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REGULAR ARTICLES

All authors are responsible for submitting manuscripts in comprehensible US or UK English and ensuring scientific accuracy.

Original pictures on front cover: *Orthometopon romanicus* n. sp.
(Crustacea, Isopoda, Crinocheta: Agnaridae) (male)

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Antibiotic sensitivity pattern of bacterial isolates and physico-chemical composition of maize flour sold in major markets in Benin City, Midwestern Nigeria

Emmanuel Esosa Imarhiagbe[1,✉], Omoregbe Nosa Obayagbona[1], Osayomwanbo Osarenotor[1] and Aimuanmwosa Frank Eghomwanre[1]

SUMMARY. Samples of maize flour sold in selected markets in Benin City, Midwestern Nigeria were evaluated for antibiotic sensitivity pattern, bacteriological and physico-chemical qualities. The adoption of pour plate technique revealed a relatively high bacterial count in order of 10 - 10^3. Nil *Salmonella* counts were recorded in this study. *Bacillus* spp. was the most predominant (41.6 %) and the least predominant among the bacterial isolates was *Pseudomonas aeruginosa* (7.9 %). The various isolated bacteria showed variable patterns to the evaluated antibiotics, with zones of inhibition ranging from 0 mm to 20 mm. pH value ranged from 6.3 to 6.5; percentage moisture content ranged from 11.87% to 12.31%. There was a slight variation in the titratable acidity of the samples (2.10 to 2.56). Percentage fat content ranged from 5.10 % to 5.32 %, while the percentage protein and ash contents had a range of 8.45 % to 9.0 % and 0.85 % to 1.28 % respectively. This study revealed that maize flour from Benin metropolis markets harbored high bacterial counts with an array of antibiotic resistant bacteria. From a public health point of view, the bacterial quality of this relish food item sold in Benin City markets is indeed alarming and as such stringent measures should be adopted to manage the quality and curtail its possible role increasing the incidence of antibiotic resistance among population. However, this flour type was also observed to possess good basic dietary nutritional requirement (pH, moisture content, protein, fat and ash).

Keywords: antibiotic-sensitivity-pattern, bioteriological, maize-flour, markets, physico-chemical.

Introduction

Flour has been described as a microbiologically safe product due to its low water activity property (ICMSF, 1998); however, this property excludes the growth and survival of pathogenic bacteria that contaminate flour (Berghofer *et al*., 2003). Deibel and

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Swanson (2001) reported that cereals and cereal products is a notable food resource for the world's population and also a substrate for the proliferation of several spoilage micro-organisms under improper storage conditions. Nutritious traditional cereal foods are well sort after delicacies and they play very important role in diets in the South-Western geopolitical zone of Nigeria in particular and cereal producing zones of Africa in general.

Nutritionally, flour is known to contain a high proportion of carbohydrates, and relatively low in minerals, fats and proteins (Effiuvwevwere and Akoma, 1997; Batool *et al*., 2012), with a percentage moisture content ranging from 11 – 14 % (Batool *et al*., 2012) but the stipulated limit is 15% (WFP, 2012).

Maize flour is produced by the removal of the outer skin of the seed kernels. The removal of the outer skin of the grain during milling reduces the microbial load, ash protein and fat content (Fandohan *et al*., 2006). This has a profound effect on the physico-chemical and microbiological quality of the flour. Flour with low ash content (biomass), like extra super maize meal (refined) and cake flour have reduced fat and protein content and concomitantly low microbial load (Sperber, 2007).

The main aim of this study was to evaluate the antibiotic sensitivity pattern of maize flour borne bacterial isolates and ascertain the physicochemical qualities of maize flour sold in some major markets in Benin City, Edo State, Nigeria.

Materials and methods

Sources and collection of samples

A total of one hundred (100) samples were collected for this study from five (5) selected markets with Geographical Position System (GPS), namely; Uselu (N06.37359°, E005.61517°), Oka (N06.29031°, E005. 66377°), Ekiosa (N06.32025°, E005.63661°), Ekiuwa (N06.35192°, E005.61398°) and New Benin Market (N06.35168°, E005.63047°), located in Benin City, Edo State, Nigeria (Figure 1). Adopting standard aseptic and safety precautions, twenty (20) samples were collected per market (approximately 500g/product) using sterile polyethene bag. They were transferred to the laboratory for analysis within 24 hrs.

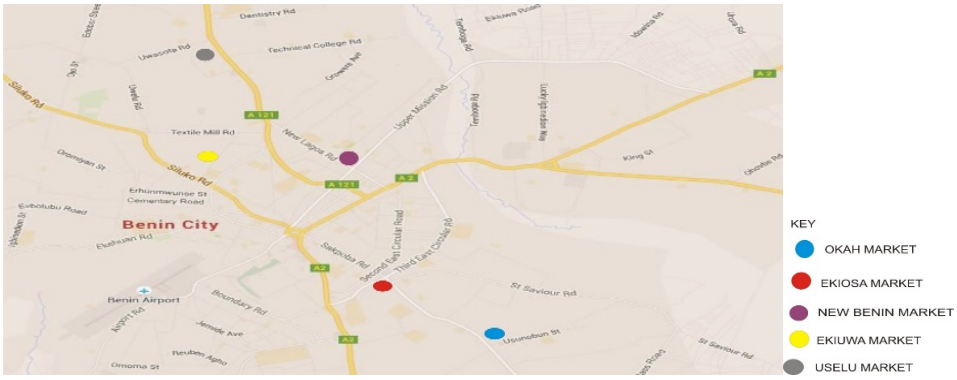

Figure 1. Map of studied area showing sampling markets (Credit: Google)

Enumeration and cultural methods

Twenty grams of each maize flour sample was aseptically weighed and homogenized with 180ml of sterilized 0.1% peptone water to produce a 10^1 homogenate. Subsequent serial dilutions up to 10^6, were aseptically made from the initial homogenate. The total heterotrophic bacterial counts were determined by the pour plate technique using 1.0ml of appropriate serially diluted samples in nutrient agar (Oxoid) and incubated at 30°C for 48 hrs. For total coliform plate counting, 1.0ml of the serially diluted samples were plated on MacConkey Agar (Oxoid) and incubated at 37°C for 24 hrs. Total Staphylococcal plate counts were determined using mannitol salt agar (Oxoid) at 37°C for 72hrs. Total *Salmonella* counts were determined by homogenizing 25 g of each maize sample into 225 ml lactose broth for 2 mins and then incubated for 24 hrs at 37 °C. This was followed by subculturing of 1 ml of the overnight incubated culture into selenite F broth (Oxoid) and was incubated for 24 hrs at 37 °c. After which 1 ml of pre-enriched culture was re-suspended into deoxycholate citrate agar (DCA) (Oxoid) for 24 hrs at 37°C. Triplicate plates of appropriate dilutions were made. The means of colony counts were used to compute colony forming units per gram (cfu/g).

Purification and identification of isolates

Bacterial purification was done by sub-culturing the various isolates onto nutrient agar plates and Gram-stained (Cheesbrough, 2000). Phenotypic profiling of both Gram-positive and Gram-negative bacteria was undertaken using API 50CHB and API 20E strips (BioMerieux, Marsielle, France). Additional tests of spore stain and oxidase were also performed.

Antibiotic sensitivity assay

Antibiotic sensitivity patterns of the isolated bacteria were determined by disc diffusion method (Cheesbrough, 2000). The test bacterial suspension were inoculated unto Muller-Hinton agar and followed by application of the discs (Oxoid) impregnated with different antibiotics. Antibiotic disc contained the following antibiotics: Amoxicillin (25µg), Streptomycin (10 µg), Ceftriaxone (30 µg), Ofloxacin (5 µg), Gentamicin (10µg), Cotrimoxazole (25 µg), Pefloxacin (5 µg), Ciprofloxacin (10 µg), Erythromycin (10µg), Chloramphenicol (30µg).The diameter of the zone of inhibition of each antibiotic disc was measured and recorded (mm).

Determination of physicochemical qualities

pH, titratable acidity, moisture, protein, fat and ash content were analyzed according to AOAC (1998).

Results and discussion

Generally, the results indicated a high total viable count (Table 1A). The mean range of bacterial counts was in the order of 10 - 10^3. The mean total heterotrophic bacterial counts ranged from 5.0×10^3 cfu/g to 7.1×10^3 cfu/g. The mean total coliform counts ranged from 3.0×10^2 cfu/g to 4.2×10^2 cfu/g, while the mean staphylococcal counts ranged from 3.0×10^2 cfu/g to 4.1×10^2 cfu/g. Nil *Salmonella* counts were recorded. Ntuli *et al*. (2013) reported that flour is known to be a safe commodity due to its low water activity, but the current study revealed the presence of microbial contaminants in the retail maize flour which are of public health concern. The high bio-load and variety of microorganisms detected and isolated from these market maize flours may be attributed to the poor sanitary and handling practices following production. These production practices may include spreading on the floor, measurement with the aid of bare hands, coughing, sneezing, exposure of the flour to moisture during retailing and others as strong contribution factors (Imarhiagbe and Emoghene, 2006). The result from this study is in agreement with previous works done by Ntuli *et al*. (2013), Aydin *et al*. (2009) and Berghoefer *et al*. (2003). Total bacterial counts and coliform counts are important parameters which are indicative of the hygienic properties of the food. *Salmonella* spp. in food items is known to account for more than 50 % of all food poisoning cases (WFP, 2012). *Salmonella* spp. causes diseases such as typhoid, paratyphoid and food poisoning (Prescott *et al*., 2002).

Table 1A.

Microbial load of maize flour in selected Benin City Markets

Markets	Total Heterotrophic Count (x 10^3 cfu/g dry weight of flour)	Total Coliform plate Count (x 10^3 cfu/g dry weight of flour)	Total Staphylococcal Count (x 10^3 cfu/g dry weight of flour)	Total *Salmonella* Count (x 10^3 cfu/g dry weight of flour)
Uselu	7.1	4.2	4.1	0.0
Oka	6.5	3.1	3.0	0.0
Ekiosa	5.2	3.0	3.0	0.0
Ekiuwa	5.0	3.0	4.0	0.0
New Benin	6.7	3.7	3.6	0.0

Table 1B revealed the percentage frequency of occurrence of bacterial isolates obtained from the maize flour samples. The result showed that *Bacillus* spp. was the most predominant (41.6 %) followed by *Enterobacter aerogenes* (16.9 %), *Micrococcus* sp. (13.5 %), *Staphylococcus* spp. (11.2 %), *Staphylococcus aureus* (9.0 %) and the least predominant among the bacterial isolates was *Pseudomonas aeruginosa* (7.9 %). Most of the bacteria isolated in this study are enteric organisms and also found in common proportion in soil, hence this food type and other cereal products are prone to microorganisms during pre- and post-harvesting stages. The presence of high counts of *Bacillus* spp., *Staphylococcus* spp., may be related to their ability to withstand dryness

and other harsh environmental conditions such as low water activity (Prescott *et al,* 2002). Some of these organisms may have found their way into this food sample through cross contamination by handlers. Certain strains of *Bacillus* have been known to cause food poisoning (Prescott *et al*., 2002).

Table 1B.

Percentage frequency of the maize flour bacterial isolates

Bacterial isolate	No of isolates	Percentage frequency of occurrence (%)
Bacillus sp.	22	22.5
Bacillus subtilis	19	19
Enterobacter aerogenes	17	16.9
Micrococcus sp.	13	13.5
Staphylococcus aureus	9	9.0
Staphylococcus sp.	11	11.2
Pseudomonas aeruginosa	7	7.9
	n=98	**100**

The measured antibiotic inhibitory zones (mm) revealed variable antibiogram patterns of the tested isolates (Table 2). It was also observed that all the bacterial isolates were sensitive to chloramphenicol with a zone of inhibition ranging from 12 mm to 20 mm, and there were no zone of inhibition around cotrimoxazole (which indicate possible resistance to the antibiotics). Omogbai and Ikenebomeh (2013) reported the increased incidences of bacterial resistance to antibiotics are indicative of the prevailing trend of antibiotic abuse and misuse by the society. This phenomenon is basically due to the fact that individuals purchase and consume antibiotics without necessary medical advice. Bacterial groups co-habiting a common environment may express a similar antibiotics pattern if they share in a common pool of R-factor plasmids (Spanggard *et al.,* 1993).

Table 2.

Diameter (mm) of zones of inhibition of antibiotic

Isolate	AMX 25µg	STR 10µg	CRO 30µg	OFL 5µg	CHL 30µg	GEN 10µg	COT 25µg	PFX 5µg	CPX 10µg	ERY 5µg
Bacillus sp.	0	0	0	0	13	10	0	0	0	0
B. subtilis	0	0	0	0	13	0	0	0	0	0
Enterobacter aerogenes	15	11	0	14	18	18	0	12	8	0
Micrococcus sp.	0	12	0	12	12	0	0	12	0	0
Staph aereus	0	0	0	0	13	0	0	0	0	0
Staph sp.	0	0	0	0	12	0	0	0	0	10
P. aeruginosa	0	0	13	8	20	0	0	12	12	0

KEY: AMX (amoxicillin), STR (streptomycin), CRO (ceftriaxone), OFL (ofloxacin), CHL (chloramphenicol), GEN (gentamicin), COT (cotrimoxazole), PFX pefloxacin), CPX(ciprofloxacin), ERY(erythromycin). B (*Bacillus*), *Staph aereus* (*Staphylococcus aereus), P. aeruginosa (Pseudomonas aeruginosa*)

The physico-chemical qualities of any food items are principal determinants of consumer acceptability and safety. Table 3 showed the physico-chemical composition of maize flour from the selected markets in Benin City. The pH value ranged from 6.3 to 6.5; the percentage moisture content also range from 11.87 to 12.31.The shelf life and microbial growth are basically influenced by factors such as pH and moisture (Batool *et al*., 2012). Thus, pH and moisture are important parameter to be considered in the evaluation of the quality and acceptability of a food item such as maize flour. Also, high moisture content is undesirable in food and it potentates microbial growth (Kordylas, 1990). The percentage moisture content of the samples was relatively high (ranging from 11.87 to 12.31 %). The high count recorded in all samples may be indicative of favorable pH and moisture condition for growth and survival. Flour is susceptible to spoilage especially when stored in moist conditions; especially when stored over a longer period of time (Victor *et al*., 2013). Slight variations were also detected in the titratable acidity of the samples (2.10 to 2.56). Omonigho and Ugbo (1998) reported that increase in titratable acidity might be due to the increased microbial activities of the micro-organisms. Percentage fat content ranged from 5.10 to 5.32, while the percentage protein and ash contents ranged from 8.45 to 9.0 and 0.85 to 1.28 respectively. WFP (2012) had proposed 8.0 % protein content as minimum requirement for maize flour quality. Thus, the relative high protein contents (8.45 to 9.0 %) observed in this study is an indication that maize flours from this region are of good quality. It had been ascertained that ash content in maize flour infers the level of husks (Ekinci and Unal, 2003), and the flour's mineral composition (Victor *et al*., 2013). It was observed that percentage ash content of all analyzed maize flour samples were below the threshold of recommended limit of 3.0 % (WFP, 2012).

Table 3.

Physico-chemical qualities of maize flour sold in Benin City markets

Markets	pH	Titratable Acidity (0.1m NaOH)	Moisture Content	Fat	Protein	Ash
			(%)			
Uselu	6.5	2.43	12.10	5.18	8.72	1.28
Oka	6.5	2.10	12.31	5.32	9.0	1.14
Ekiosa	6.3	2.43	12.00	5.10	8.82	1.21
Ekiuwa	6.4	2.47	11.87	5.29	8.80	0.85
New Benin	6.3	2.56	12.00	5.18	8.45	1.20

NB: Overall mean value

Conclusions

This study revealed that maize flour from Benin metropolis markets harbored high counts of bacteria with array of antibiotic resistant bacteria. When considered from the public health point of view, the bacterial quality of this relish food item sold

in Benin metropolis markets is alarming and as such stringent measures should be adopted to manage the quality and curtail its possible role in increasing the incidence of antibiotic resistance among population. However, this flour type was also observed to possess good basic dietary nutritional requirement (pH, content of moisture, protein, fat and ash).

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Application of dry cell battery dust to cultivated fluted pumpkin (*Telfairia occidentalis*) as a pest management strategy: implications for both the plant and the consumer's health

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SUMMARY. The study investigated the possible impact of dry cell car battery dust (DCD) on production of fluted pumpkin (*Telfairia occidentalis*). The study aims to investigate the possible implication of the application of DCD to fluted pumpkin as pest practice by some farmers in the study area. Therefore, the experiment included three different treatments: the first examined the effect of DCD on the plants after they were sown in DCD-polluted soils (DSBS); while the second was the investigation of plants on which DCD was applied after 2 weeks after sowing (DPAS). The third group was the control, wherein DCD was applied to neither plant nor soil. Results showed that plant yield, expressed in the study as the number of leaves per plant and leaf size, was better with those plant on which DCD was applied after plant establishment (32 – 48 leaves, leaf area 98.5 – 126.5 cm^2) compared with the control (13 leaves, leaf area 43.1 cm^2). Leaves in the control as well as the DSBS plants were characterized with brown spots, chlorosis, necrotic spots as well as evidence of insect pest attack. No visible sign of insect or pest attack was noticeable in the DPAS plant leaves. Although there was no significant change in proximate content of the leaves of both DCD-treated plant and the control, the leaves of DCD-impacted plants however accumulated significantly higher amounts of Pb (40.25 – 77.17 mg/kg) and Zn (13.35 – 45.87 mg/kg) than the control.

Keywords: dry cell car battery, heavy metal, hyperaccumulation, phyto-accumulation, *Telfairia occidentalis*

Introduction

Due to rapid urbanization the demand for food crops is rising day by day, and as the vegetables can be grown in small fields with intensive use of inputs within shorter period, its cultivation is gaining popularity and fetching profitability

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in urban, peri-urban and rural areas. This is a matter of serious concern as vegetables particularly leafy once, being accumulators of heavy metals provide an easy entry into the food chain. The excessive intake of these elements from the soil creates dual problems; first the harvested crops get contaminated, which serve as a source of heavy metal in our diet, and secondly the crop yield decline due to the inhibition of metabolic processes (Sanders and Adams, 1987; Singh and Agrawal, 2003).

Solid waste management constitutes a big challenge to the developing countries, especially Nigeria, Edo State inclusive because waste sorting and separation is not practiced. Thus a material that has lost its value is sent to the waste dump sites and disposed improperly. Some farmers have resorted to cultivating their vegetables on contaminated lands due to its high organic content in order to increase their yields and gains, and as a result, human health is being endangered due to heavy metal uptake by vegetables grown in soils contaminated with heavy metals (Carlson and Bazzaz, 1977; Alloway, 1996).

At a 2-Day National Training Workshop on Fruits production, concentrates processing and marketing, organized by Raw Materials Research and Dev. Council, Abuja in conjunction with the Edo State Ministry of Commerce and Industry held at Bishop Kelly Pastoral Center, Airport Rd., Benin City on November 22-23, 2012, the participants had the opportunity to discuss issues relating to possible accumulation of metals in fruits and harvestable plant parts and thus care was needed in collection fruits for processing. Particular attention was drawn to a publication by Ikhajiagbe *et al.* (2013), who reported heavy metal contents and microbial flora of fresh leaves of fluted pumpkin (*Telfairia occidentalis*) collected from road-side open markets in Benin metropolis, midwestern Nigeria. The argument was whether leafy vegetables on sales by the roadsides had the capacity for metal accumulation from nearby vehicular fumes or whether there were other sources of metal accumulation. A member of the All Farmers Association of Nigeria (AFAM) at the workshop insisted that the accumulation may be from the cropping system adopted by most farmers in growing fluted pumpkin. Accordingly, they use dry cell battery dusts on the plant stands to ward off insects and other pests. This was corroborated by most of the farmers at that workshop; insisting that the practice maid for larger and greener vegetables. Vegetable crop plants have high ability to accumulate metals from the environment and have however, been found to absorb heavy metals from the soil as well as from surface deposits on part of vegetables exposed to polluted air, which may pose risks to human health when they are on or near contaminated lands and consumed (Yusuf *et al.*, 2003). Up till the time of writing up the design for this study, the practice is still commonly practiced by a number of small scale commercial and subsistence farmers in Benin City. The present study thus becomes imperative.

The choice of *T. occidentalis* for the study is because it plays an important role in human and livestock nutrition and is an extremely important vegetable predominantly grown by small scale farmers in any available space especially in abandoned dump sites

where suspected heavy metal laden materials are found in large quantity. *T. occidentalis* is a good accumulator of heavy metals (Erhenhi and Ikhajiagbe, 2012; Ikhajiagbe *et al.*, 2013).

Toxic metals may be absorbed by this vegetable through several processes and finally enter the food chain at high concentrations capable of causing serious health risks to consumers. Their toxicity can damage or reduce mental and central nervous function, lower energy levels, and damage blood composition, lungs, kidneys, liver, and other vital organs.

This study was undertaken to evaluate heavy metal concentration of the soil polluted with dry cell battery dust and *T. occidentalis* grown on such soil as well as draw conclusions from the results obtained on the suitability or otherwise of the plant cultivated for its phytoremediation ability and for human consumption as well. Also, the question the research intended to answer was whether it was possible for any vegetable plant sown in heavy metal polluted soil within 3 months of pollution could survive and give the necessary nutritional benefits required by the body.

Materials and methods

Collection and preparation of materials for the experiment

Viable seeds of pumpkin (*Telfairia occidentalis*) were obtained from New Benin Market, Benin City, Edo State. Dry cell battery dust was obtained from dry cell car batteries collected from a mechanic shop (deals in petrol-engine cars) at Spare Parts Market, Evbarekare, off Textile Mill Road, Benin City, Edo State.

Dry cell battery dust (DCD) was obtained by crushing the dry cells from car batteries irrespective of voltage or electricity or electricity capacity. The cells were carefully crushed and then grounded into near-powdery form and then stored in a closed container before use. The dust was analyzed for composition of heavy metals (see Table 1).

Top soil (0 – 10 cm) of physiochemical property predetermined (Table 1) was collected from a cleared field, beside the Botanic Garden. Thereafter, 20 kg of the sun-dried soil was collected using shovel and hand trowel, weighed and placed into buckets. The buckets were grouped into three experiments code-named as CTR, DSBS, and DPAS respectively. CTR was the control (consisting of 4 buckets). DSBS-group comprised of those buckets containing soil that was polluted with dry cell battery dust before sowing *T. occidentalis* seeds (consists of 16 buckets). The Pap-treatment comprised of those that received dry cell battery dust in already planted (2-weeks old) *T. occidentalis* stands (consists of 16 buckets).

DCD was applied to either soil (DSBS) or plant (DPAS) in 4 concentrations; 1, 5, 10, and 25% w/w, amounting 0.2, 1, 2, and 5kg of DCD added to 20kg soil or plant stands as the case may be (Fig. 1). The entire set up was exposed in the Botanic Garden (Department of Plant Biology and Biotechnology, University of Benin,

Benin City) for 3 months under prevailing weather. Afterwards, plant leaves were randomly collected and taken to the laboratory for analyses. Plants were observed for physical morphological presentations as well as nutrient contents of the leaves. The chlorophyll content index of leaves of the test plant were estimated at 10 weeks after sowing using a chlorophyll content meter; CCM-200 plus®. CCM-200 is a non-destructive chlorophyll content measuring meter that uses absorbance to estimate the chlorophyll content in leaf tissues. The detector analyzes the absorbance ratio of both wavelengths and calculates a CCI value that is proportional to the amount of chlorophyll in the sample (Apogee Instruments Inc.). Proximate analysis of the leaves was carried out according to the methods of AOAC (2005). Accumulated heavy metal (HM) contents in plant leaves were determined by atomic absorption spectrophotometry (model, Buck Scientific 210 VGP), according to the methods of SSSA (1971) and AOAC (2005). For HM determination, plants were divided into 3 partitions; the old partition comprising of entire leaves within the plant axil measuring 45 cm from the soil level; the upper (new) partition comprised of all leaves within plant axil measuring 45 cm from apical meristem, whereas the middle partition was the portion in-between the old and new partitions (Fig. 2).

Figure 1. (a) The researcher applying DCD to plant stands (b) Plant stands at 3 weeks (c) Plant stands at 4 weeks (d) plant stands at 8 weeks

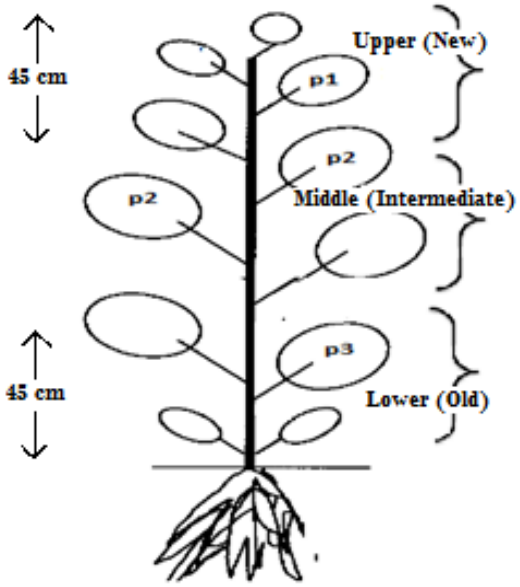

Figure 2. Leaf partitioning used in the study

Results and discussion

Physicochemical quality of soil and DCD used in the study has been presented (Table 1). This study relied on information provided by local farmers on the use of dry battery cell dust (DCD) as an alternative to insect and pest control during cultivation of fluted pumpkin. This is even more predicated on the fact that the poor economic situation in the country (Nigeria) has resulted in increase in prices of agricultural inputs including fertilizers and pesticides. The results of the study thus confirms the fear that over-reliance on this abnormal practice would only lead to catastrophe, beginning first with biomagnification of metals in the DCD-impacted plants by consumers and subsequent accumulation in the food chain.

On the 13th week, some morphological parameters of the plant were measured (Table 2, Figure 3). Stem length in the 0.2 kg DSBS plant was the highest in that category (435.42 cm), compared to 330.32 cm in 5 kg DSBS. Plant height in the plants broadcasted with DCD (DPAS-group) ranged from 474.22 cm in those plant stands that received 1kg of DCD directly (1 kg DPAS) to 300.34 cm in those plant stands dusted with 5kg of DCD (or 5 kg DPAS) respectively. The control plant was 215.65 cm. There were more leaves per plant in the plants sown in DCD-polluted soil (DSBS), ranging from 36 – 68 leaves, as well as those on which DCD was applied (DPAS-plants, 32 – 48 leaves), compared to control plants (13 leaves).

Table 1.

Physical and chemical properties of soil and dry cell battery dust used in the study

Parameters	Original soil used	Dry cell dust
pH	5.58	4.21
Electric conductivity (µs/cm)	300	ND
Total Org. carbon (%)	0.41	ND
Total Nitrogen (%)	0.10	ND
Exchangeable acidity (meq/100g)	0.20	ND
Na (meq/100g)	10.90	ND
K (meq/100g)	1.65	ND
Ca (meq/100g)	15.60	ND
Mg (meq/100g)	11.30	ND
Cl (mg/kg)	1666.00	ND
P (mg/kg)	153.00	ND
NH_4N (mg/kg)	25.40	ND
NO_2 (mg/kg)	15.01	ND
NO_3 (mg/kg)	30.75	ND
SO_4 (mg/kg)	14.63	ND
Clay (%)	4.43	NA
Silt (%)	7.82	NA
Sand (%)	87.82	NA
Fe (mg/kg)	1009.21	ND
Zn (mg/kg)	38.03	161.75
Cd (mg/kg)	0.01	7.75
Pb (mg/kg)	3.84	231.50
Ni (mg/kg)	BDL	8.85

BDL Below detection (<0.001 mg/kg); ND Not determined; NA Not applicable

Table 2.

Some measurable morphological parameters of *T. occidentalis* at 13 weeks after sowing

Parameters/ Treatments	Stem length (cm)	Stem girth (cm)	No. of leaves (Plant Yield)	Leaflet area (cm^2) (Plant Yield)	Tendril length (cm)	No. of tendrils/ plant	No. of primary stem branches	Plant fresh weight (g)	Root Length (cm)	No. of Sec. Root	Fresh root weight (g)
Control	215.65	2.3	13.2	43.1	21.3	87.7	3.72	98.63	10.3	14.6	32.6
5 kg DSBS	330.32	4.0	45.5	96.0	32.6	76.3	6.54	220.32	35.5	11.3	91.2
2 kg DSBS	340.34	3.5	36.3	86.7	28.1	84.4	5.56	254.34	25.2	12.7	53.2
1 kg DSBS	313.23	3.5	43.6	80.4	36.3	81.2	8.54	265.44	48.6	11.1	62.3
0.2 kg DSBS	435.42	4.3	58.4	93.6	45.7	119.7	5.87	258.34	50.4	10.2	80.5
5 kg DPAS	300.34	2.7	31.5	98.5	15.6	51.4	4.85	268.14	65.6	10.5	43.1
2 kg DPAS	460.35	3.2	38.3	108.1	30.7	74.8	4.76	258.43	55.2	14.7	50.6
1 kg DPAS	476.22	4.1	48.4	121.2	35.2	80.2	8.53	280.43	60.7	15.4	106.4
0.2 kg DPAS	343.75	3.5	36.3	126.5	38.4	43.5	6.75	251.34	90.3	10.8	93.6
LSD (0.05)	64.86	1.4	9.36	12.73	14.42	22.75	2.73	32.15	8.74	4.35	19.64

DSBS dry cell dust applied to soil before sowing pumpkin on polluted soils; **DPAS** dry cell dust applied to plant stands sowing in clean soils. **Stem girth** was obtained at 5cm above ground.

However, with regards to leaf size, the DPAS-plants were broader (88.5 – 142.5 cm^2) than the DSBS-plants (80.4 – 96.00 cm^2). Leaf area of control plants was 43.1 cm^2. Obviously, sprinkling DCD unto plant leaves reduced factors than retarded foliar development. Root length was lowest in the control plant (10.3 cm) compared to the DCD-exposed plants (Table 3). In the DCD-exposed plants, those that were exposed after planting (DSBS-plants) had longer roots (55.2 – 90.3 cm) compared to the DSBS-plants (25.2 – 50.4 cm). Similarly, fresh root weight was highest in 1 kg DPAS (106.4 cm) compared to the control (32.6 cm).

Accordingly, *T. occidentalis* were partitioned into new, intermediate and old leaves (Table 3). The old partition was taken as plant portion covering 45 cm from soil level; the new partition covered plant parts 45 cm from the plant apex, whereas the intermediate partition was taken as the middle leftover portion between the old and new partitions. More leaves of the old partition senesced, particularly those of the DSBS-pant category. However, more intermediate leaves were senesced in the DPAS-category (5 – 12 leaves) than in the DSBS-category (2 – 5 leaves). Similarly, more leaves senesced in the newer partition when DCD was applied unto plants (DPAS). The results also show increased CCI in leaves of DCD-impacted plants. There were no significant changes in CCI content from one partition to the other.

Table 3.

Leaf senescence and chlorophyll content index observed within partitions of *T. occidentalis*

Parameters/ Treatments	**No. of senesced per plant (from 5 – 13 WAS)**			**Chlorophyll content index (CCI) (only at 10 WAS)**		
	New partition	**Intermediate partition**	**Old partition**	**New partition**	**Intermediate partition**	**Old partition**
Control	00*	00	28	24.32	29.53	20.43
5 kg DSBS	01	05	45	36.44	38.42	34.21
2 kg DSBS	02	02	41	32.22	33.56	32.54
1 kg DSBS	02	03	35	38.42	34.29	30.29
0.2 kg DSBS	00	04	32	31.32	32.28	31.27
5 kg DPAS	09	12	25	44.34	41.69	34.35
2 kg DPAS	07	09	09	43.12	42.33	43.24
1 kg DPAS	03	10	12	38.42	39.74	40.76
0.2 kg DPAS	01	05	25	43.86	44.24	42.32
LSD (0.05)	02	03	11	5.52	5.26	6.34

*mean of 3 replicates rounded off to the nearest whole number. **WAS** weeks after sowing. **DSBS** dry cell dust applied to soil before sowing pumpkin on polluted soils; **DPAS** dry cell dust applied to plant stands sowing in clean soils; The old partition was taken as plant portion covering 45 cm from soil level, the **new** partition covered plant parts 45 cm from the plant apex, whereas the **intermediate** partition was taken as the middle leftover portion between the old and new partitions.

Physical observations of *T. occidentalis* performance in the designated plant partitions have been presented on Table 4. It was generally observed that there were no visible signs or presence of insects perching or feeding on the plant leaves on which DCD was applied after sowing (DPAS-group). Emphatically, results earlier showed that those plants treated with DCD after sowing showed better physical growth parameters – improved leaf size, reduced foliar spoilage due to insect attack. However, when applied to soil before sowing, morphological presentations of the plants declined significantly. HM toxicity diminishes plant growth and vigour, leading to death in extreme cases when significant interference of HM exists with photosynthesis, respiration, and plant-water relation (Smith *et al.*, 1989; Burd *et al.*, 2000; Chaperon and Sauvé, 2008; Ikhajiagbe and Anoliefo, 2010). It is possible that the impact on growth and morphology in the DPAS-plants was less significant compared to the DSBS-plants because of the effects of washing by rain. Since the study was carried out in the open field, there were a couple of times when it rained after DCD application. DCD which ordinarily would act on the leaf from its surface may have been reduced in concentration due to washing, thereby decreasing its effects on morphology. Baker (1981); Carlson *et al*. (1991); Haanstra and Doelman (1991); Gonzalez (1996); Halim *et al.* (2003); Ortiza and Alcañiz (2006); Wyszkowska *et al.* (2006); Martin and Griswold (2009); Nanda and Abraham (2011); Shittu and Ikhajiagbe (2013) had previously reported that effects of HM on plant growth was concentration-dependent.

Decline in growth parameters as reported in the study may be due to the inhibition of metabolic processes at higher concentrations of dry cell battery dust as earlier suggested by Sanders and Adams (1987); Singh and Agrawal (2003). Elevated concentrations of both essential and non-essential heavy metals in the soil however, can lead to toxicity symptoms and growth inhibition in most plants (Hall, 2002; Li *et al.*, 2005a,b). One possible route for HM toxicity in the plant studied is the binding of HM to sulphydryl groups in proteins; this results in impaired protein activity or disruption of structure. In some other cases, there is the displacement of an indispensable element. This cascade of activity results in deficiency effects.

Leaf senesce is one of several mechanism adopted by plants during HM stress. In the study, more leaves of the old partition senesced, particularly those of the DSBS-pant category. These are those that are close to the soil, since the only route from which phytoaccumulation of HM from soil is the root. Hence, plant leaves closer to the leaves are most likely to be more impacted. However, in the DPAS-plants, the number of senesced intermediate leaves was more. Actually, since application was from top of plant down to the bottom, it is most likely that the intermediate leaves would receive more DCD from the ones washed by rain from the upper leaves, including the ones initially applied.

The productivity of the DCD-impacted plants may have been better than in the control, given the increases chlorophyll content index. Evidently, DCD may contain some growth-promoting substances to have enhanced chlorophyll concentration in the leaves. Improved chlorophyll content index in plants implies an enhanced photosynthetic capacity of the plant during high and low sunlight.

Table 4.

Physical observations of *T. occidentalis* partitions into new, intermediate and old

	New	Intermediate	Old
5 kg DSBS	Presence of chlorosis, senesced leaves, stems and tendrils, insect eating, few brown spots, leaves become dark green, growth increases and stem wilts.	Senesced leaves, stems and tendrils, few insect biting, presence of chlorosis stem wilts.	Insect biting, branches increases, senesced leaves, stems and tendrils, leaves become dark green and tendril length increases.
2 kg DSBS	Smaller leaves, tendril length increases, few insect biting and brown spots.	Brown spots	Presence of chlorosis, senesced leaves, stems and tendrils and dryness at all parts of the plant.
1 kg DSBS	Few insect biting, wider and dark green leaves.	Branches increases	Senesced leaves, stems and tendrils.
0.2 kg DSBS	Leaf folding, increased number of tendrils and few brown spots.	Brown and cream spotting, no branching, insect and animal biting and leaf buning,	Cream colour spotting, increased branches, senesced leaves, stems and tendrils, few insect biting, wider and dark green leaves.
Control	Insect biting, cream spots, presence of chlorosis and light green leaves.	Presence of chlorosis, wilting, cream and brown spots.	Senesced leaves, stems and tendrils, cream and brown spots.
5 kg DPAS	Smaller leaves, senesced leaves, stems and tendrils.	Few brown spots on leaves; leaves become smaller and dark green.	Senesced leaves, stems and tendrils, few brown spots on leaves.
2 kg DPAS	Insect biting, brown spots and wider leaves.	Presence of chlorosis, wider and darker leaves.	Senesced leaves, stems and tendrils, drying of leaves.
1 kg DPAS	Wider and darker leaves, few brown spots.	Presence of chlorosis.	Senesced leaves, stems and tendrils, drying of leaves.
0.2 kg DPAS	Few insect biting with mucor on leaves, wider and darker leaves.	Senesced leaves, presence of chlorosis and leaf folding.	Senesced leaves, stems and tendrils, smaller leaves, few cream spots.

DSBS dry cell dust applied to soil before sowing pumpkin on polluted soils; **DPAS** dry cell dust applied to plant stands sowing in clean soils; The **old** partition was taken as plant portion covering 45 cm from soil level, the **new** partition covered plant parts 45 cm from the plant apex, whereas the **intermediate** partition was taken as the middle leftover portion between the old and new partitions.

This perhaps explains the significant changes in growth and yield of DCD-impacted plants compared to the control. It is suggested that further research could be done to isolate and characterize possible growth-promoting substances in DCD. Berndt (1997) and Vincent and Scrosati (1997) had earlier reported that dry cell batteries contained a cathode rod made up of ammonium chloride. In order to get the DCD, the entire contents, excluding the plastic casing of the battery was grounded into powered. It is suggested that the ammonium content of the DCD may be one of the growth-promoting substances for which DCD-impacted plants performed better than the control. Middleton and Smith (1979); Rhodes (1987)

reported that when conditions for plant growth become unfavourable, such as acidic soils, HM toxicity, or other restrictive factors for nitrification, ammonium is the major nitrogen source for the affected plant. Most of the nitrogen from ammonium is converted to nitrate by the activities of nitrifying bacteria in the soil, which is then transported to the plant shoot, where it is reduced to ammonia and assimilated into amino acids. Blanke *et al.* (1996) reported that ammonium nutrition in pants significantly enhanced chlorophyll production.

The study also investigated heavy metal contents of soil and test plant after harvest, including proximate contents (Tables 5-7). Prior to sowing pumpkin seeds in the DCD-contaminated soil, heavy metal (HM) content of soil after 60 days was determined (Table 5). Metal concentration generally increased with increased concentration of DCD in soil. Significant increases in metal concentrations compared to the control was recorded ($p<0.05$). Zn levels ranged from 61.14 – 102.52 mg/kg in the DCD-spiked soils compared to the control (34.99 mg/kg). However, Zn level in soil was below ecological screening value (ESV) for both microbial activity and phytotoxicity. After harvest, the levels of Pb in soil were beyond ecological screening value for plants survival in Pb-contaminated soil. This value was higher before the introduction of the test plant (see Table 5), and yet higher than ESV after removal of plant, thus suggesting tolerance of the test plant to Pb.

Table 5.

Heavy metal contents of soil after test plant was harvested

	Zn	**Cd**	**Pb**	**Ni**
	Soil HM conc. before sowing test plant, DSBS			
Control	34.99	0.03	2.99	BDL
25%	102.52	6.07	337.04	6.13
10%	87.88	4.97	247.65	5.84
5%	71.26	4.02	199.90	4.54
1%	61.14	3.00	65.82	4.26
	HM conc. after plant harvest at 13 weeks, DSBS			
Control	15.07	1.04	1.14	BDL
5 kg DSBS	9.59	0.71	64.27	0.14
2 kg DSBS	11.16	0.68	53.35	0.27
1 kg DSBS	21.01	0.93	79.46	1.39
0.2 kg DSBS	9.23	1.13	101.65	0.89
5 kg DPAS	12.13	1.14	56.34	2.04
2 kg DPAS	10.05	0.28	56.80	0.57
1 kg DPAS	8.79	0.86	90.97	0.37
0.2 kg DPAS	19.82	1.08	57.14	2.43
LSD (p, 0.05)	**5.32**	**1.07**	**34.42**	**0.87**
ESVm	100.00	20.00	900.00	90.00
ESVp	50.00	4.00	50.00	30.00

DSBS dry cell dust applied to soil before sowing pumpkin on polluted soils; **DPAS** dry cell dust applied to plant stands sowing in clean soils; **BDL** beyond detectable limit (0.001mg/kg).

Concentrations of Cd in leaves of the test plant after harvest was generally below detection limit for the DSBS-plants. However, in the DPAS-plants, evidence of Cd absence in new leaves of plants exposed to low concentrations of Cd was shown (Table 6). Similarly, Cd concentrations were higher in older leaves than in the new and intermediate leaves respectively; showing thus that accumulation of Cd in plant leaves depended on the age of the leaves. Zn accumulation in the leaves of the test plant was relatively minimal from each other; no significant differences existed among the 3 partitions with respect to Zn accumulation. This shows that at any given time, the plant balances the accumulation of Zn in its leaves, irrespective of age. In the DPAS-plants and with respect to Pb accumulation, there were higher concentrations of Pb in the upper leaves than in the intermediate and the lower. Suggestively, plant rate of metabolism is higher with new plants or plant parts than with the old. This may have some consequence on Pb accumulation. There was no significant difference in the divided accumulation of Ni in the partitioned plant leaves.

Results showed lower levels of Cd were obtained in those plants sown in DCD-polluted soils. However, irrespective of the mode of DCD application to soil or plant, harvested vegetable leaves contained very high levels of Pb. When in soil, most heavy metals become bound to organic and inorganic compounds in the soil, whereas a smaller proportion remains in the available form; thses available metal forms are either adsorbed against soil colloids, or they are dissolved in soil water (Punz and Sieghardt, 1993). Consequently, metal solubility and mobility becomes highly predisposed to their affinity for other ions or compounds within the soil matrix (Punz and Sieghardt, 1993). The availability of lead in higher proportions in the study may not be unconnected with any of these factors. Being a week Lewis acid, Pb forms durable bonds with the soil organic matter (Begonia *et al.*, 1998; Päivöke, 2002; Sharma and Dubey, 2005). It also forms complexes with sulfur (Xintaras, 1992), and freely precipitates as carbonates, phosphates and hydroxides (McBride, 1994).

Although Xiong (1997); Chantachon *et al.* (2004) have reported the rhizoextraction of Pb from soil, the movement of this metal within the plant is less well characterized. Therefore, Pb translocation to harvestable plant parts, like the leaves in this study, may be limited by binding at root surfaces or in the cell walls of roots as earlier reported by Pahlsson (1989). Pb accumulation in the cell apoplast has been reported by Tung and Temple (1996) in *Zea mays,* Wierzbicka (1998) in *Allium cepa* and *Pisum sativum* and by Piechalak *et al.* (2002) in *Vicia faba*. Sharma and Dubey (2005) also reported that Pb radially moves through the root apoplast across the cortex.

The higher values for Pb in plant tissues as reported in this study were higher than statutorily provided limits for edible vegetables (Agrawal *et al.*, 2007). This has great implications for human health. These accumulated metals are non-biodegradable. Consequently, then can accumulate in body tissues and organs like the bones, kidney and liver, for a very long time, where they can a series of metabolic damage. Although

the rate of toxicity of these accumulated metals depend, to a very large extent, on the level of intake of the vegetable, the fear of possible significant biomagnification of the HM in humans still exists, particularly given the fact that this vegetable is one of the most commonly sought after leafy vegetable in this part of the world (Nigeria), where it is useful as a pot vegetable as well as for ethnomedicinal purposes. Consumption of HM-laden vegetable results in decreases in blood pH, cancers, kidney failure, to mention a few (Varathon, 1997; Martin and Griswold, 2009).

The effects of dry cell battery dust on the moisture, ash, lipid, crude fibre, protein and carbohydrate contents of the plant are shown in Table 7. The percentage crude protein component of the plant decreased with increasing HM (obtained from dry cell battery dust) concentrations of the soil. Conversely, there was concomitant increase in total carbohydrate with DCD increase in soil or plants. Concentrations of carbohydrates in plants exposed to 5 kg DPAS and 5 kg DSBS did not differ from the control (carb. = 9.30%, $p < 0.05$).

Figure 3. Plants harvested at week13 (a) 1% (b) 5% (c) 10% (d) 25% (pollution before sowing)

Table 6.

Heavy metal content of *T. occidentalis* leaves (in partitions) after harvest

		Zn	Cd	Pb	Ni
Control	New leaves	21.15	BDL	0.07	4.18
	Int. leaves	17.42	BDL	0.10	4.14
	Old leaves	14.79	BDL	0.27	4.17
0.2 kg DSBS	New leaves	41.63	BDL	40.25	3.36
	Int. leaves	42.69	BDL	40.16	3.54
	Old leaves	35.76	0.01	48.64	2.65
1 kg DSBS	New leaves	45.33	BDL	56.98	1.98
	Int. leaves	43.78	0.01	61.78	2.99
	Old leaves	39.05	0.07	53.90	3.64
2 kg DSBS	New leaves	20.59	BDL	66.09	5.12
	Int. leaves	19.65	0.11	63.56	4.27
	Old leaves	13.35	0.19	65.08	3.67
5 kg DSBS	New leaves	45.87	BDL	62.03	3.91
	Int. leaves	41.35	0.05	68.65	3.07
	Old leaves	38.88	0.28	71.65	4.95
0.2 kg DPAS	New leaves	37.65	BDL	56.20	3.48
	Int. leaves	41.25	0.09	55.70	1.71
	Old leaves	29.71	0.29	53.67	2.36
1 kg DPAS	New leaves	38.77	0.05	63.76	3.58
	Int. leaves	38.81	0.23	53.70	4.12
	Old leaves	35.98	0.67	43.42	4.03
2 kg DPAS	New leaves	42.75	0.02	67.24	4.11
	Int. leaves	41.25	0.25	51.85	3.36
	Old leaves	32.98	0.67	33.85	2.96
5 kg DPAS	New leaves	40.25	0.03	77.17	3.59
	Int. leaves	47.32	0.34	44.75	3.63
	Old leaves	37.83	0.81	43.97	3.17
LSD (p, 0.05)	-	9.87	0.06	14.64	1.09
Limit (WHO/FAO)*		60.00	0.20	0.30	NA
Indian Standards*	-	50.00	1.50	2.50	NA

*(Agrawal *et al.*, 2007). **DSBS** dry cell dust applied to soil before sowing pumpkin on polluted soils; **DPAS** dry cell dust applied to plant stands sowing in clean soils; **BDL** beyond detectable limit (0.001mg/kg). **NA** not available

Table 7.

Proximate contents of *Telfairia occidentalis* leaves according to designated plant partitions

Treatments	Moisture (%)	Ash (%)	Lipid (%)	Crude fibre (%)	Crude protein (%)	Total carbohydrate (%)
Control	77.96	6.30	1.84	1.70	2.90	9.30
0.2 kg DSBS	78.40	5.06	6.77	1.21	2.99	9.63
1 kg DSBS	82.70	4.02	4.76	1.40	3.40	6.74
2 kg DSBS	73.90	6.70	3.30	1.54	2.30	13.26
5 kg DSBS	78.67	11.80	4.30	1.72	2.70	10.08
0.2 kg DPAS	70.64	9.30	7.03	1.76	2.68	8.59
1 kg DPAS	80.10	8.30	4.10	1.62	3.00	6.88
2 kg DPAS	78.20	5.40	6.23	1.33	2.59	6.25
5 kg DPAS	68.49	6.00	5.20	1.60	3.51	10.20
LSD (p, 0.05)	18.96	1.45	1.62	1.01	1.68	3.84

DSBS dry cell dust applied to soil before sowing pumpkin on polluted soils; **DPAS** dry cell dust applied to plant stands sowing in clean soils; **BDL** beyond detectable limit (0.001mg/kg). **NA** not available

Although significant morphological changes like stunted growth, chlorosis and necrosis are one of the most important parameters used to indicate HM presence in plants (Ikhajiagbe, 2016); this is even more difficult in plants that have inherent mechanisms for detoxifying accumulated metals and thus don't show clear-cut morphological changes. In such plants, it is usually difficult to tell if they were faced with HM toxicity by mere morphology. The application of HM-containing DCD to *T. occidentalis* (DPAS-group) did not significantly impair morphological development, but rather preserved the foliage from insect and pest attack, thus making it difficult to select out such vegetables from the markets.

Conclusions

The study confirms that sprinkling DCD on already growing fluted pumpkin as practice by a number of local farmers in Benin City, Nigeria, may have improved the plant's physical appearance, which is actually a yardstick for improved sales in the market, as the leaves did not show any visible evidence of pest attack; the leaves were larger and greener. However, significant accumulation of HM in the leaves beyond safe limit was also reported. The practice should, as matter of urgency, be stopped since obviously metal hyper accumulation by plants does not always imply dried-out plants. Further study is necessary to investigate the presence of likely growth-enhancing substances in the dry cell battery dust, which perhaps may have been the reason for its continual use by a few in this part of the globe.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 2, 2016 (p. 31-39)

Lycopene and Phycocyanin - biological properties in experimental diabetes: 1. Effects on blood parameters and liver carbohydrates

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SUMMARY. Type 2 diabetes is one of the fastest rising metabolic diseases of our time, mainly due to an unhealthy lifestyle, diet and lack of exercise. While medical treatment does indeed exist and the quality of life is improved under medication, one must take into account that the predominant geographical areas in which diabetes is on the rise are underdeveloped and as such, access to modern medicine may be hindered. The purpose of our study was to confirm the hypoglycemic and antioxidant effects of two biomolecules in the pathology of diabetes. For this study we used 40 albino male Wistar rats, divided into four groups. The control group (C) received a normal diet and tap water. The untreated diabetic group (D) was intravenously injected with a dose of 50mg/kg streptozotocin after a 12 hour fasting period and given a normal diet and water. Diabetes was induced in the same way for the two treated groups, with their diets being supplemented with 10 mg/kg lycopene (DL group) and an equivalent of 200 mg/kg phycocyanin in the form of *Arthrospira* powder (DS group). Blood was drawn every 7 days do determine glycemic status and after 14 days the animals were killed under anesthesia, with blood and liver being collected for morphological and biochemical analysis. Blood glucose significantly dropped in the DL group on the 7th day of treatment, with both treatments reducing fasting blood sugar to normal levels on the 14th day. Hepatic glucose was normalized in both DL and DS groups without any significant change in glycogen concentrations. RBC counts revealed a tendency of the erythrocytes to increase in numbers in the treated groups and lycopene appears to restore WBC numbers to normal levels. Taking into account these results, it can be said that both biomolecules have a potent hypoglycemic effect in diabetic hyperglycemia, while also improving carbohydrate metabolism in the liver. Lycopene seems to be the more effective antioxidant of the two, preventing oxidative-induced hemolysis of the red blood cells and restoring normal PCV and hemoglobin levels.

Keywords: *Arthrospira* powder, diabetes, lycopene, oxidative stress

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Introduction

Diabetes is probably the most common metabolic disease of our time, with the number of patients diagnosed having skyrocketed to 422 million in 2014 from 108 million in 1980 (World Health Organization, 2016). Statistics like these, and the fact that the most significant rise in the number of diagnosed patients occurs in underdeveloped areas of the world, motivate the search for cheap and effective diet supplements that can act as adjuvants in diabetes therapy.

Lycopene is a carotenoid that naturally occurs in red fruit and vegetables, where its main role is that of photoprotection. Recently, lycopene has been investigated for its antioxidant effect in cardiovascular disease (Arab and Steck, 2000) and especially cancer (Basu and Imrhan, 2007; Luo and Wu, 2011). As a therapeutic agent against diabetes, lycopene has proven to lower blood sugar levels (Bayramoglu *et al.*, 2013; Guo *et al.*, 2015) as well as stimulate and complement the antioxidant systems (Ali and Agha, 2009).

Phycocyanin is the main proteic compound in cyanobacteria such as *Arthrospira platensis* contributing to approximately 15-40% of their dried biomass. Structurally, it resembles bilirubin thus hinting towards its antioxidant capacity, especially considering protein and lipid oxidation. As an adjuvant therapeutic agent in diabetes, it was tested amongst others by Zhou *et al.* in 2005, who noticed a decrease in blood sugar concentrations and improved oxidative status.

Testing both compounds and observing which parameters they do or do not alter, one can draw conclusions as to how and at what level they function, considering their complex metabolism is not yet fully understood. Another possible conclusion arising from such studies could lead to complex supplement formulations that contain different biomolecules capable of complementing each other.

This study is part of a systemic study that also took into account the therapeutic effects of lycopene and phycocyanin on the liver, brain, kidney and pancreas of diabetic rats.

Materials and methods

All reagents used in this study were of analytical grade and were purchased from Sigma-Aldrich Chemie GmbH, Germany, Nordic Invest S.R.L., Romania and S.C. BioZyme S.R.L, Romania. Lycopene was from König Laboratorium, Canada, and *Arthrospira* poweder was from Adams Vision, Romania.

The experimental model consisted of 40 adult male Wistar rats distributed into four groups as follows: a control group (C, n=10), an untreated diabetic group (D, n=10), a diabetic group treated with lycopene 10 mg/kg (DL, n=10) and a diabetic group treated with *Arthrospira* powder 200 mg/kg (DS, n=10). Three days before

the treatment, diabetes was induced by a single dose of intravenously administered streptozotocin (50 mg/kg dissolved in ice cold 10 mM citrate buffer) in 3 of the groups (D, DL and DS). All the animals had *ad libitum* access to tap water and were fed a standard diet (S.C. Siamond Prod. S.R.L., Cluj Napoca, Romania) according to their weight, with the DL and DS groups having their diets supplemented with the aforementioned doses of lycopene and *Arthrospira* powder (AP) respectively.

The animals were sacrificed by exsanguination under anesthesia 14 days after the confirming their diabetic status. For the purpose of this experiment, blood samples were harvested by retro orbital bleeding at the start of the treatment and seven days into the treatment as well as blood and liver samples on the 14th day for the following biochemical and morphological analysis: RBC, WBC, PCV, hemoglobin, blood glucose and liver glucose and glycogen.

Blood and liver glucose concentrations were measured using the Somogy-Nelson method (Nelson, 1944). Glycogen concentration was determined by the Montgomery method (1957) modified by Lo *et al.* (1970). Hemoglobin concentration was measured using the Drabkin assay (1935). RBC and WBC were counted in a Thoma counting chamber and a Burker-Turk counting chamber respectively. PCV was measured using centrifuged glass capillaries filled with blood. Eosin – hematoxylin staining was used on fresh blood smears to determine the differential WBC count.

Results were analyzed using the two tailed *t* test and considered statistically significant at $p \leq 0.05$.

Results and discussion

This study was conducted in an effort to establish the therapeutic effect that natural supplements have in the case of experimental diabetes in adult male rats. Both compounds were investigated for their capacity to reduce blood sugar and to prevent oxidative stress.

Blood glucose was measured three times during the experiment to establish the way in which the two supplements affect diabetes-induced hyperglycemia. The results (Table 1, Fig. 1) confirm literature data concerning the hypoglycemic effect lycopene has (Bayramoglu *et al.*, 2013; Ip *et al.*, 2013; Guo *et al.*, 2015), shedding light on how fast it acts to reduce blood glucose. Compared to AP, lycopene acts faster, reducing the blood sugar levels below the diabetes diagnostic threshold. However, even though AP acts slower, it proves to be a more potent hypoglycemic agent with similar results being obtained by Layam and Reddy, 2006. These results correlate with measurements obtained from the tissue, where glucose concentration in the DL and DS groups dropped to normal levels (Table 1, Fig. 2a).

Considering these two supplements have complex metabolic pathways that are in no way similar to each other, a plausible explanation to their action is that they can, at least partially, restore normal function in the pancreas and thus insulin signaling.

Table 1.

Effects of lycopene and *Arthrospira* powder on blood sugar, hepatic tissue glucose and glycogen concentrations in diabetic Wistar rats

Parameter	Control	D	DL	DS
Blood glucose concentration day 0 (mg/dL)	123.72 ± 10.4	484.19 ± 37.2 ***	432.57 ± 40.42 ***	395.23 ± 23.93 ***
Blood glucose concentration day 7 (mg/dL)	123.72 ± 10.4	346.63 ± 17.87 ***	135.97 ± 29.48 ***	280.92 ± 20.42 *
Blood glucose concentration day 14 (mg/dL)	117.27 ± 9.01	210.39 ± 41.81	70.54 ± 8.42 ** *	59.07 ± 8.17 *** **
Hepatic tissue glucose concentration (mg/g tissue)	1.14 ± 0.28	9.79 ± 2.73 **	2.84 ± 0.44 * *	2.15 ± 0.73 *
Hepatic glycogen concentration (mg/g tissue)	0.49 ± 0.05	0.59 ± 0.08	0.51 ± 0.10	0.56 ± 0.13

D – untreated diabetic; DL – diabetic treated with lycopene; DS – diabetic treated with *Arthrospira* (Spirulina) powder containing phycocyanin; Results are expressed as mean ± SE. Comparisons made: black – *vs* Control group; red – vs D group; * - p<0.05; ** - p<0.01; *** - p<0.001

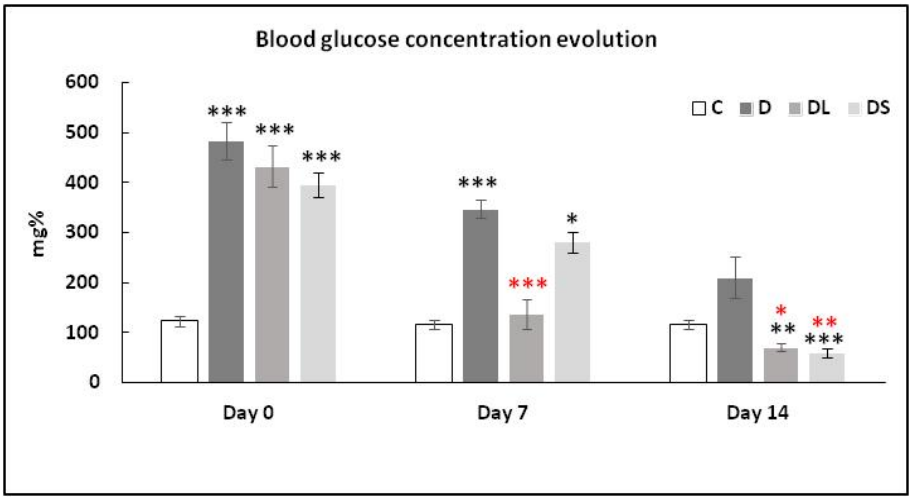

Figure 1. Effects of lycopene and *Arthrospira* powder on the concentration of blood glucose. The results are expressed as mean ± SE. Comparisons made: black - vs Control group; red - vs D group; * - p<0.05; ** - p<0.01; *** - p<0.001

A recent study has tried to explain the hypoglycemic effects of AP by its capacity to intervene in the pentose phosphate pathway and increase the activity of hexokinase by modulating the NADPH/$NADP^+$ ratio (Farouk *et al.*, 2013). Evidence of lycopene playing a role in glucose metabolism has emerged in 2016 when Eze *et al.* discovered that it can activate and stimulate glucokinase in the absence of insulin signaling. Interestingly, glycogen concentrations appear to not have been affected (Table 1, Fig. 2b).

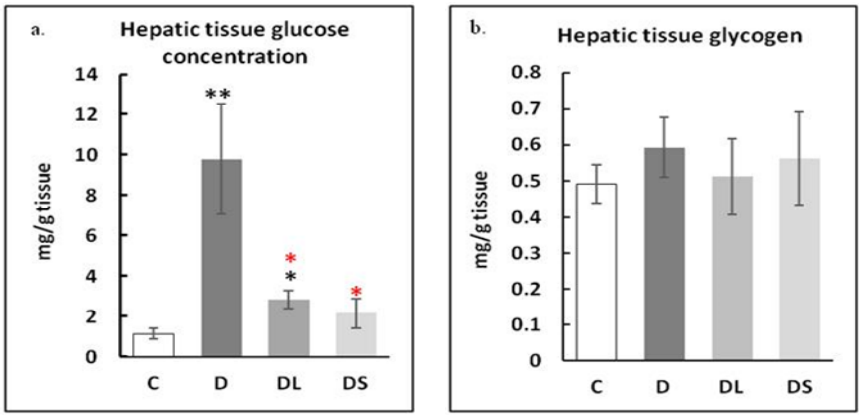

Figure 2. Effects of lycopene and *Arthrospira* powder on the concentration of hepatic glucose (a) and hepatic glycogen (b). The results are expressed as mean ± SE.

Morphological aspects of the blood (RBC, WBC, PCV) seem to have a tendency to normalize in the treated groups, especially in DL.

Lycopene has the intrinsic capacity to neutralize reactive oxygen species (ROS) in the plasma as described by Holzapfel *et al.* in 2013 as well as to prevent ROS-induced hemolysis *in vitro* (Chiste *et al.*, 2014) thus explaining the increase in RBC and consequently in PCV and the concentration of hemoglobin (Table 2, Figs. 3 a,c,d). The effect of AP on these parameters is not as potent, with only a slight statistically significant increase in RBC (Fig. 3a) and a tendency of the hemoglobin concentration to increase (Fig. 3c) probably due to the high concentration of bioavailable iron ion (Fe^{2+}) in AP (Khan *et al.*, 2005). Similar results were obtained by Romay and Gonzalez in 2010 on cultured human erythrocytes.

WBC counting data reveals that of the two supplements only lycopene acted as an immunostimulant by restoring leukocyte numbers to normal levels (Table 2, Fig. 3b). Lycopene has been proven to stimulate the secretion of cytokines (Luo and Wu, 2011) and consequently stimulate the proliferation of leukocytes. AP appears to have no effect on WBC numbers in the blood of diabetic rats, despite sources claiming it should (Milaðiu *et al.*, 2004).

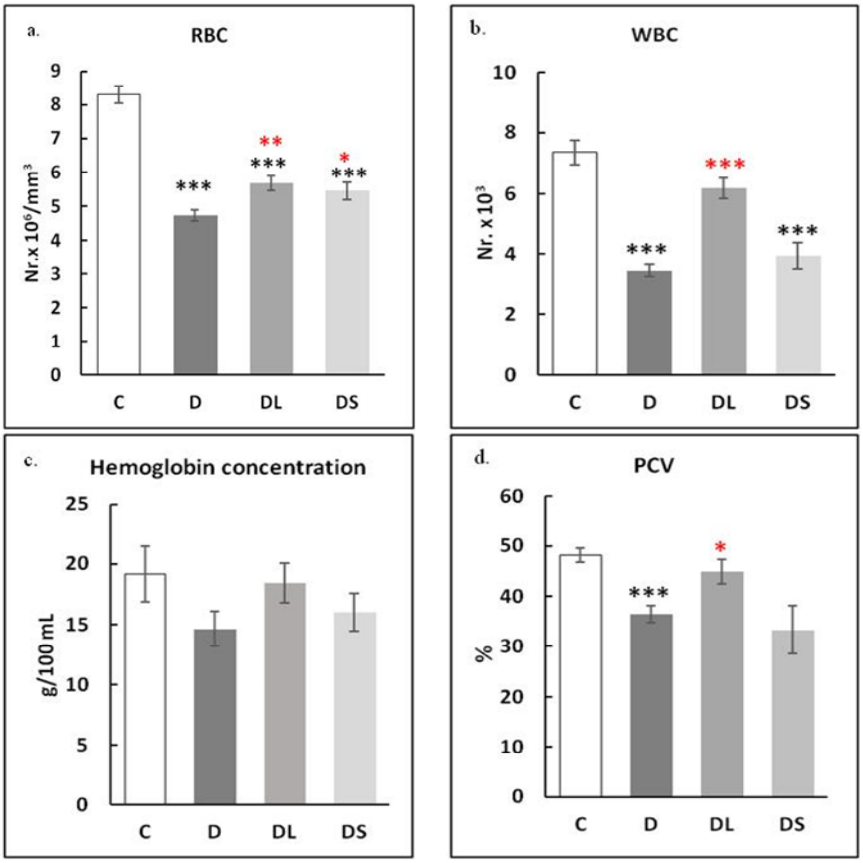

Figure 3. Effects of lycopene and *Arthrospira* powder on the number of red blood cells (a), white blood cells (b), the concentration of hemoglobin (c) and the packed cell volume (d). The results are expressed as mean ± SE.

The differential WBC count (Table 2, Figure 4) reveals a drop in the percentage number of lymphocytes in the untreated diabetic group as well as a normalization in the DL group and what appears to be an overstimulation of the lymphoid progenitor in the DS group. Even though the exact mechanism of phycocyanin activation of lymphocytes is unknown, data concerning is capacity to activate and modulate lymphocytes towards NHEJ DNA repair has been published recently (Stankova *et al.*, 2011). Phycocyanin does seem to be a more potent inhibitor of the hyperglycemia-induced hyperactivation of neutrophils (Xiu *et al.*, 2014) thus preventing neutrophil degranulation and oxidative burn. Lycopene lowered the number of neutrophils but in a lesser degree. Neither compound appears to have any effect on monocyte or eosinophil numbers in the DL and DS groups when comparing them to the untreated group.

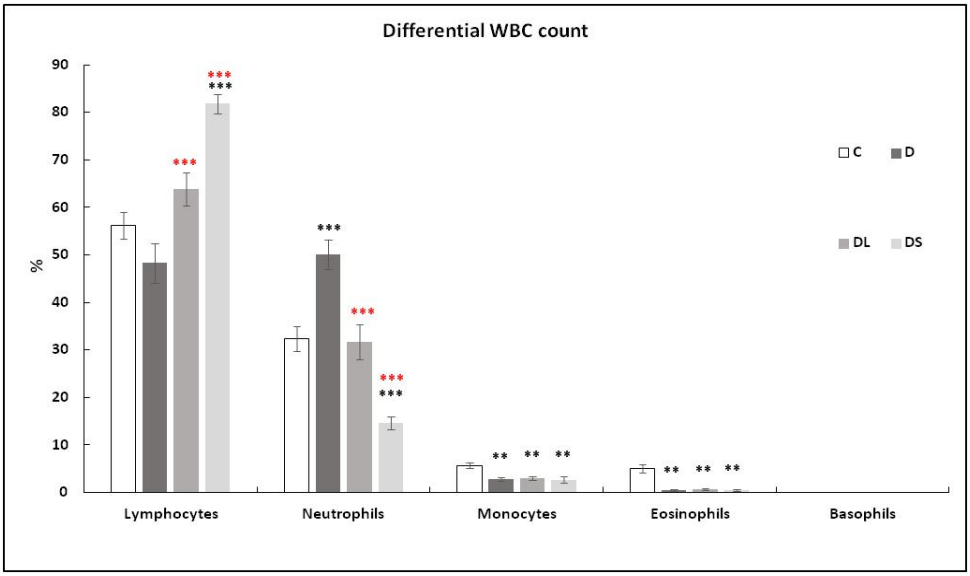

Figure 4. Effects of lycopene and *Arthrospira* powder on the differential WBC count. The results are expressed as mean ± SE.

Table 2.

Effects of lycopene and *Arthrospira* powder on morphological and biochemical parameters in the blood of diabetic Wistar rats

Parameter	Control	D	DL	DS
RBC (x 10^6/mm^3)	8.3 ± 0.26	4.72 ± 0.15 ***	5.68 ± 0.21 *** **	5.44 ± 0.26 *** *
Hemoglobin concentration (mg/dL)	19.14 ± 2.29	14.62 ± 1.44	18.41 ± 1.62	15.99 ± 1.55
PCV (%)	48.12 ± 1.43	36.51 ± 1.66 ***	44.91 ± 2.41 *	33.33 ± 4 .77
WBC (x 10^3/mm^3)	7.34 ± 0.41	3.44 ± 0.2 ***	6.19 ± 0.34 ***	3.93± 0.43 ***
% Lymphocytes	56.26 ± 2.81	48.29 ± 4.17	63.83 ± 3.44 ***	81.83 ± 2.03 *** ***
% Neutrophils	32.3 ± 2.64	50.08 ± 3.01 ***	31.69 ± 3.63 ***	14.62 ± 1.38 ***
% Monocytes	5.65 ± 0.59	2.83 ± 0.37 **	3 ± 0.34 **	2.63 ± 0.65 **
% Eosinophils	5.09 ± 0.85	0.46 ± 0.15 **	0.62 ± 0.25 **	0.47 ± 0.22 **
% Basophils	0	0	0	0

RBC – red blood cells; WBC – white blood cells; PCV – packed cell volume; D – untreated diabetic; DL – diabetic treated with lycopene; DS – diabetic treated with *Arthrospira* powder; The results are expressed as mean ± SE.

Conclusions

Both supplements had an impressive capacity to lower blood sugar concentrations in rats affected by experimental diabetes. While lycopene is a faster acting hypoglycemic agent, *Arthrospira* powder proved to be more potent in the end. The supplements seem to facilitate the internalization of glucose into the liver and stimulate glycolysis even in the absence of insulin signaling. In regards to oxidative stress, lycopene was superior to AP in almost all categories, managing to normalize to an extend all the parameters we have tested. Besides being a powerful antioxidant, lycopene also seems to be a potent modulator of the immune response, whereas AP did show promise in the well established process of hyperglycemia induced hyperactivation of neutrophils.

These results, we hope, validate the status of both supplements as hypoglycemic agents, and antioxidant in the case of lycopene.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 2, 2016 (p. 41-54)

Lycopene and Phycocyanin - biological properties in experimental diabetes: 2. Effects on biochemical, enzymatic and histological parameters

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SUMMARY. The economic impact of diagnosing, treating and monitoring diabetic patients is huge, for both patients and medical systems involved. Adequate diets are hard to follow especially in poor countries, and stress life significantly contributes to diabetes development. In addition, access to relevant treatment may be limited for many patients and some medical policies do not help.

Dietary supplements are a key to help patients raising their quality of life, to alleviate symptoms and in some cases, to reduce insulin doses.

40 adult male Wistar rats were used, divided in 4 groups of 10 animals each: Control (C), Diabetic (D), Diabetic+Lycopene (DL) and Diabetic+Spirulina (DS). Diabetes was induced with a single dose of streptozotocin (50 mg STZ/kg body weight) in the tail vein. The DL group was given 10 mg lycopene/kg/day, while the DS group received 200 mg phycocyanin/kg/day in the form of *Arthrospira* (Spirulina) powder containing 15% phycocyanin.

Treatment with *Arthrospira* powder reduced the activity of seric alanine aminotransferase (ALT) and seric aspartate aminotransferase (AST) in diabetic animals (DS). Serum catalase (CAT) activity in the DS group was significantly reduced, compared to both C and D groups. Hepatic CAT activity in both treated groups increased as compared to the control and diabetic group. Furthermore, phycocyanin and lycopene stimulated serum and hepatic lactate dehydrogenase (LDH) activity in DL and DS rats.

Some other effects of lycopene and Spirulina powder, such as lowering blood and hepatic cholesterol concentrations and normalizing the histological structure of brain, liver, kidney and pancreatic tissues, support the assumption that both compounds have a potential therapeutic role as adjuvants.

Keywords: diabetes, lycopene, phycocyanin, streptozotocin

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Introduction

Diabetes is a complex of metabolic disorders mainly characterized by high blood sugar concentration, either because cells cannot respond to insulin, or because insulin production is insufficient (NIH, 2014).

Using adjuvant treatments in early stages of diabetes can improve patients' life and prevent, or slow down diabetes evolution. According to Pribac *et al.* (2011) vegetal compounds found in *Ganoderma lucidum* (reishi mushroom) or *Trigonella foenum-graecum* (fenugreek) could improve biological parameters and restore β-cells function in diabetic rats.

Arthrospira platensis (commercial name: Spirulina) is an aquatic cyanobacteria, rich in nutrients, vitamin B complex, minerals (Fe, Se, Cr etc.), proteins, gamma linoleic acid and antioxidants (vitamin E and β-carotene) (Layam and Reddy, 2006).

Spirulina contains large amounts of phycocyanin, a phycobiliprotein (PBP) with a chemical structure similar to bilirubin. PBPs are proteic aggregates soluble in water and responsible for light absorption (Devendra *et al*., 2014). PBPs are used as fluorescent markers, nutritive supplements, antioxidant and anti-inflammatory agents, and natural dyes. Cyanobacteria are grown at industrial scale as a source of many useful compounds, including phycocyanin (Hirata *et al*., 2000).

According to Nishanth *et al.* (2010), phycocyanin inhibits cyclooxygenase-2 (COX-2), stimulates cytokine expression and enhances expression of superoxide dismutase (SOD) and CAT, two enzymes that are essential for mammalian natural antioxidant system.

Selenium binds to phycobiliproteins, increasing their antioxidant properties. Chromium stimulates carbohydrate metabolism and increases insulin activity (Belokobylsky *et al*., 2004).

Lycopene is a carotenoid without provitaminc function found in tomatoes and other (mostly red) vegetables and fruits. Lycopene increases transcription nuclear factor E2 (Nrf2), which regulates oxidative response (Kensler and Wakabayashi, 2009). It was shown by Bayramoglu *et al*. (2013) that lycopene has hypoglycemic effect in diabetic rats, also lowering total cholesterol and triglyceride plasma concentrations. Ali and Agha (2009) reported normalization of antioxidant enzymes activity in diabetic rat erythrocytes. Lycopene also improved carbohydrate metabolism, serum lipid profiling, and enhanced antioxidant enzyme activities in the kidney of STZ-induced diabetes mice (Guo *et al*., 2015).

While certain beneficial effects of lycopene and phycocyanin have been documented in various pathologies, little attention has been given to their antidiabetic promise. This study was undertaken to investigate the biological properties of lycopene and Spirulina powder (containing 15% phycocyanin) in experimental diabetes, in order to document their high potential as adjuvants in diabetes treatment.

Materials and methods

All reagents used in this study were of analytical grade and were purchased from Sigma-Aldrich Chemie GmbH, Germany, Nordic Invest S.R.L., Romania and S.C. BioZyme S.R.L, Romania. Arthrospira (commercial name: Spirulina) powder was from Adams Vision, Romania and Lycopene was from König Laboratorium, Canada.

The study was conducted on 40 male Wistar rats, weighing 150±50 g, divided into four groups: Control (C), Diabetic (D), Diabetic+Lycopene (DL) and Diabetic+Spirulina (DS). Each group included 10 individuals.

All animals had *ad libitum* access to tap water and received a standard diet. Those in groups DL and DS received the standard diet supplemented with lycopene, or Spirulina powder containing 15% phycocyanin. Lycopene and Spirulina were both administered orally: 10 mg/kg body weight lycopene, and the amount of Spirulina corresponding to 200 mg/kg body weight phycocyanin.

Diabetes was induced with a single dose of streptozotocin (50 mg/kg b.wt.) dissolved in 10 mM sodium citrate, pH 4.5, injected in the tail vene. The control group received only the vehicle.

On the 14^{th} day of the experiment, the animals were sacrificed under anesthesia. Blood and liver samples were harvested, to quantify the following parameters: whole cholesterol, protein concentration, and the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and catalase (CAT). Tissue pieces of liver, kidney, brain and pancreas were taken for histological examination.

Total protein concentration was determined by Bradford (1976) colorimetric assay using the Bradford "ready-to-use" reagent. The colorimetric assay based on the reaction with iron chloride in presence of sulphuric and acetic acids was used for cholesterol measurement (Zlakis *et al*., 1953).

For CAT activity, the decay of H_2O_2 was monitored at 240 nm (Vives-Bauza *et al*., 2007). The activity of LDH was measured as the oxidation rate of NADH at 365 nm (Bergmeyer and Bernt, 1974). Reitman and Frankel (1957) photocolorimetric assay was used for the determination of AST and ALT activities.

The results were expressed as mean values±SE and multiple comparisons between experimental groups were made using the *t* test: all groups *vs* control group; DL and DS *vs* D group. Differences were considered statistically significant at $p \leq 0.05$.

Results and discussion

The diabetic condition became evident 3 days after STZ administration, with glycaemia values ranging for 400 to 600 mg/dL. Water consumption and diuresis of diabetic animals increased dramatically, while body weight decreased (data not shown here).

STZ intoxication, raised **cholesterolemia** of rats in D group with 32.5%, as compared to the control group (Table 1). Vornoli *et al.* (2014) also reported a 4.5-fold increase in plasma total cholesterol, in STZ-injected rats put on a high fat diet for 8 weeks. In groups receiving either lycopene (DL) or Spirulina (DS), blood cholesterol concentration returned to control values (Table 1). Earlier works (Heber and Lu, 2002) reported that lycopene and carotenoids moderately lowered cholesterolemia, by inhibiting 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis. Sheu *et al.* (2013) showed an improvement of lipid profile and cholesterol reduction by C-phycocyanin extracted from *Arthrospira sp.*

Table 1

Effect of lycopene and phycocyanin on total cholesterol and total protein concentration, in serum and liver tissue

Parameter	Control	D	DL	DS
Cholesterolemia (mg/dL serum)	88.83±5.44	117.74±19.55	89.02±5.86	89.58±12.13
Hepatic cholesterol concentration (mg/g tissue)	4.70±0.50	8.31±1.05 **	4.21±0.25 **	5.22±0.50 *
Proteinemia (mg/dL serum)	1.77±0.04	1.69±0.02	1.69±0.03	1.62±0.04
Hepatic protein concentration (mg/g tissue)	144.48±4.72	133.47±5.51	144.68±4.70	147.47±47

D–Diabetic group, DL–Diabetic+lycopene, DS–Diabetic+Spirulina. Multiple comparisons were made: black - *vs* Control group; red - *vs* D group; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Liver total cholesterol increased with 76.8% in the liver of STZ-diabetic animals, while both Spirulina and lycopene reversed cholesterol values to the control (Table 1). We believe this parameter is relevant because excessive intracellular accumulation of cholesterol is the result of imbalance between plasma intake and cellular synthesis. Lowering of cholesterol synthesis by inhibiting HMG-CoA reductase, and suppression of the expression of LDL receptors in cell plasma membranes may prevent abnormal cholesterol accumulation (Palozza *et al.*, 2012). Our data are consistent with those reported by Xia *et al.* (2016), showing that C-phycocyanin prevented cholesterol accumulation in the liver of mice with subacute alcohol-induced injury. Ou *et al.* (2012) found a significant decrease of plasma and liver cholesterol in alloxan-induced diabetic mice pre- and post-treated with 200 mg/kg phycocyanin. Young rats fed for 5 weeks with tomato powder (10% of the diet) or lycopene (0.62g/kg diet) showed significant reduction of liver total cholesterol and LDL-cholesterol, in both presence and absence of 1% H_2O_2 in the drinking water (Alshatwi *et al.,* 2010). Although most authors emphasize the role of lycopene in reducing blood cholesterol in various chronic diseases, fewer data are available concerning liver total and LDL-cholesterol. However,

the distribution of absorbed lycopene in organs indicated liver as a major retention site (Kong *et al.,* 2010). Moreover, Kim *et al.* (2012) reported that lycopene-enriched tomato wine failed to decrease liver, plasma and faeces cholesterol in rats fed a high-fat diet. This inconsistency between studies may result from different factors such as the type and duration of dietary intervention, animal species, gender and age, lycopene source and processing.

Serum and liver total protein content were not influenced in a significant manner by STZ diabetes, spirulina or lycopene treatments (Table 1). However, in diabetic animals, Spirulina and lycopene administration totally reversed the 7.62% reduction of liver protein, as compared to the control group. Although there is no evidence of a direct stimulation of liver protein synthesis by lycopene or Spirulina, it is well-known that oxidative stress accompanying various diseases, including diabetes, deeply alter nucleic acids and protein structure and synthesis. Proteins that are rich in tyrosine, tryptophan, histidine and cysteine are particularly affected and subjected to proteasome degradation (Cichoz-Lach and Michalak, 2014). Consequently, compounds possessing antioxidant properties may protect both nucleic acids and proteins from oxidative damage.

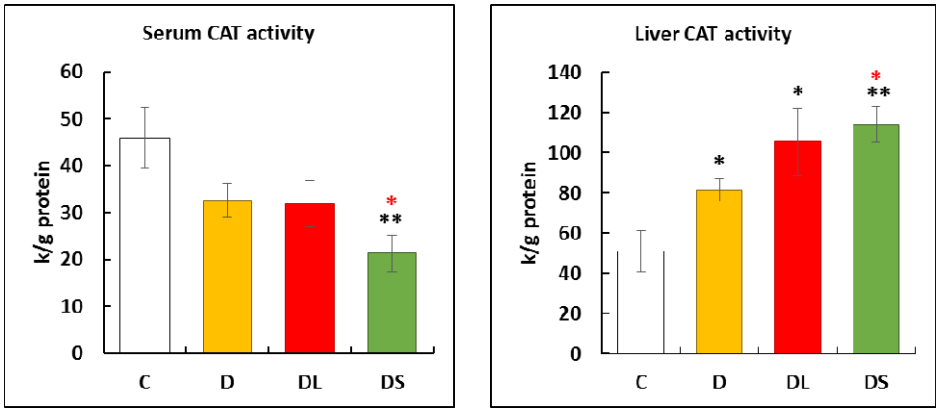

Figure 1. Effect of lycopene and phycocyanin on seric and hepatic CAT activity. Multiple comparisons were made: black - vs C group; red - vs D group; *p<0.05; **p<0.01. Results are expressed as k/g of protein in the sample. k=the rate of a first-order reaction.

Catalase (CAT) activities in sera and liver homogenates are shown in Fig. 1. The enzyme is ubiquitous in most tissues and catalyzes the decomposition of H_2O_2 to H_2O and O_2, consecutive to the dismutation of superoxide anion to molecular oxygen and H_2O_2 in the superoxide dismutase reaction. Therefore, CAT is a crucial enzyme in protecting the cell from oxidative damage. In humans, it is believed that a decreased blood CAT activity may be associated with type 2 diabetes (Góth, 2008). Serum CAT

activity decreased with 21% in all three diabetic groups as compared to control; lycopene did not restore the control value, and Spirulina decreased enzyme's activity even more than diabetes. At a first glance, these results seem hard to explain; we have to take into consideration several factors that may influence CAT activity. First, most of the blood enzyme is located into erythrocytes; even a slight hemolysis of the serum can alter the measurements. Second, CAT is not the only enzyme that scavenges H_2O_2; it shares this function with glutathione peroxidase, and the contribution of each enzyme may change according to H_2O_2 concentration (Mueller *et al.*, 1997; Selvan *et al.*, 2011).

However, most of CAT is located into the liver. There was a significant increase of its activity in the liver of diabetic animals, as compared to C group, probably as a reaction to the increased reactive oxygen species (ROS) formation that accompanies diabetes development. Lycopene administered to diabetic animals slightly increased CAT activity above the one in the D group, while Spirulina was even more effective. Previous research provide good evidence for the antioxidant properties of lycopene and Spirulina. Kong *et al.* (2010) and Heber and Lu (2013) stated that lycopene is the most efficient antioxidant among carotenoids, in trapping singlet oxygen and reducing thiobarbituric acid reactive substances (TBARS). However, Alshatwi *et al.* (2010) believe that tomato powder is more efficient than lycopene supplement against lipid peroxidation in rats. A strong correlation between oxidative stress and diabetes has been demonstrated by Ou *et al.*(2012) in alloxan-injured mice. They also found that phycocyanin from Spirulina had a preventive effect on ROS generation, reducing malondialdehyde formation in liver, kidney and pancreas. In golden Syrian hamsters fed a hypercholesterolenic diet, phycocyanin enhanced SOD, CAT and glutathione peroxidase activities in the liver (Sheu *et al.*, 2013).

LDH activity in the liver was tremendously stimulated by both lycopene and Spirulina (Fig. 2), in diabetic rats. These data are in accordance with the considerable decrease in liver glucose found in DL and DS groups (see Moldovan *et al.*, 2016). Ou *et al.* (2012) reported that phycocyanin from Spirulina enhanced liver glucokinase (GK) activity in diabetic mice. Several years later, Eze *et al.* (2016) found a similar action of lycopene in diabetic rats. Liver cells contain the highest amount of GK, the enzyme accounting for 95% of the hexokinase activity in these cells. Phosphorylation of glucose by GK provides substrate for both glycogen synthesis and glycolysis. As in our experiment liver glycogen content was not affected, we believe that most of the glucose was directed *via* glycolysis. The rise in serum LDH closely parallels enzyme's activity in the liver.

Serum transaminases are specific (ALT) and non-specific (AST) markers of hepatocytes plasma membrane integrity. High activities of these enzymes usually account for altered membrane integrity and/or permeability. In our experiment, serum transaminases significantly increased in diabetic animals (Table 2) and lycopene administered for 2 weeks in a daily dose of 10 mg/kg could not reverse this effect.

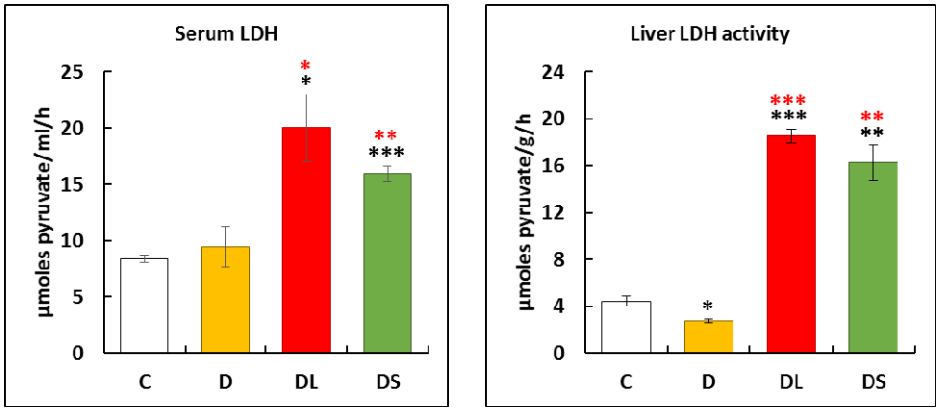

Figure 2. Effect of lycopene and phycocyanin on and hepatic LDH activity. Comparisons: black - vs C group; red - vs D group; *p<0.05; **p<0.01; ***p<0.001.

Table 2.

Effect of lycopene and Spirulina on membrane integrity biomarkers

Parameter	Control	D	DL	DS
Seric ALT activity (µg pyruvate/mL/ hour)	45.46±10.57	183.33±21.86 ***	218.89±26.27 ***	134.83±21.32 **
Seric AST activity (µg pyruvate/mL/ hour)	204.75±13.25	242.84±9.57 *	247.31±12.42 *	192.52±13.51 **
Hepatic ALT activity (µg pyruvate/g tissue/ hour)	127.83±3.52	138.20±3.48 *	143.55±4.95 *	143.10±6.15
Hepatic AST activity (µg pyruvate/g tissue/ hour)	48.70±1.30	44.72±1.46	44.51±3.38	49.97±4.70

D – Diabetic group, DL – Diabetic + lycopene, DS – Diabetic + Spirulina. Comparisons: black - *vs* Control group; red - *vs* D group; * p<0.05; ** p<0.01; *** p<0.001.

However, there is strong evidence (Eze *et al.,* 2016) that lycopene in higher doses (up to 40 mg/kg) and given for a longer time (4 weeks) is capable to reduce serum ALT and AST. Interestingly, Baymaroglu *et al.* (2013) reported similar results with 2.5 mg/kg lycopene, in a 7-day experiment. Only Spirulina (in a daily dose corresponding to 200 mg phycocyanin/kg) significantly reduced seric transaminases in DS group, as compared to D group. Our data are consistent with the ones reported by Gargouri *et al.* (2016), El-Sheekh *et al.* (2014), and Anwer *et al.* (2013).

Liver ALT activity (Table 2) increased with 8.1% in diabetic animals, and with 12.3% in DL and DS groups, showing the implication of this enzyme in the reversible conversion of glucose-derived pyruvate to alanine.

Liver AST is usually involved in the malate-aspartate shuttle, providing NAD^+ for glycolysis in the cytosol, and NADH in the mitochondria, for the electron transport chain. It seems that nor lycopene, neither Spirulina have not enhanced the shuttle's activity, as the elevated LDH (Fig. 2) could maintain an appropriate NAD^+ level in the cytosol.

Histology of pancreas, liver, kidney and brain tissues

These tissues were chosen for histological examination due to their involvement in the pathology of diabetes.

In experimental diabetes, STZ enters β-pancreatic cells *via* the low-selectivity GLUT-2 glucose transporters and alkylates DNA molecules and proteins. DNA fragmentation and depletion of NAD^+ and energy stores ultimately result in β-cell necrosis (Lenzen, 2008; Wu and Yan, 2015).

Diabetes induced severe alterations in the ***pancreas*** (Fig. 3B): smaller and fewer Langerhans islets, with low cellularity and an obscure demarcation between islets and the acinar tissue. Similar modifications were reported by Ou *et al.* (2012) in the pancreas of alloxan-induced diabetic mice. Although the mechanisms by which alloxan and streptozotocin cause experimental diabetes are different, it seems that structural alterations are alike. Lycopene was not effective in reducing the dystrophy of Langerhans islets (Fig. 3C), while Spirulina (Fig. 3D) improved the appearance of pancreatic tissue, showing fewer, but larger islets, with better cellularity.

The ***liver*** of control rats had normal structure, with lobules separated by fibrous septa (Fig. 4A), while in the diabetic liver areas of micro- and macrovesicular steatosis, accompanied by biliary stasis, could be noticed (Fig. 4B). Similar alterations were reported by Salih *et al.* (2009) in STZ-induced diabetic mice. Left ventricle failure, specific for diabetes associated diseases, can be recognized by greatly dilated centrilobular veins (Fig. 4B). Both lycopene and Spirulina improved the histological appearance of hepatic tissue; however, small inflammation foci were still present in the DL group (Fig. 4C), while enlarged spaces between the cell strands could be noticed in the DS group (Fig. 4D).

Kidney tissue in control rats had normal Bowman spaces, glomeruli and parenchima (Fig. 5A). The diabetic animals, (Fig. 5B) showed enlarged, or even disrupted Bowman capsule; tubular necrosis could also be seen. Lycopene and Spirulina visibly improved the renal tissue appearance: fewer altered renal corpuscles were present in DL group (Fig. 5C) without disrupted capsules; in the DS group (Fig. 5D) the renal corpuscles were quite normal, while small areas with tubular necrosis were still present. Our observations are consistent with those of Ahmed *et al.* (2015), who examined the alterations of liver and kidney tissue in STZ-induced diabetic rats and noticed a significant attenuation of these modifications by antioxidants Coenzyme Q10 and vitamin E. We believe that the beneficial effects of lycopene and Spirulina on tissue structure is also due to the free radical scavenging properties of these natural products.

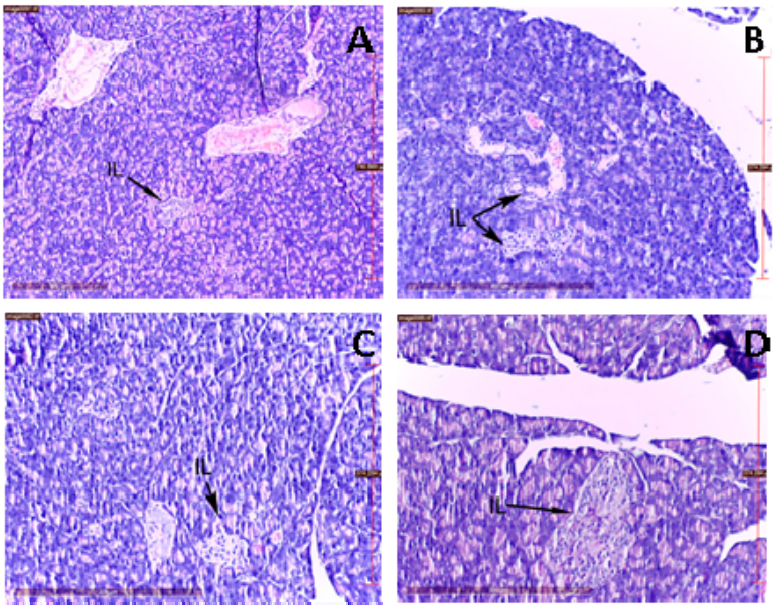

Figure 3. Histological aspects of pancreatic tissue in control (A), diabetic (B), DL (C) and DS (D) rats. IL – Langerhans islets. Bar – 300 µm.

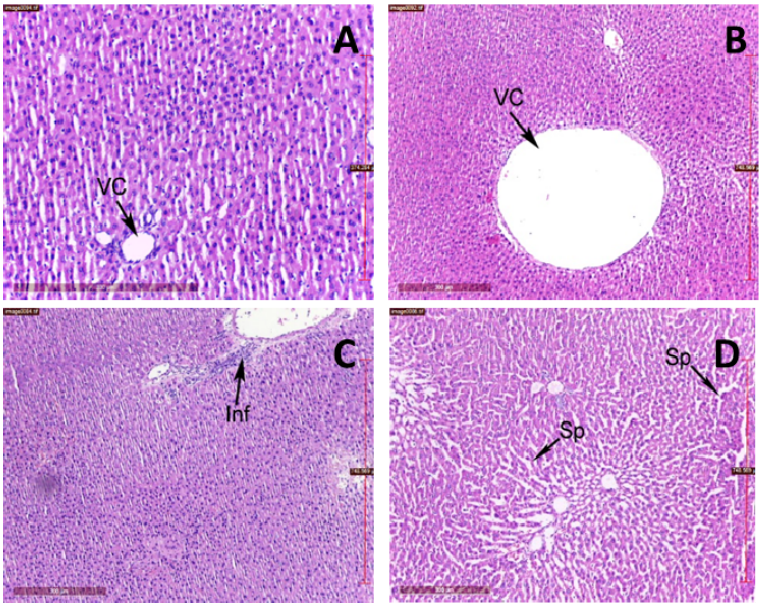

Figure 4. Histological aspects of hepatic tissue in control (A), diabetic (B), DL (C) and DS (D) rats. VC – centrolobular vein; Inf – inflammation focus; Sp – spaces between cell strands. Bar – 300 µm.

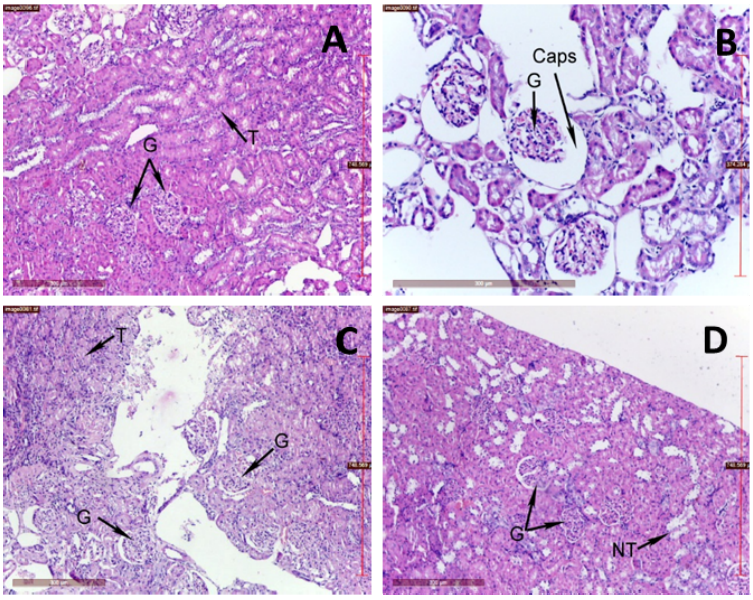

Figure 5. Histological aspects of kidney tissue in control (A), diabetic (B), DL (C) and DS (D) rats. G – glomeruli; T – tubule; Caps – Bowman capsule; NT – tubular necrosis. Bar – 300 µm.

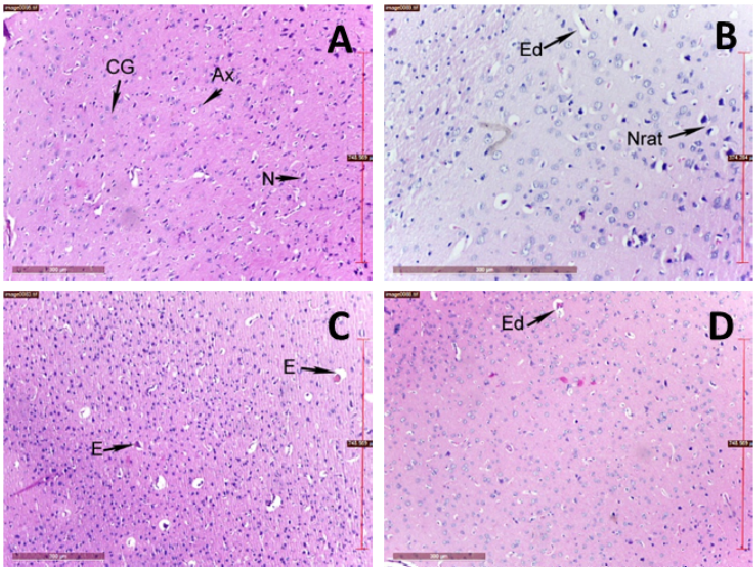

Figure 6. Histological aspects of brain in control (A), diabetic (B), DL (C) and DS (D) rats. CG-glia cell; Ax-axon; N-neuron; Ed, E-edema; Nrat-shrinked neuron. Bar-300 µm.

Diabetes brings about structural changes in the ***brain***, leading to cognitive dysfunctions, in both humans (Biessels *et al.*, 2014; Wrighten *et al.,* 2009) and rodents (Huang *et al.,* 2012). In our experiment, STZ-induced diabetes caused edema and neuronal damage (shrinking) (Fig. 6B), similar to the modifications reported by Huang *et al.* (2012): demyelination and axonal degradation, dystrophic neurons and abnormal oligodendrocytes. In the DL group (Fig. 6C) edema were also evident, while Spirulina (DS group, Fig. 6D) visibly reduced the extent of these alterations.

Conclusions

According to our results we can say that lycopene and spirulina treatments improved biochemical and enzymatic parameters. The oxidative stress was reduced and membrane integrity partly restored. Glycolysis was stimulated by both adjuvants.

Histological examination of pancreas, liver, kidney and brain tissues confirm the biochemical and enzymatic results, by showing certain improvements of diabetes-induced alterations after lycopene and spirulina administration.

As lycopene and Spirulina have specific and different mechanisms of action, even though their effects are roughly similar, further directions of research would be to test them together, as a cocktail, on diabetic animals, for various periods of time and also as pre-treatments.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 2, 2016 (p. 55-62)

Effects of glycerol on *in vitro*-grown *Amaranthus retroflexus* L.

Cristian Banciu[1,✉], Anca Manole[1] and Gabriel-Mihai Maria[1]

SUMMARY. Our experiments used a robust plant species (*Amaranthus retroflexus*), that grows on various types of substrate and in a variety of stress conditions (drought, frost, flooding, etc.) to test its survival on culture media with glycerol as a substitute of sucrose. After aseptic seed germination, plantlets were grown on a calus induction culture medium (Gamborg basal medium enriched with NAA and kinetin). The non-morphogenetic calus was cultured on four culture media with ascending glycerol concentration replacing sucrose as a carbon source. The survival and the growing rate of the caluses are factors that lead to the conclusion of succesful degradation of glycerol by *A. retroflexus*. Further biochemical analyses will reveal the biodegradation pathways and the secondary compounds production.

Keywords: *Amaranthus retroflexus*, callus, glycerol, morphogenesis.

Introduction

Glycerol is a secondary product from biodiesel production that is transformed in other useful compounds for polymer industry. The two enzymes that are involved in this process are lipase and decarboxylase.

Amaranthus retroflexus, is an annual species belonging to Amaranthaceae family. It is native to the tropical Americas, but is widespread as an introduced species on most continents in a great number of habitats. The plant is monoecious, with individuals bearing both male and female flowers. The inflorescence is a large, dense cluster of flowers interspersed with spiny green bracts. The fruit is a capsule less than 2 mm long with a "lid" which opens to reveal a tiny black seed (Pammel, 1903). *A. retroflexus* is an annual weed which reproduces only by seeds. It is a prolific seed producer, one single vigorous plant being capable of producing between 230,000 and 500,000 seeds (Stevens, 1957). Seed production is in decline when light is limited

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(MacLachlan *et al.,* 1995). Germination requirements and dormancy patterns are also variable depending on species distribution and local climatic and ecological conditions so, generalizations should be avoided. Recent studies have suggested that germination is stimulated by light intensity and higher levels of temperature (Gallagher and Cardina, 1997; Oryokot *et al*., 1997).

A. retroflexus is an aggressive and competitive weed in a variety of row crops. It causes substantial damages in soyabean, maize, cotton, sugarbeet, sorghum, and many other vegetable crops (Weaver and MacWilliams, 1980). It is edible for animals and has a nutrient composition and digestibility at a level equivalent to that of alfalfa (Marten and Andersen, 1975; Moyer and Hironaka, 1993). Previous studies on *in vitro* multiplication and callus induction of other species of *Amaranthus* were performed with notable results (Flores *et al.,* 1982; Bennici *et al*, 1992). The authors obtained callus starting from hypocotyl and stem tissues but also on leaf disks on *A. cruentus, A. tricolor, A. hypochondriacus, A. caudatus* and *A. hybridus.* Other studies have revealed antioxidant properties and antimicrobial effects on *A. lividus* respectively on *A. spinosus* (Ozsoy *et al*, 2009; Vardhana, 2011).

The main objective of this study was to evaluate the capacity of this species to survive and grow on a culture medium containing glycerol as a carbon source. Further studies will determine the products resulted from glycerol degradation and their use in polymer industry.

Materials and methods

Seeds of *A. retroflexus* were sterilised with succesive washes in 70% ethanol for 30 seconds, 5 minutes with sterile distilled water, 5 minutes with Na hypoclorite 5% and 10 minutes with sterile distilled water. Then, seeds were transfered to (MS) culture medium (Murashige and Skoog 1962). After two weeks of aseptic seed germination, plantlets were grown on a callus induction culture medium B5 (Gamborg basal medium enriched with 0.1 mgL^{-1} NAA and 0.2 mgL^{-1} Kinetin) (Gamborg *et al.,* 1968). The non-morphogenetic calus was grown on four variants of the same medium but with ascending glycerol concentration (from 0 to 30 g L^{-1}) replacing the sucrose as a carbon source (Table 1).

The growing rate was calculated as the weight (g) difference between the weight of callus at the end of experiments (40 days after transfer to media supplemented with glycerol) and the initial weight of callus (at the begining of the experiments). The difference was considered as the callus growth under the influence of glycerol addition in culture medium comparing with control.

In our experiments calluses grown on the four media variants have been tested first for survival and then for growing with a specific rate in 5 repetitions each variant.

Table 1.

Variants of culture media tested

Media/supplement (g* L^{-1})	**1**	**2**	**3**	**4**
Glycerol	0	10	20	30
Sucrose	30	20	10	0

Results and discussion

Our experiments used seeds from *Amaranthus retroflexus*, that grows on various substrate types and in a variety of stress conditions (drought, frost, flooding, etc.) to test its survival on culture media with carbon source replaced by glycerol.

As other authors explained, there is a metabolic pathway in plants that converts glycerol in glucose via gluconeogenesis (chlamypw.mpimp-golm.mpg.de; biologydiscussion.com). In this way, it can be used as a carbon source replacing sucrose in culture media when it is the only source available. For this reason we tested four culture media variants in order to evaluate the capacity of survival and growing rate on a medium with less or without glycerol, forcing the callus to adapt to another carbon source and to swich the metabolic pathway to convert glycerol.

As a control we used a culture medium with 30 g L^{-1} sucrose without addition of glycerol, the rest of culture conditions remaining unchanged. The other three variants were media with a succesive addition of glycerol replacing sucrose in order to reach the same concentration of 30 g* L^{-1}. It is known that glycerol is used in microbiology as an alternative carbon source, replacing sucrose with very good results (Stasiak-Rozanska *et al.,* 2014; Chen *et al.,* 2012).

As it can be observed in Figures 1-4, the calluses survived, after 40 days of cultivation, on all four variants of culture media, keeping the same colour and texture.

The growing rate varies from one medium to another having in general decreasing values with decreasing sucrose concentration and higher levels of glycerol concentrations. As it was expected, the best growing rate was obtained on standard medium without glycerol addition with an increase of 9.98 g (Fig. 1). Other high growing rates were recorded on medium with 10 g* L^{-1} glycerol and 20 g* L^{-1} sucrose with a gain of 7.40 g in the 40 days period (Fig. 2). The increase of callus mass with 6.47 g was recorded on the third medium variant containing only 10 g sucrose and 20 g glycerol (Fig. 3). The most interesting result is the survival and the weight gain on the fourth medium tested where the carbon source was completely replaced by glycerol, forcing the cells to adapt to the alternative metabolic pathway converting glycerol to glucose to have an efficient energy source (Fig. 4).

The survival and the growing rate of the calluses are factors that lead to the conclusion of succesful degradation of glycerol by *A. retroflexus* (Fig. 5). Further biochemical analyses will reveal the biodegradation pathways and the secondary compounds production.

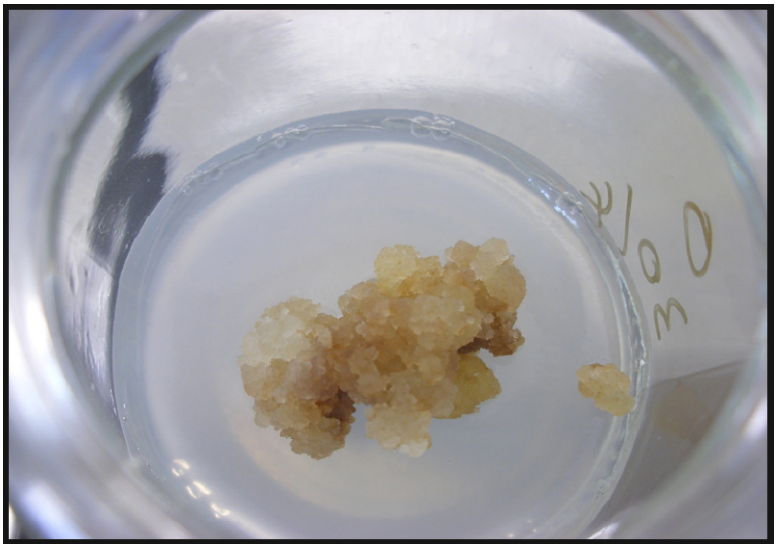

Figure 1. Callus grown on control medium (no glycerol)

Figure 2. Callus grown on culture medium with 10 g* L^{-1} glycerol

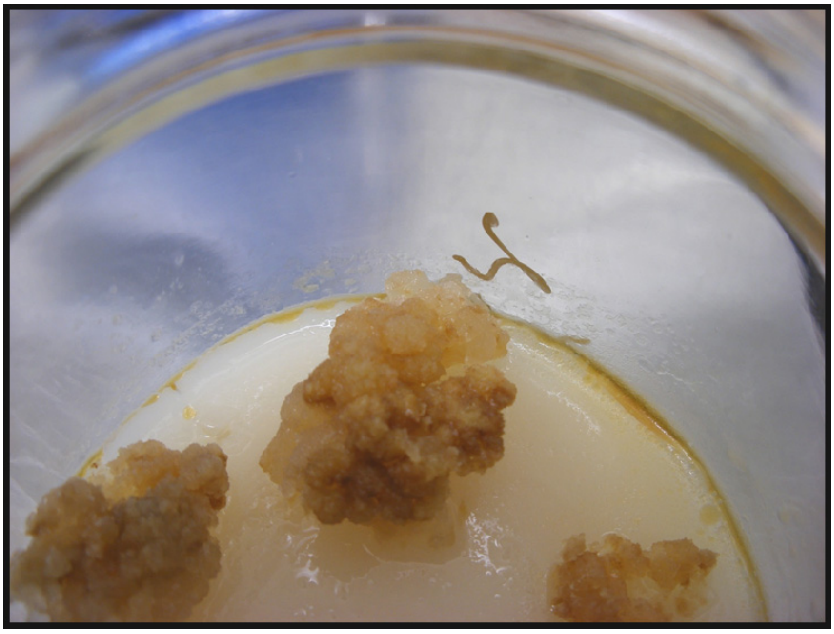

Figure 3. Callus grown on culture medium with 20 g* L^{-1} glycerol

Figure 4. Callus grown on culture medium with 30 g* L^{-1} glycerol

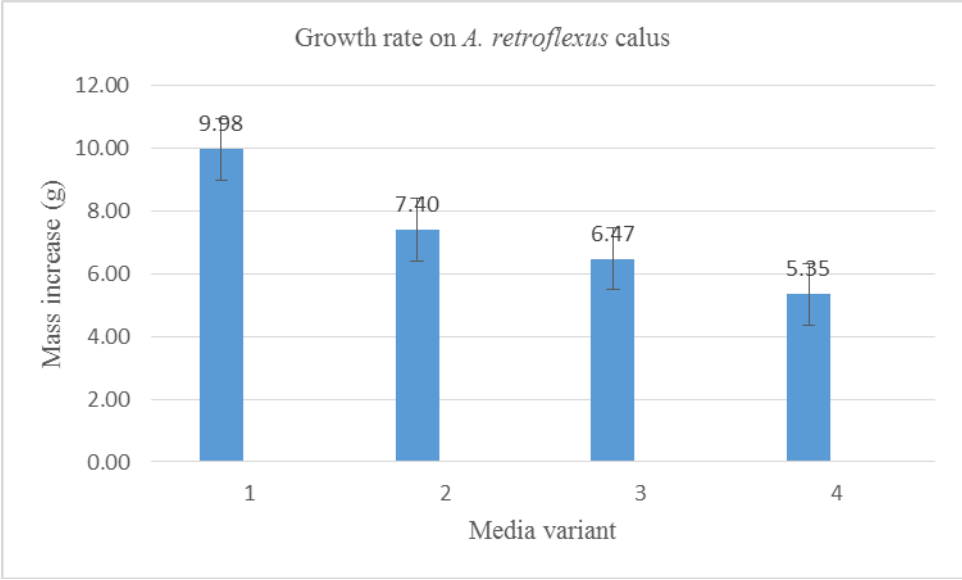

Figure 5. Growth rate of *A. retroflexus* callus on the four essayed media variants.

Conclusions

The results presented above reveal a successful growth rate of callus on culture media with glycerol as an alternative source of carbon replacing sucrose in standard medium. It means that plant cells have switched their metabolic pathway to glycerol degradation to glucose and other similar compounds as energy source. These results may be used in industrial applications and could be a viable solution especially for the polymer industry that produces polyglycerol (one of the sub-products). It offers a solution for consuming the big quantities of produced glycerol affecting the waste waters and the natural habitats. It could be a challenge for plant biotechnologies to help the industry having a positive impact to the environment.

Acknowledgements

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 2, 2016 (p. 63-68)

Orthometopon romanicus n. sp. (Crustacea, Isopoda, Crinocheta: Agnaridae) in Romanian fauna

Nicolae Tomescu[1,✉] and Lucian Alexandru Teodor[1]

SUMMARY. We have identified a new isopod species, from the genus *Orthometopon*, among specimens collected from Sacalin Island, Portita and Grindul Lupilor, during research performed in the Danube Delta (Dobrogea region). We named this species *Orthometopon romanicus* **n. sp.** The present paper describes the species' characters.

Keywords: isopods, *Orthometopon*, specific characters.

Introduction

Eight species are known from the genus *Orthometopon* (Schmalfuss, 2003). Of these, five species occur only in Greece (Schmalfuss, 1993, 2003). The species with the largest distribution is *Orthometopon planum* (Budde-Lund 1885), known from France, Slovakia, Croatia, Czech Republic, Hungary (Frankarberger, 1959, Korsós *et al.*, 2002, Schmalfuss, 1993, 2003, Sechet *et al.*, 2012, Tuf and Tufová, 2005, Vandel, 1962, Wächtler, 1937). The characters of this species were described by several authors (Frankenberger, 1959, Vandel, 1962, Wächtler, 1937). Schmalfuss (1993) described specific characters for other 7 species belonging to the genus *Orthometopon*, from Greece, of which 3 were new for science. No species, belonging to the genus *Orthometopon*, was previously described from Romanian fauna (Radu, 1985, Tăbăcaru and Boghean, 1989, Giurgincă and Curćic, 2003). In our research performed in the period: 1991-1994, in the Danube Delta, we identified a new species, belonging to the genus *Orthometopon*, among specimens collected from Sacalin Island, Portita and Grindul Lupilor, which we named *Orthometopon romanicus* **n. sp.** The present paper describes the species' characters.

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Materials and methods

The investigated biological material was collected in august 1994, from three locations in the Danube Delta: Sacalin Island, Portita and Grindul Lupilor. All specimens were collected from areas with sandy soil and herbaceous vegetation, reed and sedges (hygrophilous plants). On the Sacalin Island specimens were collected only from areas covered with sedge, with moist soil and moderate temperature. The specimens density was approximately 200 specimens/m^2. In Portita locality specimens were collected from areas covered with reed and rich in detritus, the soil was sandy and moist and temperature moderate. Here too, the density of *Orthometopon* was high, approximately 1000 specimens/m^2. In the Grindul Lupilor area, specimens were collected from similar habitat to the one on Sacalin Island. Totally 19 males, 106 females and 16 juveniles were collected. Six males were dissected in the laboratory and prepared on microscope slides in Canada balsam and Euparal. Whole males and 4 females were studied and photographed dorsally. Microscope slides were analysed under the stereomicroscope and microscope and photographed. The photographs were used to describe the species. The slides ad specimens conserved in ethanol 70^0 will be donated to the Zoological Museum of the "Babeş-Bolyai" University, Cluj-Napoca.

Results and discussion

Species description

Size: males 5 x 2 mm – 7.4 x 3 mm, females 6 x 2.8 mm – 8.2 x 3.5 mm.

Colour: The cephalon is dark brown with small yellow-orange dots (Fig. 1, c). On the pereional tergites there are large dark brown spots, which form one longitudinal line on each side at the basis of the coxal plates and two lines in the median region. Between these lines there are dark brown and yellow-orange spots, which vary in form and size (Fig. 1, a, b). Within populations of *Orthometopon* we found variability in the colour of the pereional tergites. The pleon is mostly dark brown with a row of small yellow-orange spots in the median region and on the sides (Fig. 1, a, b, e).

Somatic characters

Cephalon. The lateral cephalic lobes are reduced and triangle-shaped. The median lobe is short and has the form of an obtuse triangle (Fig. 1, c).

Pereion. The posterior end of the coxal plates of the tergites 1, 2 and 3 is straight. In the case of the tergites 4-7, the posterior end of the coxal plates is curved, more marked on the tergites 6 and 7. The noduli laterales are placed at the same distance from the lateral edges of the coxal plates (Fig. 1, d).

Pleon. The pleotelson is short, triangle-shaped (Fig. 1, e).

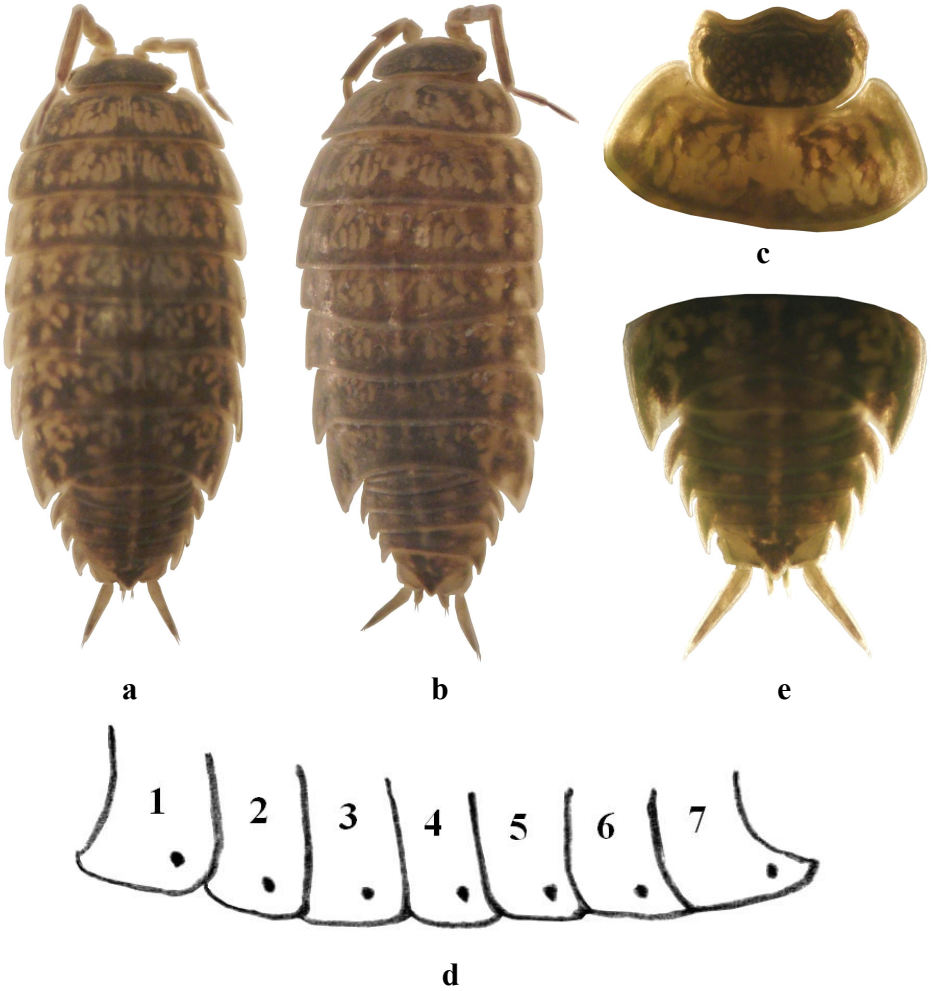

Figure 1. *Orthometopon romanicus* nov. spec., Holotyp, male and female dorsal view: **a.** ♂ 6.5 x 3 mm, **b.** ♀ 8.2 x 3.5 mm, **c.** cephalic lobes, **d.** coxal plates of the pereion and noduli laterales, **e.** pleon.

Appendages

Antennae. The last two antennal segments have approximately the same length (Fig. 2, a). In two males we found abnormal antennae, one of them with shorter and less pigmented segments.

Pereiopods. Pereiopods 1-3 have numerous thorns on the merus and carpus (Fig. 2, b, c, d). In males, the 7thpereiopod's ischium is slightly concave on the ventral side, on the carpus there is a short triangular crest of the carpus proximal region (Fig. 2, e).

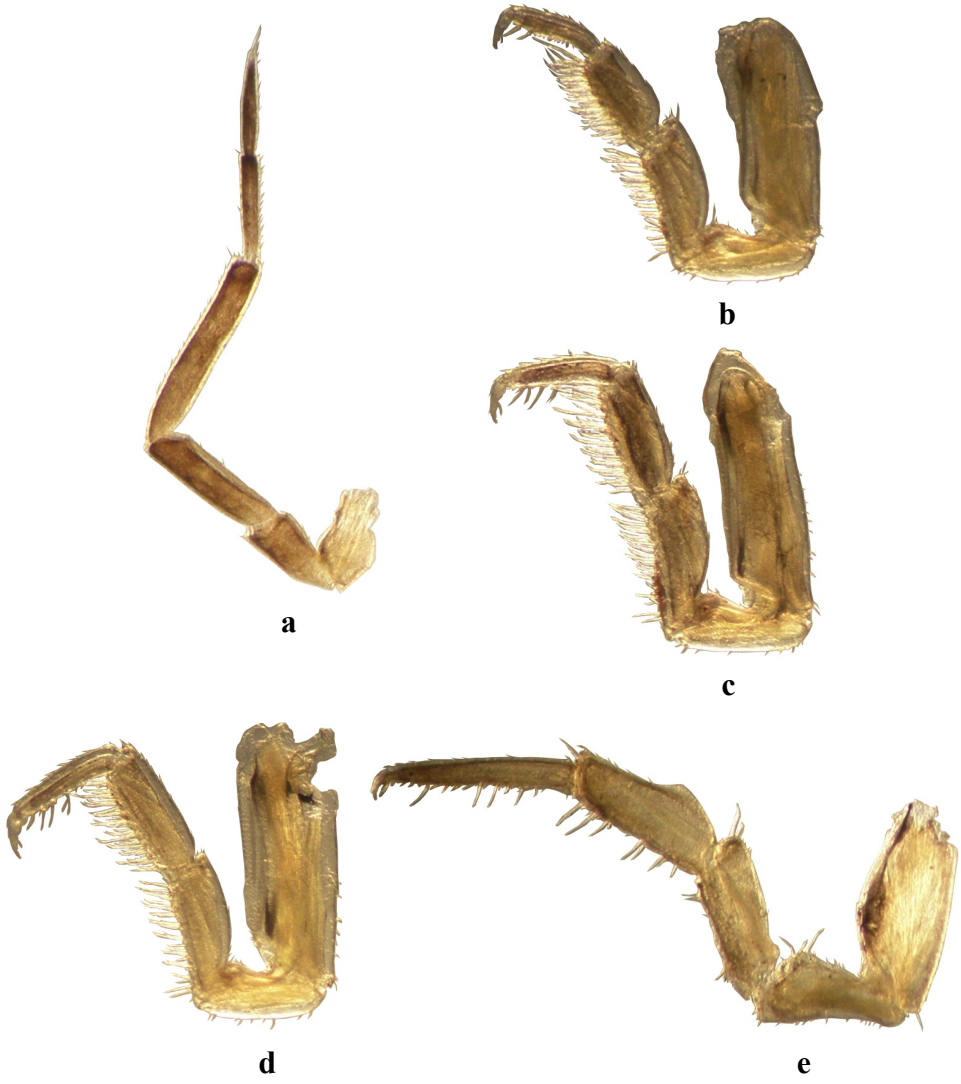

Figure 2. *Orthometopon romanicus* nov. spec., Holotyp, ♂ 6.5 x 3 mm **a.** antenna, **b.** pereiopods 1, **c.** pereiopods 2, **d.** pereiopods 3, **e.** pereiopods 7.

Pleopods. Exopods of the 1st pleopods of males have their inner side oblique, their external side slightly curved in the distal region. The posterior extremity is slightly concave (Fig. 3, a). The exopods of the pleopods 2-5 have rare short thorns on their external side (Fig. 3, b, c, d, e).

Endopods in males have a wide basal half, with the external side slightly curved (Fig. 3, f). The extremities of the endopods have each a tuft of short thorns (Fig. 3, g).

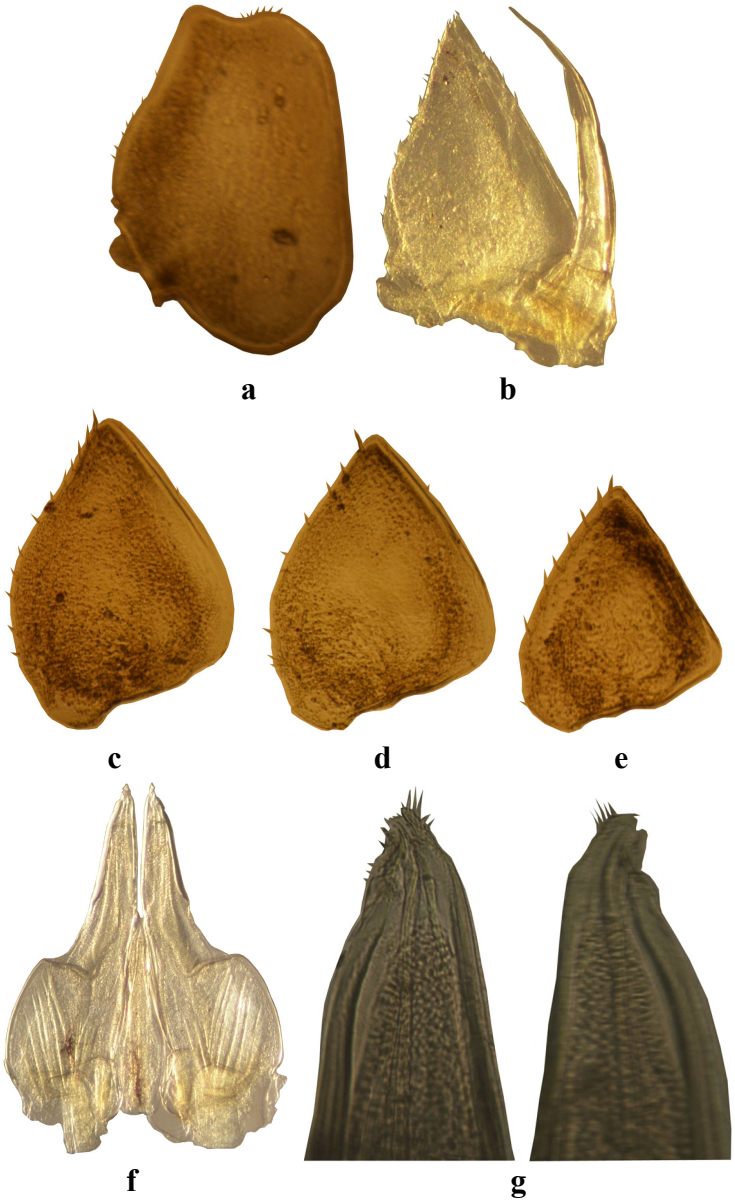

Figure 3. *Orthometopon romanicus* nov. spec. Holotyp, ♂ 6.5 x 3 mm, **a.** exopod pleopods 1, **b.** exopod and endopod pleopods 2, **c.** exopod pleopods 3, **d.** exopod pleopods 4, **e.** exopod pleopods 5, **f.** endopod pleopods 1, **g.** apex of the endopod pleopods 1.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 2, 2016 (p. 69-80)

Fish communities of the small rivers of Turda and Tureni Gorges

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SUMMARY. In 2014 the fish fauna of watercourses from the following two Natura 2000 SCIs were investigated: Tureni Gorge (ROSCI0034) and Turda Gorge (ROSCI0035). The research focused on fish species of community interest (Habitats Directive, Annex II), their position in the ichthyocoenose (numerical abundance, biomass, ecological significance and IBI). Ten fish species were captured in the investigated SCI Rivers, half of them being fish species of community interest present in Annex II of the Habitats Directive.

Keywords: abundance, biomass, fish ecological parameters

Introduction

The Tureni Gorge (ROSCI0034) includes a small SCI area with a total surface of 134 ha, out of which aquatic surfaces represent less than 1%. The area has a small number of habitats (according to standard Natura 2000 Form), crisscrossed by the small Valea Racilor River. The Tureni Gorge protected area is the result of karst phenomena manifested in Jurassic limestone (tithonic) located at the contact of the Trascău Mountains with the Turda-Alba Iulia Depression (part of the Transylvanian Basin). The appearance of the area is that of a karst canyon, V-shaped, with limestone walls rising between 20 meters (in Tureni quarry right at the entrance to the gorge) and 105 meters high within the gorge. The distance between the walls increases in the second half of the gorge, reaching 160 meters at the exit towards the Copăceni locality. The length of the watercourse of the Valea Racilor River is 1850 meters.

The Turda Gorge (ROSCI0035) has been recognized since 1938 as a natural reserve of national importance following the proposal of Professor Alexandru Borza. Within a relatively small area (324 ha), it hosts primarily scientific treasures – of botanical, zoological, ecological, geological, historical and spelunking value. The

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Turda Gorge protected area is located 7 km west of Turda locality, on the eastern edge of the Trascău Mountains along the Hăşdate River watercourse (tributary of the Arieş River). The Turda Gorge is an epigenetic gorge consisting of limestone of Jurassic age, its walls reaching 300-320 meters above the current level of the Hăşdate River, mostly very steep, almost vertical. It contains interesting endokarstic phenomena (caverns, caves), which mark the landscape value of this area.

All Romanian SCIs jointly contain 26 fish species of community interest (Tatole *et al.,* 2009) in accordance with the Habitats Directive 1992 and Law 49/2011 completing O.U.G. 57/2007, but in both studied SCIs just 1 species of community interest was present in Natura 2000 Standard Form: *Cobitis taenia (C. elongatoides)* (Nalbant, 1993; 1994; 2003), in accordance with the nomenclature adopted under the Habitats Directive species lists.

Both of the small rivers (Hăşdate River in the Turda Gorge and Valea Racilor River in the Tureni Gorge) are tributaries to the Arieş River which discharges in the Mureş basin (part of the Transylvanian basin) that belongs to the Danube basin.

Some historical data about fish fauna are found in Bănărescu (1964), but only for the Hăşdate River (with 6 fish species found: *Cobitis taenia, Barbus meridionalis, Barbatula barbatula, Gobio obtusirostris, Squalius cephalus* and *Phoxinus phoxinus*). Fish data is lacking from the Valea Racilor River, which confers greater importance to this paper - especially for future studies.

Materials and methods

Investigations were conducted during July 2014 in both of the studied Natura 2000 SCIs: ROSCI0034 Tureni Gorge and ROSCI0035 Turda Gorge.

Due to the rivers being small in the studied area, only electrofishing was used for sampling with a SAMUS 725MP device using a 12V accumulator and 5-60 Amps with an output of 600 W. The electrofishing was carried out during the day in 2 sampling sites for Tureni Gorge and 3 for Turda Gorge (Table 4). The fish fauna community was assessed, especially the presence of species of community interest (Directive Habitats, Annex II), quantitative structure (numerical abundance, biomass), ecological indices (Table 1), IBI (biological integrity index) (Table 2, Table 3), specimen dimensions, overall status of aquatic habitats in terms of existing anthropogenic pressures.

The captures were sorted by species (fish species identification using Bănărescu, 1964 with updates after Bănărescu, 1994; 2004; Nalbant, 2003; Lelek 1987; Kottelat, 1997; Kottelat and Freyhof, 2007; Froese and Pauly, 2016) weighing and length measurement were performed. The numerical abundances and biomass were determined for each species and site, in order to find the status of species in the fish community, using CPUE ("Catch per Unit Effort", means individuals / hour or individuals / 100 m²). After field measurements, the remaining individuals were released into the river. Human impact was also estimated.

Table 1.

Quantitative population indices
(Botnariuc and Vădineanu, 1982, Gomoiu and Skolka, 2001, Muhlenberg, 1993, Odum, 1975, Sârbu and Benedek, 2004, Schwerdtfeger, 1975, Simionescu, 1984, Şindrilariu *et al.,* 2002)

Dominance (D)			Constancy (C)		Ecological significance (W)	
Class		**%**	**Class**	**%**	**Class**	**%**
sporadic	D1	<1	very rare	C1=0-10	accidental	W1< 0.1
subrecedent	D2	1 (2^0) - <2	rare	C2=10.1-25	accessory	W2=0.1-1
recedent	D3	2 (2^1) - <4	widespread	C3=25.1-45	associate	W3=1-5
subdominant	D4	4 (2^2) - <8	frequent	C4=45.1-70	complementary	W4=5-10
dominant	D5	8 (2^3) – 16	very frequent	C5=70.1-100	characteristic	W5=10-20
eudominant	D6	>16 (2^4)			main, leading	W6>20

Table 2.

Criteria for determining fish IBI
(Ureche, 2008 after Battes, 1991, Karr, 1986 and Miller, 1985)

PARAMETERS CATEGORIES	PARAMETER	EVALUATION INTEGRITY CLASS		
		5	**3**	**1**
Composition and abundance of species	1. Total number of fish species	> 90% (abund.)	50-90 % constant	<50% (rare)
	2. Total number of cyprinids	> 45%	20-45%	<20%
	3. Total number of salmonids	> 5%	1-5%	<1%
	4. Others fish species	> 20%	5-20%	<5%
	5. Total number of native fish species	> 68%	35-67%	<34%
	6. Total number of non-native species	<1%	1-10%	>10%
	7. Total number of disappearing fish species	<1%	1-10%	>10%
Composition of the food fish populations	8. Proportion of zoobenthivorous species	> 45%	20-45%	<20%
	9. Proportion of carnivore species	> 5%	1-5%	<1%
	10. Proportion of carnivore and planctivorous	<20%	20-45%	>45%
	11. Proportion herbivorous and detritivores	<25%	25-50%	>50%
Stock and general state of fish populations	12. Numerical Stock (ex./100 m²) (ex./100 m linear / collectors)	> 100 ex (>20 ex)	10-100 (5-20)	<10 (<5)
	13. Gravimetrical Stock (g/100 m²) (g/100 m linear / collectors)	> 1000 g (>5000 g)	100-1000 (500-5000)	<10 (<5)
	14. Proportion of hybrid individuals	0%	0-1 %	> 1%
	15. Proportion of ill individuals	0%	0-1 %	> 1%

Some specimens were collected and preserved in liquid for more detailed taxonomical analysis for species for which correct identification was doubtful, according to the latest systematic reviews. Water temperature was identified using an electronic thermometer, water depth and turbidity with a Secchi disk, and geographic coordinates with GPS Garmin e-trex 30 (Table 4).

Table 3.

Framing levels of the evaluation integrity degree in fish ecosystems
(Ureche 2008 after Battes, 1991, Karr 1986 and Miller, 1985)

No.	APRECIATION	SCORE Small rivers (Miller A, 1985)	Medium and big rivers and reservoirs		EVALUATION INTEGRITY CLASS
			Karr J. R. & Co., 1986	Battes K. W., 1991	
1	Excellent	37-40	57-60	70-75	I
2	Excellent-good	34-36	53-56	66-69	II
3	Good	30-33	48-52	59-65	III
4	Moderate-good	28-29	45-47	55-58	IV
5	Moderate	23-27	39-44	47-54	V
6	Poor-Moderate	21-22	36-38	43-46	VI
7	Poor	16-20	28-35	35-42	VII
8	Poor-Very low	12-15.	24-27	20-34	VIII
9	Very low	<12	<23	<20	IX

Results and discussion

In the Turda Gorge, 3 sites were sampled between 46.56946º - 46.5611º lat. N and 23.69058º – 23.6675º long. E, with water depths less than 70 cm, turbulent water, a 20ºC water temperature and the bottom mostly boulders and stones, with only stones upstream (Table 4).

In the Tureni Gorge 2 sites were sampled between 46.60455º - 46.36556º lat. N and 23.71501º – 23.42361º long. E, with water depths less than 100 cm, clear water, 20ºC water and the bottom mostly boulders and stones, rarely mud (Table 4).

In the summer of 2014, 10 fish species were captured, including 5 species of community interest and 4 according to Natura 2000 Standard Form, meaning *Barbus meridionalis* (present in Romania after Kotlik *et al.*, 2002, Iftime 2004, Antal *et al.*, 2015)*, Romanogobio (Gobio) kessleri, Romanogobio vladykovi (Gobio albipinnatus)* and *Rhodeus amarus* added. A usually common species for 2014 catches and Natura 2000 Standard Form (2007) is *Cobitis taenia*, which was absent in the Turda Gorge (Table 5). It is hard to say what caused *C. taenia* to be missing from the Turda Gorge. The fact that *Phoxinus phoxinus* was also absent indicates that there may be some negative impact affecting these two species. Other possibilities might be bad sampling time for those species or that they could not be observed in turbulent waters (0.6-0.8 in T/D report in Table 4).

Table 4.

Geographic coordinates and some physical parameters
from the sampled points of the Turda and Tureni Gorges

SITE CODE	Tureni14E1	Turda14E3	Turda14E2	Turda14E1
GEOGRAPHIC COORDINATES	46.36556º	46.56946º	46.5611º	46.56352º
	23.42361º	23.6675º	23.69058º	23.68709º
DATE	15.07.2014	16.07.2015	16.07.2015	16.07.2015
Beginning time hour	10:00	14:30	12:30	11:00
Working Time (h:minutes)	0:15	0:15	0:15	0:15
Surface of fishing (m^2)	25.00	25.00	25.00	25.00
BOTTOM	boulders and stones, rarely mud	stones	boulders and stones	boulders and stones
T°C water	20	20	20	20
WATER DEPTH (D) (cm)	50	50	40	70
TRANSPARENCY (T) (cm)	50	30	30	50
T/D	1.0	0.6	0.8	0.7

Tureni14E2
46.60455º
23.71501º
15.07.2014
13:30
0:15
25.00
boulders and stones, rarely mud
20
100
100
1.0

Table 5.

Species richness in both studied Romanian SCIs
(1=presence species, bold are species of community interest)
Legend: carn=carnivorous, erb=herbivorous, zoobent= zoobenthivorous, ihti-ichthyvorous (piscivorous), omni=omnivorous, detrit=detritivores

No.	Species	Feed	Bănărescu 1964	Tureni Gorge	Turda Gorge
1	*Abramis brama*	Zoobent		1	
2	*Alburnoides bipunctatus*	Omni, zoobent			1
3	*Barbatula barbatula*	Zoobent, detrit	1		1
4	***Barbus meridionalis***	**Zoobent**	**1**	**1**	**1**
5	***Cobitis elongatoides (Cobitis taenia)***	**Omni, zoobent,**	**1**	**1**	
6	*Gobio obtusirostis*	Omni, zoobent	1	1	
7	*Phoxinus phoxinus*	zoobent	1		
8	***Romanogobio vladykovi (Gobio albipinnatus)***	**Omni, zoobent**		**1**	**1**
9	***Romanogobio kesslerii (Gobio kessleri)***	**Omni, zoobent**		**1**	**1**
10	***Rhodeus amarus***	**erb**		**1**	**1**
11	*Squalius cephalus*	Carn, Ihti	1	1	1
	TOTAL		6	8	7
	Community Interest Species		**2**	**5**	**4**

Fish abundance and biomass are presented in percentages in Fig. 1 and Fig. 2 for each studied SCIs.

In the Tureni Gorge in the Valea Racilor River *S. cephalus* is numerically dominant, while two fish species of community interest, *C. taenia* and *B. meridionalis,* are also with high values, but in biomass *S. cephalus* is far ahead of *B. meridionalis* and others species (Fig. 1).

In the Turda Gorge in the Hășdate River *B. meridionalis* is numerically dominant, followed by *S. cephalus* and *A. bipunctatus*, but in biomass *S. cephalus* is far ahead of *B. meridionalis* and *A. bipunctatus* (Fig. 2).

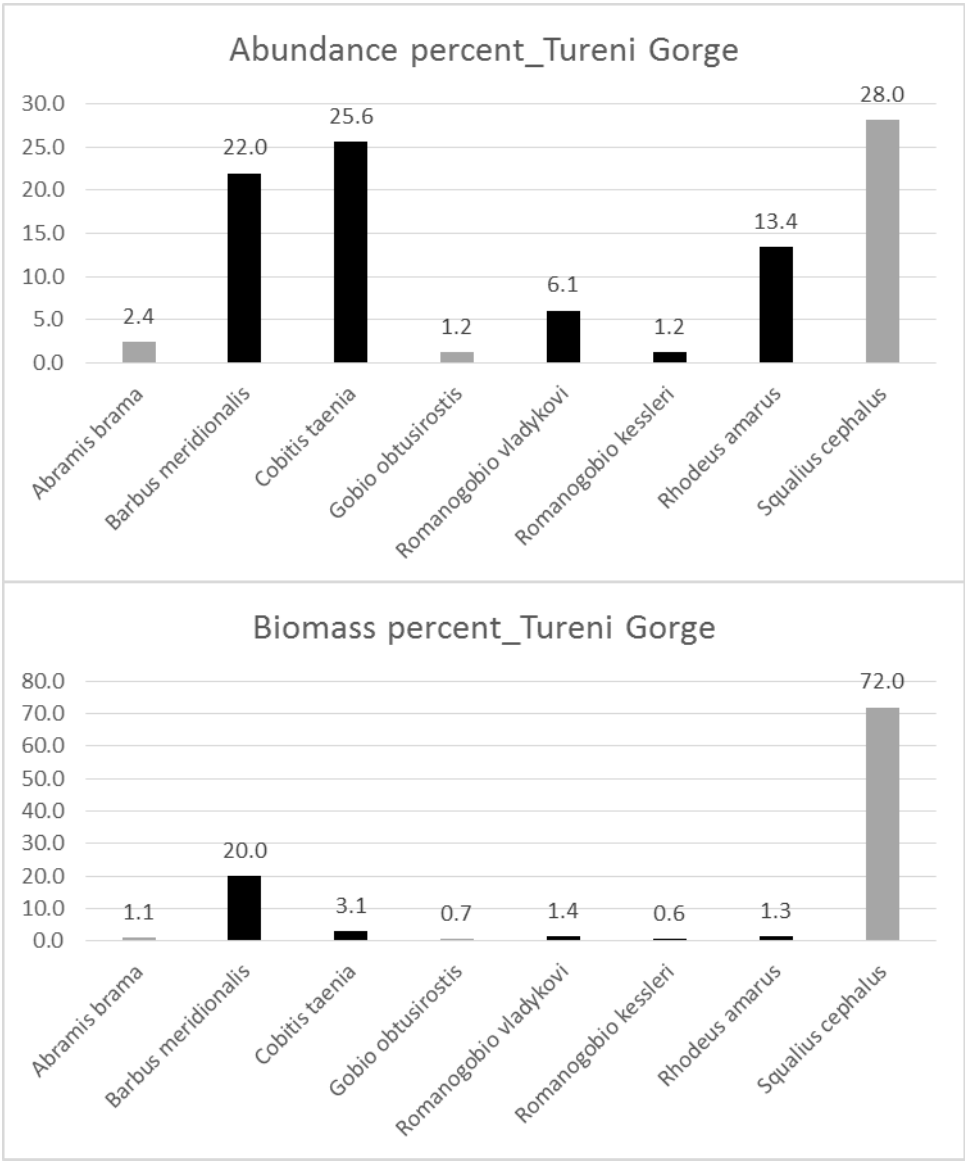

Figure 1. Fish abundance (top figure) and biomass (bottom figure) percentages from ROSCI0034 Tureni Gorge (black columns are fish species of community interest)

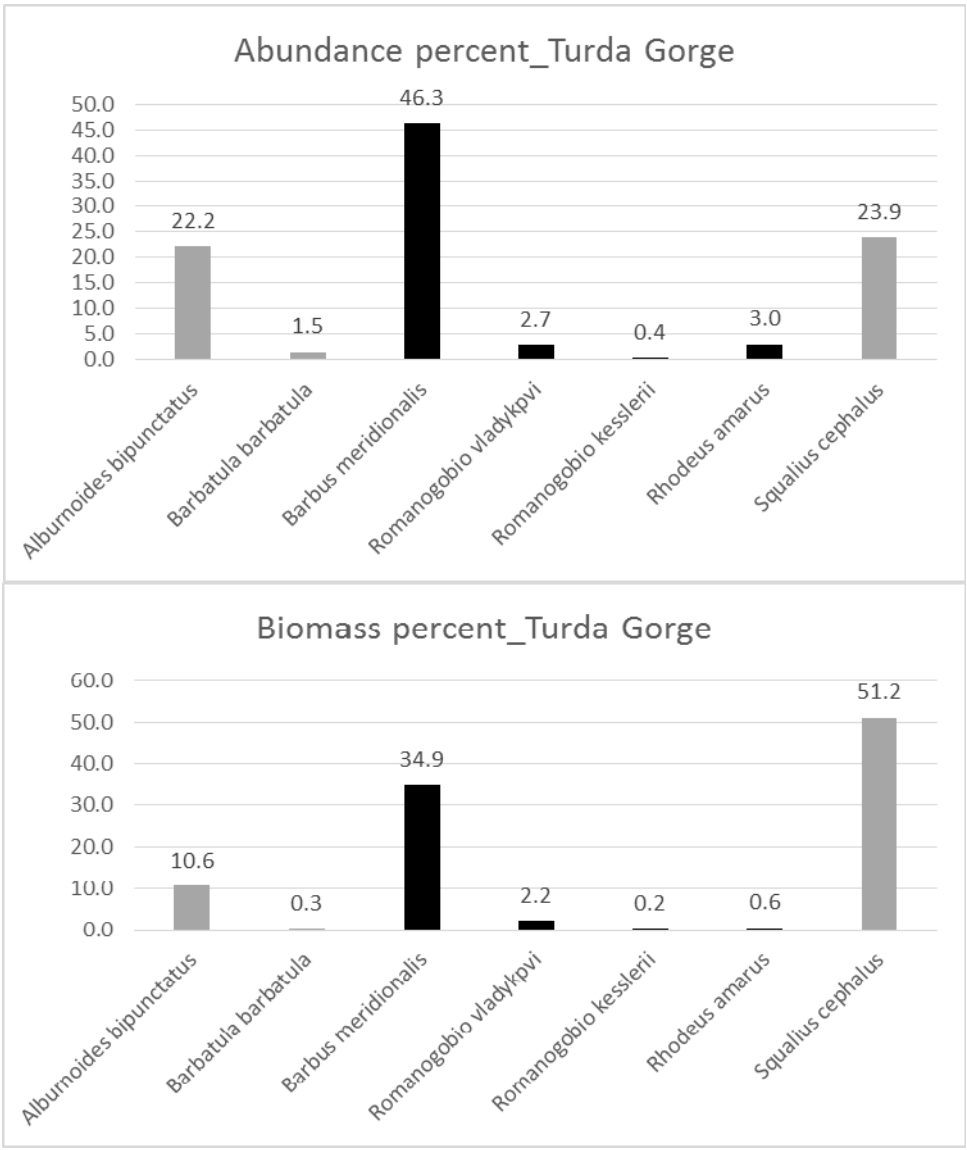

Figure 2. Fish abundance (top figure) and biomass (bottom figure) percentages from ROSCI0035 Turda Gorge (black columns are fish species of community interest)

ROSCI0034 Tureni Gorge

The fish fauna from this SCI is composed of 8 fish species including the presence of 5 species of community interest, versus SCI Standard Form only with *Cobitis taenia*. The fish fauna is dominated in abundance and biomass by *Barbus meridionalis,*

Cobitis taenia and *Squalius cephalus* **(**Fig. 1). This SCI River is affected mostly by tourists and pollution from households but with a small negative impact.

ROSCI0035 Turda Gorge

In this SCI 7 fish species were captured, including 4 species of community interest. The newly captured species were *Barbus meridionalis, Romanogobio kessleri, R. vladykovi* and *Rhodeus amarus* compared with existing Standard Form of SCI with *Cobitis taenia* which wasn't found during the sampling period. The fish fauna is dominated in abundance by *Barbus meridionalis, Squalius cephalus* and *Alburnoides bipunctatus* **(**Fig. 2). Regarding anthropogenic impacts, disturbances are caused mostly by angling, tourists and sheepfolds (sheep enter the water to drink and disturb water quality), with a small to medium negative impact.

The ichthyocoenosis from the Valea Racilor River of Tureni Gorge is characterized by the main, eudominant species *S. cephalus*, but an accidental fish species is *Abramis brama* (Table 6).

The ichthyocoenosis from the Haşdate river of Turda Gorge is characterized by the main, eudominant species *S. cephalus, B. meridionalis* and *A. bipunctatus,* no fish species is accidental, but most are accessory-associate fish species (Table 6).

Table 6.

Ecological significance from the rivers of the Tureni and Turda Gorges

		Tureni Gorge			**Turda Gorge**		
No.	**Species**	**D class**	**C class**	**W class**	**D class**	**C class**	**W class**
1	*Abramis brama*	D2	C2	W1			
2	*Alburnoides bipunctatus*				D6	C5	W6
3	*Barbatula barbatula*				D2	C4	W3
4	*Barbus meridionalis*	D6	C3	W5	D6	C5	W6
5	*Cobitis taenia*	D6	C4	W6			
6	*Gobio obtusirostis*	D2	C2	W2			
7	*Romanogobio vladykovi*	D4	C4	W3	D3	C3	W2
8	*Romanogobio kessleri*	D2	C4	W2	D1	C2	W2
9	*Rhodeus amarus*	D5	C5	W5	D3	C3	W3
10	*Squalius cephalus*	D6	C5	W6	D6	C5	W6

According to IBI the scores for both studied areas are classified as good (Table 3) mean values are 63 for Turda Gorge and 61 for Tureni Gorge in evaluation marks class III (Table 7).

Table 7.

IBI results for fish fauna from the rivers of the Tureni and Turda Gorges

Category	Parameter	Evaluation marks Turda	Evaluation marks Tureni
Composition and abundance of species	1	5	5
	2	5	5
	3	1	1
	4	3	3
	5	5	5
	6	5	5
	7	5	5
Food composition for fish populations	8	5	5
	9	5	5
	10	1	1
	11	3	3
Stock and general state of fish populations	12	5	3
	13	5	5
	14	5	5
	15	5	5
SCORE		63	61
Appreciations		Good	Good
Evaluation integrity class		III	III

Conclusions

In 2014, in both the Tureni and Turda Gorge SCIs, 10 fish species were captured - including 5 species of community interest. Four more species of community interest were captured: *Barbus meridionalis, Rhodeus amarus, Gobio (Romanogobio) kessleri* and *Gobio albipinnatus* (*R. vladikovy)* versus Natura 2000 Standard SCI Form, which had only one species *Cobitis taenia*. The species of community interest *Cobitis taenia* was found only in the Tureni Gorge, absent in the Turda Gorge. Fish abundance and biomass are both dominated by *Barbus meridionalis* and *Squalius cephalus*, with small differences between sites. Turda Gorge is closer to the Trascău mountains proven by the presence of submountain fish species in the area like *Barbatula barbatula* and *Alburnoides bipunctatus*, also in the past was present *Phoxinus phoxinus*. Anthropogenic influence occurs only with low to medium negative impact represented by angling, tourism, household pollution and sheepfolds in the neighborhood that disturb water quality. Fish associations are represented by the main species *S. cephalus* and *B. meridionalis* with small differences between sites, and an accidental species, *Abramis brama*. Ichthyocoenosis for both the small rivers studied are less affected, appreciation category is good and with class III Evaluation marks according to IBI.

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Distribution of *Amelanchier ovalis* Medik. in the Romanian Carpathians – a critical overview

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SUMMARY. This study proposes a critical analysis of the distribution of the rare plant species *Amelanchier ovalis* Medik. in the Romanian Carpathians.The species was mentioned as sporadically distributed in this part of Carpathians, but we found no evidence that the plant was ever collected and deposited in public herbaria. Besides herbarium material, a critical analysis of available botanical literature concurred to the same conclusion: the presence of *Amelanchier ovalis* in all previously mentioned localities in the Romanian Carpathians can not be supported by any concrete data and, therefore, can be considered as doubtful. The paper reports the recent discovery of *Amelanchier ovalis* in a new area in the Eastern Carpathians (Vrancea Mountains, Putna-Vrancea Natural Park). A detailed description of plant communities in which the species grows is also provided.

Additionally, new sites with *Hieracium telekianum* Boros & Lengyel (Eastern Carpathian endemic and rare species) are also reported in Vrancea Mountains.

Keywords: *Amelanchier ovalis,* Chorology, Red Book, Romanian Carpathians, threatened species

Introduction

Romanian flora includes many species whose presence is uncertain and should be reconfirmed (e.g. *Dianthus diutinus* Kit., *Euphorbia paralias* L., *Thesium ebracteatum* Hayne, *Ledum palustre* L., *Ophrys fusca* Link, *Osmunda regalis* L., etc.) (Sârbu *et al.* 2013). Two of these species (*Saussurea porcii* Degen and *Jasione orbiculata* Griseb. ex Velen.) have been recently reconfirmed in Rodna (Eastern Carpathians) and, respectively, in Retezat Mountains (Southern Carpathians) (Mátis *et al.*, 2014; Bartók 2014).

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One of this species is *Amelanchier ovalis* Medik., which until recently was not confirmed by herbarium material in Romanian flora, although in some botanical works is listed as sporadically distributed (Ciocârlan, 2009; Oprea, 2005).

Genus *Amelanchier* was described in 1789 by Friedrich Casmir Medicus, German botanist and physician (Medicus, 1789). *Amelanchier* is a genus of shrubs and small trees belonging to the subfamily *Pomoideae* of the *Rosaceae* and includes ornamental species (chiefly from North America) and a single species widely distributed in Europe - *Amelanchier ovalis* (Jones, 1946).

This is a slender often scaly-barked shrub. Leaves are 2-5 x 1.5-3 cm, ovate, oblong to obovate or almost orbicular, rounded or emarginated at apex, lanate beneath when young. Flowers are entomophilous, in erect lanate racemes (rarely solitary or paired) appearing shortly in advance of the foliage, or as the leaves unfold. Pedicels (1-2 cm long) are bracteate at the base and bearing a second bract at or near the middle. Hypanthium is campanulate or urceolate, lanate at first, soon glabrous. Petals 5, white, or rarely pink, oblanceolate to narrowly oval, are 4-6(-8) times longer than sepals. Sepals are lanceolate, 2-2.5 mm long. Stamens (10-20) are short, inserted on the rim of the calyx. Styles 2-5, are free; carpels 2-5. Fruit is globose, 6-8 mm in diameter, glabrous or slightly tomentose at apex, red when young, bluish-black and with bloom when ripe (Strid, 1986; Franco, 1992).

Amelanchier ovalis naturally occurs in Western, Central and Southern Europe, Asia Minor and along the north coast of Africa, in the forest zone, rocky slopes along streams, and grassy subalpine meadows on calcareous bedrock, at 400-1100-2200 m (Strid, 1986). The flowering period is April to June.

In the Romanian Carpathians, *A. ovalis* was reported to grow in several massifs (Rodna, Ceahlău, Hăşmaş, Bârsa, Retezat, Trascău and Bihor Mountains - Buia 1956, Oprea 2005), but most of these localities were recorded in XIXth century, without recently confirmed populations. In the Romanian Floras and checklists it was considered as a sporadic element (Oprea, 2005; Ciocârlan, 2009) or a rare species (Sârbu *et al*., 2013). In the Red Lists elaborated for Romanian Flora, *A. ovalis* is considered a "Rare Species" (Oltean *et al*., 1994; Dihoru and Dihoru, 1994) or placed in the "Insufficiently Known" (K) sozological category (Boşcaiu *et al*., 1994) The species is not included in the Red Book of Vascular Plants of Romania (Dihoru and Negrean, 2009).

The main goal of this study was to clarify the distribution of *Amelanchier ovalis* in Romania, based on a detailed review of the available data concerning the presence of the species in this part of Carpathians. Moreover, the discovery of new sites for this species is reported and the phytocoenotic context in which *A. ovalis* grows is characterised by original field data.

Material and methods

Our investigations were based on recent field studies, analysis of herbarium material stored at CL, BP, BUC, BUCA, BUCF, BVS, SIB, I, IAGB, IASI, CRAI (acronyms according to Thiers 2015), as well as literature data.

All existing herbarium material was revised and all available information from botanical literature was critically compiled in order to clarify the distribution of *Amelanchier ovalis* in the Romanian Carpathians. Several field surveys were made between 2003 and 2016 in Ceahlău Mts., Hăşmaş Mts., Rodna Mts. (Eastern-Carpathians); Bucegi Mts., Piatra Craiului Mts., Piatra Mare Mt., Postăvaru Mt., Retezat Mts. (Southern Carpathians); Trascău Mts., Bihor Mts. (Apuseni Mountains) where the species was reported.

Phytosociological characteristics of newly discovered sites with *Amelanchier ovalis* were studied according to the Braun-Blanquet approach (Braun-Blanquet, 1932). Species names follow Flora Europaea (Tutin *et al*., 1992-1994) when author's name not indicated.

Results and discussions

1. *Distribution of Amelanchier ovalis in the Romanian Carpathians*

1.1. *Historical considerations*

1.1.1. **Munţii Rodnei** (Rodna Mountains, Eastern Carpathians)

This important hotspot of alpine flora in Romania is very well studied from botanical point of view, but no botanist cited the species in Rodna Mountains. A single herbarium material was found in BUCA (no. 52130), with this information on the voucher's label: "Munţii Rodnei, leg. I. Prodan, 1896". Carefully studying the label, it was clear that it was not the Prodan's handwriting. Most probably the specimen was mislabelled and the plant has not been collected from that range. Moreover, Prodan (1939) did not mention *A. ovalis* from Rodna Mountains in a work published after the supposed collections. In the same publication he clearly stated that, in Transylvania, the plant is possibly present only in Bihor Mountains.

The species was not mentioned in other botanical monographs of famous botanists like Porcius (1878), Schur (1866), Simonkai (1886), Soó (1944), Coldea (1990). We could neither find *A. ovalis* in different parts of this mountain range that were investigated (A. Bartók, pers. obs. 2003, 2004, 2005, 2009, 2010, 2012, 2013, 2014, 2015, 2016).

1.1.2. **Munţii Ceahlău** (Ceahlău Mountains, Eastern Carpathians)

Ceahlău Mountains represent maybe the most intensively investigated region by the botanists, considering the whole range of Eastern Carpathians. The first mentions of *A. ovalis* from this massif date back to XIXth century, when J. Edel (1853) cited the species from the higher part of Ceahlău, but without exact location. In his floristical synthesis, Brândză (1883) mentioned *A. ovalis* in Ceahlău Mountains quoting Edel, from rocky cliffs in forests, without exact locality.

Later, Grecescu (1906), in his article about vascular plants of Ceahlău, specified that serviceberry is wrongly published by Edel from this area because, in his opinion, the species does not grow spontaneously in Carpathians.

Despite that, in Romanian botanical literature (e.g. Prodan, 1939; Buia, 1956; Dumitriu-Tătăranu, 1961; Zanoschi, 1971; Beldie, 1977; Chifu *et al*., 1987; Mititelu *et al*., 1989; Manoliu *et al*., 2002; Oprea, 2005; Chifu *et al*., 2006; Ciocârlan, 2009; Sârbu *et al*., 2013) snowy mespilus is listed for the Ceahlău Mountains, but the data referred to the same old and doubtful sources.

In this mountain range the presence of snowy mespilus was never confirmed by herbarium material. We could not trace any herbarium specimen of *Amelanchier ovalis* from Ceahlău Mountains. Also, we could not find serviceberry in this mountain group (A. Bartók, pers. obs. 2003, 2004, 2005, 2006, 2007, 2009, 2015, 2016).

1.1.3. **Munţii Hăşmaş** (Hăşmaş Mountains, Eastern Carpathians)

Amelanchier ovalis was first listed in this mountain group by Schur (1859) from Öcsém Peak (*Ecsém Teteje*). Even Schur no longer mentioned the species in Hăşmaş Mountains in his *Enumeratio Plantarum Transsilvaniae*, published in 1866. Moreover, according to Soó (1940), Schur's data from Öcsém Peak should be removed. Recently, in a comprehensive monograph of flora and vegetation of Hăşmaş Mountains Nechita (2003) mentioned the snowy mespilus's presence in this mountain range, but only referring to the Schur's data (Schur, 1859b).

The flora of Hăşmaş Mountains is relatively well explored and other floristical or phytosociological publications (Soó, 1943; Nechita and Mititelu, 1996; Nechita, 2000) did not mention the occurrence of *A. ovalis* in this mountain range.

We could not find snowy mespilus in Hăşmaş Mountains (A. Bartók, pers. obs. 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2011, 2013, 2014, 2015) and also we were unable to trace herbarium specimens of this taxon from that mountain range in any public herbaria.

1.1.4. **Munţii Bârsei** (Bârsa Mountains, Southern Carpathians)

Schur (1866) listed *Amelanchier ovalis* (under *Aronia rotundifolia*) in this mountain group, around Braşov.

Bârsa Mountains (including Piatra Mare and Postăvaru) represent one of the most intensively investigated region by botanists, considering the whole range of Carpathians, but *A. ovalis* was not mentioned in any subsequent monographs about the flora of this area (Römer, 1905; Fink, 1975, 1977; Buiculescu, 1989; Danciu and Parascan 2000; Danciu and Pop 2008).

Only Dumitriu-Tătăranu (1961), Hager (1985) and Oprea (2005) cited snowy mespilus from Bârsei Mountains according to Schur (1866).

Amelanchier ovalis could not be observed in Bârsa Mountains (A. Bartók, pers. obs. 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015) and also we were unable to trace herbarium specimens of snowy mespilus from that mountain range in all checked herbaria.

1.1.5. **Munţii Retezat** (Retezat Mountains, Southern Carpathians)

Baumgarten (1816) was the first and only author that mentioned snowy mespilus (under *Amelanchier mespilus*) in this mountain range, but without exact locality. Simonkai (1886) cited this taxon in Retezat Mts. after Baumgarten, but he questioned its presence in that range.

Other more recent publications about the flora of Retezat (Csűrös, 1956; Nyárády, 1958; Csűrös, 1971) did not mention *A. ovalis* in this mountain group. We could neither find any herbarium voucher of this species from the Retezat Mts. or in the field, in different investigated parts of this mountain range (A. Bartók, pers. obs. 2003, 2004, 2005, 2006, 2007, 2009, 2010, 2012, 2013, 2014, 2015).

1.1.6. **Munţii Trascău** (Trascău Mountains, Apuseni Mountains)

Amelanchier ovalis was mentioned twice in flora of this mountain group, by Schur (1859a) and Pavai (1862). Schur cited the presence of snowy mespilus in Piatra Cetii Peak. Pavai noticed the species on Piatra Caprei Peak, in a phytocoenosis composed by *Quercus cerris* L., *Sorbus aria* (L.) Crantz, *Sorbus torminalis* (L.) Crantz and *Rhamnus saxatilis* Jacq.

Csató (1896) mentioned *Amelanchier ovalis* in the monograph of Alsófehér vármegye (Alba Iulia county), but he only refered to the Pavai and Schur's data.

The flora of Piatra Cetii and Piatra Caprei Peaks is relatively well studied (e.g. Ghişa *et al*., 1965; Pop *et al*., 1960) but the species was not found by any botanist in the last century.

We could not trace any herbarium specimen of *A. ovalis* from this area. Also we could not find snowy mespilus near Piatra Cetii and Piatra Caprei Peaks nor in other parts of this mountain range (A. Bartók, pers. obs. 2003, 2004, 2005, 2006, 2007, 2009, 2010, 2011, 2012, 2013, 2014, 2015). Several other recent botanical investigations in these points failed as well to find this species (A.S. Bădărău, P.D. Turtureanu - personal communications).

1.1.7. **Munţii Bihor** (Bihor Mountains, Apuseni Mountains)

Kerner (1863) recorded for the first time this species in Bihor Mountains, but without exact locality. He specified only a phytocoenosis composed by *Cytisus falcatus* Walst. & Kit., *Cotoneaster tomentosus* Lindl., *Spiraea ulmifolia* Scop., *Sorbus aria* (L.) Crantz, *Salix silesiaca* Willd and *Amelanchier ovalis* Medik.

Later, Hayek (1916) mentioned a list of species including *A. ovalis* from this mountain range (Piatra Batrina, Piatra Galbina, Piatra Muncelu). Similarly, Jávorka (1927) referred to Batrina (Piatra Bătrâna) as the certain place in Transylvania where the species occurred.

The subsequent botanical works no longer mentioned *A. ovalis* in Bihor Mountains (Simon, 1966; Pop and Hodişan, 1962; Pop *et al*., 1965; Coldea *et al*., 2008).

We could not trace any herbarium specimen from the Bihor Mountains in all checked herbaria. Moreover we could neither find *A. ovalis* in this mountain range (A. Bartók, pers. obs. 2009, 2013, 2014; P.D. Turtureanu and M. Pușcaș, pers. obs. 2016).

As a synthesis, the present investigations suggest that the occurrence of snowy mespilus in the aforementioned mountains remains unproved by clear data. Confusion of the species, mislabelled material make all these sites to be uncertain for the actual *Amelanchier ovalis* chorology.

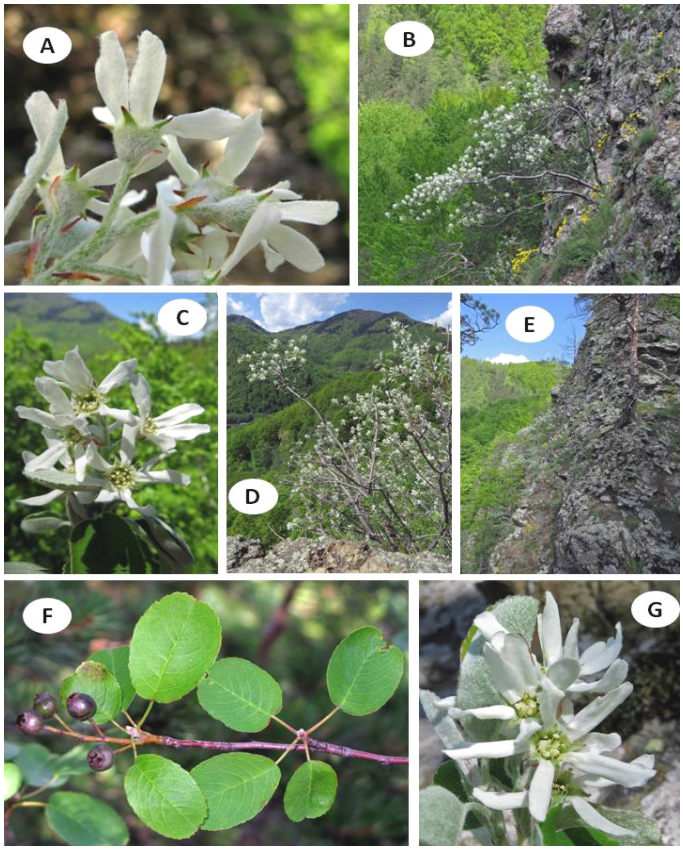

Figure 1. *Amelanchier ovalis* in Vrancea Mts.: A, C, G – The flowers, B, D, E – Habitus and habitat, F – Fruits (Original photos by A. Bartók and A. Indreica).

1.2. *New localities for Amelanchier ovalis* in *South-Eastern Carpathians*

Munţii Vrancei (Vrancea Mountains, Eastern Carpathians)

Botanists never mentioned the species in this mountain group. The area is relatively well studied from botanical point of view (e.g. Paşcovschi and Leandru, 1955, Mititelu *et al*., 1996; Ştefan *et al*., 1997) but *A. ovalis* is not listed among the present species.

Amelanchier ovalis was discovered by A. Indreica in Vrancea Mountains in 25.07.2011, close to Lepşa locality (Vrancea county), on the left side of Putna river cross to the Putna Waterfall, on a ridge under Locoţeilor Peak. The site was revisited in 2015 by A. Indreica and A. Bartok. In august 2016, the species was observed by P.D. Turtureanu and M. Puşcaş on Piatra Ciutei, a site not far from the former. Currently these are the only certain locations of *Amelanchier ovalis* in the Romanian Carpathians.

The collected material has been deposited in the Transilvania University of Braşov - Faculty of Silviculture Herbarium, Braşov (BVS no. 64671, 64672) and in the „A. Borza" Botanical Garden Herbarium, Cluj-Napoca (CL no. 665394, 666333).

2. *Habitat description by recent field observations*

In Romanian botanical literature, *A. ovalis* is ascribed to *Berberidion*, *Quercetalia pubescentis* or *Fraxino-Cotinetalia* syntaxa (Chifu *et al*., 2006; Sârbu *et al*., 2013). From Natura 2000 habitats perspectives, this taxon is considered character species for the habitat 40A0 Subcontinental peri-Pannonic scrub (Gafta and Mountford, 2008); other authors (Oprea *et al*., 2010) included *A. ovalis* in the species list of the habitat 9410 Acidophilous *Picea* forests of the montane to alpine levels (*Vaccinio-Piceetea*), probably based on the note of Brandza (1883) who indicated as habitat for *Amelanchier* "*rocky clefts inside the subalpine forests*".

We discovered recently *A. ovalis* in the Putna-Vrancea Natural Park between Lepşa and Tulnici localities. Here it grows in communities on rocky sites in a forested landscape, on steep conglomerate rocks and cliffs, with south or south-west aspect and altitudes between 600-700 m. In the investigated phytocoenoses (Table 1), *Amelanchier ovalis* and *Carex humilis* are dominant species seconded by *Aurinia saxatilis*, *Thymus comosus*, *Seseli gracile*. Since the cover of shrub layer is very scarce and the number of releves is small we refrain to define such communities as a shrub association. Without any doubt, these phytocenoses with *Amelanchier* belong to dry and saxicolous type, as it is revealed by accompanying species, community structure, aspect and slope of the site. This makes them closer to *Berberidion vulgaris* Br.-Bl. ex Tüxen 1952 alliance. At the level of association, the decision is hard to take since in Europe *Amelanchier ovalis* is quite frequent in many vegetation types, especially in southern and western Europe (Faucault and Julve 2001).

The snowy mespil also grows inside forest habitats, on rocky outcrops and steep slopes. One relevee of oak forest belonging to *Cytiso nigricantis-Quercetum petraeae* Paucă 1941 is given below:

Releve number (EU-RO-007 database) 5276, altitude 640 m, aspect S, slope 50°, rocks at surface 15%; releve area 400 m^2; date 25.07.2011; location between Lepșa and Tulnici (Vrancea county).

Tree layer (80%): *Quercus petraea* 5, *Fraxinus excelsior* +, *Pinus sylvestris* +, *Carpinus betulus* +.

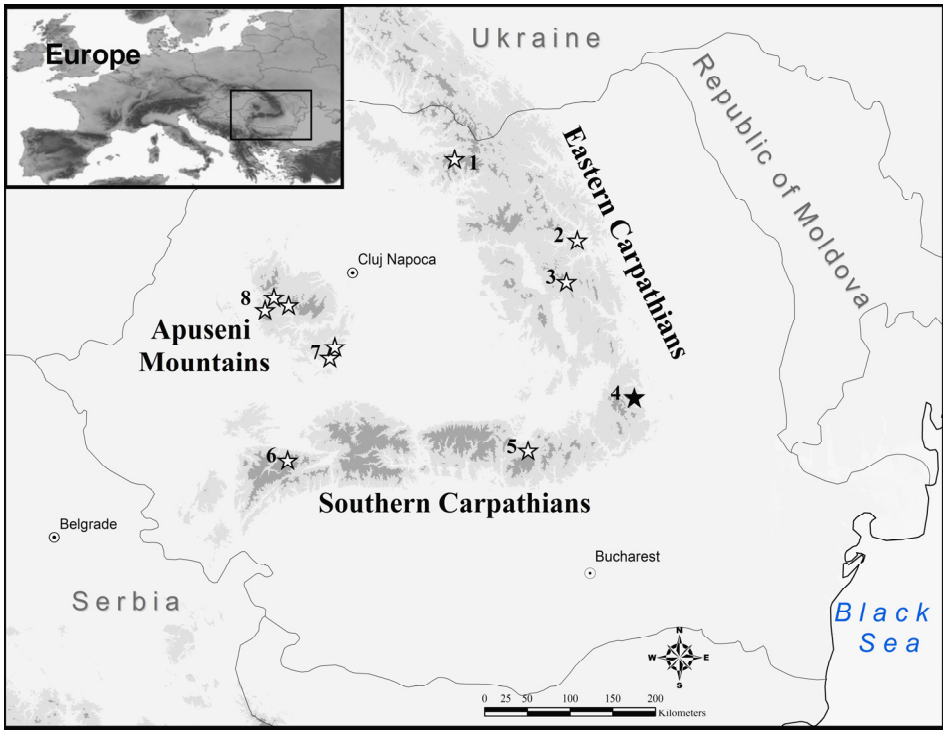

Figure 2. Chorological map of *Amelanchier ovalis* in the Romanian Carpathians: 1-Rodna Mts.; 2-Ceahlău Mts.; 3-Hăşmaş Mts.; 4-Vrancea Mts.; 5-Bârsa Mts., 6-Retezat Mts., 7- Trascău Mts., 8-Bihor Mts. The black star represents the certain location, the white stars represent the uncertain locations.

Shrub layer (1%): *Amelanchier ovalis* +, *Rosa canina* +, *Corylus avellana* +.

Herb layer (40%): *Luzula luzuloides* 2, *Poa nemoralis* 1, *Silene nutans* ssp. *dubia* 1, *Hieracium murorum* 1, *Campanula persicifolia* 1, *Genista tinctoria* 1, *Calamagrostis arundinacea* 1, *Lychnis viscaria* 1, *Lembotropis nigricans* +, *Chamaecytisus hirsutus* +, *Sedum telephium ssp. maximum* +, *Digitalis grandiflora* +,

Hieracium sabaudum +, Hieracium umbellatum +, Hieracium lachenalii +, Galium schultesii +, Veronica chamaedrys +, Clinopodium vulgare +, Trifolium medium +, Coronilla varia +, Galium mollugo agg. +, Campanula rapunculoides +, Seseli gracile +, Asplenium trichomanes +, Asplenium septentrionale +, Polypodium vulgare +, Thymus comosus +, Cardaminopsis arenosa +, Erysimum odoratum +, Rubus canescens +, Verbascum lychnitis +, Solidago virgaurea +, Salvia glutinosa +, Phleum montanum +, Cnidium silaifolium +, Lapsana communis +, Festuca rupicola +, Hypericum perforatum +, Galeopsis tetrahit +, Fallopia dumetorum +.

Table 1.

Communities with *Amelanchier ovalis* on rocky sites near Lepşa (Vrancea county)

Relevé no.	1	2	3
Altitude (m, a.s.l.)	680	700	700
Aspect	SSW	WSW	SSW
Slope (°)	70-80	70	70
Cover (%)	15	15	15
Shrub layer			
Amelanchier ovalis	1	2	1-2
Pinus sylvestris	.	+	.
Juniperus communis	+	.	.
Fraxinus excelsior	.	.	+
Rosa tomentosa	+	.	.
Sorbus aucuparia	+	.	.
Herb layer			
Lembotropis nigricans	+	+	+
Aurinia saxatilis	1	1	+
Carex humilis	2	1	1
Sempervivum zeleborii	+	+	1
Dianthus spiculifolius	+	1	+
Galium album	+	+	+
Asplenium septentrionale	+	+	+
Thymus comosus	1	2	.
Seseli gracile	1	1	.
Sedum telephium ssp. *maximum*	+	+	.
Erysimum odoratum	+	.	+
Chamaecytisus hirsutus	+	+	.
Peucedanum oreoselinum	+	+	.
Poa nemoralis	+	.	.
Silene nutans ssp. *dubia*	+	.	.
Hieracium telekianum	.	+	.
Cardaminopsis arenosa	.	+	.

Place and date of relevés: 1-3. Vrancea Mts. (Eastern Carpathians), Putna-Vrancea Natural Park, near Putna waterfall, conglomerate rocks, 9.V.2015. Releve area: 25 m^2.

Moss layer (30%)*: Hypnum cupressiforme* 2*, Polytrichum juniperinum* 2*, Rhytidium rugosum* +*, Leucodon sciuroides* +*, Abietinella abietina* +*, Polytrichum formosum* +*, Leucobryum glaucum* +*, Dicranum scoparium* +.

In Vrancea Mountains *Amelanchier ovalis* starts to bloom in late April (early May) and fructifies starting with late June.

It is important to underline that during our research in Putna-Vrancea Natural Park we discovered two small populations of *Hieracium telekianum* Boros & Lengyel, an Eastern Carpathians endemic plant, known to the present only from Harghita and Ciomatu Mountains. One population has been identified together with *A. ovalis* and the other was discovered in Tişiţei Gorges by S. I. Bartók. The collected material has been deposited in the „A. Borza" Botanical Garden Herbarium, Cluj-Napoca (CL no. 665395).

3. *Recommended IUCN threat category*

Only one small area that contains populations of *Amelanchier ovalis* is certainly known in the Romanian Carpathians, in a restricted part of the Vrancea Mountains. The place where the species occurs is included in Putna-Vrancea Natural Park.

On the basis of new chorological data and estimation of the number of individuals (between 100 and 200) and populations condition, we can define *Amelanchier ovalis* as IUCN CR C2a(i) (IUCN, 2012) in Romania.

We therefore recommend the establishment of a special protected area for this species and the inclusion of *Amelanchier ovalis* in the next edition of the Romanian Red Book of Vascular Plants as Critically Endangered (CR).

Conclusions

Based on the critical analysis of the occurrence of *Amelanchier ovalis* in the Romanian Carpathians it appears that the species is very rare and deserves more attention from botanists and nature conservation authorities.

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Climatic and habitat suitability of *Leontopodium alpinum* Cass. populations in the Romanian Carpathians

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SUMMARY. Species distribution models (SDMs) are widely used to obtain distributional knowledge in the context of incomplete biogeographic data sets. In this study we modeled the potential climatic niche of edelweiss in the Romanian Carpathians and the Alps for present and future environmental conditions. The prime objective of the study was to identify the possible impact of climate change over the distribution of this species with focus on the Carpathians. Ensemble models were fitted using the BIOMOD modeling tool in R software, for current conditions and two possible future scenarios (RCP pathways 6 and 8.5 for the year 2050). The results show that climate suitability is expected to decrease by 2050 in most of the locations considered in the study. These results outline the species sensitivity to climate warming, in agreement with previous studies.

Keywords: climate change, edelweiss, species distribution model (SDM).

Introduction

Leontopodium alpinum Cass.*,* commonly known as edelweiss, can be found in Asia and in Europe predominantly in the alpine area. Its European range is stretched from the Carpathian range in the East to the Pyrrenees in the West, and the Apennini and the Bulgarian mountains in the South (Tutin *et al*., 1976). In the Alps, edelweiss is found almost exclusively in the alpine area. In the Swiss Alps for example, species distribution models showed a high habitat suitability at high elevations, dry areas and steep slopes (Ischer *et al.,* 2014). In the Romanian Carpathians however, the species can be found as low as 440 m a.s.l. (the Lotrului Mountains). In a similar fashion to the Alps, the species is also distributed in the subalpine zone (e.g. the Piule-Iorgovanu Mountains, Gurgan area – 1900 m altitude), and in the alpine zone (e.g. the Bucegi Mountains, Omu Peak – 2500 m altitude). The species prefers rocky areas but it can also be found in mountain and alpine steep meadows, with calcareous substrate.

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Anthropogenic factors have caused a visible decrease of the species population in many European countries (IUCN Red List, 2003). The species is collected by tourists in many European countries as it is considered to be iconic for alpine flora. This situation led to early conservation measures in countries such as Switzerland, where the edelweiss has been protected since 1878, in Austria, since 1887 (Pop, 1939) and Slovenia, since 1896 (Skoberne, 2004). Currently, in Europe, the edelweiss is listed as *Least concern,* according to the IUCN Red List of Threatened Species, but the plant has strikingly different conservation statuses depending on the country where it occurs. Thus, the plant is listed as *Least Concern* in Switzerland (Moser *et al*., 2002), *Vulnerable* in Slovakia (Institute of Botany of Slovak Academy of Sciences, 2001), *Endangered* in Bulgaria (Petrova and Vladimirov, 2009) and Germany (Bundensamt für Naturschutz 2012), *Critically Endangered* in Ukraine (Kriscfalusy and Budnikov, 2007) and is protected in Montenegro. In Bulgaria, *in vitro* seed propagation methods have been used for *ex situ* conservation (Kozuharova, 2009).

In Romania, the negative effect of harvesting on edelweiss was observed by professor Alexandru Borza at the beginning of the 20th century. He took measures to protect edelweiss populations by declaring the plant a "natural monument" in 1933 (Borza, 1964).

Adding to the anthropic pressure, global temperatures have increased over the past hundred years by about 0.74ºC (1906 – 2005) (Trenberth *et al*., 2007). Following the temperature increase, plants are expected to migrate upwards on mountain slopes, a process associated with a reduction in available suitable habitat (Walther *et al*., 2005; Parolo and Rossi, 2007; Kumar, 2012; Gottfried *et al.,* 2012). A similar impact is expected for edelweiss, but its magnitude may be argued to be smaller, given the persistence of the species in low altitude locations across the Carpathian range. The article will try to assess all the known locations for the species in the Romanian Carpathians in regards to their climate suitability in current and future climate conditions. Our objective is to respond to the following questions: i) What is the current potential environmental niche for edelweiss in the Romanian Carpathians? ii) What is the impact of climate change on the potential niche? iii) What are the implications of these findings for the conservation of *Leontopodium alpinum*?

Materials and methods

Study area and distribution data

The Carpathians, part of the Alpine orogenic system, influence the climate, vegetation and soil through their altitude, fragmentation and structure (Badea *et al*., 1983). The presence of limestones and conglomerates generates a specific structural relief on the surface of which rocky meadows, preferred by edelweiss, are grouped.

Field activities were carried out in twelve mountains from the Romanian Carpathians (Fig. 1). 430 GPS presence points were collected in the Romanian Carpathians during the summer months of 2012 – 2015, with a Garmin 76 GPS unit. Additionally, 26 locations were extracted from existing literature (Iancu and Decenei, 1964; Puşcaru-Soroceanu and Puşcaru, 1971; Resmeriță, 1973; Frink, 2015; Frink *et al.,* 2015).

In order to build distribution models under climate change, we needed a more complete outlook on the ecological niche of the species. Aside from the Romanian Carpathians data, 1176 presence points were obtained for the Alps from several sources (www.infoflora.ch, University of Vienna Herbarium, www.gbif.org, Reist *et al.,* 1993; Freléchoux and Gallandat, 1995; Meyer, 1995; Mingard, 1996; Hoffer and Mingard, 1997; Mingard, 1997, Mingard and Moret, 1997; Mingard, 1998; Steiner, 2002; Ciardo and Jutzeler, 2007; Bornand, 2008 and foreign contributor).

The presence data was rasterized at 1 km which caused some points from the database to be eliminated as they had location errors above 1 km, or they occurred in the same raster cell. Therefore, 692 presence points from Alps and Romanian Carpathians entered the statistical modeling process, of which 43 points from Romania (from a total of 64 points).

Environmental data

Climatic predictors with a spatial resolution of 30 arc-seconds (~1km) were obtained from the *WorldClim* dataset (Hijmans *et al*., 2005). Topographic variables (slope, aspect, roughness, Topographic Ruggedness Index (TRI), Topographic Position Index (TPI) were calculated at the same resolution using the raster package (Hijmans *et al.,* 2005) in R 3.2.1 (R Core Team, 2011). The Topographic Wetness Index (TWI) was calculated in Saga GIS (Conrad *et al.,* 2015).

Information from all climatic and topographic variables was extracted for the 692 presences of the species (*raster* package, R 3.2.1 software). The importance of each predictor was tested using a generalized linear model (GLM) in which the predictors were also included in an exponential form, in order to incorporate some of the nonlinear correlation. In order to avoid multicollinearity, all predictors were tested for pair-wise correlation using Spearman's rho correlation coefficient (0.7 threshold).

For 2050, the climate variables were obtained also from WorldClim using the *Met Office climate* model (HadGEM2-ES) (Collins *et al.,* 2011) for two Representative CO_2 Concentration Pathways: RCP 6 and RCP 8.5 (Vuuren *et al.,* 2011). RCP 8.5 scenario describes the situation with the largest emissions of greenhouse and corresponds to a future characterized by the absence of mitigation policies. Under this scenario, there will be a considerable increase in these emissions leading to a radiative forcing of 8.5 W / m2 (Riahi *et al*., 2011). Regarding the RCP 6, greenhouse gas concentrations are predicted to grow until 2100 and then to decline (Toshihiko *et al*., 2011).

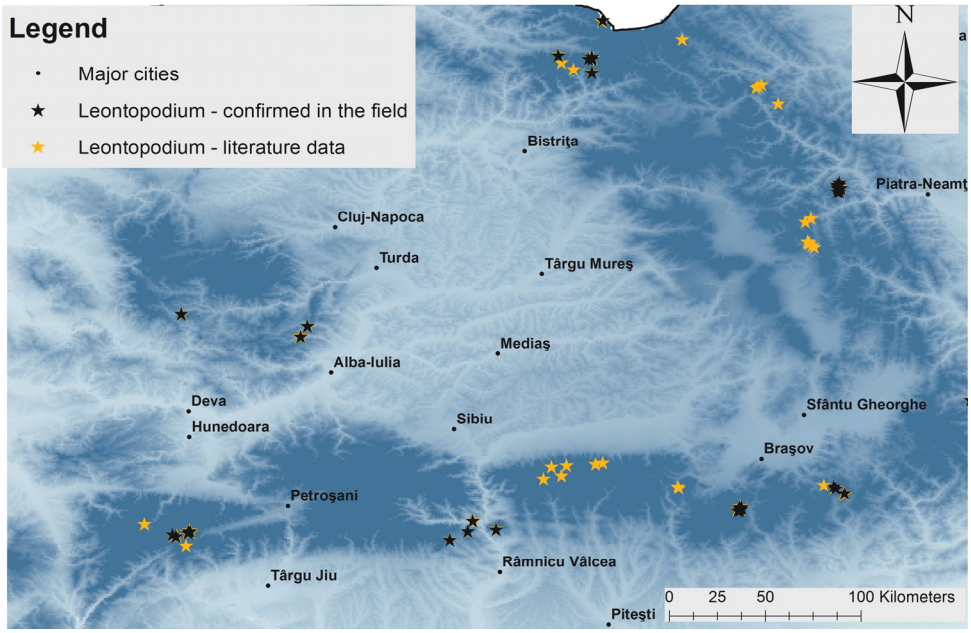

Figure 1. *Leontopodium alpinum* Cass. populations from the Romanian Carpathians considered for the current study

Modeling method

We used the BIOMOD modeling tool (*biomod2* package, R 3.2.1, Thuiller *et al.,* 2014) to fit the ensemble models for current and future conditions (2050). The ensemble models were built as an average of 9 distinct modelling methods: Generalized Linear Model (GLM), Generalized Boosting Model (GBM), Generalized Additive Model (GAM), Classification Tree Analysis (CTA), Artificial Neural Network (ANN), Surface Range Envelop (SRE), Flexible Discriminant Analysis (FDA), Multivariate Adaptive Regression Splines (MARS), and Random Forest (RF).

Information on the absence of the species was generated using the *biomod2* package, with the *sre* strategy (pseudo absences are selected outside of the broadly defined environmental conditions for the species). Ten times more absences than presences were used to create the model (Elith *et al.,* 2006; Massin-Barbet *et al*., 2012).

BIOMOD uses a repeated split-sample procedure keeping a part of the initial data out of the calibration for the subsequent validation of the predictions (Thuiller *et al.*, 2009). 10 repetitions were executed for each modeling method, with an 80/20 split (80% for calibration, 20% for validation).

Finally, all models were assembled into a single ensemble model based on their individual AUC scores (area under the receiver operating characteristic curve, Ogilivie and Creelman, 1968). The AUC has values between 0.5 (random predictions) to 1 (perfect

models). A model is considered to be good if the AUC value is higher than 0.7 (Swets, 1988). An AUC of 0.7 was established as threshold (only models with AUC values above the threshold were used in the ensemble).

Habitat data

As edelweiss has a clear preference for base-rich soils, we used existing soil and geology maps for Romania (Florea *et al*., 1994; Murgeanu *et al.,* 1966-1970) to extract substrates which are favorable for the species (e.g. purely calcareous bedrocks, mixed bedrocks with limestone such as conglomerates, etc.). Edelweiss is also a light-demanding species (Ischer *et al.*, 2014). Therefore we applied a filtering layer with non-forested areas (Forest Europe 2011) to the layer with favorable substrate.

The data with potentially suitable habitat for the species was rasterized at the resolution of 30 arc-seconds (~1km). The information was not included in the models, and was used as a final step in the processing of the model output, to filter out areas with climate and topographic suitability which lacked a suitable substrate for the species.

Results and discussion

Four predictors were selected as relevant for the species (respecting the maximum collinearity threshold): two climate predictors (BIO3 - *isothermality*, and BIO5 - *maximum temperature of the warmest month*), and two topographic predictors (*roughness index* and TWI - *Topographic Wetness Index*). These predictors were used to build the models.

The climate and topographic model (masked with suitable habitat) for current conditions predicted a rather widespread potential distribution of edelweiss in the Romanian Carpathians (Fig. 2).

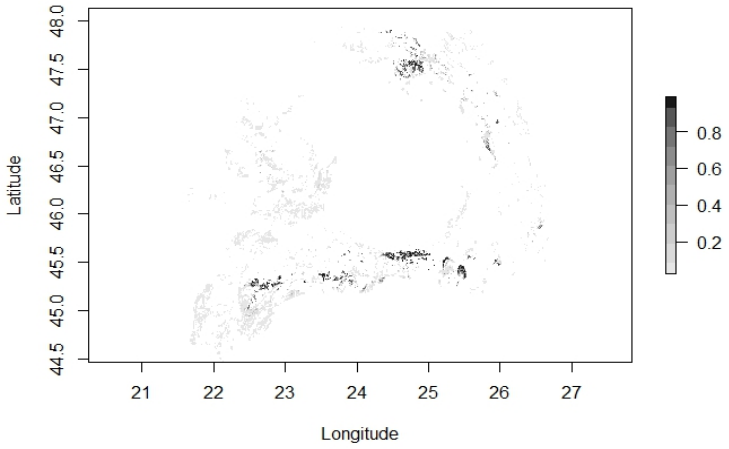

Figure 2. Species probability of occurrence in current climate conditions

As the species has an iconic status, and was intensively searched in all mountain ranges, we can assume to have quite complete information on its overall distribution in the Romanian Carpathians. We will limit all interpretations of climate change impact to the known locations of the species in the Carpathians. For these locations, we extracted the probability of occurrence in current and future climate conditions and the altitude – Table 1 (Hijmans *et al*., 2005).

According to the climate and topographic model, the highest probability for edelweiss occurrence (99%) is found above 2200 m (a.s.l.) in the Bucegi Mountains. The lowest probability is 5.8 % at 450 m (a.s.l.) in Obcinele Bucovinei. The model suggests the species is currently very near to its climatic limit in the Lotru Mts. (Doabrele), Trascău (Întregalde Gorges & Râmețului Gorges), Obcinele Bucovinei (Pietrele Lucinei), Căpățânii Mts., and locally in the Rarău Mts. (Vârful Rarău), the Ciucaș Mts. (Tesla) and the Hășmaș Mts.

Table 1.

Probabilities of edelweiss occurrence (%) for each known location and scenario

	Location	**Current cond.**	**RCP 6 (2050)**	**RCP 8.5 (2050)**	**Altitude (m a.s.l.)**
1	Bihor Mt. (Piatra Struțu)	27	5.5	4.8	936
2	Bucegi Mt. (Coștila)	99	91	77.8	2300
3	Bucegi Babele	84.8	17.9	4.7	2113
4	Bucegi Mt. (Cab.Caraiman I)	76.1	82.2	26.4	2022
5	Bucegi Mt. (Cab.Caraiman II)	99	90.4	75.6	2218
6	Bucegi Mt. (Caraiman I)	98.9	79.1	19.1	2075
7	Bucegi Mt. (Caraiman II)	98.7	77.5	22.7	2064
8	Căpățânii Mt.	27	8	7.4	901
9	Căpățânii Mt. (Narățu)	42.8	3.7	3.5	1348
10	Ceahlău Mt. 1	94.8	7.5	4.5	1670
11	Ceahlău Mt. (Detunatele)	91	4.6	4.5	1600
12	Ceahlău Mt. (Ghedeon)	61.1	13.2	4.3	1777
13	Ceahlău Mt. (Piatra cu apă)	83	4.7	4.2	1385
14	Ceahlău Mt. (Toaca 1)	92.4	4.9	4.3	1642
15	Ceahlău Mt. (Toaca 2)	90.3	4.5	4.4	1645
16	Ceahlău Mt. 2	92.1	5.1	4.8	1618
17	Ceahlău Mt.3	85.1	7.3	4.3	1688
18	Ceahlău Piatra Lată	87.7	4.1	4	1448
19	Ciucaș Mt. (Tesla)	26.5	3.5	3.4	1397
20	Ciucaș Mt. (Zăganu)	95.2	11.2	4	1723
21	Ciucaș Mt. I	76.5	6.1	3.7	1645
22	Ciucaș Mt. II	65.9	4.2	3.6	1799
23	Cozia Mt.	81.1	3.6	3.5	1296
24	Făgăraș Mt. (Culmea Podeanu)	98.8	48.4	7.8	1921
25	Făgăraș Mt. (Muchia Bâlea)	98.7	21.3	4.7	1939

	Location	Current cond.	RCP 6 (2050)	RCP 8.5 (2050)	Altitude (m a.s.l.)
26	Făgăraș Mt. (Piatra Caprei)	99	87.4	44.1	2240
27	Făgăraș Mt. (Trăsnita)	98.9	24.3	6.8	1896
28	Făgăraș Mt. (Turnurile Podragului)	98.4	8.6	4.4	1751
29	Făgăraș Mt. (Vârful Râios)	93.8	82.5	28.4	2291
30	Hăsmaș Mt. (Piatra Altarului)	38	3.4	3.3	957
31	Hăsmaș Mt. (Piatra Singuratică)	31.5	4.5	4.8	1485
32	Hăsmaș Mt. (Suhardu Mic)	35	3.4	3.3	974
33	Hăsmaș Mt. I	93.6	7.2	4.5	1686
34	Hăsmaș Mt. II	47.6	6.5	3.9	1667
35	Hăsmaș Mt. IV	22.9	4.3	4.1	1628
36	Hăsmaș Mt. Mt. III	90.1	4.8	5.1	1540
37	Lotru Mt. (Doabrele)	10.2	6.4	6	458
38	Maramureș Mt. (Coman)	86.7	4	4	1504
39	Maramureș Mt. (Farcău)	93.8	10.1	4.1	1750
40	Maramureș Mt. (Pop Ivan)	98.6	14.1	4.4	1783
41	Obcinele Bucovinei (Pietrele Lucinei)	5.8	3.5	3.5	1116
42	Piatra Craiului (zona Lespezi)	98.8	26.5	5.6	1812
43	Piatra Craiului Mt.(Umerii P.C.)	96.3	4.9	3.9	1635
44	Rarău Mt. (Pietrele Doamnei)	78.5	38	38	1513
45	Rarău Mt. (Popii Rarăului)	82.5	46	38	1285
46	Rarău Mt. (Varful Rarău)	28.3	38	36	1503
47	Retezat Mt. (Piatra Iorgovanului)	93.6	4.4	4.3	1801
48	Retezat Mt. (Piule)	98.9	20.3	6	1999
49	Retezat Mt. (Scorota I)	98.3	7.9	5.3	1850
50	Retezat Mt. (Scorota II)	71	23.5	7.1	1974
51	Retezat Mt. (Stănuleții Mari)	98.9	16.1	5.4	1966
52	Rodna Mt. (Cascada Cailor I)	88.3	5.3	5.4	1548
53	Rodna Mt. (Cascada Cailor II)	36.4	4.9	4	1248
54	Rodna Mt. (Corongiș)	93	6.7	4	1760
55	Rodna Mt. (Mihăiasa)	78.6	4.6	4.2	1645
56	Rodna Mt. (Obârșia Rebrei)	95.7	17.6	4.1	1917
57	Rodna Mt. (Turnu Rosu)	98.7	13.5	4.8	1774
58	Rodna Mt.(Valea Rea)	69.4	4.5	4.1	1385
59	Stănișoara Mt. (Bîtca Oblânc)	97	35	34	1163
60	Trascău (Întregalde Gorges)	23.9	4.7	4.3	814
61	Trascău (Râmețului Gorges)	21.4	7.8	6.8	722
62	Țarcu Mt. (Fața Fetii)	80.8	4.1	3.8	1517
63	Vâlcan Mt. (Oslea)	78.8	4	4	1747
64	Vrancea Mt. (Tișiței Gorges)	93	6.3	6.3	739

The two models for the year 2050 show a pronounced decrease of climate suitability for most of the known locations of the species. This decrease is more pronounced at lower than at the higher altitudes. In the RCP 6 scenario, the models suggest the species will be near its climatic limit in all of its locations from several massifs – such as Hăşmaş, Vâlcan, Vrancea and Ţarcu (adding to the previously mentioned ones). As expected, the RCP 8.5 scenario is more drastic and predicts a consistent decrease. In this "worst case scenario", the species will be in unfavorable climate conditions in most of its current locations, with the exception of high altitude populations such as those from the Bucegi or Făgăraş Mts., or, surprisingly, the somewhat smaller range of the Rarău Mts.

Currently, marginal populations of edelweiss persist in local environmental conditions which favor the species presence (e.g. the northern aspect of slopes and cold air currents). Small scale studies related to the viability of these populations are needed in such locations to help better understand the reason for the species resilience. These studies are even more necessary as in the next decades most of the *Leontopodium alpinum* populations from the Carpathians will be located in a sensibly warmer climate. Adding to this problem, the fragmented distribution of basic soils and the limited maximum altitude of limestone rock formations which harbor the species at lower altitudes suggest the need to establish monitoring sites for the species in multiple locations.

Conclusions

In case there is no significant change in CO_2 emissions, by the year 2050 many of the known locations for the species in the Carpathians will be near the limit of its climatic tolerance. The current study complements existing literature on this emblematic species by offering additional insight regarding the climate vulnerability of *Lentopodium alpinum* populations in the Romanian Carpathians. The results suggest there is justified concern related to the future of the species in Romania.

Therefore, we consider there is a strong need to monitor the species populations from low altitudes. Further research is needed to enhance the predictions of climate change impact on edelweiss. Physiological and demographical information could also be included in the models (Fordham et al., 2013), which would lead to a better understanding of species response to climate change.

These measures should be accompanied by public campaigns in order to create awareness among local communities. Awareness campaigns should focus on the threats the species faces, and should be doubled by punitive measures taken against any action which endangers the natural habitat of the species.

Symbol of the alpine meadows, edelweiss raises the interest and sympathy of the audience thus stimulating awareness for nature conservation. *Leontopodium alpinum* could represent a *flagship* species for the protection of vegetal associations in which it

develops, as they are varied and rich in endemic species and also subjected to anthropogenic pressure. By protecting the habitats in which edelweiss occurs, many other rare or endemic species will benefit as well.

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Physico-chemical properties of soils populated with wild halophytes in some Romanian areas

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SUMMARY. Soil salinity is one of the most important abiotic stress factor which reduces crop productivity worldwide, limits plant survival rate and restricts the use of arable land. Romanian saline soil areas are located on the seaside of the Black Sea and in different inland regions of the country. Nine Romanian sampling sites were analyzed, several obligative and facultative halophytes were identified.The main objective of the present study is to determine physical and chemical properties (pH, conductivity, salinity, redox potential) of some soil samples of saline areas from the Transylvanian Basin in order to give a better understanding about further characterization of halophytes adaptation mechanisms.

Key words: conductivity, halophytes, pH, redox potential, salinity.

Introduction

Soil salinity represents one of the most important abiotic stress factors which reduces the productivity of cultivated plants in the whole world, limits the growth and surviving rate of plants and restricts the use of tilled land. Soil salinization is one of the most serious problems of modern agriculture, because salts can also accumulate by irrigation. Soil salinity affects every aspect of plant growth and development, and is a major constraint of crop yield. Excess concentration of salt in soil has immediate effect on cell growth and associated metabolism (Munns and Tester, 2008; Muchatea *et al*., 2016). In general, plant root growth has been found to be reduced under salinity (Li *et al*., 2016; Farissi *et al*., 2013; Yan *et al*., 2016). Natural evaporation eliminates the pure water from the soil, substances concentrate, and thus salts can

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reach a damaging level, with adverse effects on the growth of plant species sensitive to salt. Plants face extreme salinity especially in the closeness of salt mines, salt lakes, sea shores and estuaries where sea water mixes fresh water. Natrium (Na^+) and chloride (Cl^-) ions are the most considerable components of soil and sea water, but other ions, like sulphate (SO_4^{2-}) and calcium (Ca^{2+}) also have an important role in salinity formation. In high-salt environments, plants initially suffer because of osmotic stress, reflecting water deficit, and subsequently experience ion-specific stress resulting from altered ion concentrations both in halophytes and glycophytes (Blumwald *et al*., 2000; Shabala and Mackay, 2011).

Some wild plant species, the so called halophytes, tolerate a large variation of soil salinity (Cadaret *et al*., 2016). These are capable to complete their life cycle in high salinity conditions, due to a series of anatomical and physiological adaptation mechanisms, developed in order to counter the ionic toxicity and water loss. Obligate halophytes vegetates on soils with high salts concentrations (> 0.5% NaCl), in order to accomplish their life cycle, they need high salt concentrations. Most of themes are succulent plants, this morphology being an adaptation to stress conditions (especially salt). Facultative halophytes do not need the salt presence by all means, but they tolerate it. These are found on the soils with lower salt concentration (<0.5% NaCl) or on the edge of saltings, where the salt concentration is lower.

Salt habitats develop on wide areas in two regions: on sea sides and inside continents, in zones with arid climate. Saline habitats vary in their salinity levels due to differences in topography, soil properties, and microclimate (Bazihizina *et al*., 2009; Kumar *et al*., 2016). The study is concentrated on continental saltings. Continental salt areas are speeded from East Mongolia, through Central Asia, Western Siberia and Transylvanian Plain up to Pannonian Plain. In West, from Carpathian Basin they are found only in isolated zones, on limited areas. Thus, the saltings in Romania are close to the Eastern limit of their range in Eurasia. These territories form in zones where due to the long warm and arid period in the summer a negative balance of phreatic water is formed. Thus, the water in the deep zones of the soil rises to surface, carrying the soluble salts in the soil. After the evaporation of water on the surface, the salts crystallize at soil level.

In the present study, physical and chemical properties of salty soils have been studied. We have also successfully screened the presence of the halophytes at the field level.

Materials and methods

The study was carried out at nine salting zones from Transylvanian Plain (Fig. 1). In the autumn of 2015, soil samples were taken to determine physical and chemical properties of soil. from these zones. Observations were also made on the vegetation characteristic to these areas of saltings.

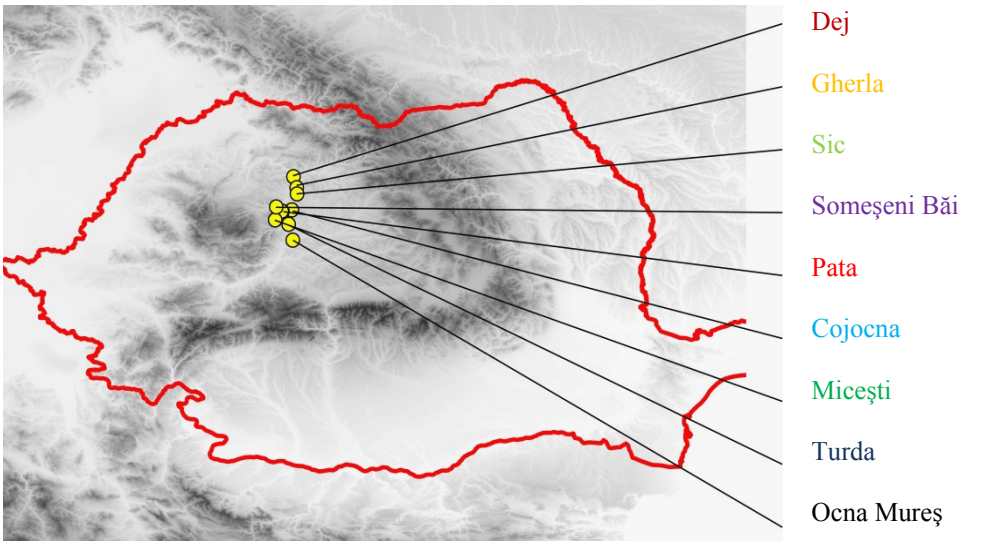

Figure 1. Soil sampling points

Sampling and processing the soil samples. The soil samples were taken form layers at different depths (0-10 cm, 10-20 cm, 20-30 cm), using a pedological bore made of inoxidable steel. Each soil sample weighted about 500 g. The samples were put in polyethylene bags, labeled, well sealed, and than taken to the Pedology Lab of the Faculty of Environmental Science and Engineering, Babeş-Bolyai University, Cluj-Napoca.

Determinant parameters: pH, electrical conductivity (EC), salinity, redox potential (ORP). For assessing the physico-chemical parameters, the soil samples were dried in open-air, at room temperature, for two weeks [US-EPA 3050B, ISO 11464] so that no parameter to be assessed is modified. After drying, the soil samples were sieved with a 2 mm sieve in order to remove the coarse material [ISO 11464].

pH, conductivity, salinity and redox potential were potentiometrically assayed in aqueous fraction (1:5) [ISO10390]. 40 g of soil were taken, prior dried in open-air (fraction < 2 mm), and put in an Erlenmeyer glass on top of which 200 ml of ultra pure water were added. The samples were stirred for two hours and that left to sedimentate for 30 min. After filtering the supernatant the mentioned parameters were analysed for each soil sample.

Results and discussion

Vegetation screening. Out of obligate halophytes the following species were identified: *Salicornia europaea* (Sic, Pata, Cojocna, Turda, Tureni, Ocna Mureş), *Triglochin maritimum* (Pata, Turda, Tureni), *Aster tripolium* (Sic, Pata, Cojocna, Turda, Tureni, Someşeni, Ocna Mureş), *Plantago tenuiflora* (Sic, Pata, Someşeni, Ocna Sibiului, Cojocna), *Spergularia salina* (Ocna Mureş, Someşeni) and *Limonium gmellini* (Sic, Cojocna, Turda, Someşeni). Out of facultative halophytes *Artemisia pontica* (Sic, Pata), *Festuca pseudovina* (Sic, Pata, Cojocna) and *Achillea setacea* (Sic, Pata) were identified. Origin of the salt tolerance of halophytes has evolved through the accumulation of adaptive mutations leading to physiological and biochemical modifications required to thrive in high salinity (Bromham, 2014; Himabindu *et al*., 2016).

Physico-chemical parameters. By accumulating highly soluble compounds called osmoprotectants, which maintain osmotic pressure, most of halophytes can tolerate high-salt conditions (Guo *et al*., 2002). Nine salting zones were chosen for taking soil samples. The results of the measuring made on soil samples taken form saltings, far all the parameters, are showed in Table 1.

Table 1.

The values of the parameters analysed at soil samples

Sample No	Sampling spot	Depth [cm]	pH	EC [µS/cm]	Salinity [‰]	ORP [mV]
1	Dej	10-20	6.63	1709	0.8	-7.1
2		0-10	6.26	553	0.2	+22.3
3	Gherla	10-20	6.86	2680	1.4	+14.6
4		0-10	7.24	1303	0.6	-67.7
5	Sic	10-20	8.36	5010	2.7	-21.5
6		0-10	8.72	4700	2.5	-116.0
7	Cojocna	10-20	7.70	3280	1.7	-50.4
8		0-10	6.98	2430	1.2	-22.3
9	Pata	10-20	7.07	3900	2.1	-21.5
10		0-10	6.38	3520	1.8	+14.8
11		10-20	8.06	20800	12.4	-71.4
12	Turda	10-20	7.78	6300	3.4	-60.0
13		0-10	6.89	6880	3.8	-11.8
14	Ocna Mureş	10-20	8.39	1993	1.0	-106.5
15		0-10	8.71	1144	0.5	-22.5
16	Micești	10-20	6.58	1602	0.8	-1.6
17		0-10	6.51	1304	0.6	+13.4
18	Someşeni Băi	10-20	7.27	1674	0.8	-11.9
19		0-10	8.39	1090	0.5	-107.1

pH values influence soil characteristics. The availability (mobility) of nutrients and pollutants is directly dependent of the pH value. Likewise, the soil organisms activity, degrading the organic substances, iron, manganese and aluminium discharge depend on pH. The soils pH falls into different reaction categories (Blaga *et al.,* 2008).

From the analyses done, the pH values indicate slight acid to moderate alkaline reactions, the values falling between 6.26 and 8.71. Out of all samples, 42 % samples are slight acid, 21% slight alkaline, 32% moderate alkaline and a single sample exhibits a neutral pH (Fig. 2).

The highest value, of 8.71, was recorded at Ocna Mureş, in the upper most 10 cm of soil, and than the pH slightly decreases with depth, reaching 8.39 in the layer of 10-20 cm (Fig. 2). Currently, the soils with pH higher than 7.2 are called saltings, because they contain in their structure high amount of salts.

The lowest pH was recorded at Dej, reaching values of 6.26 and 6.63 indicating a slight acid soil, which allow a better development of plants by higher nutrients mobility (Fig. 2). Plants rather support an acid soil, than an alkaline one.

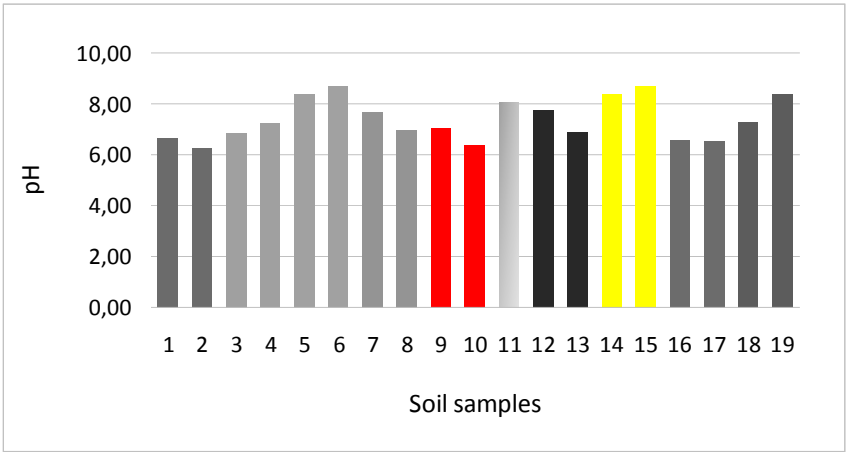

Figure 2. The values of the pH for soil samples

If we analyze the modification of pH value dependent on depth, two situations are differentiating: first, when pH decreasing along with depth, second, when it increases along with depth. As figure 2 shows, representing the samples taken from Dej, Cojocna, Pata, Turda, Tureni-Miceşti, the pH increases with depth, while in the other cases the situation reverses. These small modifications can influence the growth and development of plants in the studied areas. As a first indicator, pH can show us the presence of salts in higher concentrations in a soil, but, for a better highlight of this characteristic, the salinity was assessed.

Salinity (S) represents the accumulation of water soluble salts in soil. These salts include potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), chloride (Cl^-), sulphate (SO_4^{2-}), carbonate (CO_3^{2-}), bicarbonate (HCO_3^-) and sodium (Na^+). Salinization (primary) undertakes the accumulation of salts by natural processes due to a high salts content in the material from the origin soil or the underground waters (Krishnamoorthy *et al*., 2016). If there is a concentration of salts in soil, this will influence the water and nutrients regime, these being less accessible to plants. All the analyzed samples contain high concentrations of solute salts, showing that these soils are salty ones (Fig. 3).

The highest salt concentrations were recorded at Sic (2.7 ‰ and 2.5 ‰), Cojocna (1.7 ‰ and 1.2 ‰), Pata (2.1 ‰, 1.8 ‰ and 12.4 ‰) and Turda (3.4 ‰ and 3.8 ‰), values above 1 ‰. A very high concentration was recorded at Pata, where the value reached 12.4 ‰ (Fig. 3). It is indicated to follow the correlation between the concentration of salts and the development of vegetal associations, as well as the repetition of concentration analyses.

Except the soil samples taken from Turda, for all the other samples, higher salt concentrations were recorded in the 10-20 cm horizon, which can denote that the salts are originating from the geological sublayer, either by pedogenesis processes, either by phreatic layer.

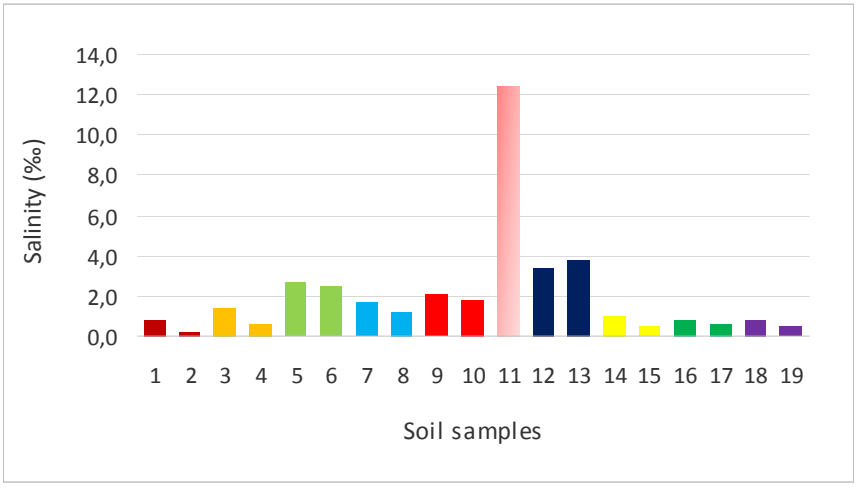

Figure 3. Salinity of soil samples

Conductivity (EC). Electric conductivity indicate the spatial distribution of different soil properties, with applications on the water course in soil, localization of zones with high salt content, determination of soil texture, identification of soil types, but also their quality. The field measurements of EC accumulate the product of static and dynamic factors: soil salinity, clay content and mineral composition of soil, water content (Wang *et al.,* 2012).

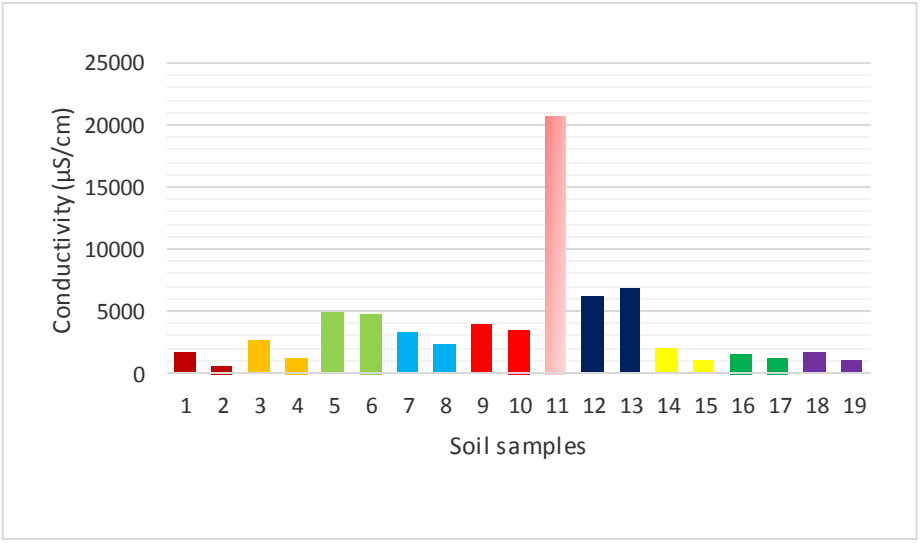

Figure 4. Soil samples conductivity

The clayish soils have the capacity to store water and they reach higher values of electric conductivity, while the sandier soils do not have this capacity and record lower electric conductivity values.

The highest conductivity was recorded at Pata, 20800 µS/cm, value which can be correlated with the high salinity of sample and will be also correlated with the clay content. The high conductivity was also recorded at the samples from Turda and Sic, values above 5000 µS/cm, which indicates the presence of dissolved soils and clay fractions, which has to be determined (Fig. 4).

Oxidoreduction potential (ORP). Oxidation Reduction Potential is a value measured in milivolts, its size indicates if a solution is oxidating or reducing. 79% of water samples show a negative ORP value and the rest of 21% represents positive values, meaning oxidant values. The samples with positive values were recorded from Dej (22.3 mV), Gherla (14.6 mV), Pata (14.8) and Tureni (13.4 mV) (Fig. 5).

A positive ORP value indicates that the solution can have negative effects on health of living organisms, because they do not possess the antioxidant capacity to neutralize the oxygen free radicals causing oxidative damages. A negative ORP value indicates that the environment is a strong antioxidant, absorbs free radicals, contributing to a better oxygenation of live organisms.

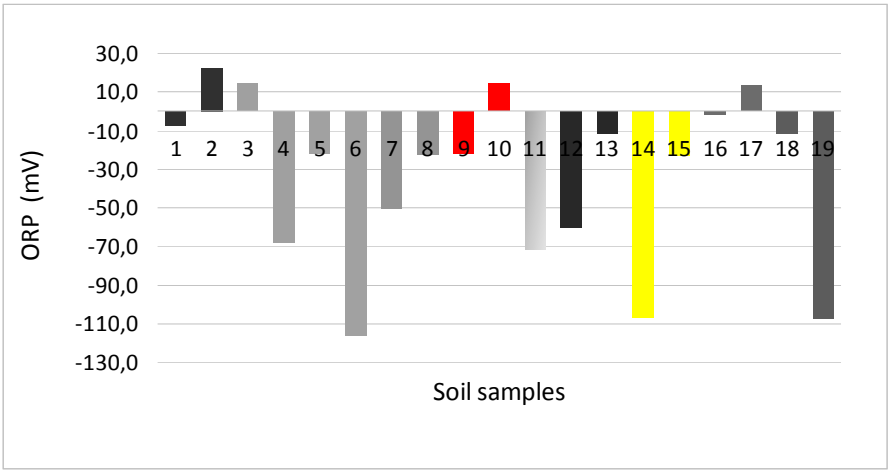

Figure 5. Oxidoreduction potential of soil samples

Conclusions

Salinity is one of the most severe abiotic environmental factors at a global scale, with deleterious effect on plant growth and development. There were identified obligate and facultative halophytes which populates Romanian salty areas. All studied physico-chemical soil parameters (pH, ORP, conductivity, salinity) vary between a broad range, depending on the location of the studied area and depth. These parameters have a decisive influence on the plant populating the upper zone of the soil. All of the measured values proved to be specific for salty areas.

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Arsenite-induced activation of JNK1/2 and p38 MAP kinases in ELM-1 murine erythroleukemia cells

Ferenc Kósa[1,✉]

SUMMARY. Arsenite stimulates different mitogen-activated protein kinase (MAPK) pathways in the murine erythroleukemia cell line ELM-I-1. The activation of JNK1/2 and p38 was determined by Western blotting with antibodies specific for phosphorylated, activated forms of these kinases. Our data indicate that arsenite stimulates rapid phosphorylation of JNK1/2 and p38 MAPKs in ELM-I-1 cells. In the concentration range of 12.5-500 μM arsenite stimulates the stress response kinases JNK1/2 and p38. Maximal JNK1/2 and p38 activation was observed at 50 μM. This concentration produces an 7-40 fold increase in the activity of the JNK1/2, and an 4.5-7.5 fold increase in the activity of the p38 MAPK. The arsenite effects were time dependent: maximal activation of the stress response kinases JNK1/2 and p38 were observed after a 60 min exposure to arsenite.

Keywords: arsenite, erythroleukemia cells, JNK1/2, MAPK, p38

Introduction

Arsenic is an important environmental toxicant (Bernstam and Nriagu, 2000; Ventura-Lima *et al.*, 2011), and is a major health concern for 200 million people worldwide (Ellinsworth, 2015). Human exposure to drinking water contaminated with arsenic is a serious global health concern and predisposes to cardiovascular disease states, such as hypertension, atherosclerosis, and microvascular disease (Ellinsworth, 2015). Epidemiological studies have established a close association between exposure to arsenic and increased incidences of cancer in arseniasis-endemic areas of the world including Taiwan, Mexico, Chile, Argentina, Thailand, India, Canada and the USA (Lau *et al.,* 2004). Arsenic is carcinogenic to humans, and targets in particular the urinary bladder, liver, kidney, skin, lung, prostate and other internal sites (Bode

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and Dong, 2002; Platanias, 2009). The Agency for Toxic Substances and Disease Registry ranks arsenic as number one on their Priority List of Hazardous Substances (http://www.atsdr.cdc.gov/spl/), and it is also classified as a Group I carcinogen by the International Agency for Research on Cancer (Ellinsworth, 2015).

The mechanism by which arsenic mediates carcinogenesis remains a subject of debate, with evidence supporting several plausible etiologies, including disruption of signaling cascades (Ventura-Lima *et al.*, 2011), elevated levels of oxidative stress (Ellinsworth, 2015), chromosomal aberrations and epigenetic changes (Faita *et al.*, 2013; Reichard and Puga, 2010).

Despite the adverse effects of arsenicals, they show great promise in the chemotherapy of certain types of human cancer (Bode and Dong, 2002; Platanias, 2009; Wang, 2001). In this regard, arsenic is an effective treatment for acute promyelocytic leukemias (Platanias, 2009; Wang, 2001). Thus, it appears that arsenic, a known human carcinogen, is also an effective chemotherapeutic (Bode and Dong, 2002; Platanias, 2009). Therefore the study of the mechanism of arsenic on the mitogen activated protein kinases could have important implications in both cancer causation and cancer chemotherapy.

The various effects of arsenite may be mediated through activation of a MAP kinase cascade (Bode and Dong, 2002; Huang *et al.* 1999; Porter *et al.*, 1999; Ventura-Lima *et al.*, 2011). Previous investigations have demonstrated that arsenite activates members of the MAP kinase family, transcription factors such as activator protein-1 (AP-1), and immediate early genes, including c-jun, c-fos and c-myc, which help to regulate the expression of transforming oncoproteins and growth factors (Bode and Dong, 2002; Li *et al.*, 2006; Platanias, 2009). Each of the three major mitogen-activated protein kinase (MAPK) pathways (ERK, JNK, p38) transduce a variety of extracellular signals that lead to diverse cellular responses such as cell growth, differentiation, proliferation, apoptosis and stress responses to environmental stimuli (Kyriakis and Avruch, 2012; Schaeffer and Weber, 1999; Tibbles and Woodgett, 1999).

The purpose of this study is to examine for the first time the MAPK signal transduction pathways induced by arsenic in our established *in vitro* murine erythroleukemia cell line (ELM-I-1).

Materials and methods

Chemicals and reagents

Anti-(p38) rabbit polyclonal IgG, anti-(JNK1/2) goat polyclonal IgG, anti-(phosphorylated-JNK1/2), anti-(phosphorylated-p38) mouse monoclonal IgG, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-(rabbit IgG) and anti-(mouse IgG) peroxidase conjugates, Bradford reagent and protease inhibitors, sodium m-arsenite ($NaAsO_2$) were obtained from Sigma (St. Louis, MO, U.S.A.);

α-minimal essential medium without nucleotides, Dulbecco's modified Eagle medium, horse serum, fetal calf serum were purchased from Life Technologies (Gaithersburg, MD, U.S.A.), analytical grade chemicals from Sigma (St. Louis, MO, U.S.A.), Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany).

Cells and culture conditions

Erythropoietin-responsive murine erythroleukemia cells, line ELM-I-1 (Schaefer *et al.*, 2004) was kindly provided by Prof. W. Ostertag, Heinrich Pette Institute for Experimental Virology and Immunology (Hamburg, Germany). ELM-I-1 cells were grown in α-minimal essential medium without nucleosides, supplemented with 10 % (v/v) horse serum and with 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified air/CO_2 (19:1) atmosphere. In the experiments, exponentially growing cells were plated at (1-2) x 10^5 cells/ml and at (4-8) x 10^4 cells/ml. Approximately 16 h later the cells were treated with arsenite. At the indicated time points, cells were harvested and analysed.

Western blot analysis

Cells were collected, rapidly washed in ice cold phosphate-buffered saline (PBS) and suspended in RIPA-buffer [5 mM Tris-HCl (pH 7.4), 15 mM NaCl, 100 mM EGTA, 1% Nonidet P-40, 10 % deoxycholic acid] containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 1 mM p-nitrophenylphosphate and 10 mM sodium pyrophosphate. The samples were lysed on ice for 30 minutes and insoluble material was removed by centrifugation at 12000 x g at 4 °C for 12 min. Protein content in the supernatants was determined using the Bradford reagent, the samples were then diluted with 3x Laemmli sample buffer [180 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 300 mM dithiothreitol, 45 % (v/v) glycerol, 22.5 mM EDTA, 0.0015% (w/v) bromphenol blue] 3 : 1 and heated to 100 °C for 5 min. 40 to 60 μg of total protein were separated on 10 % (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A) using the Mini-Protean II System of Bio-Rad (Hercules, CA, U.S.A.). The membranes were blocked with 5 % (w/v) dry milk in Tris-buffered saline [TBS: 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl] containing 0.1 % (v/v) Tween-20 and probed with anti-(p38) and anti-(JNK1/2) polyclonal antibodies as well as with anti-(phosphorylated-JNK1/2) and anti-(phosphorylated-p38) monoclonal antibody at room temperature for 1h. After washing in TBS-Tween-20, the membranes were incubated with horseradish peroxidase-conjugated second antibodies, and visualised using the ECL Western blotting system of Amersham (Buckinghamshire, U.K.). When the same membrane was sequentially probed with antibodies of different species origin, the membrane was treated with 0.5 % (v/v) H_2O_2 in TBS at room temperature for 25 min, extensively washed in TBS, then blocked and analysed with the next first and peroxidase-conjugated second antibodies as described above.

Results and discussion

Although inorganic arsenic is clearly carcinogenic in humans (Bode and Dong, 2002; Platanias, 2009), it is unclear if it acts through genotoxic or epigenetic mechanisms. Arsenic is usually assumed to act principally *via* an epigenetic effect (Bode and Dong, 2002; Platanias, 2009). An important epigenetic mechanism could be the inappropriate activation or inactivation of signal transduction pathways leading to the activation of transcription factors, which in turn modulates the gene expression (Bode and Dong, 2002; Porter *et al.*, 1999; Ventura-Lima *et al.*, 2011).

To determine whether arsenite exposure affects the activity of specific factors involved in murine erythroleukemia cell signaling, we used antibodies against phospho-p38 and phospho-JNK1/2 to examine the effect of arsenite on MAPK pathways by Western blot.

Concentration and time-dependent effects of arsenic treatment on JNK1/2 kinase activity

The JNK pathway has been implicated in the regulation of apoptosis induced by various stimuli (Kyriakis and Avruch, 2012; Muscarella and Bloom, 2002; Platanias, 2009; Qu *et al.*, 2002; Tibbles and Woodgett, 1999). To determine if JNK signal pathway may be involved in arsenic-induced effects, ELM-I-1 cells were subjected to different concentrations (1-500 μM) of arsenite for 60 min.

Dual phosphorylation of JNKs at Thr183/Tyr185 is essential for kinase activity and phosphorylation at this site is an excellent marker of JNK activity (Kyriakis and Avruch, 2012; Tibbles and Woodgett, 1999). Thus, to confirm JNK activation, the levels of phosphorylated JNK/1/2 were determined by Western blot analysis (Fig. 1A) and analyzed by scanning densitometry (Fig. 1B).

The immunoblot data revealed that sodium arsenite enhanced the JNK1/2 signal transduction pathway, as indicated by the increase in activated JNK1/2 (Fig. 1). Although at 1 μM concentration arsenite has no detectable effect, our data show that at low concentration of 12.5 μM it can produce an ~3.5-4 fold increase in the phosphorylated form of the JNK1/2. Maximal activation (7-40 fold increase over basal level) were obtained with 50 μM arsenite.

In our experiments the membranes used to define phosphorylated forms of MAPKs were stripped and then reprobed with an antibody that detects each kinase regardless of its phosphorylation state, to ensure equal loading of each lane and to serve as an internal control for subsequent quantitation. As shown in Figure 1A (bottom panel), no major differences were observed between the treated and control cells in the levels of total JNK1/2, so the increase of the phosphorylated form (Fig. 1 A, upper panel) is not the result of an arsenite-induced JNK1/2 protein overexpression.

In the following time-dependent experiments we used 50 μM sodium arsenite (Fig. 2). Phospho-JNK1 and 2 were detected 2 min after arsenite addition and maintained for at least 4 hours following exposure. Maximal JNK1/2 activation was observed after 60 min of incubation with arsenite.

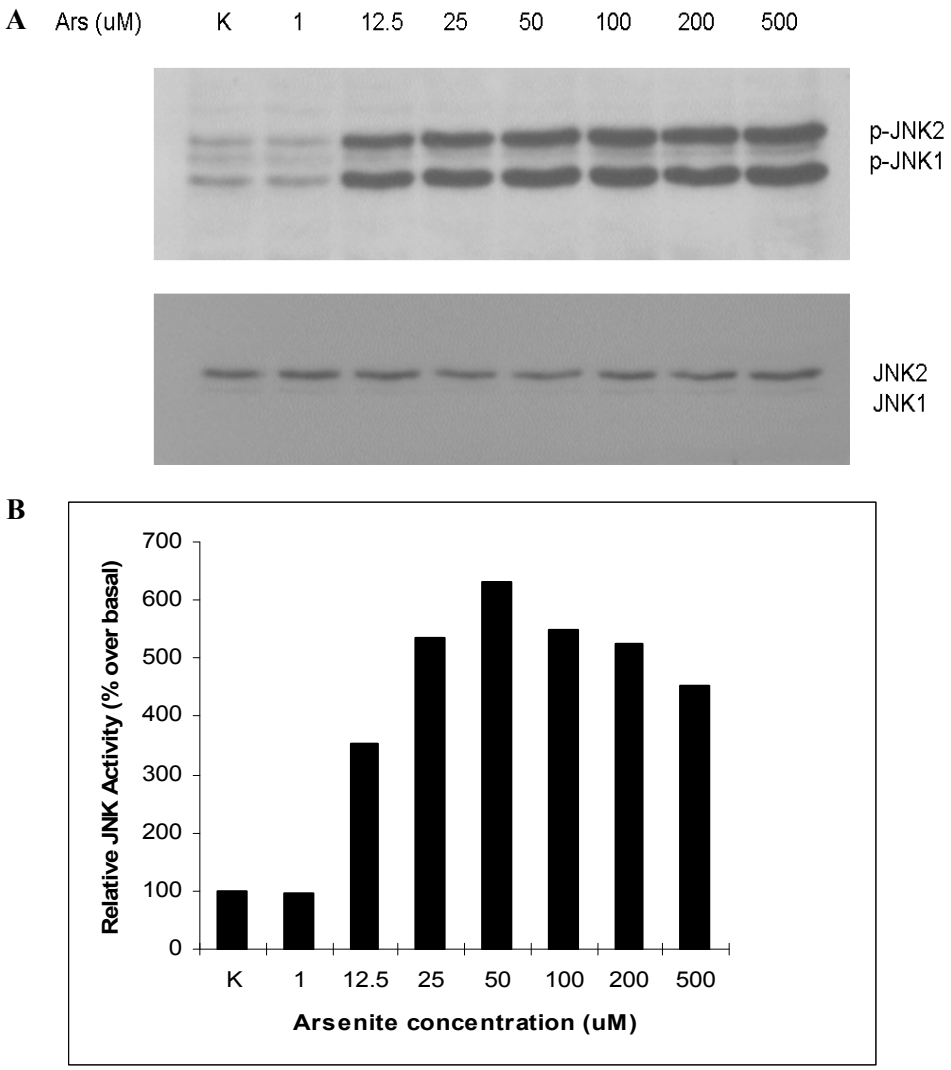

Figure 1. Dose response curve of JNK1/2 activation by arsenite (cells were treated with different concentrations of arsenite for 1 h; K - control). JNK1/2 MAP-kinase phosphorylation was determined by Western blotting (A, upper lane) and the activity is expressed graphically as fold stimulation over basal, quantified using a scanning densitometry system (B). The results shown in A are representative of three independent experiments. In the bottom lane (A) Western blots were reprobed with antibodies against JNK1/2 that recognize the non-phosphorylated JNK1/2 forms to assess the total amount of JNK1/2

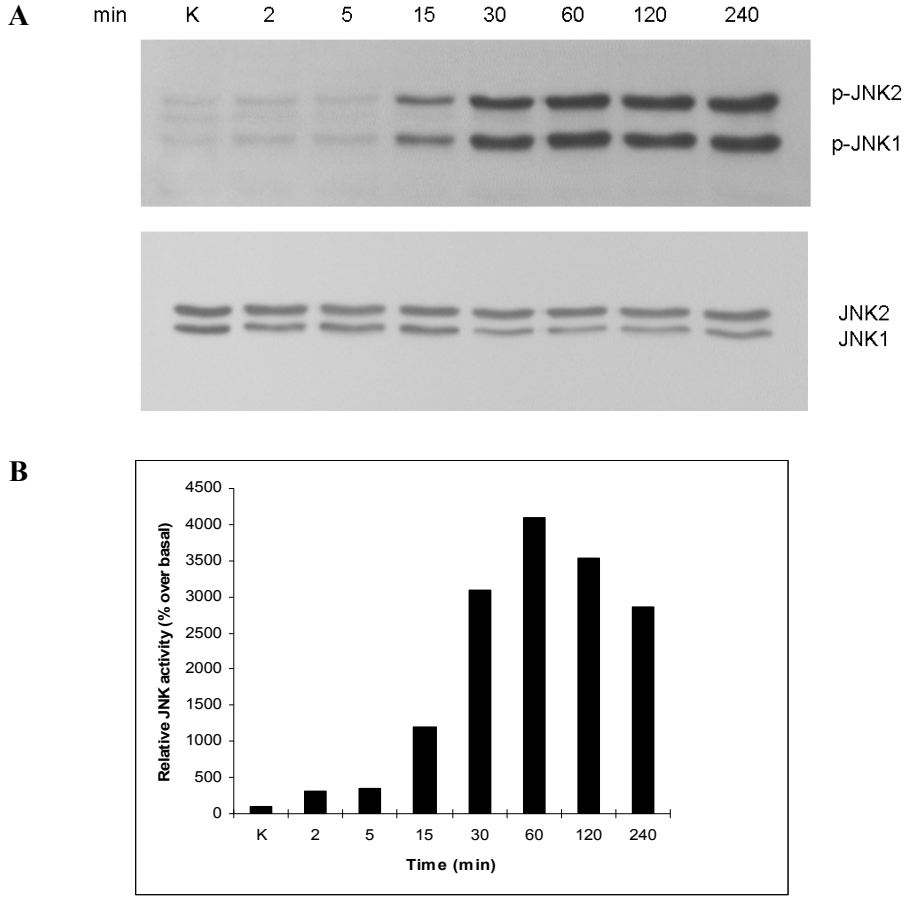

Figure 2. Time course of JNK1/2 activation by arsenite in ELM-I-1 cells (cells were treated with 50 μM arsenite for the times indicated; K - control). JNK1/2 MAP-kinase phosphorylation was determined by Western blotting (A, upper lane) and the activity is expressed graphically as fold stimulation over basal, quantified using a scanning densitometry system (B). The results shown in A are representative of three independent experiments. In the bottom lane (A) Western blots were reprobed with antibodies against JNK1/2 that recognize the non-phosphorylated JNK1/2 forms to assess the total amount of JNK1/2.

Induction of phospho-JNK1/2 in cells may be achieved in two ways: by activation of upstream kinases or by inhibition of JNK1/2 phosphatases. Arsenite has been reported to act in both ways, with several upstream kinases serving as potential targets (Meriin *et al.*, 1999; Theodosiou and Ashworth, 2002), and with a sulfhydryl-containing JNK phosphatase being especially sensitive to inhibition by this toxicant. Such phosphatase inhibition results in a reduction in the rate of JNK dephosphorylation and subsequent accumulation in the levels of phospho-JNK (Theodosiou and Ashworth, 2002).

At present, we do not know which of these potentially mechanism is primarily responsible for activation of JNK1/2 in the ELM-I-1 cell line.

Effects of arsenic treatment on p38 kinase activity

We next explored whether arsenite influences p38 MAPK activation in ELM-I-1 cells. To determine if p38 signaling pathway may be involved in arsenic-induced effects, ELM-I-1 cells were subjected to different concentrations (1-500 μM) of arsenite for 60 min. Maximal activation were obtained with 50 μM arsenite (data not shown).

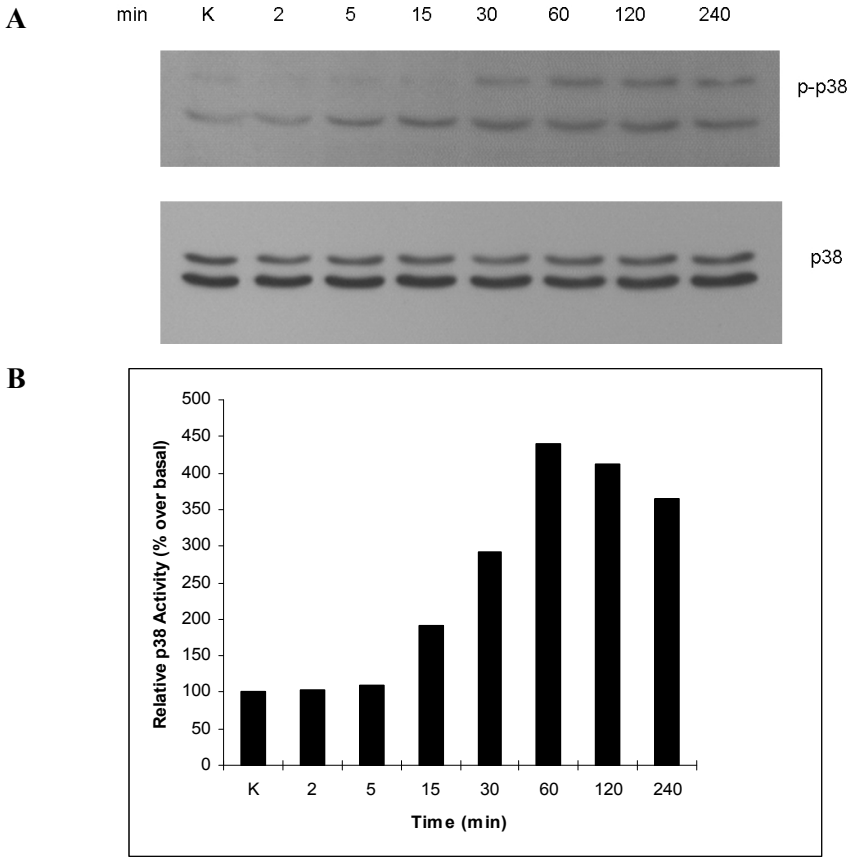

Figure 3. Time course of p38 activation by arsenite in ELM-I-1 cells (cells were treated with 50 μM arsenite for the times indicated; K - control). p38 MAP-kinase phosphorylation was determined by Western blotting (A, upper lane) and the activity is expressed graphically as fold stimulation over basal, quantified using a scanning densitometry system (B). The results shown in A are representative of three independent experiments. In the bottom lane (A) Western blots were reprobed with antibodies against p38 that recognize the non-phosphorylated p38 forms to assess the total amount of p38

For the time-dependent experiments we used 50 μM sodium arsenite. Figure 3A,B shows that arsenite (50 μM) increased ~ 2 fold the level of phospho-p38 after a 15 min arsenite treatment. Maximal stimulation (~4.5-7.5 fold over the basal level) was measured after 60 min of arsenite incubation. The phosphorylated form of the p38 was observed for at least 4 h after arsenite exposure.

Figures 3A, bottom panel, show that the level of non-phosphorylated p38 following arsenite remained the same, indicating that changes in p38 phosphorylation were not due to alterations in steady-state protein level.

Conclusions

Our data indicate that arsenite rapidly stimulates the phosphorylation of JNK1/2 and p38 MAPKs in ELM-I-1 cells. In the concentration range of 12.5-500 μM arsenite stimulates the stress responses kinases JNK1/2 and p38. Maximal JNK1/2 and p38 activation was observed at 50 μM. This concentration produces an 7-40 fold increase in the activity of the JNK1/2, and an 4.5-7.5 fold increase in the activity of the p38 MAPK. The arsenite effects were time-dependent: maximal activation of the stress response kinases JNK1/2 and p38 was observed after 60 min exposure to arsenite.

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Reactive oxygen species and anthocyanin are involved in plant response to wounding as part of insect feeding – the case of the somatic hybrids *Solanum tuberosum* + *Solanum chacoense*

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SUMMARY. The cultivated potato ranks third in agricultural crop production on global scale. Being the target of attacks by various insects it requires increased attention and protection as well as breeding for resistance. Meanwhile the Colorado potato beetle (*Leptinotarsa decemlineata*, CPB) has become the biggest enemy of the cultivated potato worldwide. An alternative method of controlling the growth of CPB population would be the use of resistant potato varieties. One of the most effective sources of host-resistance mechanisms is the wild *Solanum chacoense*. Somatic hybridization *via* protoplast fusion made it possible the introgression of valuable traits from *S. chacoense* into cultivated potato.

Adequate responses to biotic stresses are crucial for plant survival. The formation of reactive oxygen species during microbial infection is a well-known process but their role against herbivore attacks is still not outlined. The reactive oxygen species (ROS) concentration in plants is controlled by different antioxidative mechanisms in order to maintain the normal function of cells.

The aim of our study was to determine the role of ROS signaling as defense response induced by mechanical wounding in potato somatic hybrids. The correlation between CPB resistance and H_2O_2 accumulation rate were determined, as well. Also the role of anthocyanin as a ROS scavenger was established.

Based on our results we conclude that H_2O_2 accumulation highly influences somatic hybrid response to insect herbivore attacks therefore this type of ROS plays an important role in plant defense mechanism against CPB.

Hydrogen peroxide accumulation led to anthocyanin generation therefore anthocyanin plays a role as radical scavenger in potato somatic hybrids. This study is the first to point out the role of reactive oxygen species and anthocyanin biosynthesis in response to mechanical wounding in the somatic hybrids resistant or sensitive to Colorado potato beetle.

Keywords: anthocyanin, hydrogen peroxide, somatic hybrids

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Introduction

Potato (*Solanum tuberosum* L.) is considered as an extremely valuable crop especially now, when Earth's nutritional problems are increasing. Potatoes are grown in 160 countries and more than 4000 cultivars are known (Camire *et al.*, 2009). Romania is ranked on the sixth place as largest harvested area potato grower in Europe: in 2014 more than 3.5 million tons of potato were produced (FAOSTAT, 2014).

Most of the wild relatives of cultivated potato provide host-plant resistance to different pests and diseases. These wild species represent a rich and diverse source of resistant genes (Hawkes, 1990) which could be useful for potato improvement. *Solanum chacoense* is a diploid (2n=2x=24), tuber-bearing *Solanum* species. This wild *Solanum* species attracted the attention of potato breeders because possesses broad resistance against different pathogens (Sato *et al.*, 2006; Simko *et al.*, 2007). Some accessions of *S. chacoense* are highly resistant to Colorado potato beetle (CPB) (Sinden *et al.*, 1986). In order to introgress insect resistance from *S. chacoense* Thieme *et al.* (unpublished data) and Rakosy-Tican *et al.* (2004) produced somatic hybrids and backcross progenies between cultivated potato cv Delikat and Désirée and *S. chacoense* with/without DNA mismatch repair system (MMR) deficiency. The MMR system is involved in maintaining genome stability. Harfe and Jinks-Robertson (2000) observed that *MSH2* mutations, a key gene of MMR, significantly increases the rate of homeologous recombination. The high degree of MMR system effectiveness makes difficult the incorporation of useful genes into agronomic important crops. Therefore Rakosy-Tican *et al.* (2004, 2015) used MMR deficient *S. chacoense* to increase the chance of introgression of wild species genetic material into cultivated potato.

In plants the herbivore beetle attack is generally associated with wounding. Both herbivore attack and mechanical injury induce modifications of plant's wound response (Kessler and Baldwin, 2002). Immediately after wounding, plants accumulate reactive oxygen species, like superoxide anion, hydrogen peroxide and hydroxyl radical.

The role of reactive oxygen species in plant defense against herbivores is not clear, but the importance of ROS signaling in the generation of plant defense responses is supported with numerous experiments. Van Breusegem *et al.* (2001) observed that plants after wounding begin to produce superoxide anion in damaged tissue and H_2O_2 throughout the plant. The quantity of ROS accumulation has a positive correlation with plants's resistance against attacker (Moloi and van der Westhuisen, 2006). Hypersensitive response (HR) like symptoms were observed in the case of resistant willow to the *Dasineura marginemtorquens* (Hoglund *et al.*, 2005), resistant rice to the *Orseolia oryzae* (Bentur and Kalode, 1996) and bean resistant to *Apion godmani* (Garza *et al.*, 2001).

In plants ROS represent a general physiological response. Under stress condition large amounts of ROS are generated, which has an important role in plant defense response but also could affect the health of plants. Therefore a system which stabilizes the concentration of ROS is essential. Stabilization of ROS levels after pathogen attack in *Arabidopsis thaliana* is controlled by ascorbic acid and also by anthocyanin generation (Nagata *et al.*, 2003), which has ROS scavenging effects (Sanz *et al.*, 1994).

The aim of our study was to evaluate the importance of ROS signaling as defense response induced by wounding potato somatic hybrids with/without MMR deficiency and BC clones. The correlation between CPB resistance and H_2O_2 accumulation rate as a response to mechanical injury of SHs and BC clones was investigated, along with the ROS scavenging role of anthocyanin in potato somatic hybrids.

Materials and methods

Plant Material

Somatic hybrids were produced by using protoplast electrofusion technique. Thieme *et al.* (unpublished) used mesophyll protoplasts of *S. tuberosum* cv. Delikat and *S. chacoense* GLKS 30138 (*S. chc* 138) from Gross Lüsewitz Potato Collections, IPK Satellite Collections North Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Genebank, (Germany) to produce somatic hybrid plants (ex. SH 1552/1). BC1 plants were obtained after sexual backcrossing of somatic hybrids with S. *tuberosum* cv. Sonate (ex. 1552/1/2). Rakosy-Tican *et al.* (2004) used potato Delikat and Désirée cultivars in somatic hybridization as tetraploid parents and the highest leptine producer diploid *S. chacoense* accession PI 458310 (*S. chc* HL) from NPGS Sturgeon Bay, USA. MMR deficient *S. chacoense* was obtained by using *Agrobacterium*-mediated transformation. The AS construct contained the 1 kb fragment of the *AtMSH2* cDNA in antisense orientation. The DN construct contained the *AtMSH2* coding sequence with a mutation converting a strongly conserved Gly codon at position 697 to an Asp codon (Ispas, 2004). One transgenic line for AS and two for DN (DN5 and DN11) and *S. tuberosum* cv. Delikat and Désirée were used in protoplast electrofusion to produce MMR deficient somatic hybrids. The hybridity nature of regenerated plants with/without MMR deficiency was validated using SSR (Besenyei *et al.*, personal communication) and RAPD molecular markers.

The obtained SHs and BC clones resistant ability against Colorado potato beetle were evaluated using laboratory bioassay and food preference test, also known as adult choice test (Table 1) (Molnar *et al*., 2016). Several genotypes possessed antibiosis and antixenosis ability to CPB. Somatic hybrids marked with red in Table 1 proved to be as effective as *S. chacoense* in CPB resistance analysis.

Four weeks old *ex vitro* cultured *Solanum* genotypes were used in evaluating plant response to mechanical wounding (Table 1). Injury-free plants were selected and were divided into two categories: stressed and control groups. The third and fourth mature leaves of different genotypes belonging to stressed group were wounded with a sterile perforator (hole diameter = 0.55 cm), which has to simulate the physical injury during herbivore attacks. The experiments were done in triplicate.

Table 1.

Resistance of somatic hybrids between cultivated potato and *S. chacoense* with/without MMR deficiency and BC progenies to Colorado potato beetle.

	Resistant	Susceptible
Wild type SHs + BC_1	**1552/1**	1552/1/7
	1552/1/2	1552/1/11
	1552/1/4	1552/1/12
		1552/1/16
MMR deficient SHs	**De.DN5.5**	Dk.DN5.3
	De.DN11.29	Dk.DN5.6
	Dk.DN5.4	Dk.DN5.17
	Dk.DN5.7	Dk.DN11.24
	Dk.DN5.11	Dk.DN11.34
	Dk.DN11.10	Dk.AS10.5
	Dk.AS10.13	Dk.AS10.11
	Dk.AS10.35	Dk.AS10.51
	Dk.AS10.40	
	Dk.AS10.43	
	Dk.AS10.47	
	Dk.AS10.61	

Note: Red marked SHs proven to be the most toxic to CPB larvae and also had strong repellent effect against adult CPB, being as resistant as the wild species *S. chacoense*.

ROS determination

Six hours after wounding, the damaged third leaves and the same third undamaged leaves from control plants were collected and weighted for hydrogen peroxide determination. These detached leaves were placed into a 3,3 -diaminobenzidine solution (DAB 1 mg/ml, pH 7.5). The samples were incubated for 3 hours in light, then the leaves chlorophyll content was removed by heating them at 80°C in 80% ethanol. The quantity of hydrogen peroxide was determined using the protocol described by Kotchoni *et al.* (2006). After the ethanol treatment the leaves were almost transparent, only the produced polymerization products were brown. The leaves were homogenized in 5x volume compared to leaf weight of 0.2 M perchloric acid and the mixture was incubated for 5 minutes on ice and then was centrifuged (10000 RPM) for 10 minutes at 4°C. The supernatant absorbance of samples was measured at 450 nm using UV/VIS JASCO V-530 spectrophotometer. The concentration of hydrogen peroxide was determined for 1 g of plant tissue using standard calibration with 0.2 M $HClO_4$ containing different concentration of H_2O_2 (100 µM, 1 mM, 5 mM, 10 mM, 25 mM, 50 mM).

Anthocyanin content determination

Changes in anthocyanin production during wounding was determined using wounded fourth leaves from stressed group and untouched fourth leaves of control plants. Total anthocyanin content of leaves was measured using pH differential method described by Lapornik *et al*. (2005). 72 hours after wounding the leaves were collected and weighted. Detached leaves were homogenized in 5x volume compared to leaf weight of 70% methanol and were incubated for 48 hours in dark at room temperature. After two days 1 ml of the filtered extract was transferred in two sterile Falcon tubes (noted as A and B). 1 ml of 0.01% HCl in 95% ethanol was added into each tube. In A tubes 10 ml of 2% HCl (pH 0.8), in B tubes 10 ml of citrate buffer (pH 3.5) were then added. The samples were mixed and the absorbance of both types of probes (A and B) was measured at 520 nm against 70% methanol as blank. The total anthocyanin content was calculated using the formula: TAC = (A-B) x f, TAC = total anthocyanin content expressed as µg/g cyanidine, A = sample absorbance in 2% HCl (pH 0.8), B = sample absorbance in citrate buffer (pH 3.5), f =(MW x DF x CF1 x CF2)/(ε x l), MW = molecular weight of cyanidine-3-glucoside (449 g/mol), DF = dilution factor (5): 1:5 volume leaf weight: extraction solution (70% methanol), CF1 = conversion factor 1 (106 µg/g), CF2 = conversion factor 2 (1 l/ 1000 ml), ε = molar extinction coefficient of cyanidine-3-glucoside (26900 l/mol*cm), l = path length (1 cm).

Statistical analysis

Statistical analysis was performed using R statistical software. Pairwise comparison of ROS accumulation and anthocyanin content of control and wounded plants was compared using Student t-test. During this study if the P value fell below 0.05 it was interpreted as significant difference between the compared data.

Results and discussion

Because plants are sessile organisms and are exposed to different pathogen or pest attacks, they have developed various defense strategies. Inducible defenses of plants have three major levels: surveillance, signal transduction and defensive chemicals production (Dangl and McDowell, 2006).

When insect herbivores attack a plant, they first produce wounds on the plant's tissue, then inject elicitors through the generated hole. Wound- and herbivore specific elicitors activate different signaling pathways in plants (Kessler, 2002). The earliest signaling responses include ion fluxes through the plasma membrane, calcium concentration changes in cytoplasm, generation of active oxygen species (ROS) and changes in protein phosphorylation and immobilization (de Bruxelles and Roberts, 2001).

The accumulation of ROS during defense is biphasic. In the first minutes after wounding a rapid but weak transient burst of $^1O_2$ and H_2O_2 production occurs and after a few hours a second, more sustained, massive accumulation of ROS can be

observed (Liu *et al.*, 2010). The highest concentration of ROS accumulation can be observed after four-six hours from wounding (Orozco-Cardenas and Ryan, 1999). After wounding plants produce ROS locally in damaged tissue and systemically (mostly H_2O_2) throughout the plant (Kessler and Baldwin, 2002). H_2O_2 level in plants are maintained at high concentration as long as the herbivore attack persist (Orozco-Cardenas and Ryan, 1999).

The role of ROS in plant defense against herbivores is still unclear, but the quantity of ROS accumulation positively correlates with the plant's resistance to herbivore attacks. HR-like symptoms, which were induced by ROS signaling, have been observed in different resistant plants attacked by insects (Chen, 2008; Liu, 2010). Hypersensitive response to Colorado potato beetle egg masses were observed in resistant somatic hybrids between *S. chacoense*, *S. berthaultii* and *S. tuberosum* (Balbyshev and Lorenzen, 1997). In the places were CPB eggs where attached onto leaves, plants generated necrotic zones.

Bi and Felton (1995) proposed that ROS accumulation affects plant-herbivore interaction. ROS accumulation in plants during herbivore attacks also have direct effects on the insects's health. ROS is responsible of direct oxidative damages to the insect's midguts. Besides ROS damages the nutritive components of the plants, therefore the nutritive value as food decreases during herbivore attacks (Orozco-Cardenas and Ryan, 1999).

ROS level changes after wounding

ROS accumulation of wounded somatic hybrids with or without MMR deficiency and backcross progenies were evaluated during our experiments. Wounding as an experimental procedure is often used to investigate plant defense responses against herbivore attacks (de Bruxelles and Roberts, 2001). Quantitative analysis of H_2O_2 accumulation in wounded plants was performed using DAB staining, which produces brown coloration at accumulation zones of H_2O_2 in plant tissue.

During optimal conditions, plant cells produce various ROS, which play important role in normal metabolic processes. In case of non-stressed plants (control group) the average concentration of H_2O_2 ranged between 35 and 37.5 μM/g FW in our investigation.

After DAB treatment, brown coloration appeared on the wounded leaves around the induced wound (hole) and across major (and rarely on minor) veins of the leaves. Orozco-Cardenas and Ryan (1999) observed similar distribution of H_2O_2 accumulation after wounding in tomato leaves. In our experiments the concentration of H_2O_2 varied between 34.5 and 45.5 μM/g FW in wounded leaves. Intense accumulation of H_2O_2 was observed after wounding in the case of *S. chacoense*, 11 SHs and 2 BC_1 clones. (Fig. 1). Both type of MMR deficient somatic hybrids (DN and AS) were represented in this group.

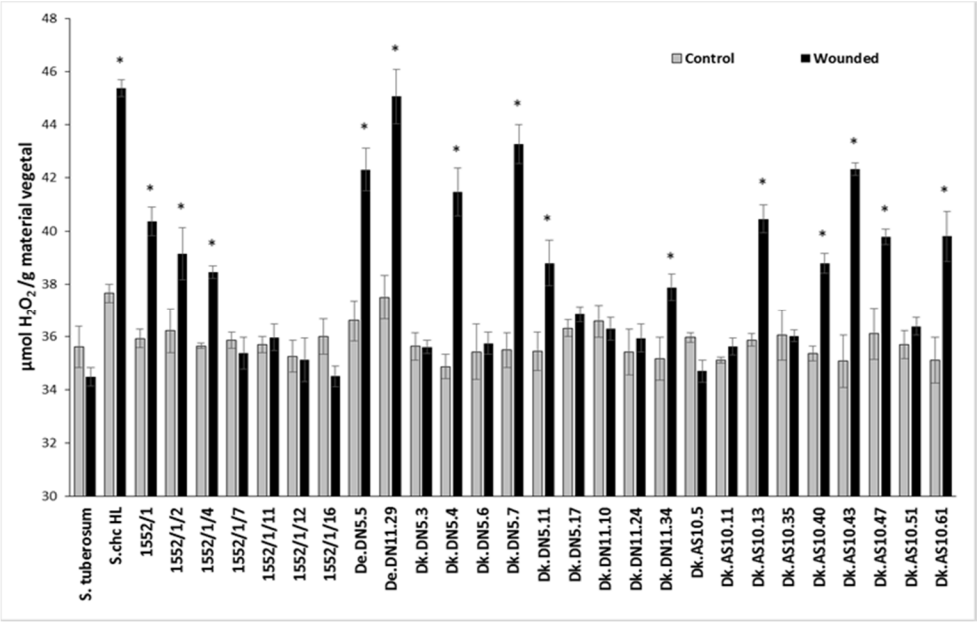

Figure 1. Quantitative evaluation of H_2O_2 in control and wounded somatic hybrids with and without MMR deficiency, their progenies and parental lines (*S. tuberosum* and *S. chacoense*); Note: * - somatic hybrids and BC clones with significant differences (n=5, t-test, $p<0.05$) between wounded and control plants

In case of marked genotypes (*) in Fig. 1, the produced H_2O_2 concentration was significantly higher than in control leaves. In case of *S. tuberosum* wounded leaves produced significantly less (t. test, $p<0.05$) amount of H_2O_2 than the wounded leaves of the marked genotypes.

The intense accumulation of H_2O_2 as a result of wounding showed high correlation with plant resistance against CPB. Most of the somatic hybrids which proved to be resistant to CPB attacks produced high concentration of H_2O_2 during mechanical stress. Only CPB resistant Dk.DN11.10 and Dk.AS10.35 hybrids have similar H_2O_2 pattern like susceptible genotypes, but these hybrids also show contradictory results after two types of CPB resistance analyses. Both SHs had antibiosis properties being toxic to CPB larvae but did not have repellent effect against adult CPB.

Resistant MMR deficient SHs produced larger amounts of H_2O_2 than resistant wild-type SHs but resistant SHs without MMR deficiency accumulated a significantly higher amounts of H_2O_2 than susceptible plants. *S. chacoense* and MMR deficient SHs with the strongest resistance against CPB like De.DN5.5, De.DN11.29, Dk.DN5.4 and Dk.DN5.7 produced the highest concentrations of H_2O_2 after wounding. Somatic hybrids which contained dominant negative sequence of *MSH2* gene accumulated higher amounts of H_2O_2 compared to SHs with antisense orientation of *MSH2* gene but these differences between the two types of SHs were not significant.

Anthocyanin accumulation

The generated ROS during different biotic and abiotic stresses need to be stabilized in order to avoid oxidative damage of the cells, which may also affect the survival of plants. Plants can protect their cells by scavenging ROS through the activation of antioxidative systems like: superoxide dismutases, glutathione peroxidases, catalases or by production of different antioxidants like ascorbate, flavonoids, anthocyanin, etc. (Nagata *et al.*, 2003). These enzymes and antioxidants optimize cellular redox state, which are essential in maintaining the homeostasis of plants (Shao *et al.*, 2008). Because the accumulation of anthocyanin takes 1-2 days after detection of stress, this antioxidant is effective against long-lived radicals like H_2O_2 (Nagata *et al.*, 2003).

In order to evaluate the antioxidative effect of anthocyanin against the accumulated active oxygen species the concentration of anthocyanin was determined and compared between wounded and control plant leaves. The basic levels of anthocyanin varied between 4.8 and 10 µg/mg FW. In all cases of tested *Solanum* genotypes, mechanical injury of leaves induced accumulation of anthocyanin in different concentration levels (Fig. 2).

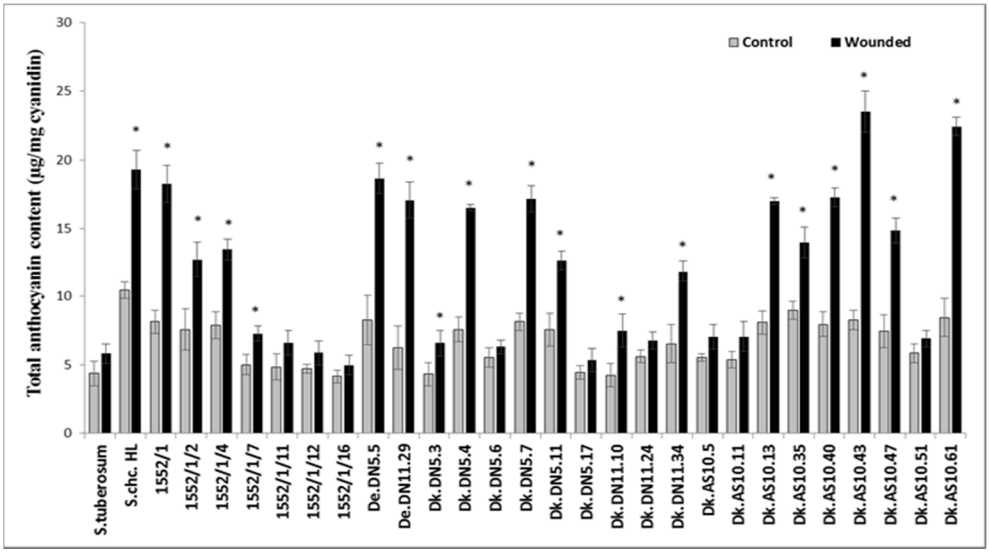

Figure 2. Anthocyanin accumulation level in control and wounded wild-type and MMR deficient somatic hybrids, backcrosses and parental lines (*S. tuberosum*, *S. chacoense*); Note: *-somatic hybrids and BC clones with significant differences (n=5, t-test, $p<0.05$) between wounded and control plants

The radical scavenging activity in wounded plants increases the anthocyanin level by a minimum of 20%, but even by 185% in affected leaves. Significant differences between stressed and control plant anthocyanin content was observed in case of genotypes

marked with * in Fig. 2. Nagata *et al.* (2003) obtained similar result: they observed a 10 fold increase of anthocyanin content, which had intensified radical scavenging activity in *A. thaliana* plants irradiated with γ-rays.

In addition, a positive correlation between the generated anthocyanin content and the accumulated ROS amount was observed (Pearson correlation coefficient: 0.731). In those cases when highest amount of ROS was synthesized after mechanical injury of leaves, a high quantity of anthocyanin was also produced. Therefore, in the case of somatic hybrids the accumulation of ROS was followed by anthocyanin synthesis in order to stabilize ROS concentration in plants.

Conclusion

Based on the results presented above, one can conclude that wound-induced hydrogen peroxide accumulation plays an indispensable role in plant response to mechanical injury and can be associated with plants defense against herbivore attacks.

The H_2O_2 accumulation ability of plants highly influences their response to insect herbivore attacks. In those cases when plants responded to mechanical injury with intense H_2O_2 accumulation they also possessed both antibiosis and antixenosis properties against CPB.

In case of potato somatic hybrids anthocyanin has an important role as radical scavenger, which provides protection against oxidative stress generated after mechanical injury of leaves.

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Contributions to the knowledge on the biocoenotic characteristics of temporary water-bodies from Northern Dobrogea (Romania)

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SUMMARY. In the present study, we analyzed the algae assemblages and environmental condition in four temporary ponds located in Dobrogea mainland (Babadag Plateau) aiming to reveal the role of those habitats as a support to the breeding period of the amphibians. The outcomes of this study were that the algae community structure and its diversity were high given the changing conditions, with a total number of 75 taxa. The highest number of taxa belonged to the phylum Bacillariophyta and Chlorophyta, driven by: temperature and local hydrology conditions. Some of the native amphibians also use this type of habitats during the breeding period, the local climatic conditions having as effect longer juvenile stages in case of each species (in comparison with the ones of populations, of the same species, present in permanent water bodies from the studied region). The novelty of our study resides in the fact that we obtained data on algae assemblage distributed in temporary ponds, information regarding the type of diversity and a better understanding of ecological value of these habitats.

Keywords: algae, amphibians, ponds, Romania

Introduction

Ponds are important habitats within many landscapes, because of the diversity of wildlife they support and their value in terms of biodiversity and socio-economic benefits (Boix *et al*., 2012). These habitats are harboring singular flora and fauna that are often exclusively or infrequently found in permanent ponds (Jeffries, 2008; Florencio *et al*., 2014). On a global scale, temporary ponds (TPs) are often neglected due to their ephemeral character, even if on a global scale, they cover a greater total area than lakes (Boix *et al.*, 2012). These ecosystems have been classified as endangered all over

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the world and many of them are at risk, mainly due to human activities, such as agricultural pollution, expansion of cropland and water resources over-exploitation (Dimitriou *et al.,* 2006). No information was recorded on the historic evolution of the TPs of Dobrogea region. Most of those habitats seemed to be not a priority in Romanian nature-conservation policy and thus there are very few information regarding their status. Nevertheless, although Romania adopted in its environmental legislation (O.U.G., 2007) the European directives that are dealing with environmental protection (Council Directive, 1992; Council Directive, 2006), there is not a clear strategy for management and conservation efforts that should be applied for protecting these habitats and avoiding their disappearances.

The temporary ponds of Dobrogea Plateau are important habitats that could be characterized by their variability in size, hydrological functioning, having a total dependence on local hydrology and in most of the cases being linked with permanent aquatic habitats in the spring time. Those ponds that are not linked with permanent water bodies usually occupy small depressions which are an important environment for amphibians and birds (Török *et al*., 2015).

Material and methods

Field surveys, in situ measurements of water temperature, dissolved oxygen concentration (DO), electrical conductivity (EC) have been performed using HANNA instruments (portable HI 98290 Multiparameter with GPS). Algal biomass using bbe- Moldaenke environmental techniques and evaluation of amphibian populations have been made from July to October 2014.

The biomass of every algae group is given by the concentration of chlorophyll ***a***. The bbe- Moldaenke environmental techniques uses the fluorescence extinction spectrum performed at 470 nm LED for green algae, 610 nm LED for blue-green algae (cyanobacteria), 525 nm LED for diatoms and 570 nm LED for cryptophyceae.

Water samples (50 to 250 ml) preserved with Lugol solution, were taken for subsequent analysis of algae taxa.

Quantitative studies have been carried out using a light microscope at low and high magnification. Filaments, colonies and coenobies were counted individually. At least 200 cells were enumerated per each sample.

To assess the trophic state of the temporary ponds, the following indices were used in the present study: delta-eutrophicity index, epsilon-eutrophicity index (Oltean, 1977); Nygaard compound index (1949), Thunmark's chlorophycean index. To determine the level of pollution of the water, the organic pollution index (Palmer, 1969) was used. The saprobic indicator values of certain algal species were considered, following Rott (1997), Hindak (1978), Sládeček (1973). Identifications were made to the species level using more identification keys (Krammer and Lange-Bertalot, 1986, 1988, 1991; Ettl, 1983).

Descriptive statistics using PRIMER 6 statistical package was applied to derive information about common characteristics of the Cerbu TPs. The spatial pattern of algal population was analyzed using Bray Curtis index of similarity.

Amphibians were assessed using the methodology developed for nation-wide monitoring of amphibians and reptiles of Community Interest (Török *et al.* 2013).

Description of the Study Area

The research-site within which ponds were naturaly dug is located in south-eastern Romania, respectively, in the central part of northern Dobrogea (a historical region delimited by the Danube River and the Black Sea), at the western limit of the forested area of the Babadag Plateau.

There are four small ponds in the landscape of the area, two of them having a significant spatial and temporal variation which mirrored the changes in water level during the summer - autumn period and the pond life during the year (Fig.1).

The taxonomy and natural history of these ponds are unknown. The ponds have no aquatic plant communities; there are fish-free habitats which supported at least four species of amphibians - during the investigations being recorded *Pelophylax ridibundus, Rana dalmatina, Bufo viridis* and *Pelobates* sp. (the tadpoles of the later taxa were assumed to belong to *Pelobates syriacus*).

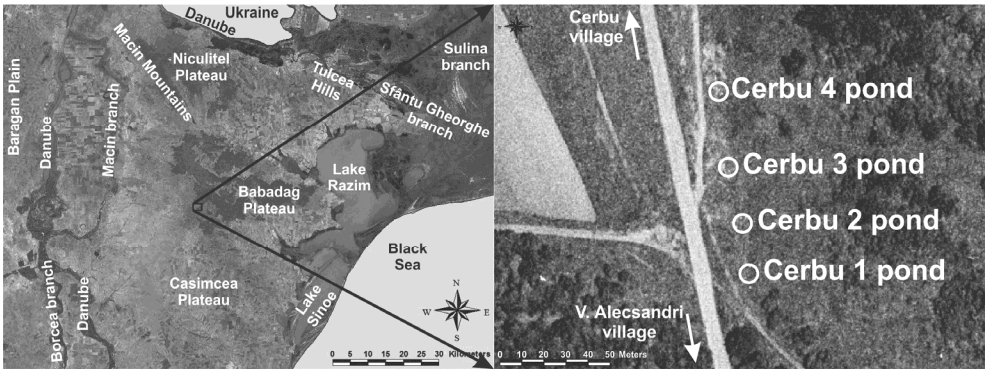

Figure 1. Location of the studied temporary ponds.

Results and discussions

Environmental characteristics of the TPs

An extensive survey of ponds during summer times has identified the ranges of temperature, DO and EC, which create the distinctive patterns of the local variation of those environmental variables that could influence the colonization rate, species richness

and composition/ structure of the algal communities. In this respect, the temperature of water ranged between 25.4°C to 31°C; DO between 4.4 to 22.46 mg/L; EC 151.8 µS to 469 µS. During autumn the temperature dropped at 17.4°C and EC ranged between 256.3 and 411 µS.

Ponds Cerbu 1 and Cerbu 3 (Fig. 1) have dried up and refilled after precipitations during the two seasons.

Phytocommunities of the TPs

The algae assemblage of a TP is a good indicator of the ecological status and could be an important tool to explore the biodiversity pattern of those kinds of vulnerable systems.

According to the similar quality of photosynthetic pigments of the Chlorophyceae and Euglenophyceae, and of Bacillariophyceae and Dinophyceae in case of evaluation of the algal biomass using fluorescence extinction spectrum, it was not possible to distinguish between the amounts of biomass produced by these taxonomic groups. Only by additionally analysis, performed by using microscopy techniques, it was possible to differentiate the contribution of those taxonomic groups to the community of the primary producers of the four TPs.

The total recorded concentration of algae biomass reached the maximum of 936 µg/L has been recorded in Cerbu 4 pond (pond no. C4 in Fig. 2). The lowest recorded concentration being 31 µg/L recorded in October in Cerbu 3 pond (pond no. C3 in Fig. 2). The results suggest a high degree of trophic conditions and heterogeneity of the pond life across the present landscape.

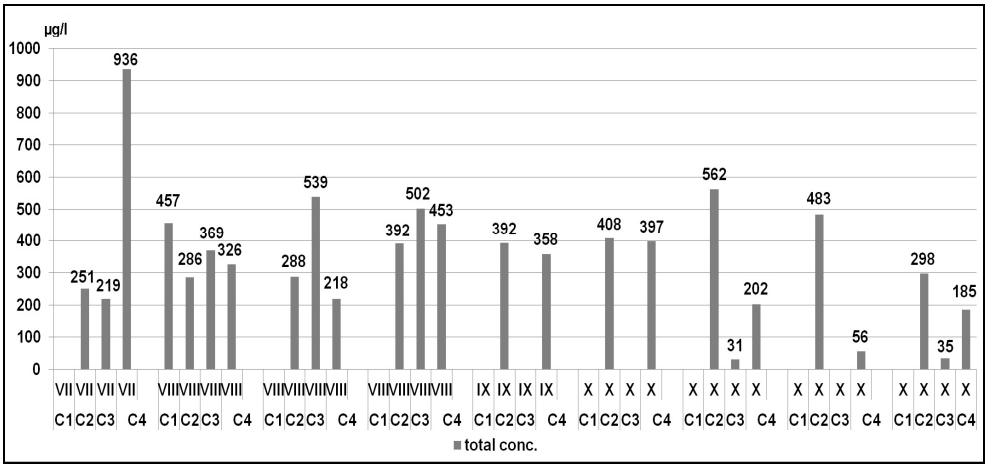

Figure 2. Variation of algae biomass during investigation period

The ponds mostly affected by the spatial and temporal variation of the hydrological events are Cerbu 1 pond (C1) and Cerbu 3 pond (C3). However, the local conditions allowed a rapid development of algal communities. A new water supply and a temporary fill up creating the opportunity to a rapid species colonization of the ponds. The first inhabitants being the groups of Cyanophyceae (blue green algae) (Fig. 3), but the most significant contribution to the maintenance of the diversity through species replacement is given by the green algae through its genera *Scenedesmus* and *Kirchneriella*.

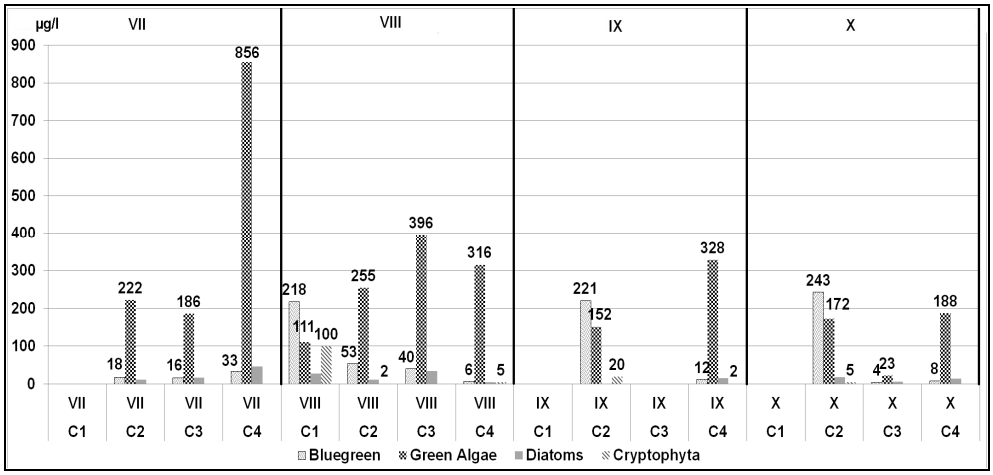

Figure 3. Biomass variation of the taxonomic groups

Algal taxonomical survey

A total number of 75 taxa were identified, belonging to the following phylum: Cyanophyta, Euglenophyta, Bacillariophyta and Chlorophyta (Tab. 1). Numerous taxa identified in the TPs are known to be found in ponds, as: *Euglena hemichromata*, *Phacus acuminatus*, *P. orbicularis*, *Trachelomonas intermedia*, *T. volvocina*, *Cyclotella meneghiniana*, *Scenedesmus intermedius*, *S. protuberans* and *Tetraëdron caudatum*. True planktonic species were also found, as *Aphanothece clathrata*, *Trachelomonas scabra*, *Didymocystis planctonica*, *Kirchneriella subcapitata*, *Scenedesmus bicaudatus* and *S. longispina*. Other taxa have a cosmopolitan distribution, as *Diatoma tenuis*, *Navicula pygmaea, Monoraphidium contortum* and *Scenedesmus opoliensis*.

Table 1

Algal taxa identified in the samples from the four temporary ponds studied in this work (abbreviations: SU-summer, AU-autumn)

TAXA	SU_C1	SU_C2	SU_C3	SU_C4	AU_C2	AU_C3	AU_C4
Phylum Cyanophyta							
Aphanocapsa cf. holsatica (Lemmermann) G.Cronberg & Komárek 1994					+		
Aphanothece clathrata West & G.S.West 1906		+					
Aphanothece minutissima J.Komárková-Legnerová & G.Cronberg 1994					+	+	
Merismopedia tenuissima Lemmermann 1898				+			
Microcystis pulchra (Kuetzing) J. Stein				+			
Phylum Euglenophyta							
Euglena gaumei Allorge & Lefèvre 1931				+			
Euglena hemichromata Skuja 1948					+	+	
Phacus acuminatus Stokes 1885		+		+			
Phacus orbicularis K.Hübner 1886				+			
Trachelomonas intermedia P.A.Dangeard 1902					+		
Trachelomonas oblonga Lemmermann 1899					+		
Trachelomonas scabra Playfair 1915					+		
Trachelomonas volvocina Ehrenberg 1834					+	+	
Phylum Bacillariophyta							
Achnanthes minutissima Kützing 1833					+		
Aulacoseira granulata (Ehrenberg) Simonsen 1979	+			+			
Caloneis molaris Krammer in Krammer & Lange-Bertalot 1985	+	+		+	+		
Cocconeis pediculus Ehrenberg 1838	+	+		+	+		
Cocconeis placentula Ehrenberg 1838	+	+		+	+		
Cocconeis placentula var. euglypta (Ehrenberg) Grunow 1884	+	+	+	+	+		
Cyclotella meneghiniana Kützing 1844	+			+	+		
Diatoma tenuis C.Agardh 1812				+			
Diploneis oculata (Brébisson) Cleve 1894				+			
Fragilaria ulna (Nitzsch) Lange-Bertalot 1980	+						
Fragilaria ulna var. acus (Kützing) Lange-Bertalot 1980				+			
Gomphonema parvulum Kützing 1849	+			+		+	

Table 1 continued

Gomphonema truncatum Ehrenberg 1832					+		
Hantzschia amphioxys Grunow in Cleve & Grunow 1880		+		+	+		
Navicula cincta (Ehrenberg) Ralfs in Pritchard 1861				+	+		+
Navicula cryptotenella Lange-Bertalot in Krammer & Lange-Bertalot 1985	+	+			+	+	
Navicula gregaria Donkin 1861					+		
Navicula lanceolata Ehrenberg 1838			+				
Navicula cf. mutica Kützing 1844	+	+			+		
Navicula phyllepta Kützing 1844			+				
Navicula pygmaea Kützing 1849	+		+		+		
Navicula radiosa Kützing 1844		+	+		+		+
Nitzschia intermedia Hantzsch in Cleve & Grunow 1880		+					
Nitzschia palea (Kützing) W.Smith 1856	+					+	
Nitzschia paleacea Grunow in Van Heurck 1881	+	+			+	+	
Nitzschia paleaeformis Hustedt 1950	+				+		+
Nitzschia perminuta (Grunow) M.Peragallo 1903	+				+		
Pinnularia borealis Ehrenberg 1843	+						
Pinnularia brebissoni (Kützing) Rabenhorst 1864	+				+		
Pinnularia microstauron (Ehrenberg) Cleve 1891	+						
Stauroneis anceps Ehrenberg 1843	+	+			+		+
Surirella angusta Kützing 1844	+	+			+		
Phylum Chlorophyta							
Closteriopsis acicularis (Chodat) J.H.Belcher & Swale 1962							+
Cosmarium subcostatum Nordstedt in Nordstedt & Wittrock 1876				+			
Crucigeniella rectangularis (Nägeli) Komárek 1974				+			
Didymocystis planctonica Korshikov 1953				+			
Kirchneriella microscopica Nygaard 1945				+	+		+
Kirchneriella subcapitata Korshikov 1953					+		
Monoraphidium contortum (Thuret) Komárková-Legnerová in Fott 1969	+	+			+	+	+
Monoraphidium griffithii (Berkeley) Komárková-Legnerová 1969					+		+
Scenedesmus acutus Meyen 1829		+					
Scenedesmus acuminatus (Lagerheim) Chodat 1902				+			
Scenedesmus bicaudatus Dedusenko 1925	+	+	+	+			+

Table 1 continued

Scenedesmus danubialis Hortob. 1970							+
Scenedesmus ecornis (Ehrenberg) Chodat 1926	+	+			+		
Scenedesmus gutwinskii var. *heterospina* Bodrogsközy 1950					+		+
Scenedesmus intermedius Chodat 1926		+	+	+			+
Scenedesmus longispina Chodat 1913							+
Scenedesmus cf. obtusus Meyen 1829	+	+					
Scenedesmus opoliensis P.G.Richter 1895							+
Scenedesmus opoliensis var. mononensis Chodat 1926							+
Scenedesmus pecsensis Uherkovich 1956							+
Scenedesmus protuberans F.E.Fritsch & M.F.Rich 1929			+				+
Scenedesmus protuberans var. *danubianus* Uherkovich 1956							+
Scenedesmus quadricauda (Turpin) Brébisson in Brébisson & Godey 1835			+	+	+		+
Scenedesmus quadricauda f granulatus Hortob. 1960							+
Scenedesmus quadrispina Chodat 1913							+
Schroederia setigera (Schröder) Lemmermann 1898		+					
Tetraëdron caudatum (Corda) Hansgirg 1888				+			
Tetraëdron minimum (A.Braun) Hansgirg 1888	+	+		+	+		
Tetraëdron muticum (A.Braun) Hansgirg 1888					+		
Tetrastrum triangulare (Chodat) Komárek 1974							+
TOTAL TAXA	**25**	**22**	**9**	**27**	**36**	**8**	**22**

The phylum Bacillariophyta dominated the algal communities, reaching a percentage of 42%, followed closely by Chlorophyta with a 40%, Euglenophyta and Cyanophyta with less percentage (11% and 7%, respectively).

The total number of taxa differed from the two seasons, the highest variation being recorded in pond C3, ranging between a maximum of 36 taxa (recorded in autumn) to a minimum of 9 taxa (identified in the summer samples). Two of the ponds are extremely vulnerable due to the variation of water level: C1 dried out in at the end of summer (and remained dry the autumn) (Fig. 4), respectively C3 which was dry for a short period at the middle of the summer, then again in different weeks of the autumn (depending on the amount of rains).

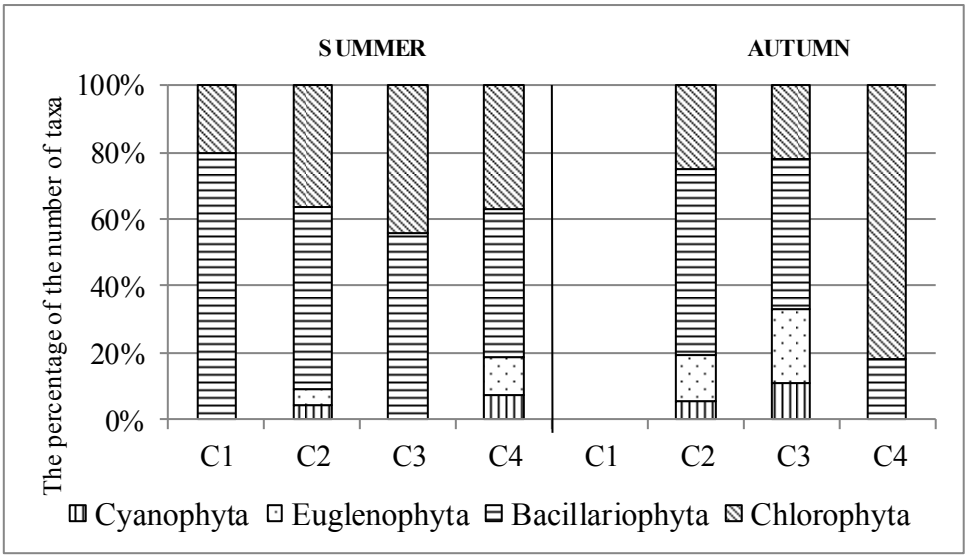

Figure 4. The distribution of phylum based on the number of species / season

A number of 25 taxa were identified in C1 in the samples collected in summer. In pond C2 the highest number of taxa was identified in autumn (36 taxa), whereas in summer only 22 taxa were recorded. In pond C3 the number of taxa was low, 9 in summer and 8 in autumn. In the TP C4 the number of 27 taxa in summer samples, dropped to 22 taxa in autumn (Fig. 5).

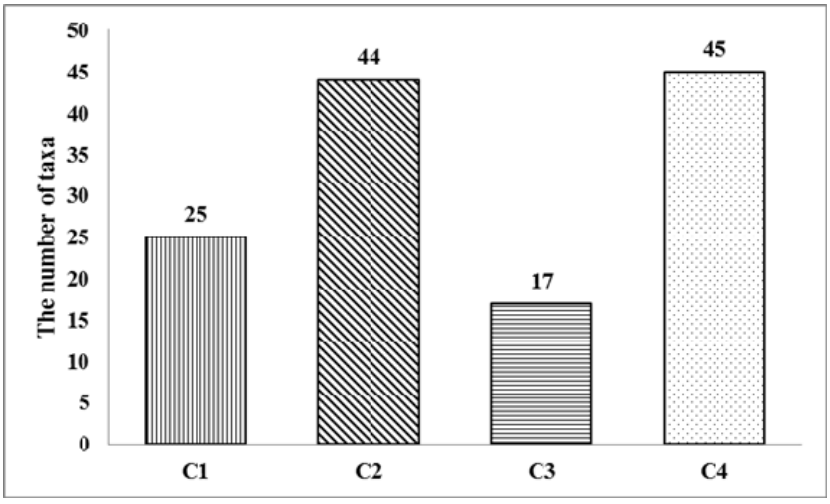

Figure 5. Number of taxa identified in each pond

The algal composition similarity between the sites from the four TPs, based on the presence – absence of the species at each site, using Bray-Curtis similarity performed with PRIMER 6 statistical package, shows that the special pattern of the algal population had a high similarity between ponds C1 and C2 (Fig. 6). Also in case of biomass, small variations were registered between ponds in late summer and autumn (Fig. 7).

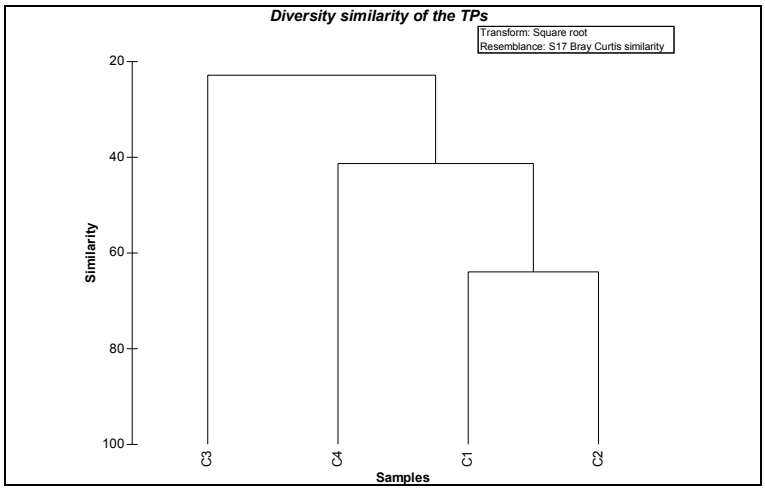

Figure 6. Diversity similarity of the temporary ponds from Cerbu area.

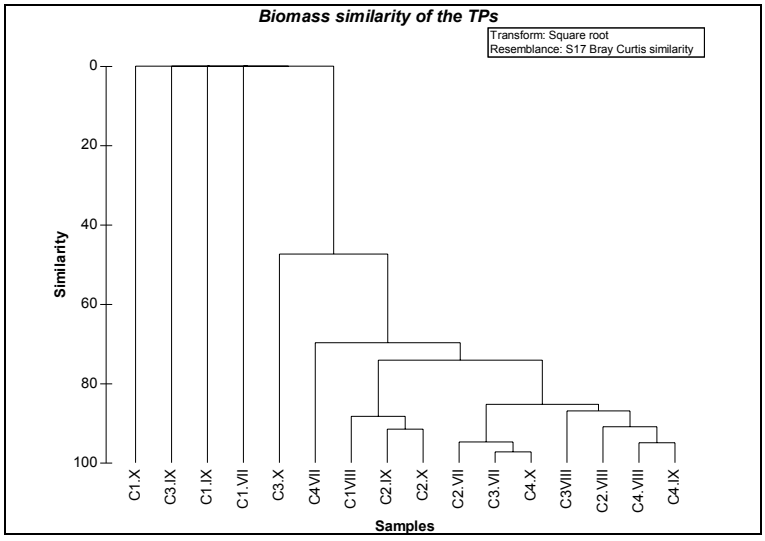

Figure 7. Biomass similarity of the temporary ponds from Cerbu area.

The highest value of phytoplankton abundance was recorded in pond C3 and C4 in summer and C2 and C4 in autumn. In those ponds there were recorded the highest amount of cells / sample.

Generally speaking when this huge development of algae occurs there is so-called algal blooms which are evident when algae color the water and the cell number reaches $5*10^6$ (Goldman *et al.,* 1983). In the studied ponds this values were associated with the decreasing trend in algal biodiversity. In summer, in pond C3 the dominating taxa were *Scenedesmus protuberans* and *S. quadricauda* with more than 90% of the total number of cells. In pond C4, in the summer samples, the highest percentage was reached by *Phacus acuminatus* (70%), other small percentages belong to *Tetraëdron* (9%), *Scenedesmus* (7%), *Monoraphidium* (5%) and *Euglena* (3%). In the autumn samples, in both C2 and C4 ponds, *Kirchneriella microscopica* was the dominant taxa (around 70% in both samples). Pond no. C2, in autumn had the highest algal-diversity with 10 species belonging to 9 genera, *Kirchneriella* was followed by *Tetraëdron* with 10% percentage (other small percentages belonged to: *Kirchneriella subcapitata*, *Scenedesmus bicaudatus*, *S. intermedius*, *S. protuberans, S. quadricauda*, *Tetraëdron minimum*, *Trachelomonas volvocina*, *Aphanocapsa* cf *holsatica*, *Monoraphidium contortum*, *Euglena hemicromata*, *Aphanothece minutissima* and species belonging to Bacillariophyceae).

Ecological status of the TPs

Each time a pond fills with water, a new episode in community ecology begins as species arrive to take advantage of the opportunity to complete the free-living aquatic stage of their life cycle (Wilbur, 1997).

Based on ecological preference of algal species the following characteristics were observed: out of the 75 taxa identified in the samples, 23 taxa are indicators of different saprobic values, a percentage of 44% indicating that the water saprobic value is β-mesosaprobic. The Palmer genus index (which refers to the organic pollution of the water) reached the highest value in pond C4: 6 genera (out of the 20 indicator algal genera) as: *Euglena, Phacus, Aulacoseira* (*Melosira*), *Navicula, Nitzschia* and *Scenedesmus* (Palmer, 1969). At genus level, the value of the organic pollution index (form 7 to 18) indicated low to intermediate organic pollution of the water in the temporary ponds studied in the present work.

According to the eutrophic classification of Oltean (1977), in pond C2, delta-eutrophic conditions were detected in autumn 2014, because of the "water blooms" with Chlorococcales (species *Kirchneriella microscopica*); epsilon-eutrophic conditions were identified in pond C4, summer 2014, due to "water blooms" with taxa belonging to Euglenophyta (specifically *Phacus acuminatus*). The values of the indices (2.04 and 10.02, respectively), together with the values of other indices: Thunmark's chlorophycean index (27) and Nygaard's compound index (42) indicate that the temporary ponds present

in this study are in an advanced state of eutrophication. This conclusion is supported by the presence of 20 taxa that indicate high levels of eutrophication, as: *Aphanocapsa delicatissima, Euglena gaumei, E. hemichromata, Phacus acuminatus, P. orbicularis, Trachelomonas intermedia, T. oblonga, T. volvocina, Aulacoseira granulata, Cyclotella meneghiniana, Diatoma tenuis, Monoraphidium contortum, Scenedesmus opoliensis* and *Tetraëdron caudatum.*

Amphibian communities of the TPs

Amphibians, having a biphasic life cycle, use for growth and development the temporary ponds. According to our results, the temporal scale of Cerbu ponds with drying periods influenced the life cycle and growth of amphibians by modification of the optimal time of metamorphosis. Previously studies showed, that the regulation of population density and the fitness of individuals are determined by complex interactions among competition, predation, and uncertainty in the length of the time ponds retain water. Anurans can have strong effects on the partitioning of the flow of nutrients through the phytoplankton vs. the periphyton (Wilbur, 1997).

In case of the four temporary waterbodies from nearby Cerbu village, the Marsh Frog (*Pelophylax ridibundus*) was the only species constantly present in the study area. Although (even if only few) adult specimens were recorded each time in the ponds filled with water, tadpoles were also observed at least till the middle of October. In the permanent aquatic systems of the region (most of them located in the valley of the Danube river, in the Danube Delta and in the Razim-Sinoe lagoonary area), tadpoles of *Pelophylax ridibundus* are usually recorded only till middle of July – beginning of August.

In case of other amphibian species, practically there were recorded only tadpoles or recently metamorphosed specimens (*Bufo viridis* and *Pelobates syriacus* till August, respectively, *Rana dalmatina* till July). Adults of these species are present in the ponds only in the spawning period (March – April). In the permanent waterbodies of the region the tadpoles of these species reach the end of metamorphosis at least one month earlier. The only adult (a *Rana dalmatina*) was observed one time (at the beginning of August) in Cerbu 2 pond, being probably a vagrant specimen, that accidentally reached, for a short time, the respective pond (in most cases, after the spawning period, the adults of this species occur practically exclusively in terrestrial, forested habitats).

Conclusions

The highest algal diversity has been recorded in pond no. C2. Even though the pond no. C1 dried out in autumn, the colonization rate was much higher than in pound no. C3, this was also dried out frequently in the two seasons. The most abundant taxa belonged to genus *Kirchneriella* and *Scenedesmus*, the environmental conditions being more suitable to their preferred ecology.

The temporary ponds are important habitats that insure the existence of local populations, even if small, of four native species of amphibians. According to the results of the present study, the metamorphosis of the amphibians recorded in the temporary ponds was completed later (with 2 or 3 months delay, depending on the species) than in case of the populations of the same species from permanent water bodies of the region.

Acknowledgements

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Ecological dynamics between Lake Sucutardul Mare and its temporary fry pond (the Fizeş Valley, Transylvania, Romania): the case of aquatic invertebrates

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SUMMARY. Temporary water bodies, connected or not to permanent wetlands, have a major importance for the regional biodiversity, acting as source of individuals for recolonization, refuge habitats or foraging sites. Colonization of zoobenthos and zooplankton from Lake Sucutardul Mare into its temporary fry pond was investigated in summer and autumn 2015. The instability of the temporary water pool, which dried out in autumn, together with its lower habitat heterogeneity led to decreased numbers of zoobenthic colonizers, that failed to survive in this "sink" habitat. No true colonization occurred in case of zooplankton, since the permanent connection between the two ponds led to similar animal communities in the water column. A rapid shift between the dominant zooplankton groups, rotifers and copepods, was observed.

Keywords: colonization, dynamics, temporary pond, zoobenthos, zooplankton

Introduction

Wetlands, man-made or natural, represent an important part of the landscape, due to their multiple values: not only hydrological and physico - chemical (water supply for different purposes, nutrient sinks or sources, flood control etc.) but also biological (maintaining species and genetic diversity, passage habitat for birds etc.) and socio - economic (supporting fisheries and agriculture, recreation or spiritual values) (Haslam, 2007).

Aquatic invertebrates characteristic to wetlands develop in benthic habitats, as well as in the water column (O'Sullivan and Reynolds (eds.), 2004). Zoobenthos comprises a high variety of taxa, from herbivores to carnivores, having different adaptations to the

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lentic environment. Zooplankton inhabits the pelagic habitats and includes mainly rotifers and microcrustaceans (cladocerans and copepods). It represents an important link in the food web, connecting the primary producers to the higher consumers (Cole, 1983).

The present study considered the aquatic invertebrate communities from a fish pond located in the Fizeș River Valley, Lake Sucutardul Mare, and the temporary pond connected to it, used to farm newly hatched fish. Together with 16 other wetland habitats, Lake Sucutardul Mare is included in a complex of aquatic and terrestrial ecosystems, Natura 2000 Special Protection Area ROSPA 0104 "The Fizeș Valley catchment area [Bazinul Fizeşului]", important as reproduction habitats and stopover sites for bird species.

Previous literature from the area focused on lake chemistry (Mihăiescu *et al*., 2008; Mihăescu *et al.,* 2010) or wetland protection and management (Maloș *et al*., 2008; Mihăiescu and Mihăiescu, 2010). Biotic communities were analyzed in relation with waterfowl ecology (Stermin *et al*., 2011) or algae (Momeu *et al.,* 1979). Fish productivity of several ponds from the Fizeș Valley was examined in an unpublished rapport following a three-year research grant, which also included physico-chemical, phytoplankton and invertebrate data (Rapport for research grant no. 2, 1986).

The present paper aimed to investigate the dynamics between a fish pond (Lake Sucutardul Mare) and its connected temporary fry pond. The latter should have been a promising habitat for aquatic invertebrates to colonize, due to its proximity and permanent connection to the fish pond. However, only zoobenthic groups colonized the temporary pond, which finally acted as a sink habitat, since it dried out completely. The present study represents the first attempt to characterize colonization of aquatic invertebrates in wetlands from the Fizeș Valley catchment area.

Materials and methods

Lake Sucutardul Mare (Sucutard II) (**L1**) is located in the Fizeș River catchment area, a tributary of the Someşul Mic River, in the center of the Transylvanian Plain, north-west Romania (N: 46º54'1.51"; E: 24º4'1.87") (Fig. 1). It is a fish pond, created in 1966 by damming the Fizeș River at the junction with the Puini Valley. The present surface of the lake is 46 ha, with a depth ranging from 4 m in 1966 to 2.5 m in 1997 (Sorocovschi, 2005). The fry pond (**L2**) (N: 46º54'17.94"; E: 24º4'11.16") represents a small water body where reproductive fish are isolated, to assure the best start in life for the newly hatched alevins. It is permanently connected to Lake Sucutardul Mare through a subterranean pipe system, where water moves gravitationally from the main lake to lower grounds. The fry pond often dries out in late summer.

Zoobenthos and zooplankton samples were taken in 2015, as follows: on June the 14th (6/14/15), June the 28th (6/28/15), July the 14th (7/14/15), July the 28th (7/28/15), August the 15th (8/15/15), September the 5th (9/5/15) and October the 3rd (10/3/15).

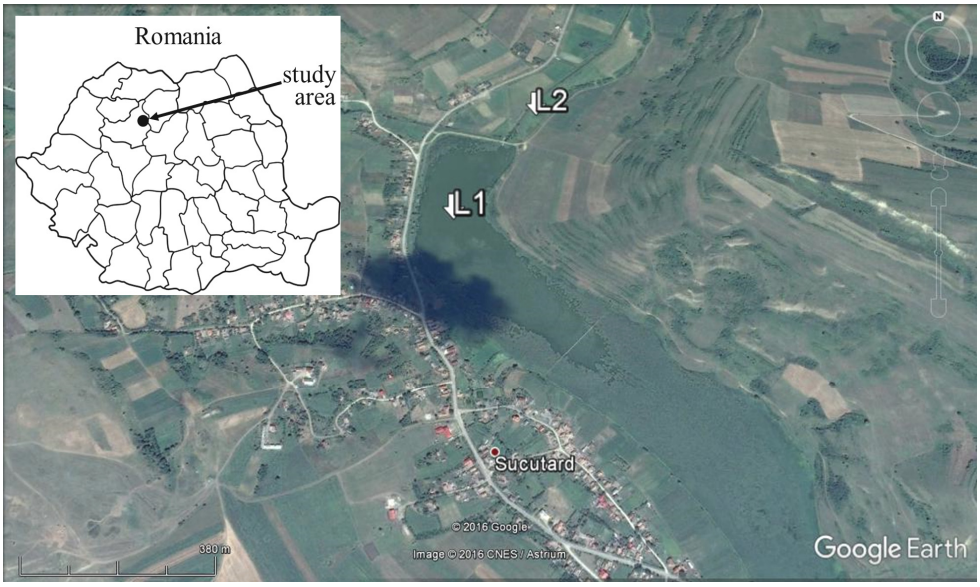

Figure 1. Location of Lake Sucutardul Mare (**L1**) and the temporary fry pond (**L2**)

Due to its shallow depth, the fry pond often dries out in summer or autumn. This was also the case in 2015, when sampling was impossible in October for both zooplankton and zoobenthos. In fact, periods of low water levels are common in the ponds of the Fizeş Valley area. Lake Sucutardul Mare was completely emptied in 2007, during a prolonged period of low rainfall, in order to maintain a higher water level in Lake Ţaga Mare, a fish pond located downstream (David, 2008).

Qualitative multihabitat samples were collected with a 250 µm mesh net for zoobenthos and a 55 µm mesh net for zooplankton; they were preserved in 4% formaldehyde. Physico-chemical parameters were measured in the laboratory using a Hanna multiparameter H198130. Benthic invertebrate identifications were made to the genus level for Ephemeroptera and Hemiptera (Heteroptera) and to various taxonomic levels for the other groups, using Sansoni (2001) and Bouchard (2004). Total length was measured for Ephemeroptera individuals (antennae and cerci excluded) (according to Petrovici, 2009), and the following size classes were considered: 1 (1 – 1.99 mm); 2 (2 – 2.99 mm); 3 (3 – 3.99 mm); 4 (4 – 4.99 mm); 5 (5 – 5.99 mm); 6 (6 - 6.99 mm); 7 (7 – 7.99 mm) and 8 (8 – 8.99 mm). In case of zooplankton, microcrustaceans were identified to the species level using Negrea (1983) for cladocerans and Damian-Georgescu (1963) and Einsle (1993) for cyclopoid copepods, while rotifers larger than 55 µm were considered as a group. The validity of all taxa was checked using de Jong *et al*. (2004). Relative abundance, frequency and the Shannon-Wiener diversity index were calculated for both zoobenthic and zooplankton communities.

Results and discussion

Physical and chemical parameters

Lake Sucutardul Mare belongs to bicarbonate - sulphate mixed class as concens the dominant anions, and to magnesium class for cations (Mihăiescu *et al*. 2010). Water temperature recorded normal values for the sampled months, ranging from 10 to 30°C, with slightly higher values in **L2**, probably due to the lower depth of the fry pond. pH values were alkaline: 8.37 in **L1** and 8.2 in **L2**, while conductivity recorded relatively high values, reaching 2.24 mS/cm in **L1** and 1.46 mS/cm in **L2** (all values recorded in August 2015).

Physical and chemical values of Lake Sucutardul Mare measured in 2015 were similar to those recorded in previous studies (Rapport for research grant no. 2, 1986; Mihăiescu *et al*., 2008), showing constant conditions over time.

Water level fluctuations caused by the drying periods can cause faster degradation of organic matter in the system (Wetzel, 2001). This could explain the moderate amount of organic matter measured in Lake Sucutardul Mare: 30 – 70 mg KMnO4/L (Rapport for research grant no. 2, 1986). The nutrients released by these oxidation processes could be, in turn, used by algal communities, since the nutrient load of the lake was low: nitrate values below 1 mg/L and total phosphorus lower than 0.2 mg/L (Rapport for research grant no. 2, 1986, Mihăiescu *et al*., 2008).

Zoobenthos and zooplankton abundances

A total of 24 zoobenthic taxa were identified in Lake Sucutardul Mare (**L1**) and only half in its temporary fry pond (**L2**) (Table 1). Several benthic groups were transported from **L1** in **L2**, through the pipe system connecting the two water pools. Most taxa that colonized **L2** were identified in the first months of the study, and only *Corixa* sp. was present in **L2** after August 2015.

A diverse zoobenthic community was present in **L1** in the sampling dates. No dominant group stood out, even if chironomids increased their percentage in autumn, while oligochaetes decreased (Fig. 2). By contrast, zoobenthos taxa depicted a well-balanced percentage distribution only in the first sampling date in **L2**, since chironomids clearly dominated the benthic communities in all other dates, with percentages exceeding 60% (Fig. 2). In fact, the Shannon-Wiener diversity index, calculated for insect orders alone, ranged between 0.4785 and 1.289 in **L1**, and only between 0.1906 and 0.8839 in **L2**.

Three zooplanktonic groups were present in the samples (Table 1): rotifers (*Asplanchna* sp., *Brachionus* sp., *Filinia* sp., *Keratella* sp., *Lecane* sp., *Polyarthra* sp.); cladocerans and cyclopoid copepods (immature and adult individuals). Cladoceran species recorded low frequencies and they were represented by few individuals, all parthenogenetic females. Only *Chydorus sphaericus* (O. F. Muller 1776) and *Moina micrura* Kurz 1875 were present in more than 50% of all samples (Fig. 3).

Table 1

List of taxa identified at the sampling dates in Lake Sucutardul Mare (**L1**) and the temporary fry pond (**L2**) (*- planktonic taxa; crustaceans and insect in phylogenetic order according to Martin and Davis 2001; Wheeler *et al.,* 2001; see text for sampling date abbreviations)

	Sampling dates						
Taxa	**6/14/15**	**6/28/15**	**7/14/15**	**7/28/15**	**8/15/15**	**9/5/15**	**10/3/15**
Annelida							
Hirudinea	L1	L1	L1	L1, L2	L1	L1	L1
Oligochaeta	L1, L2	L1, L2	L1, L2	L1, L2	L1	L1	L1
Rotifera*	L1, L2	L1, L2	L1, L2	L1, L2	L1, L2	L1	L1
Mollusca							
Gastropoda	L1	L1, L2	L1	L1	L1	L1	L1
Arthropoda, Crustacea							
Branchiopoda, Cladocera*							
Bosmina longirostris	—	*L1*	—	—	*L1*	—	—
Ceriodaphnia reticulata	—	*L1*	—	—	—	—	—
Chydorus sphaericus	*L1, L2*	*L1, L2*	*L1*	*L1*	—	—	—
Moina micrura	*L1, L2*	*L1, L2*	*L1*	*L1*	*L1*	*L1*	—
Simocephalus vetulus	*L1*	—	—	—	—	—	—
Maxillopoda, Copepoda*							
Acanthocyclops robustus	—	*L1*	—	—	—	—	—
Mesocyclops leukarti	*L1, L2*	*L1*	*L1*	*L1*	*L1*	—	—
Thermocyclops oithonoides	*L1, L2*	*L1, L2*	*L1, L2*	*L1, L2*	*L1, L2*	*L1*	*L1*
Malacostraca, Isopoda	L1	L1	L1	L1	—	—	—
Arthropoda, Hexapoda, Insecta							
Ephemeroptera							
Caenis sp.	*L1*	*L1*	*L1*	*L1*	—	*L1*	*L1*
Cloëon sp.	*L1, L2*	*L1, L2*	*L1, L2*	*L1*	*L1*	*L1*	*L1*
Odonata	L1, L2	L1	L1, L2	L1, L2	L1	L1	L1
Hemiptera (Heteroptera)							
Corixa sp.	—	—	*L1*	*L1, L2*	—	*L1, L2*	*L1*
Cymatia sp.	—	—	—	—	—	*L1*	—
Gerris sp.	*L1*	—	*L1*	—	*L1*	—	—
Micronecta sp.	*L1*	*L1, L2*	*L1*	*L1*	*L1*	*L1*	—
Naucoris sp.	*L1*	*L1*	*L1*	*L1*	*L1*	—	—
Plea sp.	*L1*	*L1*	*L1*	*L1*	*L1*	*L1*	—
Ranatra sp.	—	*L1*	—	—	—	—	—
Megaloptera	L1	—	L1	—	—	—	—
Coleoptera	L2	L1, L2	L1, L2	L1, L2	L1	L1	—
Trichoptera	—	—	L1	—	—	—	—
Diptera, Culicidae	L1	L1	L1	L1	—	L1	—
Diptera, Chironomidae	L1, L2	L1, L2	L1, L2	L1, L2	L1	L1, L2	L1
Diptera, Ceratopogonidae	L1	L2	L1	—	—	—	—
Diptera, Ptycopteridae	—	L1	—	—	—	—	—
Diptera, Simuliidae	L2	L2	L1, L2	—	—	—	—
Diptera, Stratiomyidae	—	—	L2	—	—	—	—
Diptera, Syrphidae	—	L1, L2	—	—	—	—	—

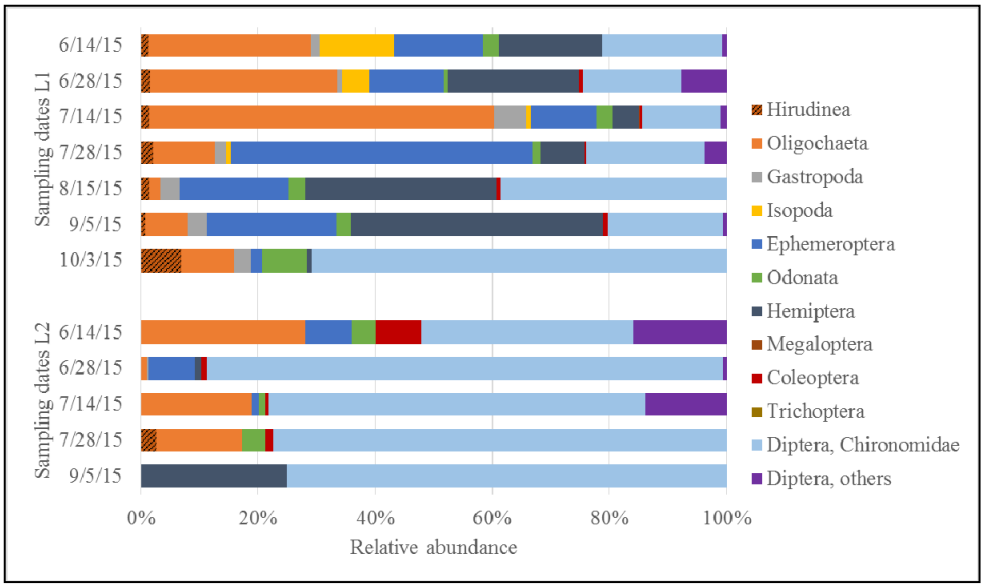

Figure 2. Relative abundance of benthic invertebrate groups in Lake Sucutardul Mare (**L1**) and its temporary fry pond (**L2**) (see text for sampling date abbreviations)

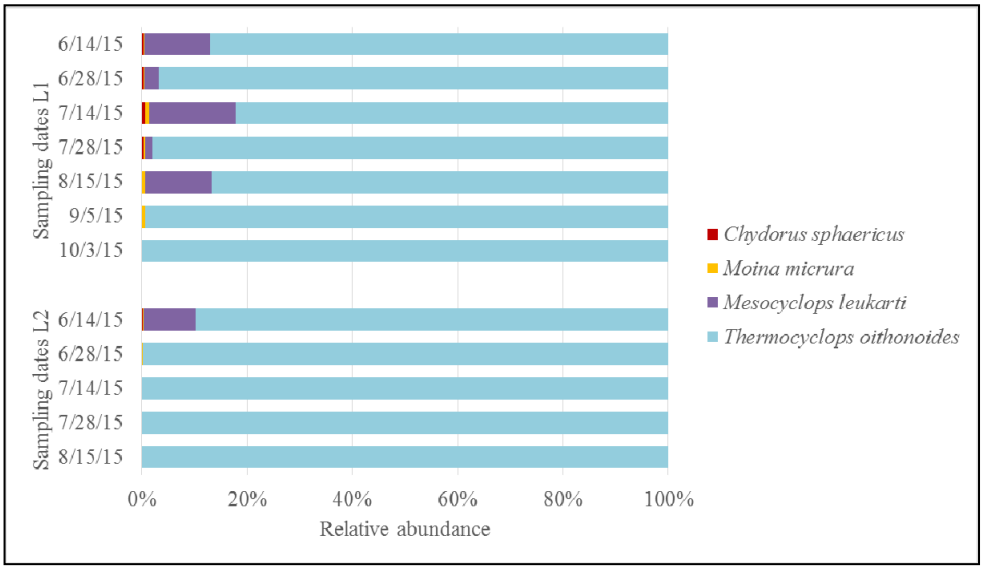

Figure 3. Relative abundance of planktonic microcrustacean species (cladocerans and copepods) in Lake Sucutardul Mare (**L1**) and its temporary fry pond (**L2**) (species with frequencies ≤ 30% excluded; see text for sampling date abbreviations)

Copepods and rotifers clearly dominated both water bodies. *Thermocyclops oithonoides* (Sars 1863) was the dominant copepod species: adults and immature individuals (copepodites and nauplii) reached high abundances in all sampling dates (Fig. 3). This was the reason why the Shannon-Wiener diversity index recorded low values for microcrustaceans: slightly higher in **L1**, ranging between 0 and 0.5238; and very low in **L2**, not exceeding 0.3448, with 0 for three sampling dates, from July to August 2015. Significant differences were recorded between the averaged diversity indices for microcrustaceans in **L1** compared to **L2** (t test = 3.211; p = 0.0014).

Zoobenthos dynamics between the two water bodies

During the sampling period, the temporary fry pond (**L2**) was colonized by zoobenthic groups coming from **L1**. In most cases, the number of individuals in **L2** was much lower than in **L1**, and they did not survive the whole sampling period (Fig. 4: A, B, C). Dipterans belonging to Chironomidae and Simuliidae represented an exception, since they were present in higher numbers in **L2** in June and July 2015 (Fig. 4: D, F). Some groups, like Culicidae, were unable to colonize **L2** (Fig. 4, E).

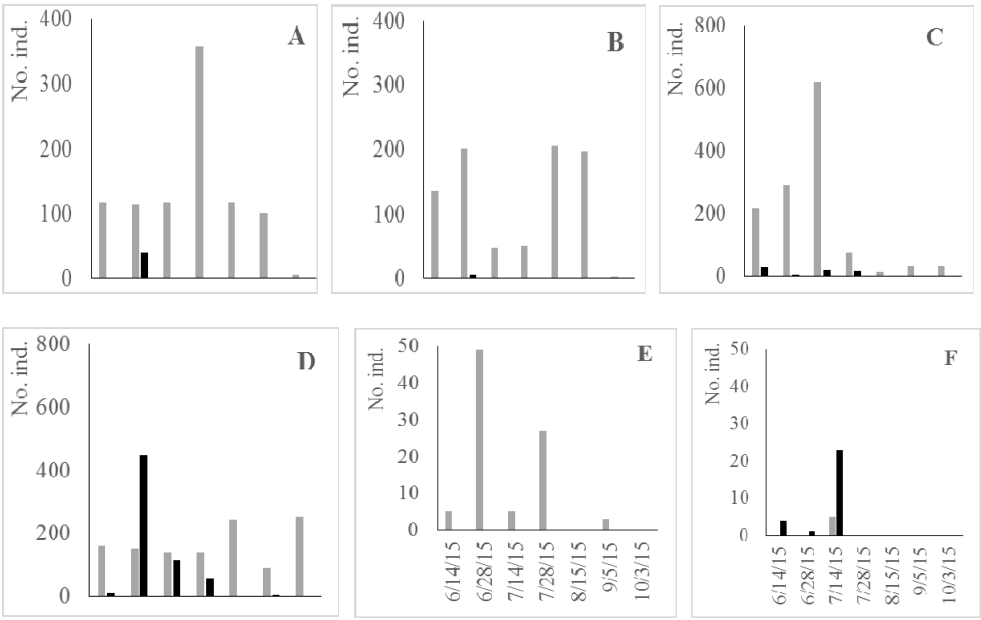

Figure 4. The number of individuals from Lake Sucutardul Mare (**L1**, gray columns) and its temporary fry pond (**L2**, black columns) for: A –Ephemeroptera; B – Hemiptera (Heteroptera); C – Oligochaeta; D – Chironomidae; E – Culicidae; F - Simuliidae (No. ind. – number of individuals; see text for sampling date abbreviations)

In case of Ephemeroptera, *Cloëon* sp. was represented in both water bodies by individuals differing in size (Fig. 5). Mayflies are known to be multivoltine, depending on local habitat conditions; development can take from several months up to a year (Bauernfeind and Soldán, 2012).

Two cohorts were visible in *Cloëon* population in **L1**: the first one with large, older individulas in late June (size classes 7 and 8), that probably colonized **L2**. The second one, characterized by larger individuals in August - September was unable to disperse in **L2**, due to the lack of favorable habitats in the fry pond, which eventually dried out.

Larger *Cloëon* individuals were present in **L2** in late June (Fig. 5), probably due to lower fish pressure, since fish fry usually feed on zooplankton and not on larger benthic invertebrates (Froese and Pauly (eds.), 2016).

Most of the *Cloëon* individuals emerging from **L2** in late June probably layed their eggs back in **L1**, since fewer individuals were found in **L2** afterwards. This could be explained by the decrease in suitable habitats for benthic invertebrates in **L2**, once the pond dried out.

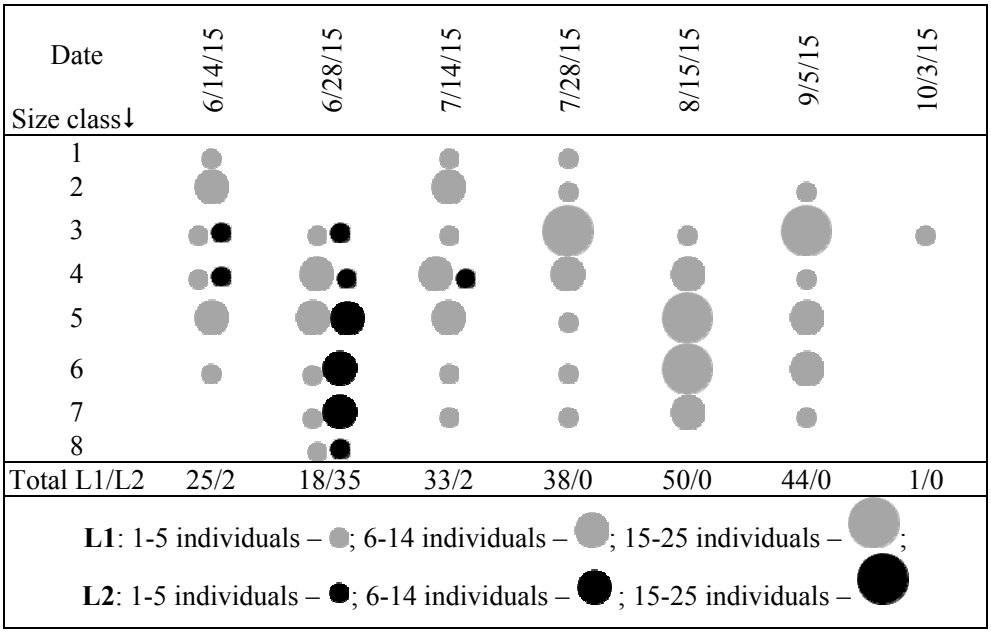

Figure 5. Dynamics of *Cloëon* sp. (Ephemeroptera) belonging to different size classes in Lake Sucutardul Mare (**L1**) and in its temporary fry pond (**L2**) (see text for sampling date abbreviations)

A high taxonomic richness was recorded in **L1** in case of true bugs, Hemiptera (Heteroptera): seven genera present, compared to only 2 in **L2** (Fig. 6). Several habitat characteristics influence true bug community structure in lakes: the presence of aquatic vegetation, accumulation of organic matter and water chemistry (Savage, 1989). Moreover, true bugs occupy different ecological regions in aquatic ecosystems, including not only benthic but also nektonic and neustonic communities. Thus, the low diversity of genera in **L2** showed the absence of suitable habitats in the temporary fry pond, compared to the main lake (Fig. 6).

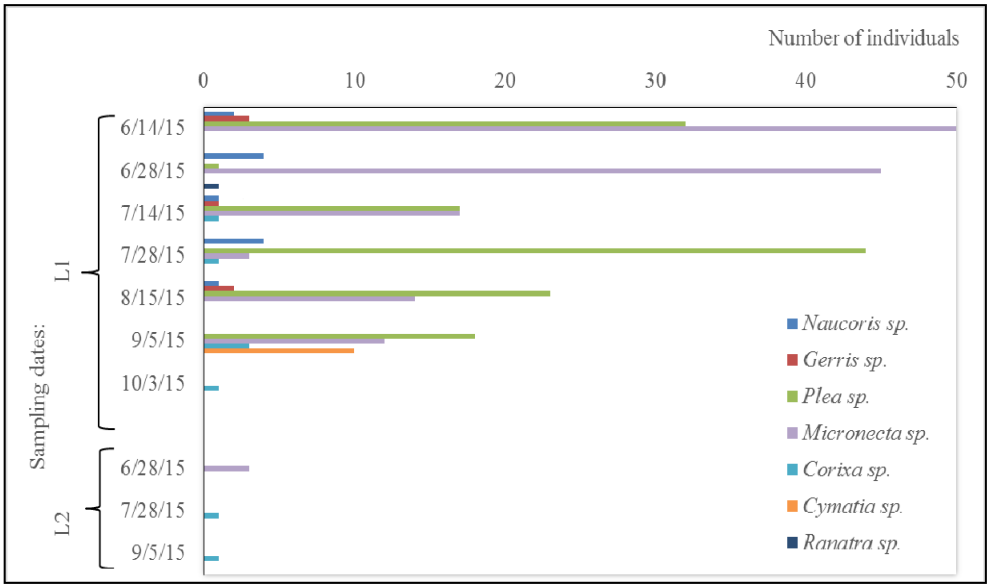

Figure 6. Number of individuals belonging to different Hemiptera (Heteroptera) individuals from Lake Sucutardul Mare (**L1**) and its temporary fry pond (**L2**) (see text for sampling date abbreviations)

Zooplankton dyamics between the two water bodies

The dominating groups of the animal plankton community changed rapidly in a short time, showing a dynamic community (Fig. 7). Rotifers, characterized by parthenogenetic reproduction under favorable conditions, were able to dominate the water column during the first sampling dates in both water bodies, outcompeting the cladocerans. However, *T. oithonoides* reached the dominant position in the water column, sometimes in only two weeks, as for the temporary fry pond (**L2**) (Fig. 7).

Sampling dates:		6/14/15	6/28/15	7/14/15	7/28/15	8/15/15	9/5/15	10/3/15
L1:	dominant group	R	R	R, *To*	*To*	*To*	*To*	*To*
	common group	*To*(i)	*To*		*Osc.*		R	R
L2:	dominant group	R	*To*(i)	*To*	*To*	-		
	common group	*To*(i)	R	R	R	*Osc.*		

Figure 7. Zooplanktonic dominant and common groups in Lake Sucutardul Mare (**L1**) and its temporary fry pond (**L2**): R – rotifers; *To* – *Thermocyclops oithonoides*; i – immature stages (nauplii and copepodites); *Osc.* – *Oscillatoria* sp. (see text for sampling date abbreviations)

Lake Sucutardul Mare had a higher zooplankton species richness compared to the fry pond: from a total of 8 microcrustacean species, 4 were found only in **L1**. However, the same dominating groups were characteristic to both **L1** and **L2** (Fig. 7), with monospecific microcrustacean communities in July and August in **L2**, when only *T. oithonoides* was identified (next to rotifers). This showed that the source of zooplanktonic individuals in **L2** was undoutably Lake Sucutardul Mare, since they could pass freely from **L1** to **L2** with the water. A two-week delay was observed in **L2** in case of a strong phenomenon of water blooming with Cyanobacteria *Oscillatoria* sp., confirming the clear connection between the two water bodies.

Colonization or seasonal dynamics?

Colonization of temporary habitats by aquatic invertebrates is mainly controlled by environmental pressures (Schneider and Frost, 1996; Incagnone *et al.,* 2015). Pond duration represents the most important factor influencing the community structure of the colonizing taxa (Schneider and Frost, 1996). Other environmental factors include habitat heterogeneity, water depth, connectivity, surface area (Studinski and Grubbs, 2007; Frisch *et al.,* 2012). Biotic interactions play an important role too, but their effect increases with increasing habitat duration (Schneider and Frost, 1996). In some cases though, some taxa cannot survive and reproduce because of short hydroperiods; these temporary ponds become "sink habitats" for those taxa (Pulliam, 1988; Schneider and Frost, 1996).

During the sampling months, zoobenthic individuals colonized the temporary fry pond (**L2**), but for a short time and in small numbers for most groups. In case of Ephemeroptera for example, more larger individuals were found in **L2** in late June than in **L1**, showing better environmental conditions in the temporary pond, probably due to lower fish predator pressure. However, decreased numbers of mayfly individuals were found in subsequent months in **L2**, as for Heteroptera, Oligochaeta, Chironomidae etc. Thus, a short hydroperiod in **L2**, together with low habitat heterogeneity, led to decreased numbers of zoobenthic colonizers from the main lake into its fry pond. **L2** would probably be a more interesting environment to colonize for benthic invertebrates if longer wet periods created more diverse habitats, like regions with macrophytes, accumultation of organic matter etc.

In case of microcrustaceans (Cladocera and Copepoda), they are considered to be well adapted to colonize and survive in temporary environments, where hydroperiod represents the main stressor, since they have short life spans, they can reproduce asexually through parthenogenesis, and they can produce resting eggs. In fact, copepod assemblages were suggested as candidates for biological indicators for environments with different wet period durations (Seminara *et al.,* 2016).

Zooplankton was more diverse in **L1** compared to the temporary fry pond. Similar results were reported in the literature, and they were explained by physical factors, like suspended solids, conductivity or pH, that made the temporary habitats unsuitable for colonizers (Simões *et al*., 2011).

However, no true colonization for zooplankton occured in the study area during the sampling dates, since the constant connection between **L1** and **L2** assured the presence of similar communities in both pools. Thus, the heterogeneity of the new habitats was less important than the constant connectivity for zooplankton, as shown also in the literature (Frisch *et al.,* 2012).

Nevertheless, the temporary ponds play an important role in preserving regional invertebrate diversity in wetlands, acting as place of refuge, source of individuals etc., provided that the hydroperiod allows taxa to survive and reproduce.

Conclusions

Zoobenthos and zooplankton communities from Lake Sucutardul Mare and its temporary fry pond were analyzed during summer and autumn 2015

A higher diversity for both communites was recorded in the main water body, due to more constant ecological conditions and a higher heterogeneity of habitats.

Colonization of the temporary fry pond with benthic invertebrates was observed, but low in intensity and duration. The temporary pond acted as “sink” for most benthic groups, when the water pool went completely dry.

No true colonization occurred in case of zooplankton, since the permanent connection of the two water bodies led to similar communities in the water column. However, rapid shifts in the dominant groups, from rotifers to copepods were observed.

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Land snail communities of Cheile Vârghişului Nature Reserve (the Perşani Mountains, Romania)

Voichița Gheoca[1,✉]

SUMMARY. This paper analyses the land snail communities of Cheile Vârghişului Nature Reserve located at the limit between Covasna and Harghita Counties (the Perşani Mountains), one of the most spectacular karst area of the southern part of the eastern Carpathians. In total 43 species of terrestrial gastropods were identified during this study and from the literature. The most abundant species were *Truncatellina cylindrica, Laciniaria plicata, Carychium tridentatum, Ruthenica filograna, Faustina faustina* and *Alopia bogatensis*. The highest diversity was found in the forest habitat with limestone outcrops, while the limestone cliffs located in the forest sheltered large populations of typical limestone species, as well as typical forest species. The restrictive environmental conditions of the exposed limestone cliffs are favourable only for a limited number of species developing large populations.

Keywords: biodiversity, community ecology, Gastropoda, limestone.

Introduction

The Cheile Vârgişului Nature Reserve represents one of the most interesting karst areas of the eastern Carpathians. The 800 ha of the reserve cover about 95% of the Cheile Vârghişului Natura 2000 site (ROSCI0036). Located at the limit between Harghita and the Perşani Mountains, the gorge, cut by the Vârghiş river into Jurassic and Cretacic limestone, is about 3.5 km long, limited by limestone cliffs of up to 200 m high (Grigore, 1989). The gorge with its 125 caves is a habitat/species management area (Category IV IUCN) and shelters valuable flora and fauna elements.

Several relatively recent studies concentrate on the vegetation (Vojtkó *et al.*, 2012) or different fauna groups (Tăbăcaru and Giurgincă, 2013; Jére *et al*., 2007; Nitzu *et al*., 2007).

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As usually in karst areas, land snails are one of the most important invertebrate taxa (Necola, 1999). During the second half of the 20th century Cheile Vârghişului area was the object of some faunistic studies concerning especially cavernicolous snail species. The most comprehensive work was published by A. Negrea (1994) - a list of the aquatic and terrestrial snail species known from the caves and adjacent areas in karst habitats of Romania, a synthesis of her previous studies and the data of Grossu (1981, 1983, 1987).

The aim of the present work is to analyse the land snail communities of Cheile Vârghişului Nature Reserve (Fig. 1).

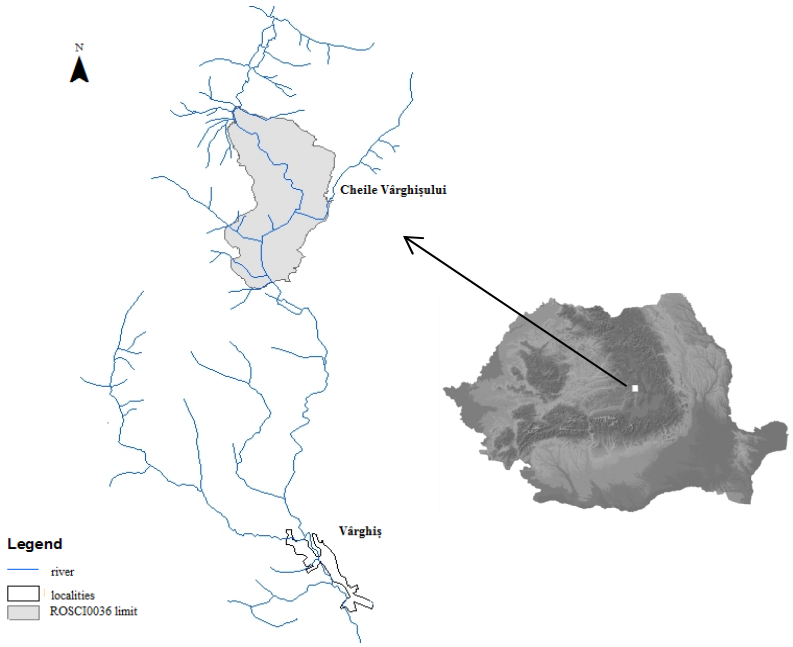

Figure 1. Location of the sampling area

Materials and methods

Three habitat types were sampled in June 2015: two of them located in an old beech forest - a rocky habitat in the forest (ST1, ST4), limestone cliffs in the forest (ST2, ST3) and exposed limestone cliffs (ST5). In the sampling point ST1 and ST4, the limestone outcrops had different sizes, smaller in ST1 and larger in ST4. The size of the limestone outcrops influences the habitat humidity. Habitats with larger outcrops have less vegetation and store more heat. We expected that species with high humidity demands are mostly found in habitats with small outcrops. Also the larger the outcrops, the more complex habitats are, providing a variety of shelters for snails.

At each site, snails from an area of approximately 200m^2 were collected by hand (two person hours by site). Additional about 20 l of leaf litter was sieved in each sampling point (Pokryszko and Cameron, 2005), and the material was sorted and identified in the laboratory. The works of Grossu (1981; 1983; 1987) and Welter-Schultes (2012) were used for species identification. The taxonomic list follows Fauna Europaea (Bank, 2013).

The list of species was registered. The number of living individuals and fresh empty shells were used to estimate snail abundance. The community structure was assessed using the relative abundance of each species. The presence/absence of snail species was used to build the Jaccard similarity diagram of the sampling stations (single linkage method, Euclidean distance). The variation of snail assemblages was analysed using Canoco 4.5 software (Ter Braak and Smilauer, 2002). An indirect gradient analysis detrended by segments (DCA) was first performed to establish the length of the gradients. Because the first gradient was 2.7 we used the linear ordination method (Leps and Smilauer, 2003). The relationships between species were investigated by means of the principal component analysis (PCA). The samples were plotted in the ordination space considering the first two axes. Species variables (the number of collected individuals) were log-transformed by the relation $y' = \log(y+1)$, and they were analysed based on inter-species correlation, divided by standard deviation, the data being centred by species. The analysis was performed on the entire snail community as well as on Hygromiidae and Helicidae species, because the representatives of the two families are gravimetrically dominant in this community. In the diagram illustrating the snail community, only the species with the best fit were represented.

Results and discussion

The systematic list is presented below. Since the only previous work referring specifically to the snail fauna of Cheile Vârghișului is that of A. Negrea (1994), the list contains also the results presented in her paper (Table 1). In total, 43 land snail species were found in the area. This number is similar to that found in the Iron Gates, for a much larger area (Gheoca, 2014). Among the identified species, 21 are listed for the first time. Also, five of the species listed by Negrea were not found during this study. Some of the differences could be due to the nature of the sampling, as a result of the fact that in the previous work were sampled the caves and the adjacent area, with some permanently wet patches. Others could be the result of errors in identification as is the most probable the case of *Cattania trizona*. Most probably its citation by A. Negrea is the result of a confusion with *Faustina faustina*, since the distribution of *C. trizona* in our country is limited to the south-western part (Banat). It is unlikely that *C. trizona* was found in Cheile Vârgișului.

The most abundant species were *Truncatellina cylindrica, Laciniaria plicata, Carychium tridentatum, Ruthenica filograna, Faustina faustina* and *Alopia bogatensis*. The highest number of specimens were found on limestone cliffs located in forest, 1634 specimens of 31 snail species, while on small rocks the diversity was higher (38 species) but with a lower abundance (834 specimens).

Among the snail species found only on small rocks scattered in the forest, were *Vertigo pygmaea, Pupilla muscorum, Ena montana, Oxychilus draparnaudi.* Due to restrictive environmental conditions of the open habitat only 9 species were found here. *Cepaea vindobonensis* was the only species found exclusively in this type of habitat.

Table 1.

The systematic list of land snail species of Cheile Vârghișului. The historical record (Negrea, 1994), the present study, and the land snail species habitat preferences (for the species found in 2015) are presented. The codes for habitats are: 1 - rocky habitat in the forest; 2 - limestone cliffs in the forest; 3 - exposed limestone cliffs.

Family/Species	Negrea 1994	Present study	Habitat type
Aciculidae			
Platyla polita polita (W. Hartmann, 1840)	-	+	1,2
Acicula parcelineata Clessin 1911	-	+	2,
Carychiidae			
Carychium tridentatum (Risso, 1826)	-	+	1,2
Carychium minimum O.F. Müller,	+	-	
Cochlicopidae			
Cochlicopa lubrica (O.F. Müller, 1774)	+	+	1
Cochlicopa lubricella (Rossmässler, 1834)	-	+	1,2,3
Orculidae			
Sphyradium doliolum (Bruguière, 1792)	+	+	1,2
Argnidae			
Agardhiella parreyssii parreyssii (L. Pfeiffer, 1848)	+	-	
Valloniidae			
Vallonia costata (O.F. Müller, 1774)	+	+	1,2,3
Vallonia excentrica Sterki 1893	-	+	2,3
Acanthinula aculeata (O.F. Müller 1774)	+	+	1,2
Pupillidae			
Pupilla muscorum (Linnaeus, 1758)	-	+	1
Pyramidulidae			
Pyramidula pusilla (Vallot, 1801)	-	+	1,2
Chondrinidae			
Granaria frumentum (Draparnaud, 1801)	+	+	1,2,3
Chondrina arcadica clienta (Westerlund, 1883)	+	+	1,2,3
Vertiginidae			
Truncatellina cylindrica (A. Férussac, 1807)	+	+	1,2
Vertigo pusilla Müller, 1774	-	+	1,2,3
Vertigo pygmaea (Draparnaud, 1801)	-	+	

Table 1 continued

Enidae			
Ena montana (Draparnaud, 1801)	-	+	1
Merdigera obscura (O.F. Müller, 1774)	-	+	1,2
Clausiliidae			
Alopia bogatensis bogatensis (E.A. Bielz, 1856)	+	+	1,2,3,
Cochlodina laminata (Montagu, 1803)	+	+	1,2
Cochlodina orthostoma (Menke, 1828)	+	+	1,2,3
Ruthenica filograna (Rossmässler, 1836)	+	+	1,2
Clausilia dubia Draparnaud, 1805	-	+	1,2,3
Laciniaria plicata (Draparnaud, 1801)	+	+	1,2,3
Vestia elata (Rossmassler 1836)	+	-	
Bulgarica cana (Held, 1836)	-	+	1,2,3
Punctidae			
Punctum pygmaeum (Draparnaud, 1801)	-	+	1,2
Patulidae			
Discus perspectivus (Megerle von Mühlfeld, 1816)	+	+	1,2
Pristilomatidae			
Vitrea diaphana (S. Studer, 1820)	+	+	1
Vitrea transsylvanica (Clessin, 1877)	+	+	1,2
Vitrea crystallina (O.F. Müller, 1774)	+	+	2
Euconulidae			
Euconulus fulvus (O.F. Müller, 1774	+	+	1,2
Gastrodontidae			
Zonitoides nitidus (O.F. Müller, 1774)	+	-	
Oxychilidae			
Oxychilus draparnaudi (Beck, 1837)	-	+	2
Cellariopsis deubeli (A.J. Wagner, 1914)	-	+	1
Morlina glabra (Rossmässler, 1835)	+	-	
Aegopinella epipedostoma Fagot, 1879	-	+	1,2
Aegopinella pura (Alder, 1830)	+	+	1
Bradybaenidae			
Fruticicola fruticum (O.F. Müller, 1774)	-	+	1
Hygromiidae			
Euomphalia strigella (Draparnaud, 1801)	-	+	1,3
Petasina bielzi (E.A. Bielz, 1859)	-	+	1,2
Perforatella bidentata (Gmelin, 1791)	+	+	1
Urticicola umbrosus (C. Pfeiffer, 1828)	+	-	
Helicidae			
Cattania trizona (Rossmässler, 1835)	+	-	
Faustina faustina (Rossmässler, 1835)	+	+	1,2
Isognomostoma isognomostomos (Schröter, 1784)	+	+	1,2
Cepaea vindobonensis (Pfeiffer, 1828)	+	+	3
Helix pomatia Linnaeus, 1758	-	+	1,2,3

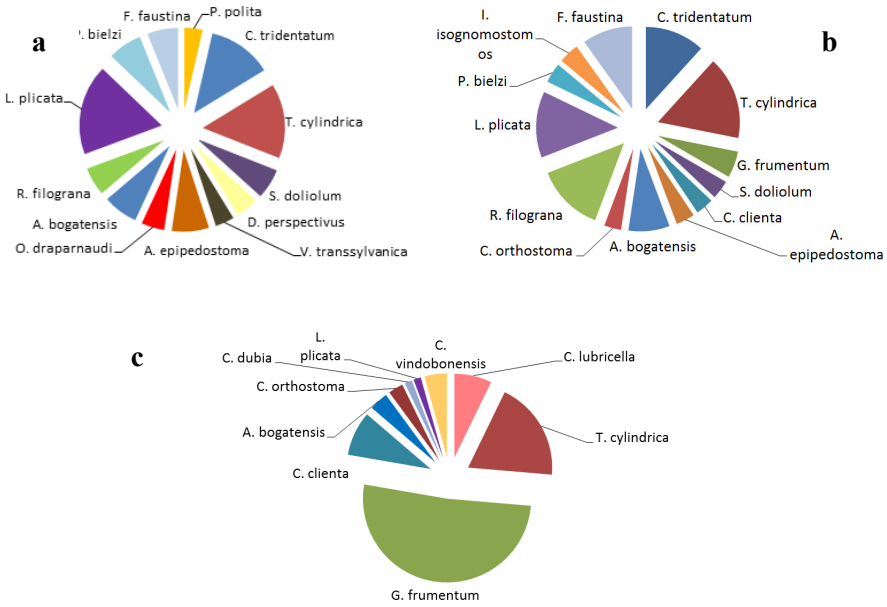

Figure 2. The relative abundance of the most common land snail species in the three habitat types a - rocks in the forest, b - limestone cliffs in the forest, c - exposed limestone cliffs . Only the species with over 2% abundance in each of the three habitat types were selected.

The relative abundance of land snail species considering the three types of habitats is represented in Figure 2. The forest habitat with scattered rocks is dominated by *L. plicata* and minute species like *T. cylindrica* and *C. tridentatum*. The limestone cliffs located in the forest offer shelter for typical forest species as *R. filograna, L. plicata, F. faustina, I. isognomostomos*, minute species as *T. cylindrica* and *C. tridentatum* and characteristic limestone species as *A. bogatensis, G. frumentum* and *C. clienta,* with large populations for most of the species. The exposed rocks are populated by typical limestone species as *G. frumentum,* which represents half of the community in this type of habitat, *T. cylindrica* and *C. clienta.* Most of the species found in the area are common land snail species, more or less confined to limestone. One of the most important exceptions is *Alopia bogatensis,* an endemic species for the Perşani Mountains. *A. bogatensis* develops large populations on limestone cliffs, being among the most abundant species in this type of habitat.

The tree diagram built on Jaccard similarity index (Figure 3), displays a cluster of the sampling points with limestone cliffs (ST2, ST3), which includes ST4, the forest habitat with large limestone outcrops. The exposed habitat is the most distinct (ST5).

Figure 4 exhibits a PCA biplot based on the abundance of land snail species. The two axes extract 72.9% of the species variation. Axis 1 displays the sampling points according to the homogeneity of the habitat.

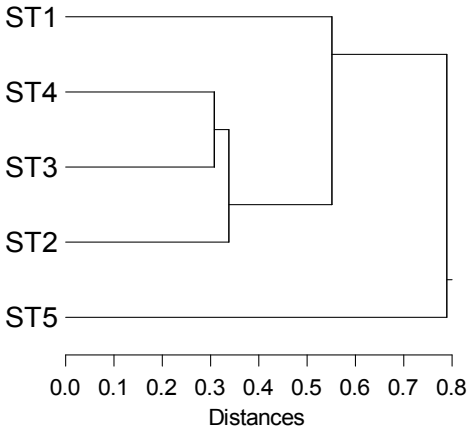

Figure 3. Hierarchical cluster analysis of sampling points based on the Jaccard similarity index between the ST1-ST5 sampling stations (Vârghișului Gorge, the Perșani Mountains, Romania).

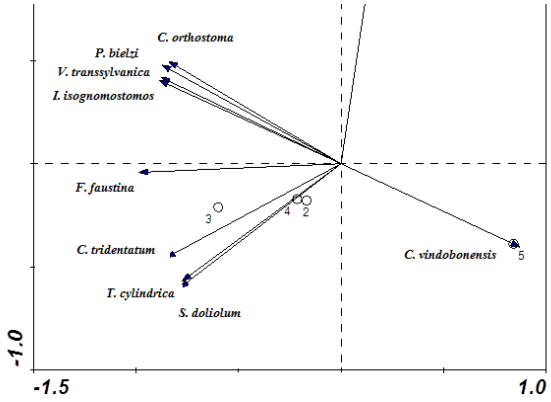

Figure 4. Result of the principal component analysis (PCA) of the land snail species from Vârghișului Gorge, the Perșani Mountains. The five sampling stations, 1-5, and the species with the best fit were represented.

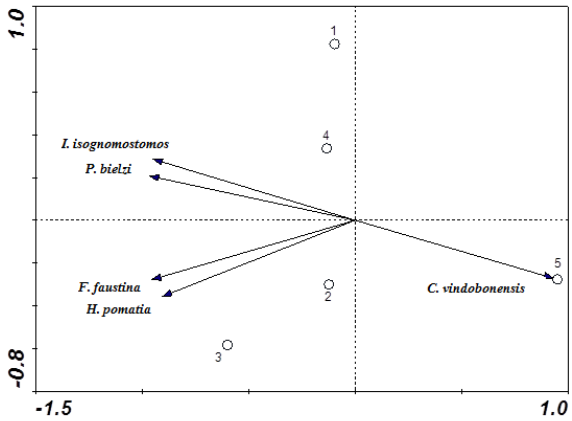

Figure 5. Result of the principal component analysis (PCA) of species Hygromiidae and Helicidae. The numbers represent the five sampling stations.

The presence of limestone blocks in the forest, combine the typical forest habitat with the diversity of microhabitats offered by these structures. Along the first axis, most of the species are associated with complex habitats. *C. vindobonensis* is the single species placed at the other extreme, being found only on exposed limestone walls.

The sampling points are displayed on Axis 2 according to their humidity. The most humid is the sampling point 1, the most typical forest habitat, while sampling point 5 is the most arid. The species' cluster display their water demands, with hygrophilous and typical forest species like *C. orthostoma, P. bielzi, I. isognomostomos,* having positive loadings on this axis, and limestone and xerophylous species (*T. cylindrica, S. doliolum, C. vindobonensis*) with negative loadings.

The PCA conducted on species of Hygromiidae and Helicidae families (Fig. 5), exhibits the discrimination among species regarding the type of the habitat. The first two axes extract 97.5% of the species variation. The first axis is given by humidity, separating the xerophylous *C. vindobonensis* from all the other species. The species' position along Axis 2 is correlated with the size of the limestone structures. *I. isognomostomos* and *P. bielzi* prefer forest habitat with small limestone outcrops, while *F. faustina* inhabit mostly cliffs. The affinity of *F. faustina* for cliffs in limestone habitat was confirmed before (Sólymos et al., 2009; Juřičková and Kučera, 2005). *H. pomatia* was found in this type of habitat together with *F. faustina*, although, the species has wide ecological amplitude.

Conclusions

The Cheile Vârghișului Nature Reserve is one of the most important karst areas of the eastern Carpathians. As usually in limestone habitats, the land snail species are developing large populations, especially the micro snails like T*runcatellina cylindrica*, and other species confined to limestone, like *Granaria frumentum*. However, the land snail communities in forest habitats, regardless the size of limestone outcrops, are dominated by clausiliids. Almost a third of the collected individuals belong to three species, *L. plicata*, *R. filograna* and *Alopia bogatensis,* the only species of the *Alopia* genus inhabiting the Perșani Mountains. The conservation of these endemic species depends on how well these limestone habitats are preserved. The diversity of the limestone habitat combined with the presence of a running water and the vegetation cover increase the species richness. Although a supplementary sampling would expand the number of taxa, we consider that our data capture an accurate image of the land snail community of this protected area.

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=== REVIEW ===

Challenges in the detection and identification of potato virus Y, an important pathogen of potato

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SUMMARY. Potato virus Y (PVY) has become one of the most important pathogens of potato. Being a RNA virus, one of the main characteristic is it's great genetic variability. Several well characterized strains du exist but the continuous emergence of new forms, the fast spreading of the existent ones and the shifting toward the increased prevalence of the recombinant necrotic strains raised a huge interest in finding ways for reducing the propagation of the disease. Two important strategies were adopted aiming for the same outcome, the reduction of PVY incidence. The first one, consists in improving the genetic background of potato and creating cultivars resistnt to PVY. This objective may be achieved by the integration of PVY resistance genes into potato gene pool. The second one, and this will be the subject of this review, relies on the finding of suitable methods for PVY detection which has to be fast and economical competitive for being applied to a large scale. The seed certification represents the most important step for multiannual and/ or interregional or international PVY spread prevention. There is no tolerance for necrotic PVY strains in seed, especially in the case of those batches that are obtained by biotechnology, and therefore, the screening for PVY became part of the protocol for potato seed certification.

Keywords: bioassay, ELISA, molecular detection, PVY^{NTN}, PVY^{N-Wi}

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Introduction

Potato is one of the most important crops ranking on the fifth place in the world as human food source after wheat, rice, corn and barley (Fageria *et al*., 1997). It is considered one of the key elements in nutrition having in the history an important contribution to the population growth and prosperity (Nunn and Qian, 2011). In the current context, in which the population is growing continuously, the need for food leads to a higher pressure for increasing the productivity in agriculture but without the possibility of expanding the cultivated areas. In addition, we assist to climate changes that endanger the crop production, due to both, abiotic and biotic stress, the last one being caused in particular by modification of plant-pathogen relationship (Halterman *et al*., 2012). The potato virus Y (PVY) has become lately one of the most threatening pathogens of potato that can lead to significant financial losses (Karasev and Gray, 2013).

PVY is transmitted mechanically but its most significant propagation is made by aphids in a non-persistent manner. An exception to this way of transmission was encountered in the case of a substrain of PVY^C (Blanco-Urgoiti *et al*., 1998 a). This mode of transmission is the main limiting factor in stopping the spread of infection by applying insecticides, a very short probing of the aphids being enough for transmitting the virus before insecticide to become effective (Hühnlein *et al*., 2013). Early PVY detection and prevention of the cultivation of contaminated potato seed represents the main strategies for limiting the PVY spread besides the utilization of PVY resistant cultivars, the last one not being the subject of this review. The PVY detection starts in the field and immediately after harvest, based on the overt symptoms (Nolte *et al*., 2004). But the detection based on phenotype manifestations is not always easy due to the variety of strains, the emergence of new recombinant PVY forms who have new symptoms, sometimes not obvious, depending on the cultivars and climatic factors (Ellis *et al*., 1996; Rykbost *et al*., 1999; James *et al*., 2003; Nolte *et al*., 2004; Schubert *et al*., 2007; Hühnlein *et al*., 2013). For an effective management of PVY control it is mandatory that precise biochemical and molecular methods of detection and identification are established, regardless on the fact that they are addressed to: the preventive screening of the potato seed (Halterman *et al*., 2012), the rapid identification during epidemics, determination of the aphid vectors in the field (Singh, 1998) or to test for the contamination of the water in the case of hydroponic cultures (Mehl *et al*., 2014).

The analysis of PVY in potato follows two close directions. The first one, based on PVY sequencing is necessary for full characterization of strains (Robaglia *et al*., 1989; Singh and Singh, 1996 b) and it's imposed each time when strains with atypical biological, serological or molecular features are found (Thole *et al*., 1993; Nie and Singh, 2003 a; Chikh Ali *et al*., 2007; Lorenzen *et al*., 2008; Hu *et al*., 2009 b; Chikh Ali *et al*., 2013 a) or when PVY incidence in a new geographical area is explored (Ohshima *et al*., 2000; Chikh Ali *et al*., 2007; 2013 a; Ogawa *et al*., 2008; Schubert *et al*., 2014).

Such studies are meant to reveal the degree of PVY variability due to mutations/ recombination (Ogawa *et al*., 2008) or the origin of certain strains (Lorenzen *et al*., 2006 a; Nie and Singh, 2002 a, 2003 a). Eventually, the findings of such studies can lead to the understanding of the molecular evolution of PVY offering the opportunity to find the important features of the virus biology, especially of the virulence determinant factors (Tribodet *et al*., 2005; Hu *et al*., 2009 b). The second direction, rely on the previous one findings, aims to establish methods for rapid identification of the virus during the routine purposes such are PVY detection and indexing in the potato seed certification protocol (Halterman *et al*., 2012). In addition, these studies follows to determine the geographical distribution of different strains aiming the localization of virus outbreaks source (McDonald and Kristjansson, 1993; Piche *et al*., 2004; Ogawa *et al*., 2008) which is an essential information for developing strategies to control the spread of infection.

In this review we will make a quick scanning of the methods addressed in time for the identification and characterization of PVY and we will highlight in particular those assays that offer the greatest advantages for the routine screening in the seed certification programs and fast PVY indexing.

The molecular structure, genetic variability and nomenclature of PVY

PVY belongs to the genus *Potyvirus*, *Potyviridae* family. All the members of this family include cylindrical, flexible viruses (Glais *et al*., 1996). PVY genome consists in a single strand of positive sense RNA molecule of about 10 kb with a VPg protein attached to the 5' end and a poly A tail to the 3' end. Viral RNA encodes a single large polypeptide molecule which is subsequently cleaved by three proteases of viral origin in ten proteins (review of Quenouille *et al*., 2013). The viral proteins (Fig. 1) are represented by P1 which is among the most variable proteins within the PVY strains (Marie-Jeanne Tordo *et al*., 1995), protease helper component (HC-Pro), protein P3, inclusion cellular protein (CI) flanked by 6K2 and 6K1 proteins, genome-linked viral protein (VPg), first nuclear inclusion protein (NIa), second nuclear inclusion protein (NIb) and coat protein (CP) (Hühnlein *et al*., 2013).

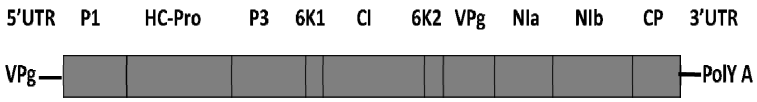

Figure 1. PVY genome structure (after Hühlein *et al*., 2013); VPg - viral genome linked protein, Poly A - poly A tail, P1- P1 protein, HCPro - helper component protease, P3 - P3 protein, Ci - cellular inclusion protein, NIa - nuclear inclusion protein a, NIb - nuclear inclusion protein b, CP - coat protein

Table 1.

The main PVY strains (after Singh *et al*., 2008, with modifications)

Strain name	Hypersensitive reaction in potato Yes (gene)/ No	Serotype	Bioassay in tobacco	PTNRD	Synonymous codes
PVY^{O}	Yes (*Ny*)	O	M, VCL	No	PVY^{O5}
PVY^{N}	No	N	VN	No	PVY^{EU-N}, PVY^{NA-N}, $NA\text{-}PVY^{N}$, PVY^{R}, PVY^{-TVN}
PVY^{C}	Yes (*Nc*)	O	M, VCL	No	PVY^{C1}, PVY^{C2}
PVY^{Z}*	Yes (*Nz*)	O or N	M, VCL	Yes, occasionally	$PVY^{Z\text{-}NTN}$
PVY^{E}**	No	N	M, VCL	Yes	PVY^{ZE}
PVY^{D}***	Yes (putative *Nd*)	NA	M, VCL	NA	
PVY^{NTN}	No	N	VN	Yes	$EU\text{-}PVY^{NTN}$, $Eu\text{-}PVY^{NTN}$, $PVY^{EU\text{-}NTN}$, PVY^{NN} $PVY^{NA\text{-}NTN}$, $NA\text{-}PVY^{NTN}$
$PVY^{N\text{-}Wi}$	No	O	VN	No	$PVY^{N\text{-}Wilga}$, $PVY^{N\text{-}W}$, PVYN-Wi-P, $PVY^{N:O}$

M – mosaic, NA – not assessed, PTNRD - potato tubers necrotic ring spot disease,
VCL – vein clearing, VN – vein necrosis
* PVY^{Z} described after Jones, 1990; Blanco-Urgoiti *et al*., 1998 b; Kerlan *et al*., 1999; 2011
** PVY^{E} described after Ggalvino-Costa *et al*., 2012
*** PVY^{D} described after Kehoe and Jones, 2015

Their role in establishing the infection, polyprotein processing, replication, translation, virion assembly, cell-to-cell and systemic movements and aphid transmission is partially known (reviewed by Dougherty and Carrington, 1988; Quenouille *et al*., 2013).

PVY was initially classified, based on the pathotype and the hypersensitivity reactions (HR) resulted by elicitation of the resistance genes present in certain cultivars of potato, in several strains named O, N, C, Z, E (review of Singh *et al*., 2008) (Table 1). PVY^{O}, the common form of PVY (O from "ordinary") is characterized by the induction of HR in cultivars carrying Ny gene, produce mosaics in tobacco plants and various degrees of mosaic in the most varieties of potato. Necrotic PVY (PVY^{N})

overcomes all known *N* genes, causes tobacco systemic vein necrosis (TVN) but in potato the symptoms can be invisible or may manifest as mild mosaic. PVY^{C} leads to HR in the cultivars having *Nc* gene and systemic mosaic in the rest of potato varieties (Karasev *et al*., 2011).

The PVY^{Z} strain was accepted based on the induction of HR in cultivars carrying the putative gene *Nz* (Jones, 1990; Blanco-Urgoiti *et al*., 1998 b; Kerlan *et al*., 1999; 2011) and now proved to be a true resistance gene (Chikh Ali *et al*., 2014). It seems to be a recombinant form between PVY^{O} and PVY^{N} having a serotype O, the restrictotype of coat protein like PVY^{O}, but a RFLP pattern at the 5' end similar with PVY^{N} (Blanco-Urgoiti *et al*., 1998 b). Another recombinant form of PVY assigned to the PVY^{E} strain overcomes all the resistance genes (*Ny*, *Nc* and putative *Nz*) in potato cultivars but in the same time it does not induce TVN (Galvino-Costa *et al*., 2012). Recently, a new isolate of Australian origin was found, manifesting new biological features. It is eliciting a putative *Nd* resistance gene apparently present in the cultivars King Edward, Russet Burbank and White Rose inducing the HR (Kehoe and Jones, 2015). Although it has a genome similar with the PVY^{C} strain based on the HR it can be classified in a new strain, PVY^{D} (Kehoe and Jones, 2015) (Table 1).

In the '80s, new PVY^{N} isolates were reported in Europe and later in North America. One of them, named PVY^{NTN}, induce tuber necrosis causing potato tubers necrotic ring spot disease (PTNRD) (Beczner *et al*., 1984). A few years later, another PVY variant was discovered, named PVY^{N-Wi} (noted after Wilga, the potato cultivar from which was isolated) which induce only mild phenotype in potato but it is responsible for braking the resistance in some potato cultivars (Chrzanowska, 1991). The new PVY strains, PVY^{N-Wi} and PVY^{NTN}, have a great variability. They are supposed to be derived by recombination between PVY^{O} and PVY^{N} strains having one or two recombination junctions in the case of PVY^{N-Wi} (Glais *et al*., 2002; Lorenzen *et al*., 2008) and 3 - 4 in the case of PVY^{NTN} giving at least nine recombination patterns (Hu *et al*., 2009a). PVY^{N-Wi} has also a great variability at the molecular level, especially in the 5' UTR (untranslated region) - P1 region resulting three lines (Chachulska *et al*., 1997; Glais *et al*., 2002; 2005). The first contains isolates that are similar to PVY^{N} at the 5' UTR - P1 end, the second includes isolates that are similar to PVY^{O} in the sequence of P1 and the third contain one isolate having a sequence PVY^{O}-like in the 5' UTR - P1 region. Despite of this heterogeneity in the region P1 of PVY^{N-Wi} the rest of the genome is similar, with that of PVY^{O} serotype. This heterogeneity resulted from the recombination at the C-terminus of HC-Pro region. To some extent there are similarities between the sequences of PVY^{N-Wi} and PVY^{NTN}, but unlike to the first one, in the case of PVY^{NTN} the recombination occurred at several points in the genome (Boonham *et al*., 2002; Glais *et al*., 2002). Almost similar with PVY^{N-Wi} (of European origin) is the American form noted $PVY^{N:O}$ (Singh *et al*., 2003; Nie and Singh, 2003 b) but this one is supposed to be derived by recombination with a different line of PVY^{O} compared with the European form (Karasev *et al*., 2011).

A different PVY^{NTN} isolate, Tu 660, originating from North America called NA-PVY^{NTN} is believed to have resulted by mutagenesis and not by recombination (Nie and Singh, 2003 a). Isolates with similar pathotype were reported in Japan also (Ohshima *et al*., 2000) but a later analysis revealed that those were non-recombinant PVY^{NTN} variants as well (Ogawa *et al*., 2008). An isolate of PVY having similarities with the European PVY^{NTN} recombinant form, induce HR in Maris Bard cultivar carrying the putative *Nz* gene, thus being classified in the PVY^{Z} group being called $PVY^{Z\text{-}NTN}$. Although it is capable of inducing PTNRD similarly to PVY^{NTN}, the TVN was not associated with it (Kerlan *et al*., 2011). It still have the molecular determinants in the HC-Pro (K-400 and E-419) that have been considered responsible for inducing of the TVN phenotype (Tribodet *et al*. 2005), but the substitution of a single amino acid (D-205 to G-205) was correlated with loss of this phenotype, so the strain causes only mosaic symptoms and mild vein clearing in tobacco rather than TVN characteristic to the PVY^{NTN} strain (Hu *et al*., 2009 b). In Syria, there were detected recombinant forms of PVY which show both genomic and biological features intermediate between PVY^{NTN} and $PVY^{N\text{-}Wi}$ (Chikh Ali *et al*., 2007 a) named PVY-SYRIII and $PVY^{NTN\text{-}NW}$ (Chikh Ali *et al*., 2010 a). These strains present three recombinant junctions HC-Pro/ P3, 6K2/ VPG, NIb/ CP, but at the 3' end the recombination junction occurs at different nucleotides resulting at least three different viral subtypes. All variants, excepting one who wasn't tested on potato yet, produce PTNRD and TVN but have O serotype (Chikh Ali *et al*., 2010 a).

The emergence of the new PVY forms, some of them presenting several recombination patters between the parental lines and inducing new phenotypes in the host plants impose an improved PVY classification which has to include serological and molecular criteria along to the first classification criteria which are based mainly on the presence or not of the HR and TVN response in the indicator plants (review of Singh *et al*., 2008; Karasev and Gray, 2013; Kehoe and Jones, 2015).

Biological assay

The main indicator plants for the presence of PVY are *Nicotiana tabacum* (Samsun varieties, NC95, NC in 2326, Burley 21), *Lycopersicon esculentum* (cv. Sheyenne), *Capsicum frutescens* (cv. Calwonder), *Solanum tuberosum*, *S. demissum*, *Physalis floridana*, *Physalis angulata*, *Chenopodium amaranticolor*, *C. quinoa* (McDonald and Kristjansson, 1993). The general symptoms induced in the indicator plants are vein clearing, vein necrosis, mottle, mosaic, interveinal necrosis, leaf drop, systemic necrosis or cupping of uninoculated leaves (McDonald and Kristjansson, 1993).

The symptoms induced in various indicator plants may give a clue about the viral strain involved. For instance, PVY^{O} and PVY^{C} induce local lesions in *C. amaranticolor* but this remains asymptomatic to the infection with PVY^{N} (Yin *et al*., 2012). For the detection of PVY necrotic forms (PVY^{N}) different species and cultivars belonging to

Nicotiana genus are used as indicators. *N. tabacum* cv. White Burley shows symptoms after 10-14 days, in the *Nicotiana affinis* cv. Lime Green the first symptoms appear after 5-10 days (Rose et al., 1987) but also *N. tabacum* cv. Samsun (10-21 days symptoms appearance) or Xanthi, can be used (Singh and Singh, 1994). *S. brachycarpum* is also a good differentiator between different PVY strains, being affected by necrosis when inoculated with PVY^{N} and mosaic in the case of infection with PVY^{O}, giving a relatively fast reaction (7 - 10 days) and offering the advantage of a lack of interference with PVX if a mixed infection do exist (Singh and Singh, 1994).

Of a particular importance for PVY indexing are the potato varieties that possess known resistance genes such are Désirée, Pentland Crown, Delikat, Maris Bard, Pentland Ivory, Allegany having *Ny* gene, Pentland Ivory or Maris Bard with *Nc*, *Ny* and *Nz* genes or King Edward, Maris Bard, Pentland Ivory, Eersteling having *Nc* gene (review by Singh *et al*., 2008; Blanco-Urgoiti, 1998 b; Kerlan *et al*., 1999; Baldauf *et al*., 2006; Chikh Ali *et al*., 2014). Depending on the resistant gene the HR appears at the interaction with specific PVY strains (Table 1). The cultivar Eva has $Ry_{adg,}$ an extreme resistance gene (derived from *S. tuberosum* subsp. andigena) that confer resistance to all the PVY strains (Baldauf *et al*., 2006) and also the Sante cultivar having the resistance gene Ry_{sto} form *S. stoloniferum* (Mehle *et al*., 2004). On the contrary, Atlantic and NY115 cultivars have no resistance to any form of PVY and may serve as susceptible controls (Baldauf *et al*., 2006).

Although important for detection and PVY indexing the bioassay tests are not infallible. Very often different strains can induce atypical phenotypes in the indicator plants. The PVY^{N} usually does not induce local lesions in *C. amaranticolor* but there are isolates that can produce these lesions (Yin *et al*., 2012). Also, the North American correspondent for PVY^{N-Wi}, $PVY^{N:O}$, usually do not cause tuber necrosis but this affection was observed under the form of an atypical PTNRD (Piche *et al*., 2004) and the uncommon symptoms were spotted later by Baldauf *et al*. (2006). Another situation is of PVY^{NTN} isolate L26, which despite of the fact that manifest an N positive serotype cannot induce TVN (Hu *et al*., 2009 b).

The biological assay can thus be inconclusive due several reasons such are the conditions in the greenhouse (Baldauf *et al*., 2006), the existence of mixed natural infections (Damirdagh and Ross, 1967), the possible contamination during analysis (Rose *et al*., 1987) or to the occurrence of new isolates that can give atypical reactions in the indicator plants (Baldauf *et al*., 2006).

Detection by immunological methods (ELISA)

As a routine, the first indicators of PVY infection are the symptoms usually present in the host. Clark and Adams (1977) established the microplate method of enzyme-linked immunosorbent assay (ELISA) for virus detection proving its versatility for a series of viruses. The method was adapted for PVY detection in potato as well.

First, there were established polyclonal antibodies PVY specific but very often the ELISA contradicted the bioassay results giving false positive or negative results and raising questions about its reliability (Rose *et al*., 1987). Subsequently, the production of a large number of more and more improved sets of monoclonal antibodies (Rose and Hubbard, 1986; Sanz *et al*., 1990; Ellis *et al*., 1996; 1997; Cerovska, 1998) and the application of different ELISA variants (triple antibody sandwich-ELISA, direct and indirect double antibody sandwich enzyme-linked immunosorbent assay, nitrocellulose membrane - ELISA) (Gugerli and Fries, 1983; Rose and Hubbard, 1986; Ohshima *et al*., 1990; Sanz *et al*., 1990; Ellis *et al*., 1996) lead to a significant increase in sensitivity and specificity being possible to distinguish between different groups of strains (Rose *et al*., 1987; Ellis *et al*., 1997; Blanco-Urgoti *et al*., 1998; Lizarraga and Fernandez-Northcote, 1989; Lorenzen *et al*., 2006). The monoclonal antibodies that give the best results in the tests were included in commercially available kits (Bioreba, Agdia, SASA, Neogen). ELISA has become a routine method for PVY detection (Nolte *et al*., 2008), now being available even immuno-strips that simplifies the test, enabling their execution in field (Halterman *et al*., 2012). By using monoclonal antibodies the serotype of PVY isolate can be determined. Accordingly with Ellis *et al*., (1997) a serotype represents a "subclass of the viral strain group with distinguishable antigenic specificities" and although the classification in strains and serotypes is arbitrary they contribute to a better characterization of the PVY isolates.

ELISA can be performed directly at the tuber level, a positive reaction being obtained after breaking the dormancy of the tubers while in the dormant state the PVY titer can be too low, sometimes under the limit of detection and leading to false negative results (Gugerli and Gehringer, 1980; De Boks and Cuperus, 1987; Barker *et al*., 1993). As a consequence, the North American Plant Protection Organization (NAPPO) and European and Mediterranean Plant Protection Organization (EPPO) which implemented ELISA tests as a mandatory step in the seed certification protocol, strongly recommend the utilization in the assays of sprouts and leaves from plants grown from sample tubers instead of using directly the sap from tubers in order to ensure a high concentration of virus and to prevent the false negative results.

However, the use of ELISA has certain limitations, by this method is not possible, for example, the differentiation of PVY^{O} from the necrotic one PVY^{N-Wi}/ $PVY^{N:O}$ (Crosslin *et al*., 2005; Lorenzen *et al*., 2006). Also, PVY^{N} and PVY^{NTN} cannot be distinguished as separate strains be ELISA (Rigotti and Gugerli, 2007; Karasev *et al*., 2010). The emergency of new recombinant isolates, other than PVY^{N-Wi} that induce TVN but not react with expected N specific monoclonal antibodies (Galvino-Costa *et al*., 2012) raises serious concerns for the efficiency of immunological analysis. A reverse situation was encountered in the case of PVY^{O5} which although have a typical PVY^{O} genotype and pathotype reacts with 1F5, a PVY^{N} specific monoclonal antibody, having apparently N serotype (Karasev *et al*., 2010). This confusion is due probably to an amino acid substitution in the CP that results in an

epitope recognized by 1F5. Luckily another N specific monoclonal antibody, SASA-N, gives the expected reaction assigning the strain to the adequate serotype, which is O (Karasev *et al*., 2010).

PVY detection/ indexing by molecular methods

The prerequisite for molecular detection of PVY is the unrestricted access to a database meant to provide the informative support. This starting point has become more and more solid, complete or partial genome sequences being available for several PVY isolates in the National Center for Biological Information (NCBI) GenBank repository. New powerful methods may be used in perspective for PVY sequencing. The next generation sequencing (NGS) which allows the whole genome sequencing became sensitive enough to identify different sequences of substrains of the same virus from mixed infections (Kehoe and Jones, 2015). Now, that it becomes more and more financially accessible it is predictable that it will be extensively used for finding the sequence of new isolates, the only limitation of the method being the huge amount of data resulted after analysis that requires a high amount of time for processing (Kehoe *et al*., 2014).

The molecular detection was a continuous process that was mainly based on PCR (Polymerase Chain Reaction) method which has to be constantly adapted to the detection of more and more PVY isolates and to assign them to the proper strain group. For the pre-PCR preparations, obtaining of a high quality RNA in regard to integrity, quantity and purity was a struggle but step by step all the drawbacks were addressed and overcome. Nowadays, this is no longer a problem regardless of the starting plant tissue (leaves or tubers), an accurate identification being possible using sap of dormant potato tuber (Agindotan *et al*., 2007), RNA extraction kits or ready to use reagents being now available (Chikh Ali *et al*., 2007 a; Kogovsek *et al*., 2008; Gawande *et al*., 2011; MacKenzie *et al*., 2015). The purification of the virus is no longer a requirement for an accurate indexing (Lorenzen *et al*., 2006 a). Of equal importance, the reverse transcription (RT) step was optimized in regard to the chosen PCR protocol, by using PVY specific primers (Glais *et al*., 1996: Singh *et al*., 1998 b; Weilguny and Singh, 1998; Moravec *et al*., 2003; Lorenzen *et al*., 2006 b) or random hexamers (Nie and Singh, 2001; 2003 b) and/ or oligo(dT) (Nie and Singh, 2000; 2003 b; Chikh Ali et al., 2010 b) in one step (Rigotti and Gugerli, 2007; Crosslin *et al*., 2005; Kogovsek *et al*., 2008) or two steps RT-PCR approaches (MacKenzie *et al*., 2015).

The first concern was to distinguish PVY among several other viruses infecting potato (Potato virus Y, Potato leafroll virus, Potato virus A, Potato virus X, Potato mop-top pomovirus, Tobacco rattle tobravirus, etc), but this wasn't such a heavy task due to the high degree of genetic variability among the species, several methods being established (Crosslin and Hamlin, 2011) including multiplex qPCR (quantitative PCR)

based ones (Boonham *et al*., 2000; Agindotan *et al*., 2007). The greatest fight was made for an accurate PVY detection/ indexing within the PVY clades and in this review the most significant molecular methods will be highlighted. For the purpose of developing methods that in perspective may be applied at an industrial level some criteria have to be accomplished, they have to be efficient in terms of time and costs requirements but without compromising the high sensitivity and specificity.

In the first approaches for PVY detection/ indexing the PCR was coupled with RFLP (Restriction Fragment Length Polymorphism) technique. Using the 5' end of PVY genome sequence, including the UTR and the P1 protein and several restriction enzymes (Taq I, Ava H and Hinc H) the assignation of o series of PVY isolates to the common PVY^{N} or PVY^{NTN} groups was accomplished (Glais *et al*., 1996). Later, more specific enzymes were found for the differentiation between common necrotic PVY forms and PVY^{NTN} (Rosner şi Maslenin, 1999) or between North American and European PVY^{NTN}, including the non-recombinant PVY isolate (Nie şi Singh, 2002 a). Blanco-Urgoiti *et al*., (1996), using available sequences of CP gene, pointed out the equal importance of RFLP in genotyping of the PVY strains. The term "restrictotype" was proposed, suggesting that the pattern resulted by RFLP can be a used for the calculation of genetic distance as a viable alternative to the classification of PVY based on sequence comparison (Blanco-Urgoiti *et al*., 1996). Although precise and bringing significant steps forward in PVY indexing the RFLP technique is considered expensive and time-consuming imposing the need to find more simple and fast ways for PVY identification (Rigotti and Gugerli, 2007).

Some studies focused on finding a specific way of identification of a particular strain of PVY while others, using combinations of primers in individual or in multiplex reactions offered solutions for indexing of a series of PVY strains or substrains. The most challenging but extremely important is the detections of the recombinant substrains or pathotypes of PVY, especially of the PVY^{NTN} which affects the tubers quality and is lately arising with an increased frequency worldwide (Weidemann and Maiss, 1996; Karasev and Gray, 2013).

Several methods were established for PVY^{NTN} indexing by RT-PCR. Targeting P1 protein using three-primer combination suitable methods were developed to differentiate detection of PVY^{NTN} of European origin and PVY^{N} (Weidemann and Maiss, 1996; Weilguny and Singh, 1998; Singh *et al*., 1998 b). Nie and Singh (2002 a) have shown, by sequencing the 5' region (comprising 5'- UTR and P1 cistron) of PVY^{N} and PVY^{NTN} from Europe and North American that the PVY^{N} and PVY^{NTN} clustered better together if they originate from the same region. A three-primer combination was established for specific differentiation of NA-PVY^{NTN} from EU-PVY^{NTN} (Nie and Singh, 2002 a). This primer set was adopted by North American Plant Protection Organization (NAPPO, 2011) for differentiation of PVY^{NTN} by other forms of necrotic PVY, EU-PVY^{NTN} including. Later, the method was improved by optimization of a multiplex PCR allowing simultaneous detection of strains or substrains of PVY

from any combination of PVY^{O}, EU-$PVY^{N/NTN}$, NA-PVY^{N} and NA-PVY^{NTN} (Nie and Singh, 2002 b). The recombination points within the CP region were also used as a marker for PVY^{NTN} indexing. By performing 4 sets of PCR primer mixes in a competitive PCR combined with mutagenically separated PCR the synthesis of enhanced specific bands was promoted leading to the discrimination between PVY^{NTN}, PVY^{O}, PVY^{N}, and PVY^{C} (Boonham *et al*., 2002). Moravec *et al*. (2003) adopting a three-primer strategy were able to differentiate recombinant PVY^{NTN} among the other PVY variants, targeting the CP region as well.

Exploiting the recombinant point within the HC-Pro/P of the PVY genome a RT-PCR reaction was designed for specific detection of PVY^{N-Wi} strain, from mixed PVY^{NTN} / PVY^{N-Wi} infections (Glais *et al*., 2005). This method is fast and can be successfully used for potato seed certification being officially applied for this purpose in France (Glais *et al*., 2005). Nevertheless, the protocol requires care, a 4114 pb product being obtained during amplification, and therefore, for preventing the RNA degradation the RT-PCR must be performed immediately after RNA isolation (Glais *et al*., 2005). For the differentiation of $PVY^{N:O}$ and PVY^{NTN} a triplex PCR was established, in which, based on the recombination sites within the PVY genome, one fragment is obtained for $PVY^{N:O}$ and three bands were amplified for PVY^{NTN} (Nie and Singh, 2003 b). The functionality of this method was validated in assays on isolates of different geographic origin and allowed the discovery of new type of isolates with an intermediate number of recombination points (Chikh Ali *et al*., 2007 a).

The most promising PCR based methods are those who facilitate the detection/ indexing of several strains or substrains in one multiplex reaction. Although multiplex methods for discriminating between PVY isolates were developed (Nie and Singh, 2002 b; 2003 b) some weaknesses were observed in identification from strain mixes from within the necrotic group (PVY^{N}, PVY^{NTN}, $PVY^{N:O}$, NA-PVY^{N} or NA-PVY^{NTN}) (Lorenzen *et al*., 2006 b). A touchdown multiplex PCR method in which eight different primers (Table 2) can be combined in different ways allows the detection of the main PVY strains (Lorenzen *et al*., 2006 b). Further, for the specific indexing of $PVY^{N:O}$ type A and B and for NA- and EU-$PVY^{N/NTN}$ discrimination specific sets of primers were in addition designed (Lorenzen *et al*., 2006 b). This became one of the methods extensively use for PVY indexing worldwide (Lorenzen *et al*., 2008; Chikh Ali *et al*., 2010 b; Karasev *et al*., 2010; Crosslin and Hamlin, 2011). In Europe, another method is that of Rigotti and Gugerli (2007) who made possible the detection of PVY^{N}, PVY^{O}, PVY^{NTN} (recombinant types), PVY^{N-Wi} and PVY^{C} in one-step triplex PCR (Table 2). One of the most comprehensive multiplex RT-PCR based detection (Table 2) developed by Chikh Ali *et al*., (2010 b) and further improved by coupling it with immunocapture instead of RNA extraction (Chikh Ali *et al*., 2013 b) allows the accurate detection of the main strains and substrains of PVY [PVY^{O} (both PVY^{O} and $PVYO^{O5}$), PVY^{N}, NA-PVY^{N}, PVY^{NTN}, PVY^{Z}, PVY^{E}, PVY-NE11, PVY^{N-Wi} and $PVY^{N:O}$].

Table 2.

Primers (after Lorenzen *et al*., 2006 b; Rigotti and Gugerli, 2007; Chikh-Ali *et al*., 2010 b) that can work in multiplex PCR for detection/ indexing of the main strains and substrains of PVY

Primer name	Sequence (5' - 3')	PCR results, amplicon/ strain
o2172	CAACTATGATGGATTTGGCGACC	181/ NTN, N:O
n2258	GTCGATCACGAAACGCAGACAT	267/ O
o2439c	CCCAAGTTCAGGGCATGCAT	398/ N
n2650c	TGATCCACAACTTCACCGCTAACT	328/ N,NA-N/NTN
n5707	GTGTCTCACCAGGGCAAGAAC	452/ NTN
o6266c	CTCCTGTGCTGGTATGTCCT	689/ N:O,O
S5585m	GGATCTCAAGTTGAAGGGGAC	
A6032m	CTTGCGGACATCACTAAAGCG	
PVYc3	CAACGCAAAAACACTCA(CT)AAA(AC)GC	440+1110/ N
PVYf	TAAGTG(AG)ACAGACCCTCT(CT)TTCTC	440+1110/ NTN(nR)
PVY3+	TGTAACGAAAGGGACTAGTGCAAAG	440/ NTN (R)
PVY3−	CCGCTATGAGTAAGTCCTGCACA	660+530/ O
CP2+	CCAGTCAAACCCGAACAAAGG	530/ Wi (Wi-P)
CP1−	GGCATAGCGTGCTAAACCCA	440+530/ Wi (N242) 660/ C
n156	GGGCAAACTCTCGTAAATTGCAG	853+532/ O
o514	GATCCTCCATCAAAGTCTGAGC	1307/ NA-N
n787	GTCCACTCTCTTTCGTAAACCTC	853+441/ NW(B)
n2258[a]	GTCGATCACGAAACGCAGACAT	1307+633+441/
o2172[a]	CAACTATGATGGATTTGGCGACC	NTN(A)
n2650c[a]	TGATCCACAACTTCACCGCTAACT	1307+441/ NTN (B)
o2700	CGTAGGGCTAAAGCTGATAGTAG	1307+633+441/
S5585m[a]	GGATCTCAAGTTGAAGGGGAC	NTN(A)
o6400	GTAACTCCTAAACAAATGGTGGTTCG	1076+633+441/ NTN-
n7577	ACTGCTGCACCTTTAGATACTCTA	NW ($^{SYR-I}$)
YO3-	CTTTTCCTTTGTTCGGGTTTGAC	1076 + 441/ NTN-NW
8648[b]	GTTTCTCCTATGTCGTATGCAAGTT	($^{SYR-II}$)
SeroN[c]		1076+441+278/ SYRIII

The need of developing detection protocols fast and safe, suitable for high throughput applications, lead to establishment of a series of methods by incorporating fluorescent dyes into the PCR assays. One of the first such methods was carried out by Walsh *et al*., (2001) who used competitive fluorescent RT-PCR assay for the differentiation of PVY^O and PVY^N. The great advantage of using fluorescent dyes lies in the possibility of performing so called "one-tube reactions" in which the detection can be made directly, no electrophoresis being required, advantage that was not provided by the pioneering method (Walsh *et al*., 2001). Later, by applying

real time PCR methods (qPCR) this disadvantage was overcome. The SNaPshot assay combined with TaqMan technology was used for discrimination between PVY^{O} and PVY^{N} (Jacquot *et al*., 2005) or PVY^{N}, PVY^{O}, PVY^{N-Wi} and PVY^{NTN} (Rolland *et al*., 2008). The high sensitivity of the methods based on fluorescent quantitative assays, regardless of the fact that TaqMan (Balme-Sinibaldi *et al*., 2006; Kogovsek *et al*., 2008), SYBR green (Hühnlein *et al*., 2013; Zhang *et al*., 2015) or multiplex qPCR (quantitative real time PCR) based on EvaGreen dye (Cheng *et al*., 2012) are involved, was pointed out during the assays, these being considered powerful tools for future PVY detection.

From the same category of the high throughput methods of detection highlighted above is the microarray test which is based on the hybridization of a probe attached to a solid surface with a target DNA (cDNA, in the case of PVY detection) which is labeled with a fluorescent dye. Its functionality was proven for the detection of several strains of PVY (Boonham *et al*., 2003). Although extremely powerful, the use of this method to a higher scale is restricted by the high costs associated to the fixation of the probe on the substrate, the purchasing of reading instruments and reagents and the maintenance procedures (Agindotan and Perry, 2007). The development of a more simple method, named macroarray (Agindotan and Perry, 2007) combined with the possibility of using synthetic probes (Bystricka *et al*., 2005; Sip *et al*., 2010) opened the way for using this procedure, in perspective, for the routine PVY detection/ indexing.

Another direction in the developing pathogen detections ways goes toward finding of easy to perform methods that can be applied directly in the field. The loop-mediated isothermal amplification (LAMP) assay, developed by Notomi *et al*., (2000), apparently qualifies for on-the-spot tests for pathogen detection (De Boer and Lopez, 2012). The method can be a substitute for PCR presenting the huge advantage of being an isothermal reaction that don't requires a termocycler and can be performed in one-tube, the versatility of the method depending only on the finding of the suitable primers. Such a method was settled for the detection of one PVY strain but although it meets all the requirements for sensibility and sensitivity the possibility of being performed in the field remains a problem to be solved (Przewodowska *et al*., 2015).

Conclusions

There has to be a continuous research activity, meant to keep up with the PVY evolution, which has to provide for the Agricultural Scientific Services reliable upgraded methods, including biochemical and molecular materials (monoclonal antibodies and specific primer sequences) for an accurate virus detection/ indexing in the continuous process of seed potato certification and quarantine. Although each assay, biological, immunological and molecular, independently offers valuable data

for PVY isolate characterization, the complete picture of a new emerged isolate that often results in a new classified strain, relies on an integrative view of the results of the combined analysis. If we refer to the main issue of the whole PVY related problem, which is the rapid detection/ indexing, the molecular methods approaches have become increasingly important and in the near future the implementation of their application in the routine screening will be mandatory due to the high variability of PVY for which accurate detection by ELISA simply will be not enough.

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=== SHORT COMMUNICATION ===

Effect of microaerobiosis on photosystem II in *Synechococcus* sp. PCC 7002

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SUMMARY. Cyanobacterial oxygenic photosynthesis transformed the early Earth's biosphere and resulted in complex aerobic life forms. During the course of evolution, cyanobacteria retained many genes that responded to specific environmental cues. Our study shows that microaerobic treatment did not significantly alter the functionality of photosystem II complex in *Synechococcus* sp. strain PCC 7002. Surprisingly, no changes in the induction levels of *psbA* genes, especially in D1' isoform, were recorded. This finding is important as signals an atypical behaviour of D1' isoform from *Synechococcus* sp. PCC 7002 to microaerobic stress as compared to other cyanobacterial strains.

Keywords: Chlorophyll, Cyanobacterium, Fluorescence, Microoxic, *psbA*, RT-PCR

Abbreviations: PSI - photosystem I, PSII - photosystem II, Q_A- first quinone acceptor in PSII, Q_B - second quinone acceptor in PSII, DCMU - (3-(3,4-dichlorophenyl)-1,1-dimethyl urea), qRT-PCR - quantitative reverse transcriptase polymerase chain reaction, O_2 - oxygen, UV-B - ultraviolet radiation B

Introduction

Cyanobacteria evolve low oxygen concentration under several habitats such as hot springs and in specially modified cells, where photosynthetic rate is limited (Voorhies *et al*., 2012). Their adaptation to these anaerobic environments and their transition to aerobic conditions and *vice versa* remains important in understanding

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the regulation of photosynthesis (Summerfield *et al*., 2011). On the scale of complexity of photosynthesis, contemporary cyanobacteria resemble closely to higher plants (Mulkidjanian *et al*., 2006). The cyanobacterial oxygenic photosynthesis is a multiplex cumulative process performed by diverse pigment-protein complexes located in thylakoid membranes inside cyanobacteria (Blankenship, 2014). These vital protein complexes include photosystem II (PSII), NADH-quinone oxidoreductase complex-1, Cytochrome b_6f and photosystem I (PSI) (Pakrasi *et al.*, 1985; Chis *et al.*, 2014). The PSII plays crucial role of catalyzing the splitting of water and leads to the generation of 4 electrons (Mulo *et al.*, 2009). The components at PSII donor side shuttles electrons to the acceptor side via different cofactors, many of them being coordinated by the core D1 protein, which is coded by the *psbA* gene (Rast *et al*., 2015).

Cyanobacteria variably contain 1–5 copies of *psbA* coding for 1–3 unique D1 isoforms per species (Wegener *et al*., 2015). The D1 isoforms are grouped as D1m, D1', D1:1, and D1:2 (Sicora *et al*., 2006). D1m is a protein isoform regularly expressed under normal growth conditions and induced under stressful conditions such as various environmental cues (Sane *et al*., 2002). The D1:1 is expressed during normal growth conditions but repressed under stress, where as in contrast, D1:2 is induced to replaces D1:1 in PSII reaction centers upon exposure to unusual growth conditions such as high light, cold temperature, UV-B radiations etc. (Sicora *et al*., 2006, Sicora *et al*., 2008, Vinyard *et al*., 2013). The D1' is always induced under low O_2 or microaerobic conditions. The previously believed silent *psbA* genes in cyanobacteria, encoding D1' isoform is a distinct functional group with a unique regulation mechanism responding to specific cellular needs. It is well distinguished from other PsbA isoforms by the consensus amino acid replacements at position 80 (Gly to Ala), 158 (Phe to Leu) and 286 (Thr to Ala) (Sicora *et al*., 2009).

Synechococcus sp. strain PCC 7002 genome contains three *psbA* genes, *a1418* (PsbA1), *a0157* (PsbA2), and *a2164* (PsbA3), encoding for three D1 isoforms. These natural variants of D1 subunits tune photochemical PSII fitness to varying solar radiations (Vinyard *et al*., 2013). The protein sequence of the PsbA1 and PsbA2 isoforms exhibits 100% amino acids similarity. *a2164* encodes a putative D1' form which exhibits these three key changes in the amino acid compositions at the respective positions (Mulo *et al*., 2009). Although the induction of D1' isoform has been studied in some cyanobacterial strains, their functional role towards photosynthesis remains widely unknown. In our study we aim to assess the expression levels of the various *psbA* genes and on the overall functionality of PSII complex, under microaerobic conditions in *Synechococcus* sp. PCC 7002.

Materials and methods

Strain, growth and treatment conditions

The wild type *Synechococcus* sp. strain PCC 7002 was grown in flasks with medium A^+ containing 1 mg $NaNO_3$ ml^{-1} at 38°C (Stevens *et al*. 1973). Light was provided by cool-white fluorescent lamps (250 μmol m^{-2} s^{-1}). The photon flux density

was measured using a QSPAR Quantum Sensor (Hansatech Instruments Ltd, Norfolk, UK) light meter while cell growth was monitored by the optical density at 550 nm (OD_{550}) with a Shimadzu UV-1700 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Microaerobiosis was achieved by argon bubbling in the culture flasks for up to 60 minutes. After 10 minutes, the oxygen concentration was found to be below 5% compared to the original value, as measured with an oxygen sensor (Mettler Toledo AG, Switzerland). The culture was returned to aerobic conditions following bubbling with air for 60 min. Subsequent to stress treatments the cells were returned to normal conditions (60 minutes recovery). 12 ml aliquots were sampled after 0, 15, 30, and 60 min of stress conditions, as well as after 30 and 60 min of recovery period for Real-Time Quantitative PCR (qPCR) measurements.

Flash-induced fluorescence measurements

Flash-induced intensification and subsequent decline of fluorescence was assessed using a double-modulation fluorometer (PSI Instruments, Brno, Czech Republic). Both measuring (2.5 μs) and actinic (20 μs) flashes were produced by red LEDs. All the measurements were completed in the interval of 150 μs to 100 s, the measuring flashes being applied in a logarithmic series and in the presence of the PSII inhibitor 3-(3′, 4′-dichlorphenyl)-1,1-dimethylurea (DCMU) at a final concentration of 10 μM, in order to block the transfer of electrons between Q_A and Q_B. To minimize the distortion of the relaxation kinetics due to the actinic effect, the intensity of the measuring flashes was adjusted to a low value. *Synechococcus* sp. PCC 7002 cells at 4 μg chl ml^{-1} were adapted to dark for 10 min prior measurements. Analysis of the fluorescence decrease was based on the model of the two electron gate as described earlier (Vass *et al.*, 1999).

Quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and dissolved in nuclease free water (Thermo Scientific, Waltham, MA, USA). Each sample was treated with 1U DNA-ase (Ambion Turbo DN-ase, Austin, TX, USA) to avoid genomic DNA contamination. The concentration of the RNA solution was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The synthesis of first strand cDNA from 1 μg of purified RNA was completed with the First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) using the random hexamer primers.

Two specific primer pairs were designed to amplify transcripts from the *psb*A genes in *Synechococcus* sp.PCC 7002. One of these primer pairs (F 5'-TTGCAGCCCACGGCTACTTC 3' and R 5'- GTTCAGAATGTCCGCCCAGGT-3') amplifies a 221 bp fragment of the genes encoding the D1 protein (SYNPCC 7002_A1418 and SYNPCC 7002_A0157), while the other one (Forward- 5'-CCACACTGTTGACCGCAACGAT-3' and Reverse- 5'-GTAGGGGCCA CCGTTGTAGAG -3') targets a 220 bp fragment belonging to the *psb*A gene responsible

for the synthesis of the D1' protein (SYNPCC7002_A2164). Another primer pair (Fwd- 5'-GCTTATCGCTGCACTGGAGT-3' and Rev- 5'-GGCCGCTTCTACTTTATTTTCC-3') was designed for the *pep*C gene encoding phosphoenolpyruvate carboxylase, to be used as a reference gene as its expression has been previously proven constitutive under conditions similar to our (Sicora *et al.*, 2006).

Results

The cells were subjected to microaerobic condition for 60 min by bubbling argon gas into the culture medium. After the treatment, the culture was returned to aerobic conditions following bubbling with air for 60 min. It is noteworthy to mention that the induction of microaerobic environment inside the culture itself is achieved by various methods including bubbling gases (such as N_2, argon etc.) or by enzymatic reactions. In our study, we used real-time qRT-PCR technique to monitor the expression levels of the two D1 protein isoforms encoded by *psbA* genes from *Synechococcus* sp. PCC 7002 under growth conditions, microaerobic treatment and during recovery, and also chlorophyll fluorescence measurements to highlight the functional characteristics of the donor and acceptor side of photosystem II during control, the microaerobic treatment and recovery.

qRT-PCR results

Our results showed significant difference between the relative amounts of *psbA* transcripts. The D1 isoform remained dominant throughout the treatment and recovery contributing to 99.9% of the total *psbA* transcripts (Table 1). In general, microaerobic treatment did not greatly alter the expression of any of the *psbA* isoform expression in our study yet minor induction at 15 min followed by slight down regulation at 60 min was noticed (Fig. 1). The changes are negligible but significant and close to error threshold of the method, in our case around the value of 2 fold. The unaltered expression of *a2164* gene during microaerobic treatment remains very unique in our study.

Table 1.

Relative transcript abundance of *psbA* isoforms under microaerobic treatment and during the recovery

psbA isoforms transcript	Microaerobic Treatment				Recovery	
	Control	15 min	30 min	60 min	30 min	60 min
D1 (%)	99.9759	99.9683	99.9552	99.9524	99.9612	99.9564
D1' (%)	0.0241	0.0317	0.0448	0.0476	0.0388	0.0436

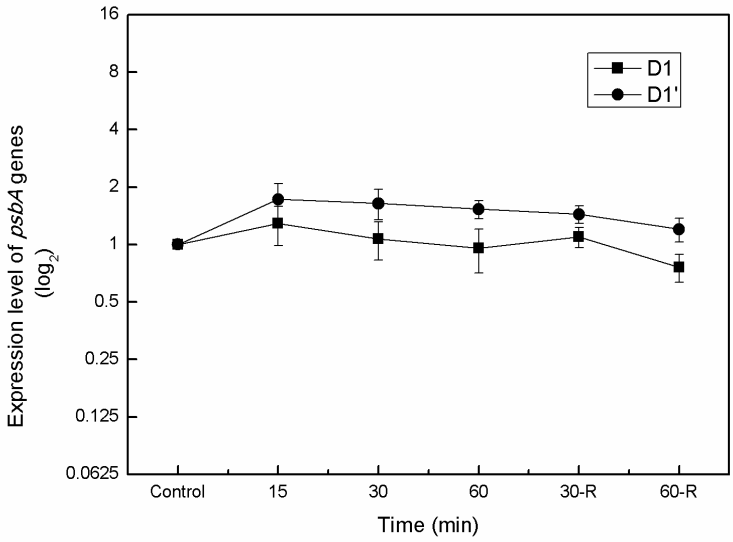

Figure 1. Real Time quantitative reverse transcriptase PCR analysis: qRT-PCR expression analysis of *psbA*-D1' (closed circle) and other D1 isoforms (closed square) under microaerobic stress (15 min, 30 min, 60 min) followed by recovery under growth conditions [90 min (30-R) and 120 min (60-R)] in *Synechococcus* sp. PCC 7002 strain.

Chlorophyll fluorescence measurements results

The functional characteristics of photosystem II were investigated using a double modulation fluorometer as described previously (Trtilek *et al*., 1997). We measured the flash-induced rise and subsequent decay of the fluorescence indicative of Q_A^- reoxidation. This gives information on the number of active PSII centers at the time of the flash as well as the efficiency of electron transfer within the acceptor side of PSII. The presence of DCMU, by synchronizing the PSII centers in a blocked state, makes the amplitude of flash fluorescence a good estimation of the potential number of active centers while in the absence of DCMU we see only the number of centers active at the time of the flash. During the 60 min of microaerobic treatment and the subsequent 60 min of recovery in normal air, at constant temperature and light intensity, we did not recorded a significant decrease in the number of active PSII centers both in the absence (Fig. 2a) or presence of DCMU (Fig. 2b). The microaerobic treatment did not significantly change the function of the PSII on the acceptor side of PSII (Fig. 2c) or donor side (Fig. 2d), an observation in accordance with previous studies performed on different cyanobacterial species (Sicora *et al*., 2009).

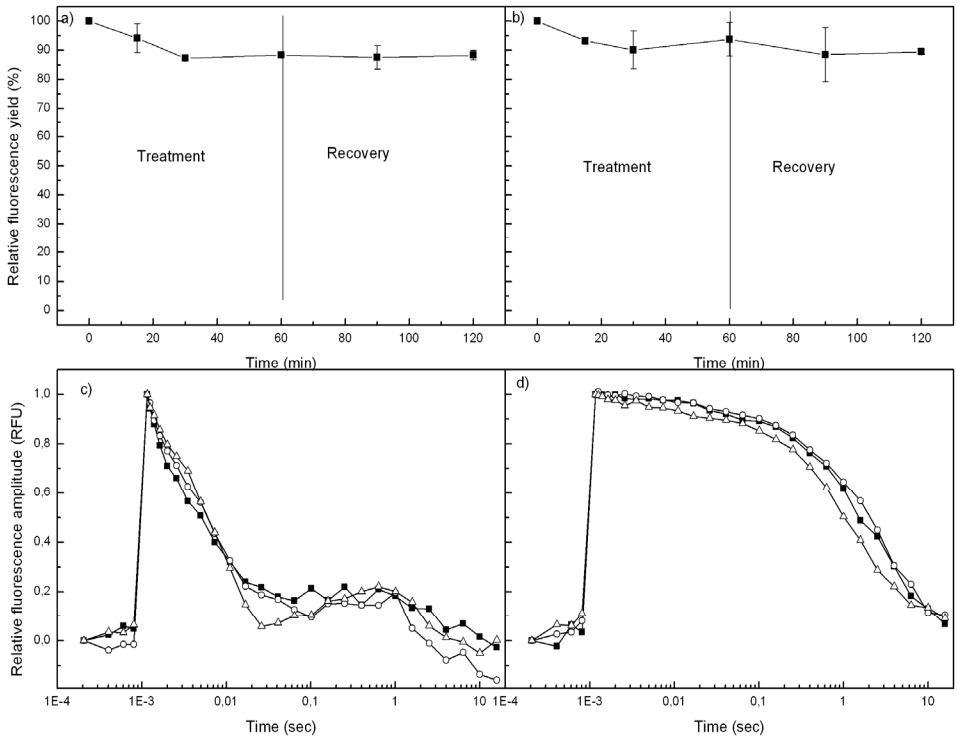

Figure 2. Influence of microaerobic conditions on the PSII function in *Synechococcus sp.* PCC 7002. Changes in the number of actual active centres (panel a) and potential active centres (panel b) at control, microaerobic treatment (15 min, 30 min, 60 min) and subsequent recovery under growth conditions (90 min and 120 min) in the absence (panel a) and presence of DCMU (panel b). The decay of flash-induced fluorescence was followed using measuring flashes on a logarithmic time scale in the absence (panel c) and presence of DCMU (panel d) at control (solid black squares), 60 min microaerobic treatment (open circles) and after 60 min of subsequent recovery (open up triangles) Changes in the shape of the curves were made evident by normalization of the decay curves to 1, during the treatment.

Discussion

Previous studies in *Synechocystis* sp. PCC 6803 had shown that D1' isoforms are induced when the cells are subjected to microaerobic conditions (Sicora *et al*., 2009, Summerfield *et al*., 2008). Sequence comparison performed on three cyanobacterial strains where microaerobical induction of D1' was recorded showed the existence of three very specific amino acid changes that are always present in D1' protein forms (Sicora *et al*., 2009). *In silico* analysis performed on different PsbA protein sequence clearly showed that *a2164* gene from *Synechococcus* sp. PCC 7002 has

all characteristic changes in the amino acids specific to a D1' isoform (Mulo *et al*., 2009). Both function of the donor or acceptor side of photosystem II complex and the amount of active PSII centers did not significantly change in *Synechococcus* sp. PCC 7002 tested under microaerobic conditions. These findings suggest that for short periods of time (60 min) microaerobic conditions did not act as a stress factor to the photosynthetic apparatus in this cyanobacterial strain. In addition, the microaerobic treatment did not influence the induction of *psbA* gene family expression. The unprecedented finding in our study is that the D1' like isoform, which responds to microaerobic conditions in other cyanobacteria, was not induced in *Synechococcus* sp. PCC 7002. It is for the first time that a member gene of the *psbA* gene family, encoding a protein isoform with the three typical amino acid mutations present, position 80 (Gly to Ala), 158 (Phe to Leu) and 286 (Thr to Ala), is not induced by short-term microaerobic treatment.

Further studies will be needed to confirm this protein as a D1' isoform or to establish it as a different and yet unknown class of D1 protein.

Conclusions

Our results indicated that *Synechococcus sp*. PCC 7002 do not increases the expression of the predicted D1' protein and there are no significant changes in the electron transfer pathways within PSII. It is for the first time that such response is recorded and allow us further speculate that *Synechococcus sp*. PCC 7002 contains an atypical, possibly entirely new form of D1 protein with, so far, an unknown function.

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=== SHORT COMMUNICATION ===

First record of *Bryocamptus (Bryocamptus) mrazeki* (Minkiewicz, 1916) in the Romanian harpacticoid fauna (Copepoda, Harpacticoida)

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SUMMARY. The harpacticoid copepod species *Bryocamptus (Bryocamptus) mrazeki* (Minkiewicz, 1916) is reported for the first time for the Romanian fauna. The species was identified in a small eucrenal spring from the Retezat Mountains, next to other taxa characteristic to the benthic habitats of clean waters. The present paper represents a necessary addition to the species distribution in Europe, since *B.(B.) mrazeki* was mostly found in mountain habitats from the Carpathian Ecoregion.

Keywords: harpacticoid copepods, new record, Romanian fauna, spring habitat, the Retezat Mountains.

Introduction

Springs, defined as the place of apearance on the surface of groundwaters as a result of sediment permeability, are unique ecotonal habitats between surface and hypogean areas, characterized by stable environmental conditions, a high biodiversity and generally good water quality (Cantonati *et al*., 2012).

Crustaceans are the dominant group in groundwaters and their related spring habitats (Stoch, 1995). Crustacean meiofauna: copepods, cladocerans or ostracods, is numerically abundant and rich in species in these environments (Dole-Olivier *et al*., 2000; Galassi *et al*., 2009). Harpacticoid copepods dominate the benthos of both lotic and lentic water bodies, with aproximatively 1000 species and subspecies (Dole-Olivier *et al*., 2000).

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The Retezat Mountains, and the homonymous National Park, located west in the Southern Romanian Carpathians, represent a unique area of protected ecosystems, including more than 20 mountain peaks higher than 2,000 meters in altitude and dozens of glacial lakes (Schreiber and Sorocovschi, 1993).

Previous studies on meiofauna from the region focused mainly on glacial lakes (Prunescu-Arion and Toniuc, 1967; Onciu and Radu, 2006; Battes, 2008). Godeanu (1974) reported faunal data from several habitats adjacent to Lake Gemenele: small lentic pools, slow-flowing waters with *Sphagnum* sp. or the Știrbul Rivulet. Meleg *et al.* (2014) investigated copepod diversity and distribution in relation with environmental drivers in the caves of the Romanian Carpathians, including the Retezat Mountains.

The aim of the paper is to improve the current knowledge of the harpacticoid copepod *Bryocamptus (Bryocamptus) mrazeki* (Minkiewicz, 1916) distribution, by reporting its first record for the Romanian fauna. The species was previously found in Central Europe (Slovakia, Czech Republic, Poland, Slovenia), in clean mountain waters. In Romania *B. (B.) mrazeki* was identified in a small spring from the Retezat Mountains, the Southern Carpathians.

Materials and methods

The species was identified in a spring located in the Retezat Mountains (N:45^{0}23’38”; E:22^{0}52’55”) at an altitude of 1550 m (Fig. 1) in 14th of September 2014. The spring was almost circular in shape, with gravel, boulders and coarse sand on the bottom. The width was about 0.5 m, and the maximum depth 0.1 m (Fig. 2) The water temperature reached 7^{0}C.

Figure 1. Location of the sampling site in the Retezat Mountains, Romania

Figure 2. The sampling site and strategy

The qualitative sample was collected using a 80 μm mesh size net and preserved in the field in 4% formaldehyde. Copepods were identified to the species level (Damian-Georgescu, 1970; Janetzky *et al*., 1996).

Results and discussion

Three harpacticoid copepod species were present at the sampling spring in the Retezat Mountains: *Attheyella (Attheyella) wierzejskii crenophila* Damian 1955; *B. (B.) mrazeki* and *Bryocamptus (Limocamptus) echinatus* (Mrazek 1893) (Fig. 3). Nine other taxa were identified, as follows: oligochaetes (Annelida, Oligochaeta); nematods (Nematoda); flatworms (Plathelminthes); stoneflies (Arthropoda, Insecta, Plecoptera); caddisflies (Arthropoda, Insecta, Trichoptera); chironomids (Arthropoda, Insecta, Diptera, Chironomidae); collembols (Arthropoda, Entognatha, Collembola); side-swimmers (Arthropoda, Malacostraca, Amphipoda); ostracods (Arthropoda, Ostracoda) (Fig. 3).

Up to the present, *B. (B.) mrazeki* was only found in central Europe, in Slovenia, Czech Republic, Slovakia and Poland (Sowa, 1965; Drzycimski, 1985; Illyová, 2001; Novikmec *et al*., 2007; Illyová *et al*., 2011; Boxshall, 2013; de Yong *et al*., 2014; Hřívová and Zhai, 2016). *B. (B.) mrazeki* is not included in the Romanian key for harpacticoid copepods (Damian-Georgescu, 1970), nor in later updates of the Romanian copepod fauna (Iepure, 2007; Iepure *et al.,* 2016).

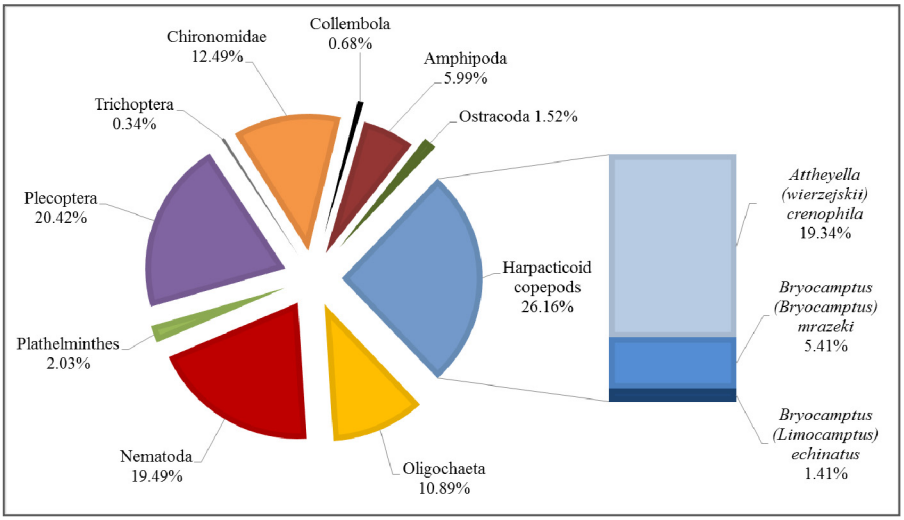

Figure 3. The relative abundance of all taxonomic groups present in the sampling site, next to the percentage of harpacticoid species

In the Retezat Mountains, Godeanu (1974) found cyclopoid and harpacticoid copepods in several aquatic habitats adjacent to Lake Gemenele. Three harpacticoid species were recorded: *Bryocamptus (Arcticocamptus) bryobates* (Monard, 1928) and *Bryocamptus (Arcticocamptus) cuspidatus* (Schmeil, 1893) in shallow habitats dominated by *Sphagnum* sp., and *Canthocamptus (Canthocamptus) staphylinus* (Jurine, 1820) in small stagnant pools and in the Știrbul Rivulet.

In the sampling spring, *B. (B.) mrazeki* was represented by 44 females, 19 males and 1 copepodite. Figures 4 - 6 depict several taxonomic features of the species. The swimming legs (Fig. 4 A - D) include P1 with a 3-segmented endopodite, different from *Bryocamptus (Rheocamptus) zschokkei* (Schmeil, 1893), and with the second segment of the exopodite bearing a long hair on the inner side, differing from *Bryocamptus* subg. *Limocamptus* Chappuis, 1929. The swimming legs P2-P4 have 2-segmented endopodites (Fig. 4). The swimming leg P4 in males has the last segment of the endopodite with 3 hairs, unlike *Bryocamptus (Bryocamptus) vejdovskyi* (Mrazek, 1893) (Fig. 5 A). Other distinctive features are the seminal receptacle in females (Fig. 5 B) and the anal somite (Fig. 6).

B. (B.) mrazeki clearly prefers clean, high altitude waters. It has been already recorded in interstitial habitats from the Eastern and Western Carpathians in Slovakia (Illyová, 2001; Novikmec *et al*., 2007; Illyová *et al*., 2011), from headwater streams in the Western Carpathians in the Czech Republic (Hřívová and Zhai, 2016), from spring fens in the Western Carpathians (Zhai *et al*., 2015) or in lake benthic habitats in the Tatra Mountains, Poland (Sowa, 1965). Thus, its distribution in the Carpathian mountain range should not be considered accidental, and new citations from Ukraine or the Romanian Eastern Carpathians could be expected.

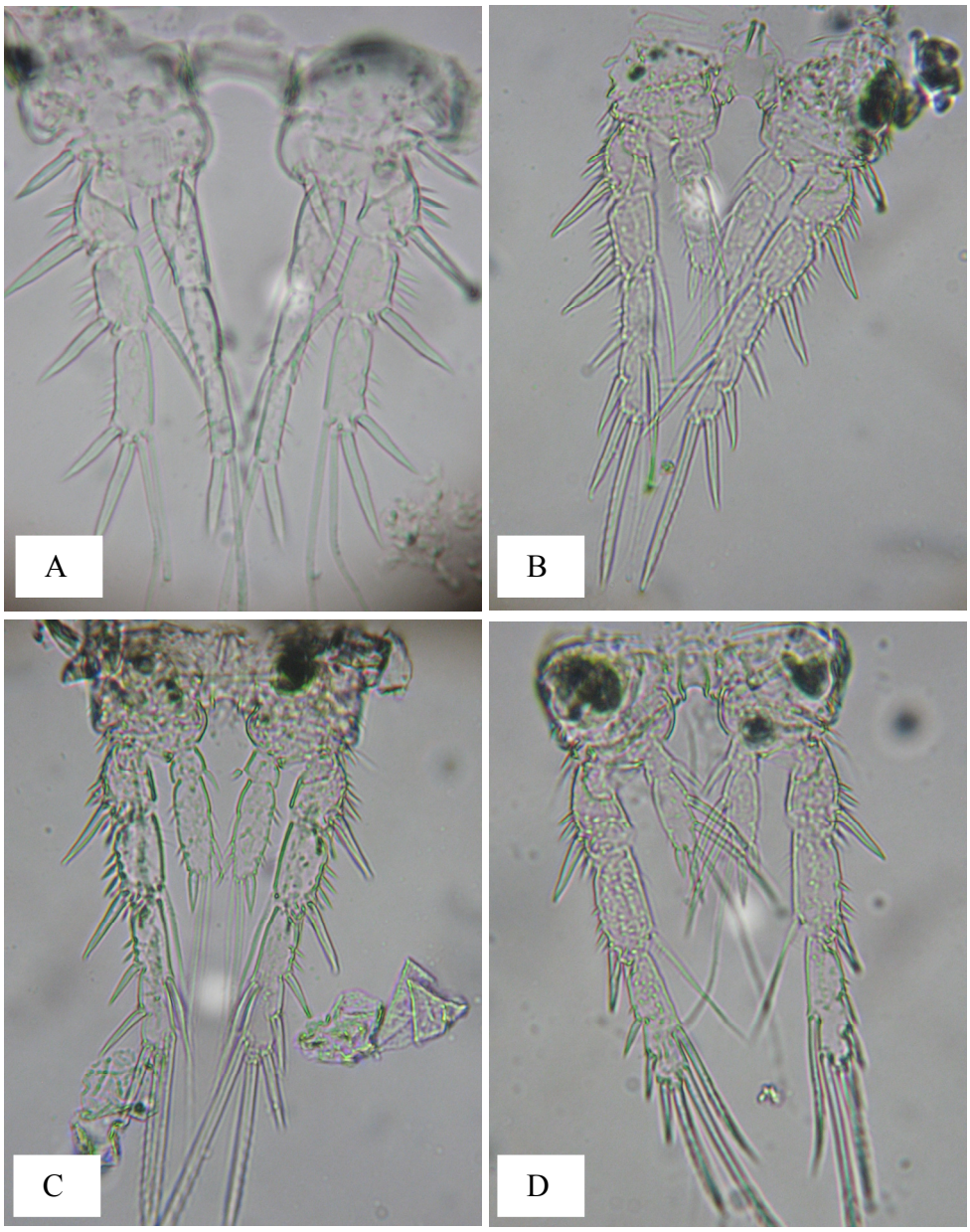

Figure 4. *B. (B.) mrazeki* ♀ from the sampling spring in the Retezat Mountains: the swimming legs: **A**: P1; **B**: P2; **C**: P3; **D**: P4

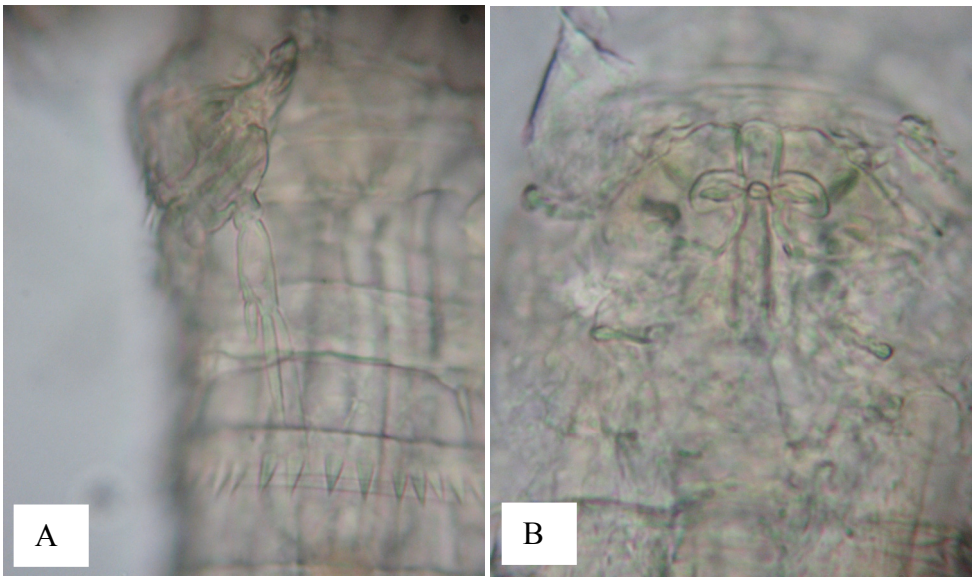

Figure 5. *B. (B.) mrazeki* from the sampling spring in the Retezat Mountains: **A**: ♂ - swimming leg P4, endopodite; **B**: ♀ - genital area

Figure 6. *B. (B.) mrazeki* ♀ from the sampling spring in the Retezat Mountains: the anal somite with 4 distinct strong teeth; and the caudal rami (the line represents approx. 100 µm)

Conclusions

The present paper represents the first record of *B. (B.) mrazeki* in the Romanian harpacticoid fauna. The species was identified from a small eucrenal spring from the Retezat Mountains. The species distribution in Europe include different clean water habitats from mountain regions, mostly from the Carpathian range, thus its citation in the Romanian Retezat Mountains cannot be accidental.

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