountain areas are important sources of natural resources, the practical importance of which goes well beyond the territorial scope of the mountain;

• Vulnerability of the territories to contemporary geo-ecological issues and processes, such as floods, landslides, fires, droughts, invasive species, etc.;

• High dependency of local people on local resources and the quality of their management;

• Rich cultural, historical and natural heritage, including the presence of historically established forms of land use and representative landscapes and protected areas;

• Potential for regional cooperation in the management of the territory at various administrative levels .

The fact that both mountain region are peripheral in reference to the planning regions which they are parts of and, therefore, the possibility of application of comparative analysis in their planning and management, plays an essential role in the final selection of the study sites. At the same time, one of them (Western Rhodopes) is also peripheral to the territory of the country, while the other (Central Balkan) is located at its center (Fig. 1 and Fig. 2).

Due to the applied aspect of the survey results, the data are analyzed on the scale of the concrete administrative units. At this stage of the study, these include the Municipalities of Smolyan and Apriltsi, and the Kalofer Mayoralty.

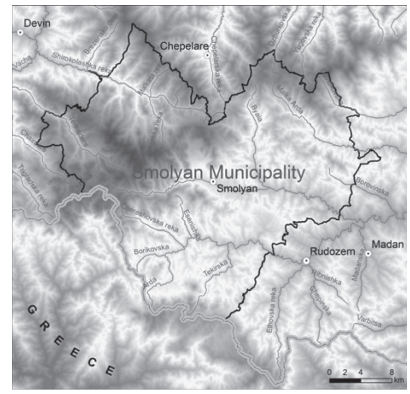

Fig. 1. Area of study – Smolyan municipality, Western Rhodopes

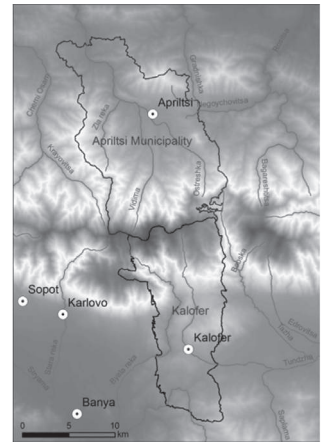

Fig. 2. Area of study – Apriltsi municipality and Kalofer Mayoralty, Central Balkan

Methods

The study has an interdisciplinary focus, in which connection the methodological apparatus includes: systematization of the information from scientific and other specialized literature, strategic and planning documents at different administrative levels, statistical and other information about the development of the study area; Field observations, incl. population surveys; Specialized methods for landscape differentiation; Cartographic, mathematical and statistical methods in GIS environment, incl. implementing a series of landscape assessments.

Starting point for the realization of the study is tracking of the conditions and relationships in the systemic organization of the landscapes. The lack of a commonly accepted classification system landscapes in Bulgaria and the commitment of our country to the European Landscape Convention[4] [3] predetermined our research choice towards

4. The European Landscape Convention is ratified by Bulgaria on October 13th 2004 and enters into force on March 1, 2005.

adaptation of the new trends in European landscape-ecological studies - The European Landscape Character Initiative (ECLAI) [11], [7] to Bulgarian geographical conditions and the current problems of regional development in the country. This decision is simultaneously directed towards several tasks, important for this research:

(1) Development of an integrated database in GIS that allows for ongoing updating with new thematic criteria, expansion of the data range and high potential applications for planning and management purposes;

(2) The development of integrated (landscape-ecological) cartographic basis for carrying out of a series of assessment studies and their cartographic interpretation for the needs of spatial planning and territorial management;

(3) Establishment of a basis for comparison of the characteristics of the Bulgarian landscapes with those of the European landscape map and on that foundation creation of conditions for cooperation, both in terms of new forms and approaches to planning and management of the territory and in specific sustainable development projects.

Our study applies the classic landscape research "genesis approach" for systematization and classification, which allows to take into account all possible factors of landscape differentiation (natural and anthropogenic) under the direct influence of the existing zonal and azonal physical geographic patterns. In the preparation process, precise adherence to the principle of "conjoint spatial dimension" of the classification categories that was justified in Popov's [8] proposal for geoecological classification of Bulgaria, received special attention. In our study, the observance of this principle means that, given the scope of the study area, the scale of the survey, and the degree of landscape diversity, landscape units are defined according to diagnostic criteria, characterized by a specific degree of indicators aggregation. In this context, the spatial generalization of the data applied in the presented study, is consistent with the level of landscapes' micro-structures in (according to Popov, [8]) or on chorological level of analysis (according Makhdoum, [5]). The study uses watersheds as natural systems that have functional integrity and naturally determined borders to generalize the information and create a new perspective on landscape functions' analysis. We adhere to the principle of geo-ecological informativeness of landscape classification by inclusion of the "hemeroby" index to the criteria basis for differentiation, as an indicator of anthropogenic transformation of landscape systems.

Results

The results received at this stage of the study can be summarized as follows:

1. Preparation of landscape classification based on representative criteria for their structure, function, dynamics, current state;

2. Development of landscape maps in GIS environment (Fig. 3 and Fig. 4).

On this information basis, a comprehensive landscape assessment of the current landscape potential (natural capital), identification of conflicts in land use and development of recommendations for the sustainable management of the territory, based on specific spatial indicators, will be carried out.

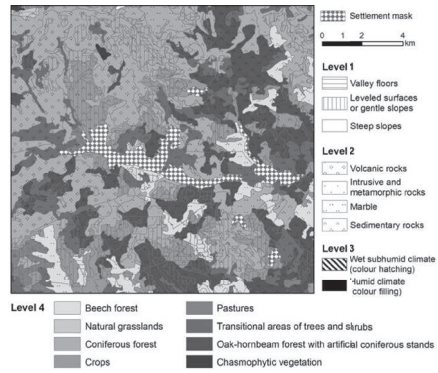

Fig. 3. Landscape map – fragment, Smolyan municipality, Western Rhodopes

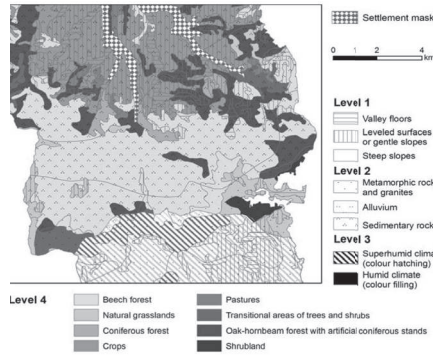

Fig. 4. Landscape map – fragment, Apriltsi municipality and Kalofer Mayoralty, Central Balkan

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ДОКЛАДИ/REPORTS

Тематично направление:
ЕКОЛОГИЯ И ОБРАЗОВАНИЕ

Topic:
ECOLOGY AND EDUCATION

"THE HUMAN – AN INSEPARABLE PART OF NATURE" AN ENVIRONMENTAL EDUCATION MODEL

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Aim: The main objective of the work is to elaborate and introduce a project-based learning (PBL) model engaged in the field of ecology and education. The final product, expected as an outcome of the completion of this project, is a set of photo-portfolios.

Material and Methods: The interdisciplinary connections between ecology, arts (photography), psychology have a key role in the project. The target groups are high-school students from ninth to eleventh grade in arts schools. The students have to craft a portfolio, containing photos, shot by each member of the group.

The results are photographs on the topic of "The Human – An Inseparable Part of Nature" and should be accompanied by a short description (when and where they were taken and what the author's motive was).

Conclusion: this PBL would develop in the participants not only biological competences but also key competences such as: communicative skills in the native language, basic knowledge in the field of science and technologies, digital competence, skills for self-dependent learning and researching and cultural competences.

Keywords: ecology, education, photography, project-based learning, human, nature

Introduction

The Bulgarian educational system mainly offers a theoretical approach to education and the high school graduates are faced with a paradox – they have knowledge which they cannot apply in real-life practice. This inevitable discrepancy poses a threat to the whole educational system which cannot, therefore, meet the requirements of the European Union. Given that, it is not unexpected for young people to be generally apathetic and indifferent [3, 4, 5]. The education in biology, based on the constructivist and humanist theories is a process focuses on active student-knowledge interactions, which leads to the integration of new concepts and skills in the already existing conceptual frameworks. This way the new knowledge can be applied in new context and to be demonstrated in a new way. Experimenting or learning through experience, problem based learning are typical constructivist methods for active construction and integration of knowledge and skills [2, 3].

Humankind constantly tries to distance itself from nature but they are inseparable and constantly interact with each other in positive and negative ways. Therefore, the right course of action is to reconnect with nature in order to learn how to preserve and protect it more successfully. Due to the industrial and technological revolution of modern times humanity has turned its back on the environment. The belief that people are independent and in control of nature has led to irreversible loss of species and natural habitats. To prevent further damage on the planet's natural resources the problem should be introduced to people starting from adolescence. In this paper we would introduce an alternative method of teaching through task-based learning. Therefore, this paper firstly focuses on the methods and materials that were used for this educational project, and secondly the focus is shifted on the expected and actual results.

Material and Methods

Our motivation to create this project was to engage high school students in untraditional learning activities which would allow them to better perceive the teaching material and be additionally motivated to study. This project is designed as an alternative stimulus for the high school students. Firstly, we wanted to determine whether any students would be interested in participating in the project and if there were any – how many of them would be boys

and how many will be girls. As the final product, expected as an outcome of the completion of this project, is a photo-portfolio and to engage in this project eleventh grade students class "A" from an art high school – 1-st High School "Pencho Slaveikov", Sofia. The research was conducted to determine the most suitable class to work with. Eventually we chose to work with this particular class because our research showed that some of the students in this class have an interest in the field of photography – three girls, in particular. Project-based learning has a huge and very positive impact on the motivation [3], communication skills and teamwork. At all the stage of the project, which are setting goals, planning, realizing the project and presenting it, the students work in groups and discuss and evaluate the reliability of their own knowledge, deductions and hypothesis, they create group strategies. It is in fact the teamwork during the project's development that creates the perfect conditions for the individual contribution of every student to be acknowledged, depending on his knowledge and abilities [1, 3]. Before starting the project we arranged meetings with their biology teacher and their class teacher to receive their approval of the project and to familiarize them with the details. With the support of the Department of Pedagogy were able to make the initial contact with the two teachers. Both of them (the teachers) were very enthusiastic and supportive as well. They assisted us in the process of presentation of the project to the students and additionally motivated them by promising an extra excellent grade for every student who takes place in the project and to arrange an exhibition of their works in the main hall of the high school. The students, on the other hand, didn't seem very enthusiastic or eager to participate in anything that involved effort of any kind on their behalf. The only ones that seem to show even the slightest interest were the three girls who were non-professional photographers. A problem occurred when we told the students that they had to form separate groups which would compete with each other during the project because the students were used to working in opposing teams. We explained to them that these conditions were essential for the project's goals to be met. After a brief discussion they decided to participate in the project under the set of conditions. They were given two weeks to form the teams (each team had to have one of the amateur photographers in it) and to choose the subtopic of their group's portfolio.

Two weeks later an e-mail from their biology teacher was received with the full list of the teams, their members and the subtopics they had chosen. The students divided into three teams – two of the teams had three members while the third one had five members. Contrary on our initial expectations, two boys actually joined team number three. The three teams chose the following subtitles for their portfolios:

Team one (Nikol Popova, Emilia Panaiotova, Dora Miteva): "Man's best friends"; Team two (Ekaterina Kovacheva, Elina Krasteva, Beatris Georgieva): "Nature and art"; Team three (Alexander Vodenicharov, Alexandra Hadrian, Georgi Yotsov, Vesela Iordanova, Martina Mladenova): "Nature and us".

Results

The final stage of our project was to inspect and evaluate the final product of the students. For that purpose, on 20 May 2014 we went to First High School "P. Slaveikov" to meet with the teams. Just one of the teams (team two) managed to present to us a draft of

their photos. Than we asked the other two teams to at least describe the idea they were working on and we were given just a vague impression of what they were going to present. After a brief discussion we decided to give them a little more time (until the May 25, 2014) to finish their portfolios and to send them to us by e-mail. The idea for the exhibition remained and the students promised to send us invitations for the exhibition itself. By the end of May 25, 2014 we had received all of the three portfolios (Fig. 1, Fig. 2 and Fig. 3).

Nikol Popova

Dora Miteva

Emilia Panaiotova

Fig. 1. Portfolio of the Team one

Ekaterina Kovacheva

Elina Krasteva

Beatris Georgieva

Fig. 2. Portfolio of the Team two

Alexander Vodenicharov

Alexandra Hadrian

Georgi Yotsov

Vesela Yordanova

Martina Mladenova

Fig. 3. Portfolio of the Team three

Discussion

Taking into consideration that the current project is our first one to apply into practice there were bound to be during its execution. Despite this, the final results are in

support of what we stated earlier: Bulgarian students are well prepared theoretically but are incapable to apply this knowledge in practice. When in an unexpected situation in which they should make a decision outside of the routine, most of them are unable to react adequately.

One of the main causes for the unsatisfactory results, as far as the products are concerned, is the fact that even though they were provided with a full set of instructions they didn't bother to follow or even read them. This led to a series of misunderstandings and as result, the students did not finish their portfolios on time. When the portfolios were finally finished, the majority of photographs did not meet the set requirements. From an objective point of view, we also could have averted this situation. After evaluating every aspect of the issue we concluded that such educational projects need more strict supervision on behalf of the project team. If we had monitored more closely the process of the creation of the team's final products we may have avoided the misinterpretation of the requirements. We should have presented the participants with more examples that are fit for the project's purpose.

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PROJECT- BASED LEARNING ON "SOIL EROSION PROTECTION"

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Abstract: Formation of ecological awareness in students through activities for global environmental issues and skills for environmental protection is one of the main goals of teaching biology and health education.

The education, learning and training in the modern school is unthinkable without project-based learning (PBL). It gives an opportunity to use various interactive and integrative learning techniques in an original and different form of training.

The purpose of this article is to develop and present a model of project-based learning on the interdisciplinary topic "Soil erosion protection". During the PBL process, interdisciplinary links between chemistry and environmental geography and information technology are realized.

This model could develop in the students biological and chemistry competences, communicative skills, basic knowledge in the field of science and technologies, digital competence, skills for self-dependent learning and researching and cultural competences.

Keywords: ecology, education, project-based learning, soils

Introduction

The project-based learning (PBL) allows for individual expression of each student, according to his knowledge, skills and values in an environment of mutual cooperation and tolerance for other positions, and hence overall motivation for the learning process in biology.

Project-based learning is a method of teaching and learning in which students acquire new knowledge and skills in the process of designing, planning and production of certain educational product [1, 2, 3, 4, 5].

Project-based learning stimulates reflection on their own cognitive activity, a process that clearly leads to the formation of metacognitive skills. The latter are pointed to be a reliable basis for the formation of personality and self-controlling their own learning, personality, able to improve throughout their lives [1, 5, 6].

During the PBL process the roles of students and teachers are exchanging. Students entering the rolls of people who actively solve problems. They are responsible for their learning, motivation and sense of satisfaction from the completion of something useful, become active participants and creators of meaning. The teacher as coach/manager manages group dynamics, the movement supports the process forward, advise and assess, facilitate the improvement of interpersonal relationships. This leads to increased need of creating new approaches and methods of working with students to provoke their interest and formation of an active life position on environmental issues.

The purpose of this article is to develop and present a model of project - based learning on the interdisciplinary topic "Soil erosion protection". During the PBL process interdisciplinary links with chemistry and environmental geography and information technology are realized.

Material and Methods

The project-based learning influence on the student's motivation and the basic steps in planning and implementation of PBL in a selected curriculum topic. PBL gives the student an active role in creating a product [5, 7]. The impact of project based learning on motivation, communication skills and team skills of students is extremely valuable. At all stages of the project: object planning, planning, implementation, presentation, students work in groups discuss for the accuracy of their knowledge, conclusions and hypotheses, create common strategies, etc. [1, 2, 5].

The sustainable use of soil preserve soil functions, prevent threats to soil, mitigate their effects. Soil is an important natural resource basis for the existence of humans, animals and plants. In natural ecosystems or manageable it performs certain functions: providing an environment for plant development and implementation of their biomass productivity, regulates and distributes water flow, store water and serves as a buffer environment by inactivating or destroying environmentally hazardous substances. Soil maintains genetic resources and environment habitat for many living organisms. It is the physical basis of the socio - economic structure of human society, is a source of raw materials and stores genetic and cultural heritage of humanity.

Formation of environmental awareness in students is a long process but necessary to preserve life on Earth. Curriculum of Biology and health education in grade 9 is suitable for use in student projects.

That is precisely what motivates our choice of topic that applies to PBL. The experiment was conducted with students in grade 9 of the "P. Slaveykov" 1-st school, Sofia.

Results and Discussion

The sustainable use of soil preserve soil functions, prevent threats to soil, mitigate their effects. The sustainable use of soil preserve soil functions, prevent threats to soil, mitigate their effects. The organization of project-based process (PBP) includes 3 stages:

First stage of preliminary study and planning [1].

The Subject of proposed project was "Soil erosion protection".

The Project Objectives were as follows:

- Formation of an active life stance on environmental issues;
- Forming a culture of conservation and wise use of resources of the environment;
- Implementation of interdisciplinary links with the chemistry, geography, information technology.
- Development of social skills: protecting its own thesis, skills, teamwork and communication for audience;
- To increase student's interest in ecology, problems of environmental pollution.

Goals are discussed with the class.

The students have a freedom in choosing the project.

Planning activities

Activities were identified, then the most suitable of them were selected. This was done on the basis of own experience and after consulting with other teachers. It was decided how these activities to meet the plan schedule.

The tasks of **Soil erosion protection** project **were set as well.**

Degradation is damage and/or destruction of the soil, which has an adverse effect on one or more of its functions. The causes may be natural and/or anthropogenic. Therefore the task is connected with the types of degradation processes. The students choose:

- Soil erosion - is a phenomenon associated with the production and transport of soil particles by wind, rain and irrigation water during natural and anthropogenic processes. Loss of soil material has a significant impact on soil functions.

- Soil contamination - is the process of accumulation of inorganic and organic pollutants from natural or anthropogenic source whose behavior and concentrations cause damage to soil functions. The main source of pollution in urban areas is transport. It is also known soil pollution from improper use of fertilizers. The lack of a natural mechanism for the conversion of nitrates into harmless compounds makes the problem especially dangerous. Entering the body free nitrates are converted into nitrites. Nitrites can be connected with hemoglobin in the blood, which deprives animals and man enough oxygen. The soil pesticides are other major pollutants. Pesticides are chemicals for protection from pests: insects, rodents, fungi and parasitic weeds plants. The using them leads to a sharp increase in the yield of crops treated with them due to the rapid destruction of pests. Pesticides hardly biodegradable and their concentration increased significantly along the food chain. If the amount of pesticide was harmful in its original application, after the time of entering the last link of the food chain it can be deadly. Pesticides accumulate in the human body, causing severe damage in various organs. In the soil may fall heavy metals: copper, lead, chromium, manganese and others. They penetrate into the soil through irrigation with contaminated water or from industrial air pollution. Operations of automotive engines are also one of the main pollutants. Heavy metals are especially dangerous because they are sustainable. They cause diseases that affect almost every organ.

Despite the lack of systematic monitoring data are available for permanent dwindling stocks of soil organic matter in arable land. It is necessary to develop and implement a program of maintenance and improvement of soil fertility, including a demonstration of good agricultural practices for soil conservation restricted substances, together with measures to protect soil from erosion and compaction, as well as training programs for farmers.

- Loss of biodiversity - is linked to other degradation processes, and changes in the landscape, leading to the loss of the natural living environment of many species also stubble burning, destroying flora and disturbs the soil balance which causes a significant reduction in soil fertility. Biodiversity loss can be stopped by reducing the intensity of degradation processes, soil and land, changes in the landscape, leading to the loss of habitat of species and stubble burning.

- Salinization of soil - a process that increases the content of water-soluble salts, thereby increasing the amount of sodium and magnesium in the soil, and has a negative influence on productivity thereof. Improvements in saline soils involves significant investment, since it is possible to achieve after the drainage of the soil, and also to the application of other complex agronomic practices.

- Soil compaction - is the process of deformation and increasing density, thereby decreasing permeability, increases the hardness distort soil structure and changes in soil profile. Arable lands are affected by compaction as the top layer and fertile. It is the application of good agricultural practices to maintain and restore soil structure, such as the use of machinery and equipment for tillage reduced pressure and intensive measures to protect the soil from erosion and degradation of soil organic matter.

Planning assessment

The criteria included in it are generic and applicable regardless of the type of presentation of the product teams.

Second stage of practical implementation

According to schedule teams gathered, selected, presented and structured information.

Followed 1 week to complete project preparation and making presentations.

Third stage of presentation, discussion, review and evaluate the results of the study.

Presentation of finished products from each working group.

In the early hours were motivated academic activities, as has been pointed out the project objectives and tasks.

Each working group had 10 minutes to present their project. Prior we had done lot for the sequence of appearance of the teams. Students had prepared essays, poems and multimedia presentations.

After presenting the projects was organized a discussion with the class.

The activity of each group was analyzed and evaluated separately to highlight their achievements and shortcomings. Finaly, recommendations and suggestions for further work were made.

Conclusion

Limiting and overcoming degradation processes in agricultural land can be achieved through the application of good agricultural practices , including a set of measures with soil-protection and run-off action for integrated soil and water-specific soil - climatic and topographic conditions, to:

- Providing a protective cover on the soil surface by vegetation or plant debris during periods of high rainfall and erosion by wind;
- Increasing the infiltration capacity of the soil;
- Maintenance and restoration of soil;
- Increasing the stock of soil organic matter;
- Use of machines and technologies for tillage with minimal pressure on the soil surface.

On the basis of the above, we can say that educational technology called project based learning can function as a flexible integrated learning environment. This is an environment structured around learning rather than teaching, environment, provocative discussions and negotiations on the objectivity, accuracy, usefulness of individual knowledge, opinions, assumptions [1, 5].

One of the objectives of secondary education in the field of science is Scientific literacy development. The realization of this objective is placing the students in the center of learning, which is a fundamental difference between traditional and innovative models of education. The process is characterized by using of so-called active or interactive methods of teaching and learning where the student body is active in the process of their learning and personal progress. Using these methods is appropriate and successfully applied in the traditional classroom. Through it realizes relation between the duration of the activity and durability of knowledge, make connection between subject areas and real-life problems.

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КРАТКИ СЪОБЩЕНИЯ/SHORT COMMUNICATIONS

Тематично направление:
ЕКОЛОГИЯ И ОБРАЗОВАНИЕ

Topic:
ECOLOGY AND EDUCATION

„I PRACTISE SOME SPORT = I AM HEALTHY AND FIT" (A MODEL FOR BIOLOGY AND HEALTH EDUCATION)

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Aim: Our society faces some serious problems today. One of them is the increasing number of young people suffering from obesity and related diseases [2]. The main aim of our project is to encourage young people to take up some sport or other physical activity and practice it for a certain period of time. Having seen the results, they would be encouraged to go on and keep fit and healthy. We also aim at helping young people gain confidence and learn more about healthy life. Besides, the project aims to make biology classes more interesting and visited and thus solves one of the problems of education today.

Material and Methods: The project "I Practice Some Sport = I Am Healthy And Fit" is a dimension of project-based learning. According to Yotovska et. al. [3] the experimentation, learning through experience and problem-based learning are typical constructivist approaches for active construction and integration of knowledge and competences. Hegedjush [1] found that the project-based learning has great impact on motivation, working competences, and teamwork competences of those who take part in it. During all the stages of the project: aim fixing, planning, realizing, presenting, students work in groups and discuss and negotiate about the authenticity of their knowledge, conclusions, hypotheses, and create common strategies. The idea of education through creating is new to Bulgaria but it is earning more and more supporters because its application results in better performance of students and increased interest in the subject. That is why we chose it to be the main method of our research.

The target group consisted of 30 students from 9^{th} and 10^{th} grade, and the project duration was one month. The group included both boys and girls, fit and obese, sportsmen and couch potatoes (persons who spend much time sitting or lying down, usually watching television). They had to choose a sport which they would like to take up or just seemed interesting to them. Then, they had to find information on how its practicing reflects upon human body and make a poster in order to present to the class what they have learned. The most creative poster and presenter were awarded certificates. During the second phase of the project, the students had to find some people who practice interesting sports and interview them. For this purpose the students were provided with

surveys, which included questions about what is the sport, how long they have practiced, what they enjoy most about the sport, etc. Some students who initially refused to take part into the project were interested in the surveys and even took some in order to help their classmates. On the whole, boys were more interested than girls – some of them had been practicing sports before the project started. Others were too shy and we had to encourage them by sharing our personal experience and assuring them that nobody was born fit and "healthily literate".

Results: The results were surprising – more than 90% shared that they looked better. Also, many of the students claimed that they tried to eat more healthily during these 2 months, although they liked junk food a lot. About 60% claimed that they gained confidence and more than 80% made new contacts while they were practicing. We asked their teacher if they had become more interested in biology classes and she said that they asked for more "projects with the people from the university" so we assume that our work has contributed to the diversity of the traditional biology class.

Conclusion: To sum up, the idea met wide acceptance among the students and many of them wanted to take part in similar future projects. We achieved our goals and the results met our expectations. The students passed some time exercising instead of surfing the web and, in the end they agreed that it was not less exciting than chatting or watching movies. Almost half of the students claimed that they would go on practicing the same way they did during the project time and we hope that we have helped them in creating some healthy habits.

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