STUDIA UNIVERSITATIS
BABEŞ-BOLYAI

BIOLOGIA

2/2008

ANUL LIII **2008**

S T U D I A

UNIVERSITATIS BABEŞ – BOLYAI

BIOLOGIA

2

Desktop Editing Office: 51ST B.P. Haşdeu, Cluj-Napoca, Romania, phone + 40 264-40.53.52

CUPRINS – CONTENT – SOMMAIRE – INHALT

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STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 3-15

TERRESTRIAL ISOPODS FROM THE WESTERN AND NORTH-WESTERN ROMANIA

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SUMMARY. We have studied the terrestrial isopod communities from 7 natural habitats consisting in plain, hill and different intermountain depressions from the west and north-west of Romania. The isopods were collected using pitfall traps. In the plain area, the researches were carried out in grassland with a sandy soil. Here we found Metoponorthus pruinosus and Porcellio scaber, but in very small populations. In a meadow with a very humid soil, with swampy and Salix cinerea bush areas, we identified: *Porcellium collicola*, *Trachelipus rathkii* and *Armadillidium vulgare*, all with numerous populations. Present in an acacia forest are: *Protrachenonicus politus* and *Armadillidium vulgare* but with little populations. In the hilly zone, studies were made in an oak and hornbeam mixed forest where we identified the following species: *Ligidium hypnorum, P. politus*, *T.arcuatus*, *T. wächtleri*, *T. ratzeburgi* and *T. difficilis*. *P. politus* is the dominant species. Researches has been carried out also on a cleared area, were few *P. politus* individuals were collected. In the intermountain depressions, in the beech and hornbeam forests, were identified species like *Ligidium hypnorum*, *Hyloniscus transsylvanicus*, *Trichoniscus carpaticus*, *P. politus*, *T. arcuatus* and *T. wächtleri*. *P. politus* is again the dominant species. In the river meadow with alder we found the same species plus *T. difficilis* which is dominant here together with *P. politus*. The sex ratio is much in favor of the females of *P. politus* and *T. rathkii*. In the case of *P. collicola* and *A. vulgare* the ratio between the two sexes is about 1:1. Among the 14 species identified in the studied habitats, *P. collicola, T. difficilis, T. arcuatus, T. wächtleri, T. rathkii* and *T. ratzeburghi* are cited for the first time in the Western and North-Western part of Romania.

KEYWORDS: terrestrial isopods, pitfall traps, sylvan species, paludicolous species, praticolous species, synanthropic species, numeric abundance, relative abundance, sex ratio.

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Introduction

The previous ecological studies about terrestrial isopods were about the central, northern and north-eastern part of Romania (Tomescu, 1974; Dolnițchi-Olariu, Tomescu, 1997; Hotea *et al.*, 2003; Mureşan *et al.*, 2003; Tomescu *et al.*,1979; 1979; 2000; 2001; 2002; 2005). In 2004 we studied the isopods communities in different biotopes of the Western part of Romania: habitats of the Western Plain (grassland, humid meadow and acacia plantation), of a hilly zone (oak and hornbeam mixed forest and a cleared surface) and of intermountain depressions in the Western Romanian Carpathians (beech and hornbeam mixed forests and an river meadow with alder). The objectives of our research were: knowledge of the specific structure of the isopod communities in the studied habitats, the preference towards some of the habitats and the size of the populations (evaluated by the values of the abundance) and the sex ratio. The results of these investigations were compared to the results of the prior researches from other regions of Romania.

Material and methods

Habitats. The studies took place in habitats of the four stations situated in the Western part of Romania (Fig.1), at altitudes between 130 m and 520 m asl.

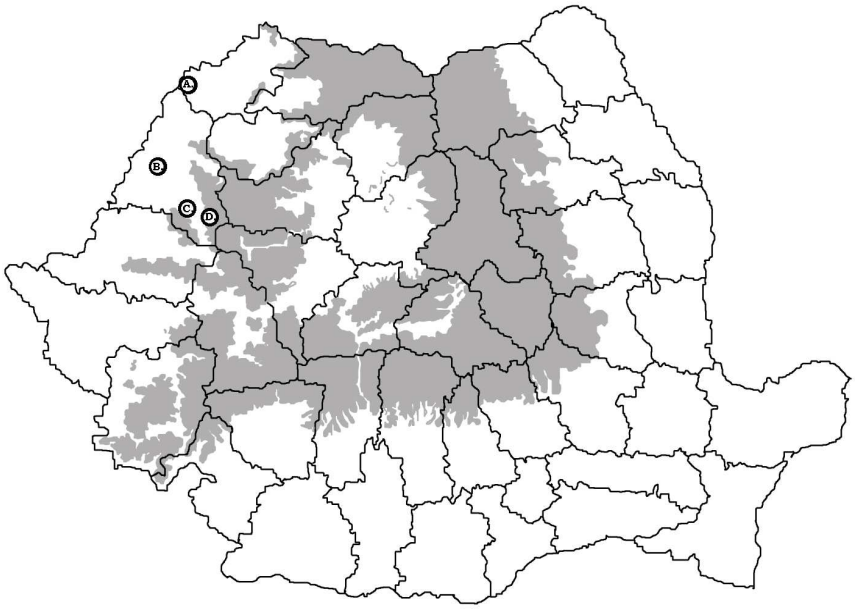

Fig. 1. Isopods sampling sites (A – Resighea station, B – Hidişel Hills, C – Tărcăița Hills, D – Băița Plai.

A. Resighea Station is situated in the Ier Plain (Fig.1 – A), a subunit of the Western Plain of Romania, at an altitude of 130 m. The researches were done in three habitats: grassland, wet meadow with Salix cinerea bushes and acacia plantation.

The grassland is near the town of Resighea, on sand dunes. The sandy soil is arid, covered with different types of xerophilous plants species and with isolated Rosa canina bushes. Sunstrokes at the soil level are very high, determining the dryness of soil and high temperatures (over 30 degrees Celsius) in the summer. These temperature and humidity conditions are below optimum for most terrestrial isopod species. We placed 5 pitfall traps.

The meadow with a humid, swampy soil is situated near a stream, on a flat site. The grass layer is very rich, with 100% coverage of the surface, made up by hygrophyte species. Large Salix cinerea bushes are to be found isolated, which in turn are made up out of 20-30 individuals. Sunstrokes are very high here as well, but don't cause drop in the humidity due to the underground water which is very close to the surface of the soil. The permanently saturated humidity contributes to the maintenance of lower temperature values compared to the grassland from the sand dunes. In the wet meadow were placed 4 pitfall traps.

The acacia plantation consists of 35-40 year old trees, on a sandy soil. The degree of coverage from the trees is about 85-90%, so the ground is mostly shaded. The grass layer is poor (15-20%) and has a patchy distribution. The litter is composed only by acacia leaves, is thin layer and discontinuous. The humidity is relatively low. Five pitfall traps were placed.

B. Hidişel Hills Station (Fig. 1 – B) is a subunit of the Padurea Craiului Hills, situated at an altitude of 170m asl. We have investigated an oak forest and a cleared surface as well.

The forest manly consists in oak trees, mixed with rare sycamore maple and hornbeam trees. The trees are about 60-70 years old and have a high degree of ground coverage, the surface of the soil being permanently shaded. The grass layer is reduced and has an irregular distribution. It can be richer on small surfaces though, where the humidity of the soil is greater. The litter is continuous and it is about 3-5 cm thick. The humidity of the soil is moderate. In the forest were placed 10 pitfall traps.

The cleared surface is situated on a flat site inside the actual forest and has an approximate surface of about 5-6 hectares. Sunstrokes on the soil are very high. The trees were cut about 2-3 years ago. The grass layer, made up by xerophytes species, has a coverage about 60-70%. The soil's humidity is very low. Here were placed 3 pitfall traps.

C. Tărcăiţa Hills Station – situated between Codru Moma Mountains and Beiuş Depression (Fig.1 – C), at an altitude of 290m. Samples were taken from a hornbeam and beech forest and also in river meadow with alder.

The hornbeam and beech mixed forest is one a 15-20 degree slope, with the eastern exposition. The density of the trees is high, thus the ground is permanently shaded. The grassy layer has a patchy distribution. The litter is continuous and has a general thickness of about 3-5 cm. The humidity of the soil is moderate. Seven pitfall traps were placed in this habitat.

The river meadow with alder, walnut, hawthorn and blackberry bushes is situated on a flat site. The age of the alder trees varies between 80-90 years. The grassy vegetation (hygrophyte species), is rich with 100% coverage of the ground. The litter is discontinuous, with a thickness of about 1-3 cm. Here were placed 4 pitfall traps.

D. Băiţa Plai Station is located in the Bihor Mountains, near Crişul Băitei River. The samples were taken from a beech and hornbeam mixed forest, from a 30 degrees slope with west exposition. The bush layer is rather rich; the litter is continuous with a thickness of 5-7 cm. The grass layer is poor, with coverage of about 10-15% and patchy distribution. In these woods were placed 4 pitfall traps.

Research methods. The isopods were collected with pitfall traps in 2004, from May until September inclusively. In the traps we placed a conserving solution – 4% formalin. The captured animals were collected from the traps monthly. The species were determined in the laboratory. The individuals were counted from each sample species and males and females were separated in order to establish the sex ratio. The results were statistically analyzed. The numeric abundance (the number of captured individuals per each sample), the relative abundance (the percentage of the number of individuals of a species divided by the total number of captured specimens), the Shannon-Wiener diversity and equitability index and also the sex ratio (for the identified species that had more than 100 captured individuals) were calculated.

The capture of isopods with pitfall traps is used with good results in quantitative ecological studies for the very mobile isopod species which live on the ground floor and was/is a method used by many other researchers [Farkas, Krčmar, 2004; Hornung *et al*., 2007; Sfenthourakis *et al*., 2005; Vadkerti, Farka, 2002). With this method, isopods can be collected for a very long period and it is also useful for determining the dynamic of a population. In these pitfall traps, very small species, with low mobility, that live in the humus layer from the surface of the soil (such as the Trichoniscus ssp, Androniscus ssp, Haplophthalmus ssp etc) or under rocks or tree bark (such as the Platyarthrus genus) cannot be captured. We consider that, in order to know the entire isopod fauna of the studied habitats, the pitfall method needs to be accompanied by hand collecting.

Results and discussions

In the habitats of the four stations in the north-west Romania were identified 14 species (Table 1.). Among these, 6 are cited for the first time in this part of Romania (Radu, 1983; 1985). The number of adult individuals was enough

to establish that *Trachelipus difficilis* Radu 1950 is not synonymous with *T. wächtleri* K. L. Koch 1841, as it is asserted in some publications (Schmidt, 1997). We will publish the evidence later, when we will conclude a research about the species of the *Trachelipus* genus in Romania.

Table 1.

The distribution of the terrestrial Isopods in the studied stations.

Nr.	Species	Ecol. category	Resighea	Hidișel Hills	Tărcăit a Hills	Băita-Plai
1	*Ligidium hypnorum* Cuvier 1792	pa	-	+	+	+
2	*Hyloniscus transsylvanicus* Verhoeff 1901	pa	-	-	+	+
3	*Trichoniscus carpaticus* Tăbăcaru 1974	pa	-	-	+	+
4	*Protrachenonicus politus* C. L. Koch 1841	sy	+	+	+	+
5	*Porcellium collicola** Verhoeff 1907	sy/pa	+	-	-	-
6	*Trachelipus difficilis** Radu 1950	sy	-	-	+	-
7	*Trachelipus arcuatus** Budde-Lund 1885	sy	-	+	-	+
8	*Trachelipus wächtleri** C. L. Koch 1841	sy	-	+	+	-
9	*Trachelipus nodulosus* C. L. Koch 1838	pr	-	-	+	-
10	*Trachelipus rathkii** Brandt 1833	eu	+	-	-	-
11	*Trachelipus ratzeburgi** Brandt 1833	sy	-	+	-	-
12	*Metoponorthus pruinosus* Brandt 1833	pr/sn	+	-	-	-
13	*Porcellio scaber* Latreille 1804	eu/sn	+	-	-	-
14	*Armadillidium vulgare* Latreille 1804	pr/sn	+	-	-	-
	Total no. of species/station		**6**	**5**	**7**	**5**

Ecological categories: eu – euritopes species, pa – paludicolous species, pr – praticolous species, sy – sylvan species, sn – synanthropic species

** - species cited for the first time in the west and north-west of Romania.*

The distribution of the species in the four stations differs. Protracheoniscus politus, a typically sylvan species, is present in the forest from all the stations. In our previous studies, we can state that it is the dominant species in the isopod communities of the deciduous, spruce fir and mixed forests, the meadow forests and the grasslands from the mountain areas with a low and average altitude (up to

900-1000m asl.) (Dolnițchi-Olariu, Tomescu, 1997; Tomescu *et al*., 2000, 2001; 2002). *Ligidium hypnorum* is present in the habitats of three of the stations, while other species, in habitats in one or two stations.

Isopod communities from Resighea station

In the investigated habitats, 528 specimens were collected and 6 species identified (Table 2.) 5 of these species live only in one habitat, proof for the big differences among the ecological conditions from the three habitats.

Table 2.

Terrestrial isopod communities in the habitats of Resighea station

	Species	Grassland on sandy soil		Bushy meadow on very humid soil			Acacia forest on sandy soil		Total individuals
		n	x	n	x	r.a.%	n	x	
1	*Protrachenonicus politus*	-	-	-	-	-	2	0.4	2
2	*Porcellium collicola*	-	-	109	27.2	21.9	-	-	109
3	*Trachelipus rathkii*	-	-	181	45.2	36.6	-	-	181
4	*Metoponorthus pruinosus*	5	1.0	-	-	-	-	-	5
5	*Porcellio scaber*	3	0.6	-	-	-	-	-	3
6	*Armadillidium vulgare*	-	-	208	52.0	41.8	20	4.0	228
	Total specimens and x	8	1.6	498	124.5		22	4.4	528
	H	0.6616		1.065			0.3046		
	e	0.9544		0.9694			0.4395		

n – total number of captured specimens, x – numeric abundance (number of individuals captured per trap), r.a. – relative abundance, H – Shannon-Wiener diversity index, e – equitability index.

In the grassland from the sandy soil situated near the locality very few individuals of *Metoponorthus pruinosus* (a praticolous and synanthropic species) and *Porcellio scaber* (an eurytopic and synanthropic species) could be collected. The values of the equitability index indicate small differences between the populations of these two species. Both *Metoponorthus pruinosus* and *Porcellio scaber* populations have however very small numbers. This fact and the absence of other praticolous species, characteristic for xeric environments (*Trachelipus nodulosus*, *Armadillidium vulgare* etc) suggest that the ecological factors from this biotope do not comply with the optimum limits of terrestrial isopods.

In the meadow with very humid soil, with *Salix cinerea* bushes, live three species of terrestrial isopods, which belong to different ecological categories: *P. collicola* – a sylvan species – lives in humid forest microhabitats, rich in humus, *T. rathkii* – a eurytope species – and *A. vulgare* – a praticolous species, typical for open and xeric habitats. All three species have large populations, just as their numeric abundance values show. The values of their relative abundance and of the equitability show little differences. The value of the Shannon-Wiener index shows a stability of the isopod community from this habitat. We must also mention that in our previous studies in meadows fof other regions, we have never encountered such a community of species, so different by the ecological point of view (Dolniţchi-Olariu and Tomescu, 1997; Tomescu, 1992; Tomescu *et al*., 1979; 1995; 2000; 2000; 2002). We also shows that, in any ours research, we never found *A. vulgare* in a meadow with a high degree of the soil humidity. The results of our study in Resighea indicate a relatively high tolerance towards for soil humidity of this species' and it is not a strictly xerophilous ones. In our studies undertaken in the Danube Delta (Tomescu, 1992) we found that *A. vulgare* populations live in the poplar plantations, under the litter, that being a proof that this species isn't a strictly praticolous species (that lives in open habitats with mostly grassy vegetation cover). The presence of *P. collicola* in this meadow shows that this species isn't a strictly sylvan species. We can therefore conclude that the terrestrial isopod species can live in other types of habitat, in which the values of the main ecological factors are found between the optimum limits, compared to the existing values for the characteristic habitats where they usually occupy.

In the acacia plantation (also on sandy soil) we managed to capture two individuals of *P. politus* and 20 *A. vulgare*. All the values of the ecological indexes show that the terrestrial isopod fauna of this acacia forest is very poor. The species present here have very small populations and don't form a stable community, a similar situation with the one of the sandy grassland. The poor isopod fauna of the acacia plantation can probably be explained by the quality of litter, which possibly does not correspond with the trophic requirements of this group. In the forest, the soil humidity higher than in the xeric meadow, while the temperature does not record high values. A fact that supports this statement is represented by very large number of Diplopoda, detritus feeders, like isopods. We also mention that in the hornbeam, beech and oak forests for example, the *P. politus* populations are very large it being considered a representative species in woods (Hotea *et al*., 2003; Mureşan *et al*., 2003).

Isopod communities of the Hidisel Hills

In the two investigated habitats were collected 319 individuals belonging to 6 species, one paludicolous and 5 sylvan species (Table 3).

Table 3.

Terrestrial isopod communities in the studied habitats of Hidişel Hills

	Species	Deciduous mixed forest			Cleared area		Total individuals
		n	x	r.a.%	n	x	
1	*Ligidium hypnorum*	2	0.2	0.7	-	-	2
2	*Protrachenonicus politus*	253	25.0	88.2	32	10.7	285
3	*Trachelipus arcuatus*	11	1.1	3.8	-	-	11
4	*Trachelipus wächtleri*	8	0.8	2.8	-	-	8
5	*Trachelipus ratzeburgi*	8	0.8	2.8	-	-	8
6	*Trachelipus difficilis*	5	0.5	1.7	-	-	5
	Total specimens and x	287	28.7		32	10.7	319
	H	0.5409					
	e	0.3019					

n, x, r. a. H, e – idem Table 2

In the mixed forest, were found all the species, while in the cleared site only one of them.

In the forest, *P. politus* has a very large population. Of the four Trachelipus species, only few individuals were captured, due to their small populations, but also because the individuals of these species prefer to live under the fallen tree barks and under rocks and less under less under litter. Their movement on the ground is much more reduced than other species and, as a consequence, the chance to fall into traps is smaller.

The presence of the 4 sylvan species from the Trachelipus genus in this forest represents a co-habiting relationship, encountered for the first time in our studies. In our prior researches in forests of other regions, we found one or two Trachelipus species in the same habitat (Tomescu, 1974; 1992; Tomescu *et al*, 1979; 1995; 2000; 2001; 2002). Their co-habitation in the forest of Hidişel Hills indicates a larger ecological diversity, due the variety of microhabitats.

The values of the relative abundance show that *P. politus* is the eu-dominant species, while the rest of the species being recendent and sub-recendent. The Shannon-Wiener index proves that there is a relatively stabile community of species, while the equitability index shows great differences between the sizes of the isopod populations from this habitat.

In the cleared site, 32 *P. politus* individuals were captured, this being the only species present here, despite the fact that this surface is surrounded by forest. The results of our study indicate that *P. politus* survives even after the forest is cut down,

where other sylvan species of *Trachelipus* genus cannot live as a consequence of the modification of ecological conditions. On this cleared surface, the temperature on the ground rises over 30 ºC, due to the strong sunstrokes, and in the same time the soil humidity drops. It is expected that sylvan species community will recover after the forest will re-grow.

Isopod communities in the studied habitats of Tărcăița and Băița-Plai Hills

In the Tărcăița Hills, we have studied isopod communities in a hornbeam and beech mixed forest and in the river meadow with alder. Overall, 219 sindividuals were collected here(x =23.8 per total species), belonging to 7 terrestrial isopod species (Table 4).

Table 4.

Terrestrial isopod communities in the habitats of Tărcăița Hills and Băița Plai

	Species	Mixed forest of Tărcăița Hills			River meadow with alder of Tărcăița Hills			Mixed forest of Băița Plai			Total individuals
		n	x	r.a. %	n	x	r.a. %	n	x	r.a. %	
1	*Ligidium hypnorum*	-	-	-	4	1.0	7.7	18	4.5	26.5	22
2	*Hyloniscus transsylvanicus*	1	0.14	0.6	3	0.75	5.8	1	0.25	1.5	5
3	*Trichoniscus carpaticus*	-	-	-	1	0.25	1.9	1	0.25	1.5	2
4	*Protrachenonicus politus*	157	22.4	94.0	19	4.75	36.5	45	11.25	66.1	221
5	*Trachelipus difficilis*	-	-	-	23	5.75	44.3	-	-	-	23
6	*Trachelipus arcuatus*	-	-	-	-	-	-	3	0.75	4.4	3
7	*Trachelipus wächtleri*	9	1.3	5.4	-	-	-	-	-	-	9
8	*Trachelipus nodulosus*	-	-	-	2	0.5	3.8	-	-	-	2
	Total individuals and x	167	23.8		52	13.0		68	17.0		287
	H		0.2461			1.292			0.8868		
	e		0.224			0.721			0.551		

In the hornbeam and beech forest, only 3 species are present: one paludicolous and two sylvan. *P. politus* is the eu-dominant species (r.a. = 94%) - 157 specimens were captured. *Hyloniscus transsylvanicus* is the sub-recendent one (r.a. = 0.6%), while *T. wächtleri* is the recendent species (r.a. = 5.4%). The small number of species and their small effective population size indicate a greater ecological uniformity in the forest of the Tărcăița Hills, compared to the oak and hornbeam forest of Hidișel Hills. The value of the Shannon-Wiener index shows that this three species do not form a very stabile community. The value of the equitability index indicates great differences regarding the sizes of the isopod populations in these woods.

In the meadow river with alder, 52 individuals were captured (x = 13.0 per total species), belonging to 6 isopod species which form a stabile community (H = 1.292). The differences concerning the effective population size of those 6 species are smaller (e = 0.721) than in the isopod community from the beech and hornbeam forest. The dominant species are *P. politus* (r.a. = 36.5%) and *T. difficilis* (r.a. = 44.3%). All the other 4 species are recendent or sub-recentend. The presence of 3 paludicolous species shows the fact that here is more than one humid microhabitat in the meadow forest. Optimum ecological conditions are found for *T. difficilis* and *P. politus*.

We consider that *T. nodulosus*, a praticolous species, appeared accidentally in our samples. The two captured individuals probably migrated from the nearby meadow into the river meadow with alder.

Isopod communities in Băița -Plai

In the Băița-Plai, we have studied isopod communities in a beech and hornbeam mixed forest, with 4 pitfall traps. Were captured 68 individuals belonging to 5 terrestrial isopod species (Table 4). Three of these species are paludicolous and two of them are sylvan, a cenotic structure that indicates a greater diversity of microhabitats in comparison with the beech and hornbeam from the Tărcăița Hills. *Ligidium hypnorum* is the dominant species (r.a. = 26.5%), while *P.politus* is eu-dominant (r.a. = 66.1%). Two other species are sub-recendent and one recendent, respectively.

If we compare the isopod fauna of the two beech and hornbeam forests in the Tărcăița and Băița-Plai we'll see the differences between the numbers of species a fact that points out a greater ecological diversity. Another visible difference is in the size of the *P. politus* population. In the forest of Tărcăița Hills, the value of the numeric abundance of *P. politus* is mach greater (x = 23.8) compared to the same index of Băița-Plai (x = 13.0), detail that suggests that in the first forest the ecological conditions favor more the survival of *P. politus* until they reach adult stage. We had similar results in some other studies, as well (Tomescu *et al*., 1995; 2000; 2001).

We can therefore conclude that in the same type of habitats, situated in the same relief unit, there are differences between the structures of the terrestrial isopod communities and also between the sizes of the population of the common species. These are cenotic differences that reflect the ecological dissimilarities which exist among habitats of the same type.

Sex ratio

The sex ratio was calculated only for those species form which more then 100 individuals were collected. (Table 5). The genetically determined sex ratio is 1:1 (males: females). In the populations of diverse habitats, it has different values because of the mortality rate, which can be smaller or greater at one of the sexes [9, 10]. The mortality rate of the two sexes in influenced by the different behavior in the mating season (ex: L. hypnorum) or by the physiological state after reproduction (ex: *P.*

politus) (Radu, Tomescu, 1976;Tomescu, 1974; 1992; Tomescu, Accola, 1992; Tomescu *et al*, 1995). There are also species at which the adult sex ratio is very close to 1:1 – for example *T. difficilis* (Accola, 1993) or *A. carniolense* (Tomescu, Accola, 1992). In the isopod populations, the sex ratio of the same species varies with the age of the individuals (Tomescu *et al*., 1995).

Table 5.

Sex ratio of isopod populations sampled from various sites from the N and NW Romania

Stations and habitats	Isopod species	Total nr. of adults	% males	% females
Resighea (meadow on humid soil)	*Porcellium collicola*	109	43.5	56.5
	Trachelipus rathkii	181	31.2	68.8
	Armadillidium vulgare	228	49.5	50.5
Hidișel Hills (oak and hornbeam mixed forest)	*Protrachenonicus politus*	207	25.6	74.4
Tărcăița Hills (beech and hornbeam mixed and the river meadow with alder	*Protrachenonicus politus*	155	25.8	74.2

In the present study in Western part of Romania, we have calculated the sex ratio for 4 terrestrial isopod species (Table 5). In the case of *A. vulgare*, the difference between the number of adult males and females is very small (1%), this showing that the mortality and the survival rates have similar values for both sexes. The results are similar with those recorded for *A. versicolor and A. carniolense* (Tomescu, Accola, 1992). Great differences were found at *P. collicola* (43.5%♂♂: 56.6%♀♀) while large differences at *P. politus* (25.6%, 25.8%♂♂: 74.4, 74.2%♀♀). For these species, the adult male mortality is much greater than in the case of adult females. Similar results were found for P.s politus, when for collecting we used Tullgren funnels, a method that ensures the capture of all the adult individuals in the litter samples (Radu and Tomescu, 1972; Tomescu, 1974).

Conclusions

In the grassland on the sandy soil live only synantrophic species (*Metoponorthus pruinosus* and *Porcellio scaber*) which withstand lower humidity rates and high temperature values on the surface of the ground. Both species have very small populations.

In the meadow with very humid soil live species with different ecological features, represented by large populations (*Porcellium collicola*, *Trachelipus rathkii* and *Armadillidium vulgare*).

In the acacia plantation, also on a sandy soil, we found *Ptotracheoniscus politus* and *A. vulgare* It is possible that the acacia leaf letter does not correspond with the terrestrial isopods trophic requirements.

In the oak and hornbeam mixed forest from the hilly zone live 6 isopod species, one paludicolous and 5 sylvan, 4 of them belonging to the Trachelipus genus. *P. politus* is the dominant species.

On a cleared surface of the forest, near an oak forest, only *P. politus* is present, in very low numbers. The Trachelipus species and *Ligidium hypnorum* are absent.

In the beech and hornbeam mixed forest from the intermountain depressions the isopod fauna is rather poor. In both woods *P. politus* is the dominant species.

In the the river meadow with alder, in the depression zone, a site with a greater diversity of microhabitats, there are 6 terrestrial isopod species; *T. difficilis* and *P. politus* being dominant ones.

The sex ratio has very close values to 1:1 in the case of *A. vulgare* (49.5: 50.5). Significant differences in favor of the females were recorded in *T. rathkii* (31.2: 68.8) and *P. collicola* (43.5: 56.6), and a great differences are recorded in *P. politus* (25.6: 74.4).

REFERENCES

Accola S., Tomescu N., Berciu C., Rusu C., (1993). The reproduction of *Trachelipus difficilis rotundatus* Radu 1950 (Crustacea: Izopoda). *Rev. Roum. Biol., - Biol. anim.*, **38**, 2, 115-121.

Dolnițchi-Olariu L., Tomescu N. (1997). Aspects ecologiques de la population d'Isopodes terrestres de la zone des Dornas. *Anuarul Muzeului Național al Bucovinei, Ştiințele naturii*, **14**, 81-92.

Farkas S., Krčmar S., (2004). Terrestrial Isopods (Isopoda: Oniscoidea) of Baranya (Croatia). *Nat. Croat*., **13**, 2, 161-170.

Hornung E., Tóthmérész B., Magura T., Vilisics F. (2007). Changes of Isopod assemblages along an urban-suburban-rural gradient in Hungary. *Europen Journal of Soil Biology*, **43**, 158-165.

Hotea R., Tomescu N., Mureşan D., Hotea M. (2003) Cercetari faunistice si ecologice asupra izopodelor terestre de pe versantul sudic al Munților Gutâi. *Analele Univ. Oradea, Fasc. Biologie*, **X**, 33-37.

Mureşan D., Tomescu N., Olariu L., Hotea R. (2003). Cercetari faunistice si ecologice asupra izopodelor terestre din sectorul mijlociu al Bazinului Arieşului. *Analele Univ. Oradea, Fasc. Biologie*, **X**, 27-32.

Radu G. V., (1983). Crustacea: Izopoda: Oniscoidea: Oniscoidee inferioare, Fauna Republicii Socialiste România, **IV**, 13.

Radu G. V., (1985). Crustacea: Izopoda: Oniscoidea: Crinochaeta, Fauna Republicii Socialiste România, **IV**, 14.

Radu G. V., Tomescu N. (1972). Studiul populatiei de *Ptotracheoniscus politus* (Crustacea: Izopoda) intr-o padure de foioase. *Studia, series biologia*, **1**, 75-82.

Radu G. V., Tomescu N. (1976). Quantitativ-ökologische untersuchungen an Landisopoden. *Pedobiologia*, **16**, 36-43.

Schmidt G. (1997). Revision of the European species of the genus *Trachelipus* Budde-Lund, 1908 (Crustacea: Isopoda: Oniscoidea). *Zoological Journal of the Linnean Society*, **121**: 129-244.

Sfenthourakis S., Anastasiou I. Strutenschi T. (2005). Altitudinal terrestrial Isopod diversity. *European Journal of Soil Biology*, **41**, 91-98.

Tomescu N. (1974). Cercetări morfologice, biologice si ecologice la izopodele terestre, teză de doctorat, pp. 224, Univ. "Babeş-Bolyai" – Cluj.

Tomescu N. (1992). Izopode terestre (Crustacea: Izopoda) din Delta Dunării, *Analele Ştiinţifice ale Institutului Deltei Dunării, Tulcea.*

Tomescu N., Schneider A. E., Weiss I. (1979). Die Isopoden einen Südhanges im Hűgelland Südsiebenbűrgens. *Muzeul Brukenthal – Sibiu, Studii şi comunicări – Şt. Nat*., **23**, 275-286.

Tomescu N., Accola S. (1992). Postembryonic development of *Trachelipus difficilis rotundatus* (Crustacea: Isopoda), *Studia, Univ. "Babeş-Bolyai", Biologia*, **37**, 2, 33-37.

Tomescu N., Accola S., Paşca C. (1995). Ecology of the population of terrestrial Isopods (Crustacea: Isopoda) in the Cheile Turzii. Studia, *Univ. "Babeş-Bolyai", Biologia,* **XL**, 1-2, 78-94.

Tomescu N., Ardelean G., Mureşan D., Popa V. (2000). Ecology of terrestrial Isopods in Nature Reserve Scărița-Belioara, Romania. *Studia, Univ. "Babeş-Bolyai", Biologia*, **XLV**, 1, 57-64.

Tomescu N., Mureşan D., Popa V. (2001). The terrestrial Isopod fauna in the superior Basin area of the Someşul Cald River, *Studia, Univ. "Babeş-Bolyai", Biologia*, **XLVI**, 2, 43-47.

Tomescu N., Dolnițchi-Olariu L., Mureşan D., Popa V. (2002-a). Reproduction and distribution of congener terrestrial Iopod species (Crustacea: Isopoda) in the Dorna Depression and the adjacent areas. In memoriam "Professor Dr. Doc. Vasile Radu", Corresponding member of Romanian Academy of Sciences, 11-18, Cluj University Press.

Tomescu N., Mureşan D., Popa V. (2002-b). Faunistic and ecological researches on the terrestrial Isopods from the superior sector of the Arieş River Basin. *Studia, Univ. "Babeş-Bolyai", Biologia*, **XLVII**, 1, 3-14.

Tomescu N., Mureşan D., Olariu L., Hotea R. (2005). Terrestrial Isopod communities (Crustacea: Isopoda) in riverside coppices and meadows of mountains, hilly and depression areas. *Studia, Univ. "Babeş-Bolyai", Biologia*, **L**, 2, 19-25.

Vadkerti E., Farkaş S. (2002). The terrestrial Isopod fauna of the Rinya region IV. Silonics-pusta, *Natura Somogyiensis*, **3**, 27-3.

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 17-24

THE AQUATIC MALACOSTRACEAN FAUNA (CRUSTACEA: MALACOSTRACA) FROM THE MEHEDINTI PLATEAU RIVERS

LUCIAN PÂRVULESCU[1]

SUMMARY. The present paper represents a biogeographically study regarding the distribution of crustacean species from the Amphipod and Decapod groups in the rivers of the Mehedinți Plateau. The georeferenced data can be used in the management policies of the natural protected areas, the *Austropotamobius torrentium* species being „a prioritary species", therefore completing the old existing data.

KEYWORDS: *Austropotamobius* torrentium, stone crayfish, amphipods, Mehedinti Plateau, distribution

Introduction

The Crustacean subphylum represents a relatively small percentage of the entire actual assembly of specific diversity of the Arthropods Phylum. At the same time, the crustaceans occupy a dominant position in the development of the trophic circuit from the aquatic ecosystems, being the main primary trophic resource converters into animal biomass. This is explained by the fact that, the actual biomass of the crustacean populations from the aquatic ecosystems of our planet surpasses the cumulate biomass of the rest of the entire metazoans group (Müller, 2002). They live in all kinds of stagnant or running waters, with shores rich in roots and oozy or stony floors.

In the lattice aquatic ecosystems, the crustaceans from the Malacostraca Class belong to the Isopods, Amphipod and Decapods orders, the Decapods also being water quality indicators and having 8 points by means of species from the *Astacus* and *Austropotamobius* genus, according to Biological Monitoring Working Party Score - BMWP (Chapman and Jackson, 1998). Among Astacidae the Stone Crayfish *Austropotamobius torrentium* is the most relevant species in relation to the habitat's health due to the fact that, it is the most sensitive to pollutants among all crayfishes (Băcescu, 1967). They prefer the upper courses of rivers, being included in the European Council's Directive 92/43 Annex 5 „Plant and animal species of common interest whose sampling from nature and exploitation is making the object of management measures" is rated as „prioritary species" (Pârvulescu, 2007). According to the Red List of the International Union for Conservation of Nature and Natural

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Resources (IUCN Red List) this species has the „Insufficiently Known" attribute (*http://iucnredlist.org*).

The existing data regarding the distribution of this species are rather old Călinescu (1929) and Băcescu (1967) and for a better management in the region, it is more than required to bring the information up to date.

The analyzed area, the Mehedinți Plateau, is a well individualized region, found at the root of the Mehedinți Mountains, between the Motru Valley in the north-east and the Danube Valley in the south, representing a passage towards the Getic Plateau. The individuality of the landscape is represented by the geographical location, under the influences of the warm Mediterranean air masses, by the direct contact with the mountains, by the geological formation (crystalline schist's and lime stone), tectonically fragmentation, the variety of the relief with gorge-like valleys, dolines, caves, etc, and the hydrographical network characteristic to karst landscapes with numerous underground drainages or dry valleys.

In this paper we intend to make a contribution to the knowledge on the structure of the benthonic fauna of the rivers and especially to the knowledge on the crustaceans from the Malacostraca Class from the area of the Mehedinți Plateau, bringing new data regarding the species distribution in the studied region.

Materials and Methods

Between July and August 2007 and August 2008 qualitative samples have been collected from the rivers and creeks from the Mehedinți Plateau, representing a total of 30 locations.

For the collection of the benthos samples, for the capture of small crustaceans, I have used a net with the dimension of the holes of 350 µm. We have gathered the samples by carting the net on the floor of the water meanwhile, up the river, the rocks and the plants were emboweled and washed. The species identification was accomplished in the laboratory using the optic microscope and the binocular magnifier. For details, micro dissections and microscopic processing were carried out. For the identification of the amphipods we used the determinative manual by Cărăuşu *et. al.* (1955), bringing up to date the systematic according to the actualized data provided by Fauna Europaea (*http://faunaeur.org*). The collected specimens were kept in alcohol 70% in the collection entitled „Specimens: Lucian Pârvulescu".

For the accomplishment of the envisaged proposes, in accordance with the decapods distribution, several stages of identification were made: consulting the reference literature regarding the presence of the species in the area, consulting, the river segments where the investigations will be carried out were determined, on a topographical map, gathering of the specimens from the water bed, gathering of the informative data from the local inhabitants (fishermen, hunters etc).

The gathering of the specimens was made directly by hand, covering approximately 200 meters in each investigated river and controlling the shores and the small spaces between the rocks. The longer rivers were investigated in at least 2

parts of the water course. We declared the species as being „*absent*" when, after covering almost 400 meters, no specimen was found, under the reserve that, when, due to the morphological conditions of the substratum, the gathering of the specimens was not possible.

The captured specimens were identified *in situ* taking into account the morphological criteria, then sexed and photographed. After the identification took place, the specimens were set free in the same place from where they were collected. Presently, the photographs, having the RAW format, are stored in the collection „Images: Lucian Pârvulescu".

For the identification, we have used the Băcescu (1967) and Ingle (1997) determinative manual. In the field, data regarding the aquatic habitats and benthonic biodiversity were collected. For the editing of the maps, we used the Inkscape 0.45 (*http://inkscape.com*) software, using as a basis, a topographical map of the region, scaled 1:50.000.

Results and discussion

Between July-August 2007 and August 2008 the lattice aquatic habitats were investigated in the geographical region of the Mehedinți Plateau, in order to list the malacostraceans species. The investigations contained the middle and upper portion of the rivers (Fig. 1). Hereinafter, we will present a general picture of the four hydrographical basins along with the investigated locations, and also, centralized data in Table 1.

The Motru River hydrographical Basin was investigated from the upper part, on the Valea Carpinei tributary (indicative location 1). At this location the *Gammarus balcanicus* Schäferna, 1922 amphipod was identified. The Left Tributary which drains into the barrier lake (indicative location 2) showed the presence of the amphipods by the means of the *G. balcanicus* species. In none of these locations, the decapods were identified. The upper portion of main course of the Motru River, towards the confluence with the Motru Sec River, was investigated (indicative location 3) and 2 species of amphipods were identified: *G. balcanicus* and *Gammarus fossarum* Koch, 1835. Taking into account the information offered by the local inhabitants, the decapods are present only in the creeks from the upper part of the river.

The Motru Sec River was investigated in three locations, on the main course near Lazului Cave (indicative location 4), on the Capra Tributary (indicative location 5) and up the river towards the Source of the river (indicative location 6). In the Motru Sec River location, the presence of the crustacean species was not identified in the collected samples, but at the other two locations, the amphipod species *G. balcanicus* was found. The presence of the decapods was not found in any of the investigated locations. According to the information provided by the rangers of the Domogled - Valea Cernei National Park, the decapods are present in most of the tributaries of the Capra River, towards the upper part.

The Brebina River (a left tributary of the Motru River) was investigated in only one location, in the lower part, towards the Obârşia Cloşani village (indicative location 7). At this location, species of amphipods by means of *G. balcanicus* were identified, as well as species of decapods, by means of *Austropotamobius torrentium* Schrank, 1803. According to the local inhabitants from the Obârşia Cloşani village (the City Hall secretary) the decapods are also present in the smaller tributaries of the Brebina River: Mazdreana creek, Comăneşti creek, Valea Seacă creek, Ogaşu Bradului creek. In the Bulba tributary (indicative location 8) the presence of the amphipods *G. balcanicus* was identified, as well as of decapods *A. torrentium*.

The left tributary of the Motru River, the Iupca River, was investigated in the upper part near the Băluţei Gorges (indicative location 9), identifying *G. balcanicus* amphipod species and *A. torrentium* decapods species. The investigation carried out on the Pistriţa River (indicative location 10), as well as those on the Rudina River (indicative location 11) did not show any presence of aquatic malacostracean species.

The Ponoarele Depression, without any exterior link to the Motru River Basin, was investigated, in tree locations. Cracu Muntelui (indicative location 12) showed the presence of amphipods *G. balcanicus* and decapods *A. torrentium*. The tributary which drains into Zăton Lake (indicative location 13) did not show the presence of any malacostraceans in the collected samples, probably due to the week, intermittent flow of the water course. The Valea Mare creek, which also drains into the Zăton Lake (indicative location 14) showed, in the collected samples, the presence of amphipods species by means of *G. balcanicus* and also of decapods, by means of *A. torrentium*.

The hydrographical Basin of the Coşuştea River was investigated in two locations, Isverna Creek towards the exit from the Isverna Cave (indicative location 15) showed richness in species of amphipods by means of two species: *G. balcanicus* and *G. fossarum*, as well as decapods species represented by *A. torrentium*. The investigations carried out on the left tributary of the Coşuştea River, Brîgleasca creek (indicative location 16) showed the presence of *G. balcanicus* and *G. fossarum* amphipods and of the *A. torrentium* decapods. The left tributary of the Coşuştea River, Turtaba creek, was also investigated (indicative location 17) showing only the presence of decapod species by means of *A. torrentium*. The main course of the Coşuştea River, upwards from Cerna Vârf village was investigated (indicative location 18) and, both amphipod species *G. balcanicus* and *G. fossarum* and decapod species *A. torrentium* were found present.

In the right tributary of Coşuştea, Coşuştiţa River (indicative location 19) the collected samples showed only *G. balcanicus* amphipods. On the upper course of the river is carried out an operation of gravel.

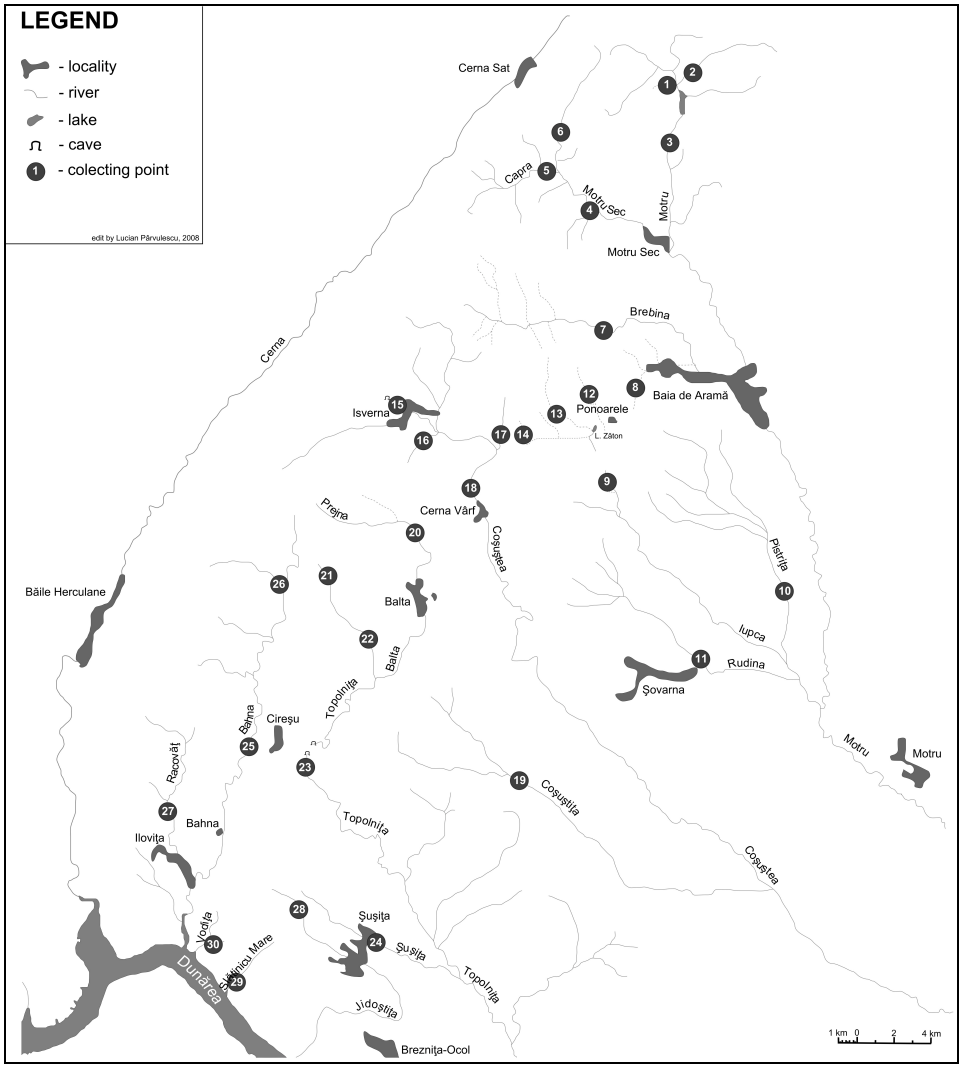

Fig 1. Geographical distribution of the points in which the sampling of the malacostraceans species was made, in the Mehedinți Plateau.

The hydrographical Basin of the Topolnița River was investigated in four locations. Prejna creek, upwards Balta village (indicative location 20), showed the presence of amphipod species *G. balcanicus* and decapod species *A. torrentium*. The locations found at the source of the Topolnița River, upwards from Mălărișca village (indicative location 21) and downwards from Sfodea village (indicative location 22) have sown the presence of amphipods species *G. fossarum*, as well as decapods species *A. torrentium.*

Table 1.

The centralized georeferenced data of the observed species from very location, in DMS system (x: present, -: absent)

Indicative location	Coordinates (DMS) N/E	*Gammarus balcanicus*	*Gammarus fossarum*	*Austropotamobius torrentium*
1	$45^0$08’16’’ / $22^0$48’13’’	x	-	-
2	$45^0$08’13’’ / $22^0$48’39’’	x	-	-
3	$45^0$06’29’’ / $22^0$48’08’’	x	x	-
4	$45^0$04’24’’ / $22^0$44’51’’	-	-	-
5	$45^0$05’34’’ / $22^0$43’33’’	x	-	-
6	$45^0$06’09’’ / $22^0$43’37’’	x	-	-
7	$45^0$01’16’’ / $22^0$42’40’’	x	-	x
8	$44^0$59’44’’ / $22^0$47’16’’	x	-	x
9	$44^0$57’11’’ / $22^0$45’26’’	x	-	x
10	$44^0$53’48’’ / $22^0$52’55’’	-	-	-
11	$44^0$52’21’’ / $22^0$48’20’’	-	-	-
12	$44^0$59’04’’ / $22^0$45’44’’	x	-	x
13	$44^0$59’06’’ / $22^0$44’37’’	-	-	-
14	$44^0$58’46’’ / $22^0$43’57’’	x	-	x
15	$44^0$58’45’’ / $22^0$37’21’’	x	x	x
16	$44^0$58’05’’ / $22^0$38’37’’	x	x	x
17	$44^0$58’16’’ / $22^0$41’26’’	-	-	x
18	$44^0$55’45’’ / $22^0$40’48’’	x	x	x
19	$44^0$49’02’’ / $22^0$40’51’’	x	-	-
20	$44^0$54’58’’ / $22^0$38’09’’	x	-	x
21	$44^0$54’09’’ / $22^0$34’12’’	-	x	x
22	$44^0$51’35’’ / $22^0$33’13’’	-	x	x
23	$44^0$48’48’’ / $22^0$33’37’’	x	x	x
24	$44^0$43’37’’ / $22^0$35’51’’	x	-	x
25	$44^0$48’51’’ / $22^0$31’07’’	x	-	-
26	$44^0$50’22’’ / $22^0$31’43’’	x	-	x
27	$44^0$46’21’’ / $22^0$27’44’’	x	x	x
28	$44^0$43’46’’ / $22^0$33’29’’	x	-	x
29	$44^0$42’12’’ / $22^0$30’36’’	x	-	-
30	$44^0$43’24’’ / $22^0$29’12’’	x	-	-

The river was also searched at the exit of the Topolnița Cave (indicative location 23). In the gathered samples amphipod species *G. balcanicus* and *G. fossarum* were found, as well as decapods *A. torrentium*. The investigations carried out on the Șușița River (indicative location 24) showed the presence of amphipods by means of *G. balcanicus* species and decapods by means of *A. torrentium* species.

The direct tributaries of the Danube were investigated, as follows: the Bahna River, near the bridge which makes the connection between Cireşu and Negruşa villages (indicative location 25), showed the presence of malacostracean species only by means of *G. balcanicus* amphipod. In the upper part (indicative location 26), in the collected samples, the *G. balcanicus* amphipod and *A. torrentium* decapod were found; the left tributary of the Bahna River, the Racovăț creek (indicative location 27), shows, in the collected samples, the presence of amphipods by means of *G. balcanicus*, *G. fossarum* and the *A. torrentium* decapod. The Jidoştița River (indicative location 28) was researched in a place located in the upper part of the Jidoştița village and displayed *G. balcanicus* amphipods species and *A. torrentium* decapod species. The investigations carried out in the Slătinicu Mare creek (indicative location 29) showed the presence of the crustaceans only by means of *G. balcanicus* amphipod. This situations is similar to the one obtained from Vodița creek (indicative location 30).

Conclusions

The qualitative researches between 2007 and 2008 in the region of the Mehedinți Plateau offer the possibility of data interpretation regarding the distribution of the aquatic malacostracean species in the studied region.

- There have been collected and identified two species of amphipods from the Gammaridae family: *Gammarus balcanicus* and *Gammarus fossarum*. The most commonly found species *Gammarus balcanicus* was identified in 23 out of 30 locations;
- No aquatic isopods have been found on samples collected in Mehedinti Plateau Rivers;
- According to the field observations, we can easily say that, the populations of the *Austropotamobius torrentium* decapods are small declining, problems being identified mostly in the extreme upper part of the Motru River, where, the species has been identified only in the extreme upper part of the river. The species is absent from the middle and lower course of the river, probably due to forest exploitations. We still have to mention that, the human impact of the near by villages is not related with the presence of the species either on the upper part or on the lower parts of the river; the presence of the species is relatively constant.

Acknowledgements

I would like to take this opportunity to give my regards to the Environmental Ministry and to Natura 2000 Network for the material support, to the Administration of the Mehedinți Plateau Geopark, represented by engineer Cornel Meilescu and to Ms. Adina Lazăr for the English translation of this text.

REFERENCES

Băcescu, M. C., *Fauna Republicii Socialiste România - Crustacea, Decapoda*, Vol. IV, pp. 209-219; Editura Academiei Republicii Socialiste România, Bucureşti, 1967.

Călinescu, R. I. (1929), *Astacus torrentium Schrank, în Munții Olteniei*, "Revista Arhivelor Olteniei", **8** (41/42), 106-109.

Cărăuşu, S., Dobreanu, E., Manolache, C., *Fauna Republicii Populare Române, Crustaceea, Amphipoda, forme salmastre şi de apă dulce*, Vol. IV, Fascicula IV, 407 pp , Academia R.P.R., Bucureşti, 1955.

Chapman, D., Jackson, J. *Biological monitoring.*, in Bartram, J., Ballance, R. (Eds.), *Water quality monitoring – A practical guide to the design and implementation of Freshwater Quality Studies and Monitoring Programmes*, UNEP/WHO, Taylor & Francis, 1998.

Ingle, R., *Crayfishes, Lobstars and Crabs of Europe, An illustrated guide to common and traded species*, pp. 33-40 ; pp. 177-191, Chapman & Hall, 1997.

Müller, G. I., In Godeanu, S. P. (Ed.), *Diversitatea lumii vii – Determinatorul ilustrat al florei şi faunei României. Vol. II – Apele continentale*, partea II, pp. 391-392, Editura Bucura Mond, Bucureşti, 2002.

Pârvulescu, L., in Combroux, I., Thiry, E., Țoia, T. (Eds.), *Caiet de habitate şi specii - Fişe pilot: Austropotamobius torrentium*, pp 47-49, Editura Balcanic Timişoara, 2007.

*** Fauna Europaea http://www.faunaeur.org (accessed in 22. 05. 2008)

*** 2007 IUCN Red List of Threatened Species http://www.iucnredlist.org (accessed in 14. 01. 2008)

Software – map editor: Inkscape 0.45 (http://inkscape.com)

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 25-38

INCORPORATING OCCUPANCY MODELS IN DESIGNING STUDIES OF ANIMAL DISTRIBUTION: A GLIMPSE ON THE HABITAT USE OF AN AMPHIBIAN IN THE SAXON LANDSCAPES OF TRANSYLVANIA

TIBOR HARTEL[1], COSMIN IOAN MOGA[1]

SUMMARY. The importance of occupancy models in studying the habitat use and distribution of organisms was only relatively recently emphasized. Their advantage is that allow to predict the site occupancy and at the same time to quantitatively estimate the detection probability of the studied organism. Here we apply for the first time in Romania these models on *Hyla arborea*, a locally and regionally common but threatened amphibian. 30 permanent ponds were studied in 2007 and 2008. Our results show that the detection probability is high (>0.7), and the differences between the found percentage of occupancy and predicted occupancy was small. However, the data not accounted for detection probability may underestimate the use of ponds containing predatory fish. According to the count data there is a sharp decline of *H. arborea* in these ponds, but the occupancy models predict no such decline, suggesting that some ponds with *H. arborea* were missed in 2008. The detection probability was positively related to the emergent vegetation cover in the ponds, but the effect of vegetation was stronger in 2007 than in 2008. We suggest the estimation of detectability on different sensitive species before their local – regional decline.

KEYWORDS: habitat, distribution, conservation, detection probability, *Hyla arborea*, Romania

Introduction

Knowing the site occupancy and distribution of organisms and also its spatial and temporal variation was and actually is an important challenge for ecologists. The range of application of such data is wide: biogeography (Bănărescu, 1970), island biogeography (MacArthur and Wilson, 1968), metapopulation ecology (Lewins, 1970;

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Hanski, 1998), community ecology (Simberloff, 2004), landscape ecology (Hartel *et al.* 2008), distributional change of organisms (Skelly *et al.* 2003) the ecology of invasive species (Simberloff, 2000), the climate change effects (Arajuo *et al.* 2006), biodiversity monitoring projects (Gibbons *et al.* 1997), conservation biology (Pellet and Schmidt, 2005) and environmental impact assessments.

When interpreting field data, researchers often assume that the detection probability of the organisms is 1 (i.e. the species, if present, is detected, otherwise not). However, a growing body of evidence suggests that the detection probability of 1 is rare in nature (Schmidt, 2004; 2005; MacKenzie 2005a;b), most of species having a detection probability less than 1. Therefore the site occupancy or demographic parameters of populations may be underestimated to an unknown degree if the detection probability is not taken into account. The costs of these biases may be high: local population turnovers (Moilanen, 2003), population trends (Funk *et al.* 2003; Schmidt, 2004) may be misestimated, important habitats for organisms may be wrongly identified (Mazerolle *et al.* 2005), local and national status of organisms may be misestimated (Schmidt, 2004), infrastructural development may be wrongly planned and finally priorities for management and conservation may be improperly set (MacKenzie, 2005a;b; Schmidt, 2005).

As the studies regarding the herpetofauna inventories are still frequent and needed in Romania (the first comprehensive results on a region were presented in Ghira *et al.* [2002]; and the numerous studies following that report for example Covaciu-Marcov *et al.* [2006], Strugariu *et al.* [2008]) our preliminary purpose is to attract attention to researchers to incorporate site occupancy models in their research design and data analysis. Researchers often use count data to express the proportion of habitat used by different species. However, the count data represent only an index of the true value of habitats occupied by the studied species (and not a "mirror" of it, as it is assumed) and are dependent on the detection probability and also on the real (but unknown) values of the measured parameter (Schmidt, 2003; 2004). The count data provide only a minimum estimate of an unknown quality (Schmidt, 2003): the researcher doesn't know how many specimens/populations he missed in his study. When the comparison of two or more counts is attempted (for example the proportion of habitat use in different years) the possibility for wrong conclusions becomes even higher because the detection probability and the true value of the habitats occupied may also change from year to year, to an unknown degree. As Schmidt (2003; 2004) noticed, amphibian ecologists seem to be unaware of pitfalls that the data unadjusted for detection probability represent. The first study that incorporated detection probability in estimating site occupancy rates in amphibians seems to be that of MacKenzie *et al.* (2002). Till then and since then many papers appeared with significant contribution on the field of amphibian ecology that not consider the detection probability in their study

design (Brönmark and Edenhamn, 1994; Hecnar and M`Closkey, 1996a,b; Hecnar and M`Closkey, 1997; Sjögren, 1991; Gagne and Fahrig, 2007 but see Mazerolle *et al.* 2005). The long term series analyses that were on the base of the "global decline" conclusion in amphibians (Houlahan *et al.* 2000) also used data that were not adjusted to detection probability. Our site occupancy studies on the Tarnava Mare basin are not exceptions (Hartel *et al.* 2006; 2007a).

In this study we will apply site occupancy model to estimate pond use and detection probability of the Tree Frog (*Hyla arborea*) in a small (30) sample of permanent ponds in the rural landscapes of the Saxon Transylvania (middle section of the Tarnava Mare basin). *Hyla arborea* is strictly protected under European (Bern Convention, Annex II, Habitat Directive, Annex IV) and Romanian level (Ministerial Order 1198/2005, Annex 3A). The population ecology of *H. arborea* is well studied in Europe (see for example Brönmark and Edenhamn, 1994; Vos *et al.* 2000; Pellet and Hoehn, 2004; Pellet *et al.* 2004; Grafe and Meuche, 2005; Pellet *et al.* 2005; Schmidt and Pellet, 2005; Van Buskirk, 2005; Vos and Stümpel, 1995; Pellet and Schmidt, 2005; Pellet *et al.* 2007; Kovács *et al.* 2007). *Hyla arborea* prefers shallow, sunny ponds (Pellet and Hoehn, 2004; Pellet, 2005) and it is sensitive to fish predation (Brönmark and Edenhamn, 1994; Van Buskirk, 2005; Hartel *et al.* 2007a) and habitat fragmentation (Andersen *et al.* 2004). Due to these features, it was proposed as umbrella species, its presence indicating amphibian communities that are species rich (Pellet, 2005; Öllerer, 2006). Many studies suggest that *H. arborea* is still widespread in Romania (for example: Ghira *et al.* 2002) including this area (Hartel *et al.* 2007a). Since the predatory fist introductions, together with other modification of the permanent pond habitats will expectedly be more and more frequent in Romania, monitoring the habitat use of this species in order to detect potential distributional changes at regional scale is urged. The specific aims of this paper are twofold:

(i) To compare the naïve (i.e. count data) and estimated values of habitat use in *H. arborea* in two years (2007 and 2008) in two pond categories: ponds without predatory fish and ponds with predatory fish.

(ii) To estimate the minimum number of site visits to conclude that the species is absent in the two pond categories.

Material and Methods

Study area and the surveys

The 30 permanent ponds surveyed for this study represented a small sample of previously surveyed (Hartel *et al.* 2007a;b) and newly located (2007) ponds. The study area is in the middle section of the Tarnava mare basin. The central section of the basin is dominated by hills ranging in elevation from maximum 600-800 m in the west to maximum 750-800 m in the east. The climate is continental (Pop 2001) with mean annual temperatures of around 6.5-9°C and mean annual rainfall ranges from 600 to 800 mm (Pop 2001). Other characteristics of the study site were previously presented (Hartel *et al*. 2007a,b).

In this study the presence of *H. arborea* in the studied pond was assessed using call surveys (Grafe and Meuche, 2005). The surveys were conducted in 2007 and 2008. Two of these surveys were conducted in May (between 10 and 18 of May) and an the other survey was conducted in June (5-7). Only night surveys were made (i.e., between 20.00-22.00). The ponds were easily accessible by car from Sighisoara. We stay maximum 5 minutes listening for frogs at the edge of the pond (or at maximum 50 meters from it). Most of time, the frogs were identified in less than one minute of listening. In this case, we stopped the listening in that pond and after noting the presence of *H. arborea* we continued the survey in the next pond. We are aware on the constrains caused by the short period of listening for calling frogs. Calling survey for relatively short time in optimal periods are frequently used in amphibian habitat use research (Pope *et al.* 2000, Gagne and Fahrig 2007 for example used 5 minutes as listening periods at each pond) and for us it was the best option to survey quickly the ponds for *Hyla arborea* in the two years.

Following Hartel *et al.* (2007a) we classified the permanent ponds in two categories: ponds without predatory fish and ponds with predatory fish. The fish species were included in predatory-non predatory category according to Hartel *et al.* (2007a). The emergent vegetation cover was quantified visually for each pond, as percentage (Hartel *et al.* 2007a).

Data analysis

The program PRESENCE (that implements the likelihood approach of site occupancy models developed by MacKenzie *et al.* 2002) was used to estimate the detection probability (p) and the proportion of sites occupied (ψ). The essence of the site occupancy models developed by MacKenzie *et al.* (2002) is that they simultaneously estimate the site occupancy, and detectability (see also MacKenzie

2005 a,b). The assumptions of this model (see also Schmidt 2005) are: (i) the sites remain occupied during the study period, no extinction, emigration or colonization happens, (ii) the detection probability is greater than zero and (iii) the detection of a species in a site is not influenced by the detection at other sites. Table 1 show a local example about how the detection histories should be used to estimate detection probability, by presenting the detection histories for $N = 30$ permanent ponds.

The detection probability (p) can be used to estimate the minimum number of visits ($N\ min$) necessary to be certain with a specified degree of confidence a species is absent from a surveyed site. The degree of confidence (α) for this estimation can be set to 0.05 (95% confident) (Kéry, 2002) or lower such is 0.01 (99% confident) (Reed, 1996). Thus

$$N\ min = \log(\alpha) / \log(1 - p)$$

where p is the detection probability (see equation (1)). The equation for $N\ min$ was solved for both 0.05 and 0.01 confidence intervals.

To calculate the probability of not seeing a species (F) after N visits following equation was used (Pellet and Schmidt, 2005):

$$F = (1\text{-}p)^N$$

F was calculated for every type of habitat that we considered in this study, for $N = 3$ (i.e. the number of visits on each site, see above). We have calculated the above parameters (ψ, p, $N\ min$ and F) separately for ponds that contained predatory fish and ponds without predatory fish (see above).

We calculated the rate of change in pond occupancy comparing the naïve estimates and the predicted estimates of the proportion of pond use in the two years. This was calculated as (site occupoancy_{2002}-site occupancy_{2001})/site occupancy $_{2001}$ (Schmidt 2005).

Table 1.

The detection histories for *H. arborea* in 2007 and 2008. "0" = the species was not detected, "1" the species was detected

Site	Visits in 2007			Visits in 2008			Status
	1st	2nd	3rd	1st	2nd	3rd	
1	0	0	0	0	0	0	"absence"
2	1	1	1	1	0	1	"persistence"
3	1	1	1	1	1	1	"persistence"
4	1	1	1	0	1	1	"persistence"
5	0	0	0	0	0	0	"absence"
6	0	0	0	0	0	0	"absence"
7	0	0	0	0	0	0	"absence"
8	0	1	1	0	0	0	"extinction"
9	1	1	1	1	1	1	"persistence"
10	0	1	0	0	1	0	"persistence"
11	1	1	1	1	1	1	"persistence"
12	0	1	0	0	1	0	"persistence"
13	0	1	0	0	1	0	"persistence"
14	1	1	1	1	1	1	"persistence"
15	1	1	1	0	0	1	"persistence"
16	0	0	0	0	0	0	"absence"
17	1	1	1	1	1	1	"persistence"
18	1	1	1	1	1	1	"persistence"
19	1	0	1	0	0	1	"persistence"
20	1	1	1	1	1	1	"persistence"
21	1	1	1	0	1	1	"persistence"
22	1	1	1	1	1	1	"persistence"
23	1	1	1	0	1	1	"persistence"
24	0	1	0	0	0	0	"extinction"
25	0	0	0	0	0	0	"absence"
26	1	1	1	0	1	1	"persistence"
27	1	1	0	1	1	0	"persistence"
28	1	1	1	1	1	1	"persistence"
29	1	1	1	1	1	1	"persistence"
30	0	0	1	0	0	1	"persistence"

Results

The detection histories for the 30 sites studied in the two years are presented in the Table 1. Two apparent extinctions and no colonization have occurred. Most of populations persisted from one year to the other.

Count data (naïve estimate of habitat use and distribution)

The naïve estimate of the habitat use shows an overall decrease in this, from 80% (2007) to 73% (2008) (Table 2), the rate of change in pond occupancy being -0.27. The percentage of pond occupancy was larger in the ponds without predatory fish than in ponds with predatory fish. No decline was registered in the habitat occupancy in ponds without predatory fish (Table 2). The ponds with predatory fish showed a loss of 13.21% of pond populations from 2007 to 2008 (Table 2) suggesting quite large rate of change in the number of occupied ponds (-0.54).

Table 2.
The parameter estimations of site occupancy in *Hyla arborea*. See the abbreviations in the "Materials and Methods, The model" section

	Naïve estimate	***Ψ*** **(SE)**	***p***	***Nmin*** **(α = 0.05)**	***Nmin*** **(α = 0.01)**	***F*** **(3 visits)**
2007						
Ponds without predatory fish	1.00	1.00 (0.00)	0.96	1	1	<0.0001
Ponds with predatory fish	0.53	0.75 (0.27)	0.33	7	11	0.30
All ponds	0.80	0.80 (0.07)	0.81	1	2	0.007
2008						
Ponds without predatory fish	1.00	1 (0.00)	0.83	2	3	0.02
Ponds with predatory fish	0.46	0.75 (0.36)	0.27	10	15	0.38
All ponds	0.73	0.75 (0.08)	0.70	2	4	0.02

Ψ, p and N min

The *Ψ* also detect a decrease in the pond occupancy in 2008 compared to 2007 (Table 2) with a rate of change of -0.25. Similarly to the naïve estimate, *Ψ* showed a lower percentage of pond occupancy for the ponds with predatory fish, compared to the ponds lacking predatory fish (Table 2). Contrary to the naïve estimation, the occupancy model showed no decrease in the occupancy of the ponds with predatory fish (Table 2). *p* was overall large for ponds without predatory fish (more than 70%) and small for the ponds with predatory fish (< 35%). Note that the *p* varied between the two years, being smaller in 2008. The values of *Nmin* suggest that 1-2 call surveys are enough to infer the absence of *H. arborea* from ponds (with 95% confidence). However, the ponds with predatory fish requires from seven to more than 10 surveys to infer the absence of *H. arborea* with the methodology presented here. The relationship between the *p* and the macrophyte coverage was positive (Figure 1). The percentage of variation explained by the reed cover was smaller in 2008 (24.7%) than in 2007 (31.5%) (Fig. 1).

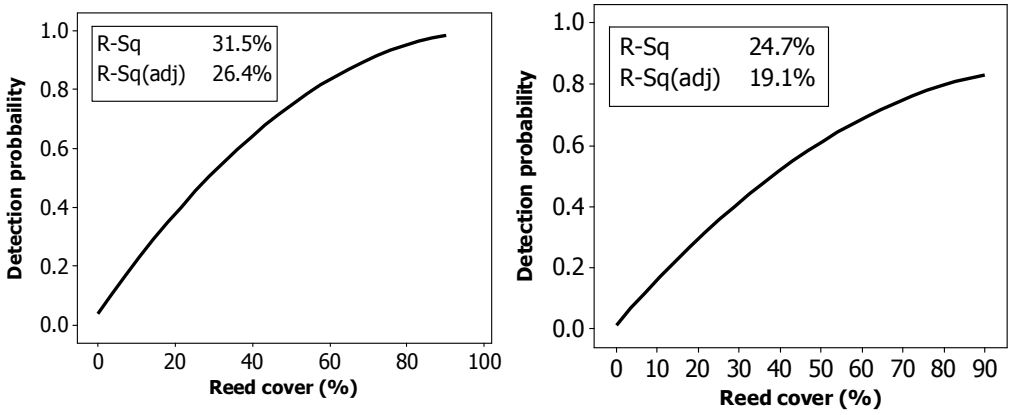

Fig. 1. The effect of reed cover (%) on the detection probability of *H. arborea*. The left figure represents data for 2007, the right figure shows the data obtained for 2008

Discussion

We estimated the detection probability in the same sample of ponds for two years, thus, it was possible to assess its variation in the two years and also between pond types. This study suggests that there are very small (data for 2008) or no (2007) differences between the count data and estimated value of habitat use in the case of *H. arborea* in these landscapes. Similarly, Pellet and Schmidt (2005) found a large detection probability for *H. arborea* ($p = 0.73$) and also they concluded that with the

sampling effort they use (average 3.7 visits per site) they detect all the populations in the studied area. In other, rarer species however, the differences between the naïve and estimated site occupancy were larger. Thus, the naïve *vs* estimated site occupancies were 0.37 *vs* 0.48 in the case of *Bufo calamita* ($p = 0.43$), 0.11 *vs* 0.26 ($p = 0.28$) for *B. variegata*, but 0.11 *vs* 0.13 ($p = 0.56$) for *Alytes obstetricans* (Pellet and Schmidt, 2005).

In agreement with a previous study that used count data for 85 ponds (Hartel *et al.* 2007), the present study also suggest that the predatory fish negatively affect pond use by *H. arborea* (see also Brönmark and Edenhamn, 1991; Van Buskirk 2005) but the increased reed cover positively affect the pond populations of this frog. Nevertheless, in both years the use of predatory fish ponds was underestimated with only three surveys per pond in a season. Recent models show that detection probability depends on the population size fluctuations (Kéry 2002; Alpizar-Jara *et al.* 2004) also. Alpizar-Jara *et al.* (2004) demonstrated that the detection probability and the probability of extinction are negatively correlated. It is possible that the *H. arborea* population sizes in some ponds were decreased after the predatory fish introductions; therefore the calling activity was not so intense in the very small populations. Our own observations suggest that the chorusing intensity of *H. arborea* may sharply decrease in just 3-4 years after massive predatory fish introductions (*Lepomis gibbosus*, *Perca fluviatilis*, see further examples in Hartel *et al.* [2007]). As calling activity is an essential feature of the reproductive and spatial (i.e. metapopulation) dynamic of *H. arborea* populations (Vos and Stümpel, 2005), the negative effects of fish introductions may likely go beyond local populations to metapopulation. Bradford *et al.* (1993) have showed that the massive fish introductions may isolate populations of *Rana muscosa* in mountain ponds. The large choruses act as important conspecific "attractors" for *H. arborea*. Individuals may disperse up to 11 km distances to find already occupied ponds, avoiding empty ponds found on the dispersal route (Vos and Stümpel, 2005). As *H. arborea* also prefer ponds with temporary character (but more constant ones, which dry only occasionally), more emphasis should be given to the creation and maintenance of such ponds in the surroundings of permanent ponds (Hartel *et al.* 2007b).

The status of a species requires knowledge about the trends in its population sizes (Houlahan *et al.* 2000) but also the trends of the number of populations (Sjögren 1991, Hecnar and M`Closkey, 1996). This study suggests that detection probability should be considered in determining status and trends of amphibian populations; otherwise the possibility to misestimate these aspects is high. The count data may suggest a sharp decline in pond occupancy from one year to the next but according to the occupancy model no such trend is obvious (see the ponds containing predatory fish in the Table 2).

The site occupancy models allow the use of many site and sampling specific covariates for accounting the detection probability: weather conditions (Pellet and Schmidt, 2005), habitat features (MacKenzie *et al.* 2002, Schmidt 2005, Mazerolle and

et al. 2005, Pellet and Schmidt 2005), survey methodology and effort (Kéry, 2002; Bailey *et al.* 2004), season (Kéry, 2002; Kéry *et al.* 2005), population size (Kéry 2002). In this study we omitted many other possible effects may potentially act on the detectability *H. arborea*. We have "standardized" our surveys by making the site visits under weather conditions that we considered as being favorable for *H. arborea*.

The results of this study raise the question: how accurate were the count data presented in Hartel *et al.* (2007a)? The surveys in this area begin many years ago, and up to five visits were made on each pond in the activity period of this frog. Moreover, the searches on each sites lasts up to one hour in many cases (instead of five minutes of calling surveys). Assuming a variable but high (>0.7) detection probability (as found by this study) we are confident that the data analyzed in that paper (Hartel *et al*. 2007a) are not biased. However, more interesting is the situation of species that are locally rare in this area (*R. arvalis*, *B. viridis*). As Kery (2000) has suggested, we also believe that special survey programs should be planned for these locally and regionally rare species.

Conclusions and recommendations

Understanding the principles of site occupancy models will undoubtly make researchers more aware regarding the design of the studies and more efficient in allocating effort, nevertheless may help researcher to better formulate the objectives of his / her study. As MacKenzie and Royle (2005) wrote: *"A good study objective should be explicitly linked to how the data will be used to discriminate between scientific hypotheses about the system or how the data will be used to make management decisions*". Accounting for the detection probability (and other parameters that can be estimated from this) is especially important in Romania because of the wide range of natural-seminatural landscapes of which biodiversity is relatively poorly known. As the biodiversity assessment requires a huge amount of effort (financial, personnel or other), and time, the biodiversity and the organisms' distribution may be strongly underestimated. Site occupancy models allow researchers to estimate these biases in quantified way. Moreover, the potential loss caused by the always growing infrastructural, agricultural, urbanistic (or other) developments may also be estimated using these models (i.e. by estimating the likelihood of not finding a species in a landscape after a given number of surveys). When a national program is promoted to assess the distribution of a certain species in Romania, researchers may gather a good image on the organisms` detection probability and estimated site occupancy in different landscapes of Romania. With care, these results can be extrapolated for wider (but structurally similar) areas and represented on the maps using GIS. In this way the status of the species will be more accurately estimated and decision makers will have a clear image about the risks that development poses to biodiversity in different landscapes/areas of Romania.

Hyla arborea is a good candidate for monitoring studies that aims to explore distributional changes on habitat use caused by anthropogenic impact. This is because it is easy to be identified in the field (i.e. using call surveys), it is widely distributed in different regions of Romania (Ghira *et al.* 2002, Covaciu-Marcov *et al*. 2006, Strugariu *et al.* 2008), locally may be still abundant and extremely sensitive to fish introductions. Considering the fact that the spatial extent of habitat use and the detection probability are variable, it is preferable to estimate detectability of species that are sensitive to human induced changes in habitat quality before their local – regional decline (Hecnar and M`Closkey, 1997; Reed 1997).

Acknowledgements
The financial support for this work was assured by the Mihai Eminescu Trust.

R E F E R E N C E S

Alpizar-Jara, R., Nichols, J. D., Hines, J. E., Sauer, J. R., Pollock, K. T., Rosenberg, Ch. R. (2004) The relationship between species detection probability and local extinction probability. *Oecologia* **141**, 652-660.

Andersen, L. W., K. Fog & C. Damgaard. (2004) Habitat fragmentation causes bottlenecks and inbreeding in the European Tree Frog (*Hyla arborea*). *Proc. R. Soc. Lond.* **271**, 1293-1302.

Arajuo, M.B., Thuiller, W., and Pearson. R.G. (2006) Climate warming and the decline of amphibians and reptiles in Europe. *Journal of Biogeography* **33**, 1712-1728.

Bănărescu, P. (1970): *Principles and Problems of Zoogeography.*

Bradford, D. F., Tabatabai, F., Graber, D. (1993) Isolation in remaining populations of the native frog, *Rana muscosa*, by introduced fishes in the Sequoia and Kings Canyon National Parks, California. *Conserv. Biol.*, **7**, 882-888.

Brönmark, C. & P. Edenhamn (1994) Does the presence of fish affect the distribution of tree frogs (*Hyla arborea*)? *Conserv. Biol.* **8,** 841-845.

Covaciu-Marcov, S.D., Ghira, I., Cicort-Lucaciu, A. Șt., Sas, I., Strugariu, Al. & Bogdan, H.V. (2006) Contributions to knowledge regarding the geographical distribution of the herpetofauna of Dobrudja, Romania. *N. West. J. Zool.* **2,** 88-125.

Funk, W. C., Almeida-Reinoso, D., Nogales-Sornosa, F., Bustamante, M. R. (2003): Monitoring population trends of *Eleutherodactylus* frogs. *J. Herpetol.* **37,** 245-256.

Gagne, S. A., Fahrig. L. (2007) Effect of landscape context on anuran communities in breeding ponds in the National Capital Region, Canada. *Landscape Ecol.* **22,** 205-215.

Ghira, I., Venczel, M., Covaciu-Marcov, S.D., Mara, Gy., Ghile, P., Hartel, T., Török, Z., Farkas, L., Rácz, T., Farkas, Z., Brad, T. (2002) Mapping of Transylvanian Herpetofauna. Nymphaea, Folia Naturae Bihariae **29,** 145-203

Gibbons, W. J., Burke, V. J., Lovich, J. E. *et al.* (1997) Perceptions of species abundance, distribution, and diversity: lessons from four decades of sampling on a government-managed reserve. *Environ. Manage.* **21,** 259-268.

Grafe, T.U., Meuche, I. (2005) Estimate of chorus tenure and population size of male European treefrogs, *Hyla arborea*: implications for conservation. Amphibia-Reptilia 26:437-444. Hanski, I. (1998): Metapopulation dynamics. *Nature* **396,** 41-49

Hartel T, Demeter L, Cogălniceanu D, Tulbure M. (2006) The influence of habitat characteristics on amphibian species richness in two river basins of Romania. In: Vences, M, Köhler J, Ziegler, T, Böhme, W, editors. Herpetologia Bonnensis II. Proceedings of the 13th Congress of the Societas Europaea Herpetologica, 31-34.

Hartel, T., Nemes, Sz., Cogălniceanu, D., Öllerer, K., Schweiger, O., Moga, C.I., Demeter, L. (2007a) The effect of fish and habitat complexity on amphibians. *Hydrobiologia* **583,** 173-182.

Hartel, T., Öllerer, K., Nemes, Sz. (2007b) Critical elements for biologically based management plans for amphibians in the middle section of the Târnava Mare basin. *Acta Scientiarum Transylvanica*, **15**, 109-132

Hartel, T. Moga, C. I., Öllerer, K., Demeter, L., Sas, I., Rusti, D., Balog, A. (2008) A proposal towards the incorporation of spatial heterogeneity into animal distribution studies in Romanian landscapes. *N. West. J. Zool.* **4,** 173-188.

Hecnar SJ, M`Closkey RT. 1996a. Regional dynamics and the status of amphibians. *Ecology* **77**: 2091-2097.

Hecnar, S. J., M`Closkey, R. T. (1996b) The effects of predatory fish on amphibian species richness and distribution. *Biological Conservation* **79,** 123-131.

Hecnar, S. J., M`Closkey, R. T. (1997) Spatial scale and determination of species status of the Green Frog. *Conserv. Biol.* **11**: 670-682.

Houlahan, J.E., Findlay, C. S., Schmidt, B.R, Meyer, A.H., and Kuzmin, S.L., 2000 - Quantitative evidence for global amphibian population declines. *Nature* **44,** 752-755.

Kéry, M. (2002) Inferring the absence of a species – a case study of snakes. *J. Wildlife Manage.* **66,** 330-338.

Kéry, M., Royle, A., Schmid, H. (2005) Modeling avian abundance from replicated counts using binomial mixture models *Ecol. Appl.* **15,** 1450–1461.

Kovács, E. H., Sas, I., Covaciu-Marcov, S. D., Hartel, T., Cupsa, D., Groza, M. (2007) Seasonal variation in the diet of a population of *Hyla arborea* from Romania. *Amphibia-Reptilia* **28,** 485-491.

Levins, R. (1970) Extinction. pp. 77–107. In M. Gesternhaber (ed.), Some Mathematical Problems in Biology. American Mathematical Society, Providence, Rhode Island.

MacArthur, R. H., and Wilson, E. O. (1967): The theory of island biogeography. Princeton, New Jersey: Princeton University Press.

MacKenzie, D. I. (2005a) What is there? Dealing with imperfect detection for species presence/absence data. The Australian & New Zealand Journal of Statistics **47,** 65–74.

MacKenzie, D. I. (2005b) What are the issues with presence-absence data for wildlife managers? *J. Wildlife Manage.* **69,** 849-860.

MacKenzie, D. I., Royle, A. J. (2005) Designing occupancy studies: general advice and allocating survey effort. *J. Appl. Ecol.* **42,** 1105-1114.

MacKenzie, D. I., Nichols, I. D., Lachman, G. B., Droege, S., Royle, A., Langtimm, C. A. (2002) Estimating site occupancy rates when detection probabilities are less than one. *Ecology* **83,** 2245-2255.

MacKenzie, D.I., Nichols, J.D., Hines, J.E., Knutson, M.E., Franklin, A.B., 2003. Estimating site occupancy, colonization, and local extinction when a species is detected imperfectly. *Ecology* **84,** 2200– 2207.

Mazerolle, J. M., Desrochers, A., Rochefort, L. (2005) Landscape characteristics influence pond occupancy by frogs after accounting for detection probability. *Ecol. Appl.* **15,** 824-834.

Moilanen, A. (2002) Implications of empirical data quality to metapopulation model parameter estimation and application. *Oikos* **96,** 516–530.

Öllerer, K. (2007) Local and landscape determinants of breeding pond use by amphibians in central Târnava Mare Valley, Romania. Master Thesis, Swedish Biodiversity Centre, Uppsala, Sweden.

Pellet, J. (2005) Conservation of a threatened European tree frog (*Hyla arborea*) metapopulation Thèse de doctorat ès sciences de la vie (PhD) présentée à la Faculté de Biologie et Médecine de l'Université de Lausanne.

Pellet J, Hoehn S. (2004) Characterization of tree frog (*Hyla arborea*) calling ponds in western Switzerland. In Glandt, D., Kronshage, A. (eds). Der Europaïsche Laubfrosh (*Hyla arborea*): Biologie, Schutzmassnahmen und Effizienzkontrollen. *Z. f. Feldherpetologie Supplement* **5,** 159-164.

Pellet, J., Schmidt, B.R. (2005) Monitoring distributions using call surveys: estimating site occupancy, detection probabilities and inferring absence. *Biol. Cons.* **123,** 27–35.

Pellet, J., Hoehn, S., N. Perrin. (2005) Multiscale determinants of tree frog (*Hyla arborea*) calling ponds in western Switzerland. *Biodiv. Conserv.* **12,** 2227-2235.

Pellet, J., Guisan, A. and N. Perrin. (2004) A concentric analysis of the impact of urbanization on the threatened European tree frog in an agricultural landscape. *Conserv. Biol.* **18,** 1599- 1606.

Pellet, J., Rechsteiner, L., Skrivervik, A. J., Zürcher, J. F. and N. Perrin. (2006) Use of the Harmonic Direction Finder to study the terrestrial habitats of the European tree frog (*Hyla arborea*). *Amphibia-Reptilia* **27,** 138-142.

Pop G P. (2001) *Depresiunea Transilvaniei*. Presa Universitarã Clujeanã, Cluj-Napoca.

Pope, S. E., Fahrig, L., Merriam, G. H. (2000) Landscape complementation and metapopulation effects on Leopard Frog populations. *Ecology* **8,** 2498-2508.

Reed, I. M. (1996) Using statistical probability to increase confidences of inferring species extinction. *Conservation Biology* **10,** 1283-1285.

Schmidt, B. R. (2003) Count data, detection probabilities, and the demography, dynamics, distribution, and decline of amphibians. *C. R. Biologies* **326,** 119-124.

Schmidt, B.R. (2004) Declining amphibian populations: the pitfalls of count data in the study of diversity, distributions, dynamics, and demography. *Herpetol. J.* **14,** 167–174.

Schmidt, B. R. (2005) Monitoring the distribution of pond-breeding amphibians when species are detected imperfectly. *Aquat. Conserv.* **15,** 681-692.

Schmidt BR, Pellet J. (2005) Relative importance of population processes and habitat characteristics in determining site occupancy of two anurans. *Journal of Wildlife Management* **69,** 884-893.

Simberloff, D. (2000) Biological invasions - How are they affecting us, and what can we do about them?. *Western North American Naturalist* **61**: 308–315.

Simberloff, D. (2004) Community ecology: it is time to move on? The American Naturalist **163,** 787-799

Sjögren-Gulve, P. (1991) Extinction and isolation gradients in metapopulations: the case of the pool frog (*Rana lessonae*). *Biol. J. Linn. Soc.* **42**, 135-147.

Skelly, D.K., Yurewicz, K. L., Werner, E.E., Relyea, R.A. (2003) Estimating decline and distributional change in amphibians. *Conserv. Biol.* **17,** 744-751.

Strugariu, A., Zamfirescu, S., Nicoara, A., Gherghel, I., Sas, I., Puscasu, C.M., Bugeac, T. (2008) Preliminary data regarding the distribution and status of the herpetofauna in Iaşi County (Romania). *N. West. J. Zool.* 4 *online first*

Van Buskirk, J. (2005) Local and landscape influence on amphibian occurrence and abundance. *Ecology* **86,** 1936-1947.

Vos, C.C. & Stümpel, A.H.P. (1995) Comparison of habitat isolation parameters in relation to fragmented distribution patterns in the tree frog (*Hyla arborea*). *Landscape Ecol.* **11,** 203-214.

Vos, C.C., Ter Braak, C.J.E, Nieuwenhuizen, W. (2000) Incidence function modelling and conservation of the tree frog *Hyla arborea* in the Netherlands. *Ecological Bulletins Copenhagen* **48,** 165-180.

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 39-50

NEST BOXES OCCUPANCY BY THREE COEXISTING DORMOUSE SPECIES AND INTERSPECIFIC COMPETITION IN THE TRANSYLVANIAN PLAIN (ROMANIA)

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SUMMARY. The composition and structure of the dormice communities (fam. *Gliridae*) as well as their interspecific relations and habitat features which shape them were studied in five deciduous forests in the Transylvanian Plain. By monthly check of nest boxes and nest tubes, 1367 records of dormice were made during three years (2005-2007). The communities were formed by two (*Glis glis, Muscardinus avellanarius*) or three species (*G. glis*, *M. avellanarius* and *Dryomys nitedula*) and were dominated by *G. glis* (81%). Interspecific competition for nest boxes appeared between *G. glis* and *M. avellanarius*, resulting in the exclusion of the smaller species. *G. glis* and *D. nitedula* showed a marked preference for nest boxes over nest tubes, while *M. avellanarius* showed no preference for either type of artificial nests. *G. glis* had the lowest occupancy rate (14.61%) in the oak forest with no shrub layer, and the highest (53.02%) in forests with shrub layer well developed, but with limited offer of natural nesting sites (tree hollows). *M. avellanarius* occupancy rates were influenced by the concurrence with *G. glis* and were highest (8.19%) in the oak-hornbeam forest (with nest tubes) and lowest (2.92%) in oak forest with no shrubs. *D. nitedula* had low occupancy rates at all sites except for the oak forest with no shrub layer (8.76%), where it probably supplements its diet with animal food.

KEYWORDS: dormouse communities, nest sites, interspecific competition

Introduction

Three dormouse species have been mentioned in the Transylvanian Plain: *Glis glis*, *Muscardinus avellanarius* (Bielz, 1888) and *Dryomys nitedula* (Sevianu and Coroiu, 2005) and all are considered vulnerable (Murariu, 2005). It is important for

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conservation to know the factors that shape the dormice communities and limit the distribution of species. Habitat selection by each species has been considered an important factor in the community dynamics (MacArthur and Levins, 1964), and species distribution and abundance is determined by the differences in resource availability and quality among habitats as a function of intrinsic habitat features and density of competitors (Guthrie and Moorhead, 2002).

Nest sites may be a potentially limited resource for small mammals (Dooley and Dueser, 1990; Bright and Morris, 1991), especially if they are cavity-nesting species. Most dormouse species (fam. *Gliridae*) prefer nesting in holes such as tree holes (Vietinghoff-Riesch, 1960; Pucek, 1981; Görner and Hackethal, 1988; Juškaitis, 1999, 2006a, 2006b; Morris *et al.*, 1990; Bright and Morris 1991, 1992; Scinski and Borowski, 2006) that satisfy the requirements of thermoregulation and protection from predators (Bright and Morris, 1992), but do not excavate their own cavities. Cavity nesting species constitute a community that interacts and competes for nest sites (Aitken *et al.*, 2002). As secondary cavity nesters, the availability of tree holes could constitute for dormice a limiting factor (Pöysä and Pöysä, 2002; Juškaitis, 2005).

Artificial nest boxes may compensate for the diminishing number of trees with holes, caused by deforestation and selective cutting of mature trees (Morris *et al.*, 1990; Juškaitis, 2005), and are a useful tool for dormice conservation (Bright and Morris, 1992). Being supplied in limited numbers, exploitation competition may appear between mammals and/or bird species that use them (Barba and Gil-Delgado, 1990; Koppmann-Rumpf *et al.*, 2003; Sarà *et al.*, 2005; Juškaitis, 2006a; Adamik and Král, 2008), but only one study (Bakó and Hecker, 2006) regarding the interaction between dormouse species with regards to nest boxes use has been undertaken.

The aims of the present study are: (1) to identify dormouse community composition and structure in areas where several species coexist, (2) to test whether interspecific competition appears over nest sites, (3) to identify the most important habitat features for each dormouse species.

Study area

The study was conducted in the west-central part of the Transylvanian Plain (Romania, 46°46'N, 24°07'E, max. alt. 500m), an hilly area scarcely forested and utilized mainly for agriculture. The mean annual temperature is 7.6°C (Pop, 2001) and the mean annual rainfall is 825 mm (Mac *et al.*, 1987).

Woodland area covered less than 10% of the surface, and forests occupied small, isolated areas, with low or no connectivity, mainly on hilltops and north slopes, while the south slopes were barren due to temperature and humidity conditions that do not facilitate the growth of woody vegetation (Pop, 2001) and to human intervention.

Five sample areas were selected for study using artificial nest boxes and nest tubes (Fig. 1).

Sample area 1-Husnar (46°59'41''N, 23°57'52''E) was an old oak forest (*Quercetum petraeae-cerris*) with numerous hollow trees and the shrub layer very well developed. The canopy species were *Quercus cerris, Q. petraea, Q.robur, Carpinus betulus, Acer campestre* and *Cerasium avium.* The shrub layer was formed by *Ligustrum vulgare, Evonymus europaeus* and *Cornus sanguinea.*

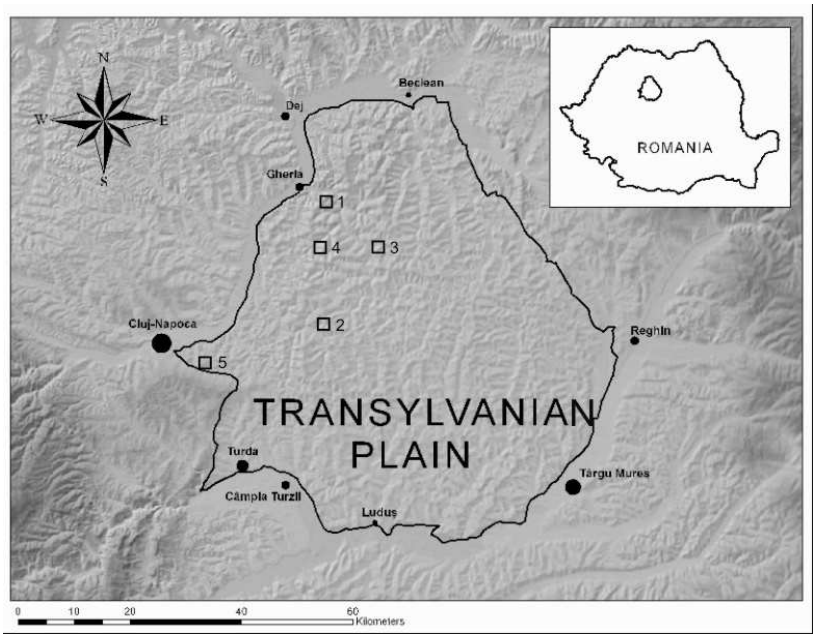

Fig. 1. Sample areas location in the Transylvanian Plain: 1-Husnar, 2-Ghiriş, 3-Ciuaş, 4-Păstăraia, 5-Zăpodie

Sample area 2-Ghiriş (46°47'48''N, 23°58'33''E) was an old clearing (30 years old) of an oak with tatarian maple forest (*Aceri tatarico-Quercetum petraeae-robori*) now dominated by field maple (*Acer campestre*), hornbeam (*Carpinus betulus*) and invaded by locust tree *Robinia pseudacacia*, with a moderately developed shrub layer formed of *Corylus avellana, Cornus mas, C.sanguinea, Clematis vitalba, Ligustrum vulgare, Staphylea pinnata*.

Sample area 3-Ciuaş (46°55'43''N, 24°05'35''E) as a forest dominated by hornbeam and sessile oak (*Lathyro hallersteinii-Carpinetum*) with a few old oaks still standing (120 years old) and a well developed shrub layer.

Sample area 4-Păstăraia (46°55'26''N, 23°57'37''E) was also a forest dominated by hornbeam and sessile oak (*Lathyro hallersteinii-Carpinetum*) with a quite well developed shrub layer.

These four study areas were located in the Fizeş river hydrographic basin.

Sample area 5-Zăpodie (46°44'30N, 23°41'29''E) was a young, single layered oak forest (*Quercetum petraeae-cerris*) with no shrub layer. Other canopy species were *Cerasum avium* and *Tilia cordata*. This forest was located in the periurban area of Cluj-Napoca city.

Material and Methods

200 large wooden nest boxes (20x20x30, hole size 50mm) were mounted in line transects (20 m apart) in sample areas 1-Husnar, 2-Ghiriş, 3-Ciuaş and 5-Zăpodie. In sample area 4-Păstăraia were mounted 50 plastic nest tubes (design after Morris and Temple, 1998) to test each species preference for different types of artificial nest sites. Nest transects were checked once a month, from May 2005 to November 2007.

An additional transect of 50 small wooden nest boxes (14x14x21, hole size 32mm) was installed in spring 2007 in sample area 3-Ciuaş, parallel with the first one, at 20 m distance, in an attempt to increase the occupancy rate of smaller dormouse species. These nest boxes were checked once a month from June to November 2007.

During the monthly check the presence of each dormouse species was recorded (animal present, used nests, identifiable signs such as food remains of faeces) and the occupancy rate was calculated (the percentage of nest boxes/tubes used by each species out of the total number of nest boxes/tubes checked).

The habitat features were categorized in terms of food and natural nest sites offer. The food offer was analyzed according to the presence and abundance of tree and shrub species known as food base for dormice (Franco, 1990; Nowakowski and Godlewska, 2006; Nowakowski *et al*., 2006; Juškaitis, 2007). The nest site offer was evaluated according to the presence and abundance of mature, hollow trees (Vietinghoff-Riesch, 1960; Juškaitis, 1999, 2006b; Morris *et al*., 1990; Bright and Morris 1991, 1992; Scinski and Borowski, 2006) and shrubs and young trees suitable for nest construction (Berg and Berg, 1998; Juškaitis and Remeisis, 2007).

Results and discussions

During the three years (2005-2007) of the research, 1367 records of dormice belonging to three species (*Glis glis, Muscardinus avellanarius* and *Dryomys nitedula*) were registered. The dormouse communities were formed of two (*Glis glis, Muscardinus avellanarius*) or all three dormouse species (*Glis glis, Muscardinus avellanarius, Dryomys nitedula)*. These species occupied the nest boxes/tubes to a different extent.

The dominant species of the community was *G. glis* with over 80% of records, followed at a long distance by *M. avellanarius* with 13% and *D. nitedula* with only 6% (Fig. 2).

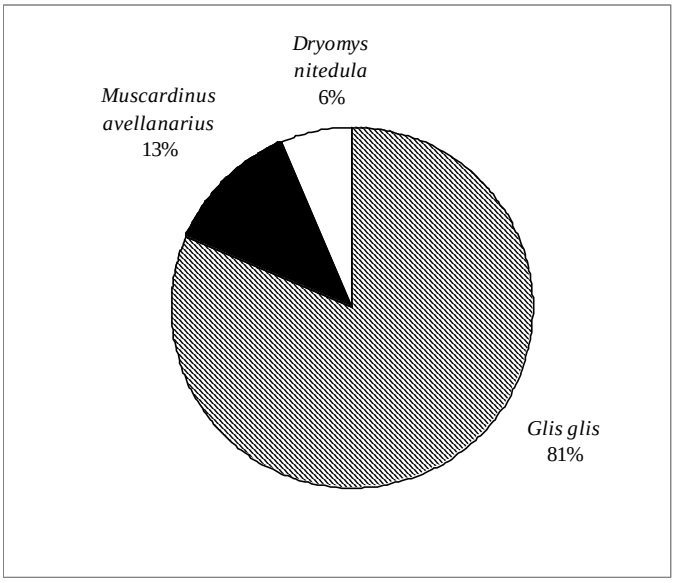

Fig. 2. Percentage of nest boxes/tubes occupied by each dormouse species

Similar results were obtained in the study undertaken in Hungary (Bakó and Hecker, 2006) where in areas with the three dormouse species coexisting, the community was also dominated by *G. glis*, although it reached there a smaller percentage (58%).

The low percentages in nest box occupancy obtained by *M. avellanarius* and *D. nitedula*, and the dominance of *G. glis* reflected the ecological adaptations of the species

regarding nest sites requirements. *Glis glis* nests exclusively in tree holes (Vietinghoff-Riesch, 1960; pers. obs), and consequently readily occupied nest boxes, as a substitute for natural tree cavities. *Muscardinus avellanarius* makes woven nests between branches in cases of limited offer of tree cavities (Juškaitis, 2005). This limitation can be either physical (forests without tree hollows) or social, caused by occupation of tree cavities by other species (Flux, 2003). *Dryomys nitedula* was reported to nest in tree hollows and nest boxes (Scinski and Borowski, 2006), but it also builds woven nests between branches (Popescu and Murariu, 2001; pers. obs.). This species had very low occupancy rates in nest boxes, reaching only 6% of all nest boxes occupied by dormice. The species might live at even lower densities than *M. avellanarius*, hence the low occupancy rates. It is possible that the smaller species (*M. avellanarius, D. nitedula*) were excluded from nest boxes as a result of the behavioral dominance of the larger specie *G. glis*, an assumption also made by Scinski and Borowswki (2006).

In order to verify the hypothesis of exclusion of smaller species from the nest boxes by the larger dormouse species by exploitation competition over a limited resource, I analyzed comparatively the monthly occupancy rates of *G. glis* and *M. avellanarius* by cumulating the results from three years of regular nest boxes check. The overall low occupancy rates of *D. nitedula* did not provide enough data for analysis.

At sites were *G. glis* had high occupancy rates, a negative correlation between the occupancy rates of the two species could be observed (Fig. 3, 4). *M. avellanarius* occupancy rate reached a peak in June, followed by a decrease during the next months, and then started to increase again in early October, reaching a second peak in late October. The occupancy rates of *G. glis* followed an inverse trend. Changes in resource use (divergence) are considered sufficient evidence for the existence of interspecific competition (Pianka, 1976; Wesolowski, 2003).

The wooden nest boxes and the plastic nest tubes were not used to the same extent by the three dormouse species.

G. glis did not breed in the nest tubes, and the occupancy rate showed a clear preference for wooden nest boxes (Fig. 5). The nest tubes installed in site 4-Pastaraia were used as day nests only by males and non-breeding females, as well as by juveniles during autumn dispersal, and the occupancy rates was the lowest of all sites investigated. This species preferred tree holes to plastic nest tubes, and colonized preferentially the natural nest sites when both were available (pers. obs.). The fact that *G. glis* avoided plastic nest tubes and preferred wooden nest boxes was also documented in the research done in Hungary (Bakó and Hecker, 2006) where the two types of artificial nest were placed in the same areas. However, this species did reproduce in nest-tubes installed in plantations in England, despite the fact that wooden nest boxes were also available (Morris and Temple, 1998).

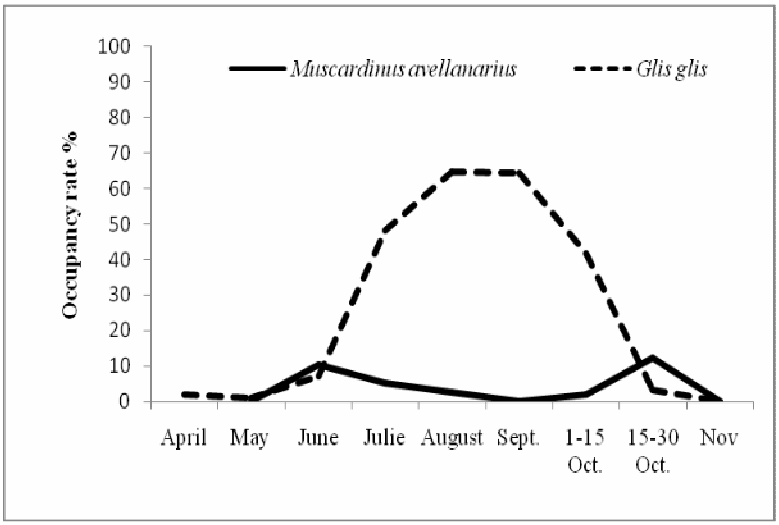

Fig 3. Mean multiannual occupancy rate in site 2-Ghiriş

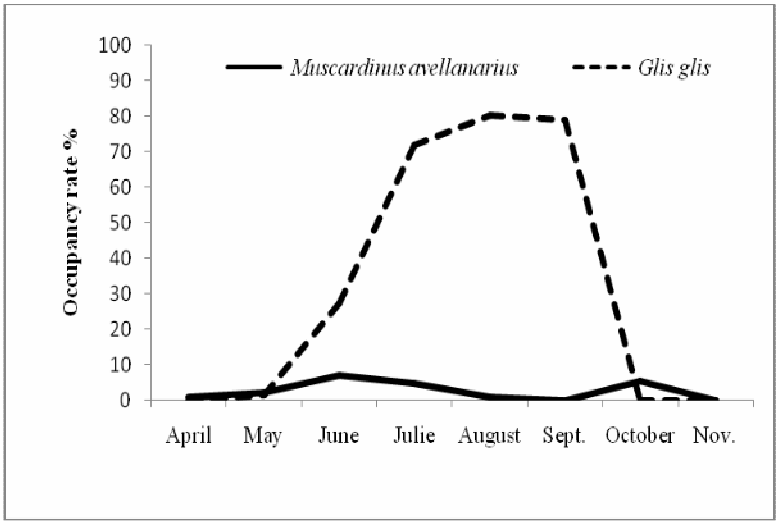

Fig 4. Mean multiannual occupancy rates in site 3-Ciuaş

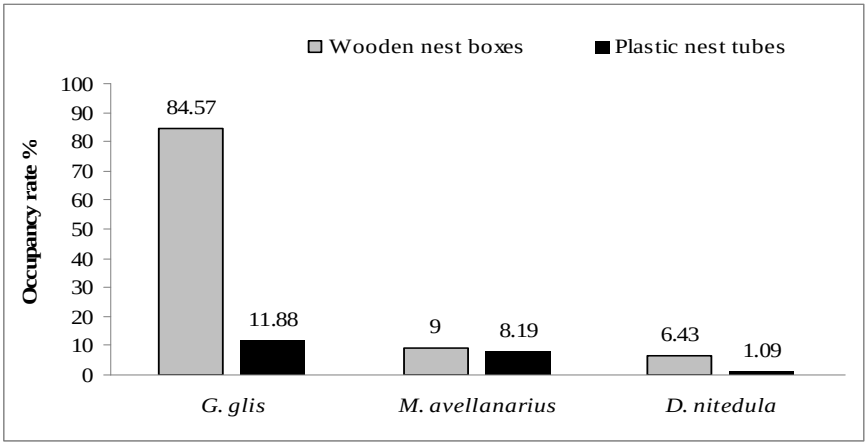

Fig. 5. Occupancy rates in wooden nest boxes and plastic nest tubes

M. avellanarius showed no preference for either type of artificial nests, and bred in both of them. These results were similar to those obtained by Hecker and Bakó in 2005 (in Bakó and Hecker, 2006). The entire *M. avellanarius* population in a given area may use the nest boxes, provided they were supplied in sufficient number (Bright and Morris, 1992; Juškaitis, 2006b), but in areas were *G. glis* was very abundant, only a few records of *M. avellanarius* could be obtained by checking nest boxes (Bakó and Hecker, 2006).

D. nitedula had low occupancy rates in the plastic tubes (slightly over 1%) and did not breed in them. The previous study conducted in Hungary suggested that *D. nitedula* showed either a clear preference for wooden nest boxes or showed no preferences at all (Bakó and Hecker, 2006).

The occupancy rates observed for the three species varied among sites where nest boxes were installed (Fig. 6).

G. glis was present in all sample sites and colonized nest boxes mostly in forests where the food sources were rich and diverse (shrub layer well developed) but timber management had led to a limited availability of nest sites (2-Ghiris, 3-Ciuas,). In forests with numerous old, hollow trees and well-developed shrub layer (1-Husnar) *G. glis* used nest boxes to a lesser extent. The lowest occupancy rate was observed in the young oak forest with no shrub layer, i.e. limited food offer (4-Zapodie), and most likely reflected low population abundance. This species actively avoided plastic nest tubes and did not breed in them (Fig. 5), hence the low occupation rate in area 4-Pastaraia where only this type of artificial nests were installed.

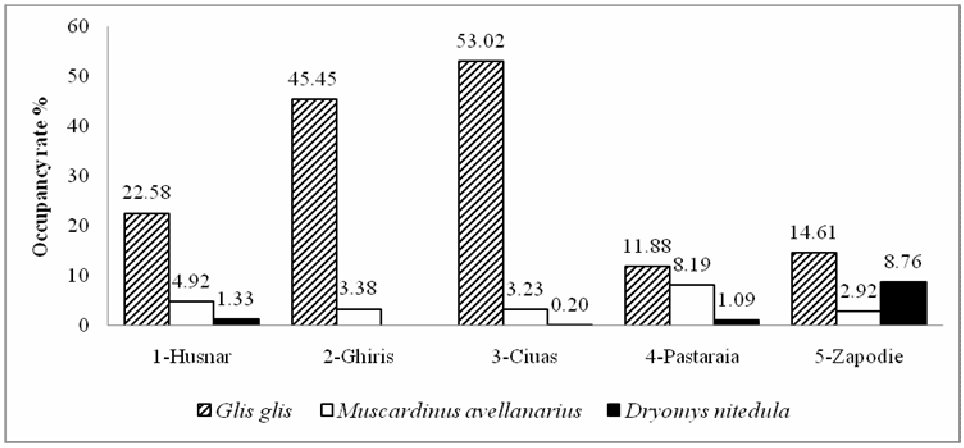

Fig. 6. Percentage of nest boxes/nest tubes occupied by each dormouse species in the five sample areas during the entire study period

M. avellanarius was present in all sample sites but had low occupancy rates, with the highest in oak-hornbeam forest (4-Pastaraia) - where *G. glis* used the nest tubes less - and the lowest in the young oak forest with no shrub layer (5-Zapodie). The high rates of occupancy observed in nest tubes that were avoided by the larger species *G. glis* was another argument for interspecific competition between these two species for nesting sites.

D. nitedula was absent in the old partial clearing (2-Ghiriş) and had very low occupancy rates in the rest of the sample areas, with the exception of sample area 5-Zapodie. Here the pressure from *G.glis* over the nest boxes was low due to the limited offer of vegetable food, and *D. nitedula* probably thrived by supplementing its diet with animal food.

The elevated occupancy rates obtained in site 4-Pastaraia by *M. avellanarius* might reflect the increase in nest tubes use in the conditions of low pressure on this resource by the larger specie *G.glis*, who actively avoided them and obtained low occupancy rates. Bakó and Heckel (2006) made the same assumption. Tree holes may be the principal nest site of *M. avellanarius* and the woven nest built between branches of shrubs and young trees is an adaptation to low availability of tree hollows (Juškaitis, 2005) because of actual scarcity or as a result of exploitation competition with other cavity nesters.

Conclusions

Dormouse communities identified in nest boxes and nest tubes installed in the Transylvanian Plain consist of three species: *Glis glis, Muscardinus avellanarius* and *Dryomys nitedula* or only two species: *Glis glis* and *Muscardinus avellanarius*.

Dormouse communities are dominated by *Glis glis*.

Interspecific competition for nest boxes appears between *Glis glis* and *Muscardinus avellanarius*. The smaller species ceases using nest boxes when the larger species occupies them extensively.

The two types of artificial nests do not meet the ecological requirements of the three species to the same extent. *G. glis* and *D. nitedula* prefer wooden nest boxes to plastic nest tubes, while *M. avellanarius* shows no preference.

The most important habitat feature for *Glis glis* and *Muscardinus avellanarius* is the presence of a rich and dense shrub layer to provide sufficient food resources.

Glis glis extensively occupies nest boxes in areas with low offer of tree hollows.

Acknowledgments.

This work was supported by two grants awarded by People's Trust for Endangered Species (to which I am greatly indebted), during 2005-2008, and a research grant awarded by CNCSIS for young scientists: TD–95, no. 351/1.10.2007. We give our warm thanks and appreciation to Mirona Stănescu for her invaluable contribution to the entire project and to all volunteers that helped during the fieldwork. We are very grateful to Alin David for the scientific review of the manuscript and for useful comments on an earlier version of the present paper.

REFERENCES

Adamik, P., Král, M. (2008). Climate- and resource-driven long-term changes in dormice populations negatively affect hole-nesting songbirds. *J. Zool. Lond*. **3**, 275, 209-215.

Aitken, K., Wiebe, K., Martin, K. (2002). Nest-site reuse patterns for a cavity-nesting bird community in interior British Columbia. *Auk*, **2**, 119, 291-402.

Bakó, B., Hecker, K. (2006). Factors determining the distribution of coexisting dormouse species (*Gliridae, Rodentia*). *Pol. J. Ecol.* **54**, 3, 379-386.

Barba, E., Gil-Delgado, J. (1990). Competition for nest-boxes among four vertebrate species: an experimental study in orange groves. *Holarctic Ecology*, **13**, 183-186.

Berg, L., Berg, Å. (1998). Nest site selection by the dormouse *Muscardinus avellanarius* in two different landscapes. *Ann. Zool. Fennici*, **35**, 115-122.

Bielz, E. A. (1888). Die Fauna der Wirbelthiere Siebenbürgens nach ihrem jetzigen Bestande. *Verh. u. Mitt. des siebenb. Vereins für Naturwiss. in Herm*., **38**, 15-36.

Bright, P., Morris, P. (1991). Ranging and Nesting Behaviour of the Dormouse *Muscardinus avellanarius* in diverse low-growing woodland *J. Zool. Lond.* **2,** 224, 177-190.

Bright, P., Morris, P. (1992). Ranging and nesting behavior of the dormouse *Muscardinus avellanarius*, in coppice-with-standards woodland. *J. Zool. Lond*. **4**, 226, 589-600.

Dooley, J., Dueser, R. (1990). An experimental examination of nest-site segregation by two *Peromyscus* species. *Ecology*, **71**,788–796.

Flux, E. C. (2003). Starlings *(Sturnus vulgaris)* making their own nest sites. *Notornis*, **50**, 4, 239-240.

Franco, D. (1990). Feeding habits of a dormouse population (*Myoxus glis*) of the Asiago Plateau (Venetian preAlps). *Hystrix*, **2**, 11-22.

Görner, M., Hackethal, H. (1988). Säugetiere Europas. Neumannn Verlag Leipzig, 372 pp.

Guthrie, C. G., Moorhead, D. L. (2002). Density-dependent habitat selection: evaluating isoleg theory with a Lotka-Volterra Model. *Oikos*, **96**, 184-194.

Juškaitis, R. (1999). Mammals occupying nestboxes for birds in Lithuania. *Acta. Zool. Lituanica*. *Biodiversity*, **3**, 9, 19-23.

Juškaitis, R. (2005). The influence of high nestbox density on the common dormouse Muscardinus avellanarius population. *Acta Theriol*. **1**, 50, 43-50.

Juškaitis, R. (2006a). Interactions between dormice (Gliridae) and hole-nesting birds in nestboxes. *Folia Zool.* **3,** 55, 225-236.

Juškaitis, R. (2006b). Nestbox grids in population studies of the common dormouse *(Muscardinus avellanarius* L.): methodological aspects. *Pol. J. Ecol*. **3**, 54, 351-258.

Juškaitis, R. (2007). Feeding by the common dormouse *Muscardinus avellanarius*): a review. *Acta Zool. Lituanica*, **2**, 17, 151-159.

Juškaitis, R., Remeisis, R. (2007). Summer nest sites of the common dormouse *Muscardinus avellanarius* L. in young woodlands of Lithuania. *Pol. J. Ecol.* **4**, 55, 795-803.

Koppmann-Rumpf, B., Heberer, C., Schmidt, K.-H. (2003). Long term study of the reaction of the edible dormouse *Glis glis* (Rodentia, Gliridae) to climatic changes and its interactions with hole-breeding passerines. *Acta. Zool. Scient. Hung*. **49**, 1, 69-76.

Mac, I., Idu, P. D., Maier, A., Ciangă, N., Sorocovschi, V. (1987). Câmpia Transilvaniei. In Oancea, D., Velcea, V., Caloianu, N., Dragomirescu, Ş., Dragu, G., Mihai, E., Niculescu, G., Sencu, V., Velcea, I., (ed.) Geografia României III. Carpaţii Româneşti şi Depresiunea Transilvaniei, 541-558.

MacArthur, R., Levins, R. (1964). Competition habitat selection and character displacement in a patchy environment. *Proc. Nat. Acad. Sci. USA*, **51**, 1207-1210.

Morris, P., Bright, P., Woods, D. (1990). Use of nestboxes by the Dormouse *Muscardinus avellanarius*. *Biological Conservation*, **51**, 1-13.

Morris, P., Temple, R. (1998). 'Nest tubes' - a potential new method for controlling numbers of the edible dormouse (*Glis glis*) in plantations. *Quarterly J. Forestry*, **3,** 92, 201-205.

Murariu, D. (2005) Mammalia. In Botnariuc, N., Tatole, V. (ed.) Cartea roşie a vertebratelor din România. Bucureşti, 44-47.

Nowakowski, W., Godlewska, M. (2006). The importance of animal food for *Dryomys nitedula* (Pallas) and *Glis glis* (L.) in Bialowieza forest (east Poland): analysis of faeces. *Pol. J. Ecol.* **3**, 54. 359-367.

Nowakowski, W., Remisiewicz, M., Kosowska, J. (2006). Food preferences of *Glis glis* (L.), *Dryomys nitedula* (Pallas) and *Graphiurus murinus* (Smuts) kept in captivity. *Pol. J. Ecol.* **3**, 54, 369-378.

Pianka, E. (1976). Competition and niche theory. In May, R. (ed.) Theoretical Ecology: Principles and Applications. Blackwell, 114- 141.

Pop, Gr. (2001). Depresiunea Transilvaniei. Ed. Presa Univ. Cj, Cluj-Napoca, 274 pp.

Popescu, A., Murariu, D. (2001). Fauna României. *Mammalia*. *Rodentia*, XVI, 2, Ed. Acad. Rom. Buc., 43-212.

Pöysä, H., Pöysä, S. (2002). Nest-site limitation and density dependence of reproductive output in the common goldeneye *Bucephala clangula*: implications for the management of cavity-nesting birds. *J. Appl. Ecol.* **3**, 39, 502-510.

Pucek, Z. (1981). Keys to Vertebrates of Poland. Mammals. PWN, Polish Scientific Publishers, Warszawa, 367 pp.

Sarà, M, Milazzo, A., Falletta, W., Belia, E. (2005). Exploitation competition between hole-nesters (*Muscardinus avellanarius*, Mammalia and *Parus caeruleus*, Aves) in Mediterranean woodlands. *J. Zool. Lond*. **265**, 347-357.

Scinski, M., Borovski, Z. (2006). Home ranges, nest sites and population dynamics of the forest dormouse *Dryomys nitedula* (Pallas) in an oak- hornbeam forest: a live-trapping and radio-tracking study. *Pol. J. Ecol*. **54**, 3, 391-396.

Sevianu, E. Coroiu, I. (2005). Small Mammals of Central Transylvania Plain: Species Composition, Diversity and Abundance. *Stud. Univ. Babeş-Bolyai-Biol*, **XLIX**, 2, 19-31.

von Vietinghoff-Riesch, A. (1960). Der Siebenschlafer (*Glis glis*). Fischer Verlag, Jena, 196 pp.

Wesolowski, T. (2003). Bird community dynamics in a primaeval forest–is interspecific competition important? *Ornis Hungarica,* **12-13**, 51-62.

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 51-62

BIRD SPECIES RICHNESS IN FIZEŞ PLAIN (CENTRAL TRANSYLVANIA, ROMANIA)

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SUMMARY. The aim of this study was to analyze the diversity of bird communities at landscape and habitat scale and to compare different habitat types according to the species richness. The study was carried out between 2002 and 2007 in the Fizeş Plain (central part of Transylvania). Four habitats types were studied (wetlands 2.19%, forests 16.5%, open areas 74.3% and human settlements 7.01%). Overall, 146 species were identified (gamma diversity) but only 108 (74%) are breeding in the area. The values of alfa and beta diversity show that wetlands are prior to conservation. The most habitat specialists has bees occurred in wetlands (27 species) and forests (26 species) and the largest number of breeding species has been identified in forests (52 species). The number of species inhabiting the four habitat types differs from the expected number. P_i index values reached its highest value in wetlands (0.9 and 0.85) and human settlements (0.62 and 0.67) and it shows negative values only in the open areas (-.28 and -0.49). All the obtained results indicate that the wetlands are of high importance sharping the species' richness at landscape (Fizeş Plain) scale.

KEYWORDS: bird diversity, species richness, specialist birds, habitats, wetlands

Introduction

The conservation value of a region can be determined by measuring or estimating its biodiversity. Often, this is done by measuring/estimating the species diversity for taxonomic groups (Sutherland, 2000). It is impossible though to obtain complete lists of species, which would include absolutely all the species belonging to every taxonomic groups from a region (Burley, 1998; Lévêque and Mounolou, 2003). Thus, the first step in the assessment of the conservation value of a site is to estimate the species richness of major taxa respectively of common or prior groups for conservation (Burley, 1998; Bleahu, 2004), that can be used as indicators of biodiversity (Hess *et al*., 2006).

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Indicators are species or taxonomic groups whose presence or diversity are associated with overall levels of biodiversity (Landres *et al*., 1988). Indicators have multiple habitat requirements, their persistence depending on high level of landscape/ habitat complementation (Hartel *et al*., 2008). The birds represent one of the groups that are mostly used as biodiversity indicators (Gregory *et al*., 2003). There are a number of reasons for this. Firstly, compared to other (vertebrate) taxa, avian communities are typically quite diverse in both number of species and variety of habitat features used, and thus provide a simultaneous assessment of a wide range of ecosystem attributes (Nuttle *et al*., 2003). Secondly, bird communities are easily monitored, using well-established and easily replicated protocols (Bibby *et al*., 2000; Sutherland *et al*. 2004).

The Fizeş Valley is part of the Natura 2000 Network, representing one of the 108 Romania SPAs (H. G. 1284/2007), but the published data are scarce. The first study from the area has been published in the second part of the 19th century (Herman, 1871), followed one century later by other two studies (Filipascu, 1968; Munteanu, Maties, 1969), these being the most recent ones, regarding only to wetland birds.

Starting with the '60s of a last century, the landscape of the Valley and the Fizeş Plain has changed radically, along with the draining of some wide marsh areas and their transformation in agricultural lands or fishponds. In that period, widespread reed surfaces were also destroyed, and the alluvial forest that extended along the Fizeş Valley was cut (Filipaşcu, 1968). Important forest areas were also cut, and in order to avoid landslides, pine forests were planted (Mac *et al*., 1987). After the '90s, part of the agricultural lands was abandoned, the forest from the Legii (one of Fizeş's affluent) was cut, and as a consequence of natural sedimentation and the clog of the ponds, the reed has begun to grow again on wide areas (Fodorean, 2007). Surely, these landscape and/or habitat changes have influenced specific diversity of bird fauna.

Under these conditions, the main aims of this study are: (1) to analyze the species richness of the avifauna from the Fizeş Plain - gamma diversity (Primack *et al*., 2002), (2) to present the the phenological status and the distribution of bird species in the four studied major habitat types, counting for alpha and beta diversity (Whittaker, 1972; Primack *et al*., 2002) and (3) to present the habitat preferences of the bird species.

Study area

The Fizeş Plain is located in the south-eastern part of the city of Gherla (Cluj county), and it contains the central-southern part of the Somes Plain (Pop G., 2001) extending on a surface of about 56171.74 ha (Table 1). The analysis of land use (Table 1) shows that the anthropic influence is high, probably a consequence of the social and economical frame of the area. The forests are present especially on slopes and hill sides, as a consequence of the massive deforestations from the past (Mac *et al*., 1987).

Table 1

Land use in the Fizeş Plain **(CORINE land cover, Romania, EEA, 2006).**

Habitat	Surface (ha)	%	Code
Discontinuous urban fabric	3934.79	7.00	112
Non-irrigated arable land	17011.95	30.29	211
Vineyards	105.8	0.19	221
Fruit trees and berry plantations	301.9	0.54	222
Pastures	9685.81	17.24	231
Complex cultivation patterns	6902.43	12.29	242
Land principally occupied by agriculture with significant areas of natural vegetation	6300.23	11.22	243
Broad-leaved forest	8704.01	15.50	311
Coniferous forest	218.65	0.39	312
Mixed forest	76.31	0.14	313
Transitional woodland-shrub	1700.42	3.03	324
Inland marshes	779.12	1.39	411
Water bodies	430.32	0.77	512
	56151.74	**100.00**	

Forests are extended over 16.5% of the area and a mainly represented by mixtures of deciduous trees *Carpino-Quercetum petraeae* Borza 1941 (syn. *Querco petraeae-Carpinetum* Soó et Pócs 1957, *Carpino-Quercetum cerris* Klika 1938 and *Quercetum robori-petraeae* Borza 1928 (Pop *et al*., 2002; Pop and Cristea, 2002), but there are small areas and plantations of Pinus *sylvestris* and *Pinus nigra* combined with *Robinia pseudacacia*.

Open habitats cover 74.3 % of the studied area. They mainly include agricultural lands, but as well pasture and meadows with *Agrostetum stoloniferae* Ujvárosi 1941, *Agrostio tenuis-Festucetum rupicolae* Csűrös-Káptalan 1962, and also shrubs *Pruno spinosae-Crataegetum monogynae* (Cristea *et al*., unpublished data).

Wetlands cover only 2.19% of the studied area and are represented by 14 fishponds, and two natural lakes (Pike Lake and Legii Lake). The floating vegetation represented by *Myriophyllo-Potamogetonetum* Soó 1934, but mainly the emergent macrophyte *Phragmitetum australis* Tx. et. Prsg. 1942, *Typhetum angustifoliae* Pignati 1942, *Typhetum latifoliae* Soó 1927 (Pop. *et al*., 2002) cover more than 770 ha.

Human settlements covered 7.01% and are mostly represented by villages with small population and semi-natural landscape.

There are three Nature Reserve in the area (Pike Lake 140 ha [H.G. 2151/2004], Legii Valley 13.5 ha [H.G. 2151/2004], Sic Reedbeds 505 ha [H.G. 2151/2004]) created for the protection and conservation of the wetland bird fauna, and the entire Fizeş Valley, with a surface of 1627 ha, has designated as Special Protected Area in 2007 (H.G. 1284/2007).

Methods

Data were collected between May 2002 and October 2007, during a total of 72 days of observation, during all the months of the year. Line transect method combined with point count method have been used (Koskimies and Vaisanen, 1991; Bibby *et al.*, 2000). Observations have been made in all four habitat types (forests, opean areas, wetlands and human settlements). Species were identified visually using the aditional optical equipment (respectively a Bushnell 7 - 21 x 40 binoculars and a Opticron 15 - 45 x 65 field scope) as well as by the sounds/songs emited by birds.

Whittaker (1972) described three terms for measuring biodiversity over spatial scale. Gamma diversity is a measure of the overall diversity for the different habitats within a region and it can be defined as "geographic-scale species diversity". Alpha diversity refers to the diversity within a particular area or habitat, and is usually expressed by the number of species (i.e., species richness) in that habitat. Beta diversity represent the change in species diversity between studied habitats.

The P_i index, initially used to analyze the food preferences (Jacobs, 1974), was calculated in order to analyze the habitat preferences of the breeding bird species. This index was calculated for each type of studied habitat by the following formula:

$$\mathbf{P_i = (X_i/Y_i - X/Y) / (X_i/Y_i + X/Y),}$$

where X_i is the number of species breeding in a given habitat, Y_i is the total number of species noted in the study area, X is the size of a given habitat and Y is the total size of the study area.

This index assumes values from -1 to +1. A value of 0 indicates that the number of species is equal to the share of the habitat in study area. A value higher than 0, indicates that there is more species in a given habitat than expected from the share of this habitat to overall surface of study area, an if the value is less than 0, the number of species in a given habitat is less than expected (Skórka *et al.*, 2006).

Results and discussions

Overall, in the four studied habitat types a total of 146 species have been identified (gamma diversity), which represent 27.86% of Europe's bird fauna and 39.24% of Romania's bird fauna. The highest species richness is in the wetland areas (alpha diversity = 64) (Table 2, Fig. 1).

Similar results regarding to gamma and alpha diversity values, as well as the relation between alpha diversity of different habitat types, have been obtained in the Crisul Repede Basin (Kovats, 1974), superior and middle basin of River Prut (Gache, 1998), Radauti Depression (Trelea, 1999), Banat (Kiss, 1999) and Tarnow Region - Poland (Skórka *et al.*, 2006).

Table 2

Bird species diversity în Fizeş Plain

	Human setlements	Forests	Opean areas	Wetlands
Gamma diversity		**146**		
Alfa diversity	**44**	**57**	**60**	**64**
Beta diversity				
Forests	17		33	
Opean areas	22	33		72
Wetlands	56	67	72	

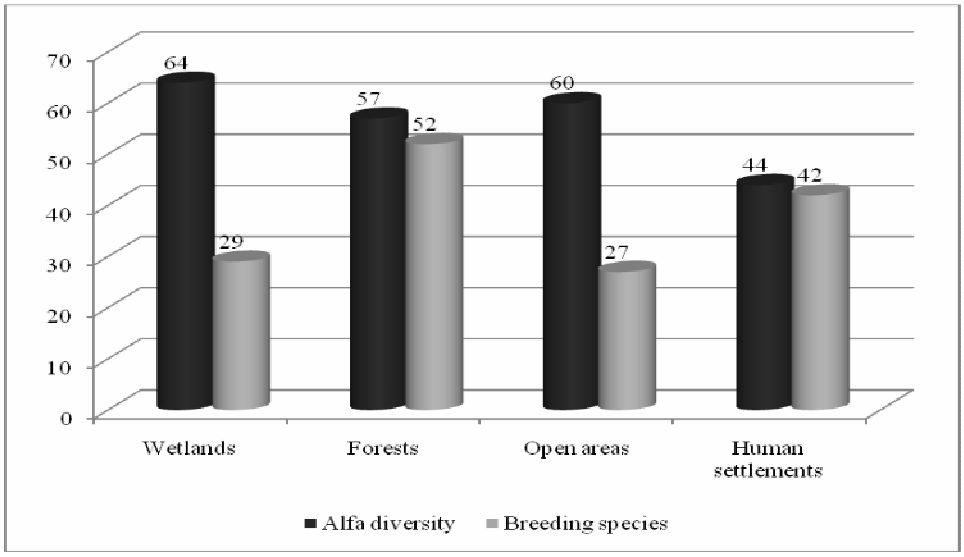

Fig. 1. Alfa diversity and the number of breeding bird species in each of the studied habitat type

The values of alpha diversity of bird fauna (species richness) in the four studied habitat types indicate that the wetlands are prior for conservation in the Fizeş Plain. From this point of view, the present conservation status from the Fizeş Plain

where all four existing protected areas were designated to protect and conserve the wetlands bird fauna is thus justified. The conservation importance of the wetlands is confirmed by the analysis of beta diversity (the gradient with which the species composition between studied habitats is changes). This shows that the wetlands bird fauna is more different from the bird fauna of the other habitat types such are forests and open areas (Table 2), whereas the last two habitats were more similar in their species composition with the bird communities of human settlements.

Actually, because of the deeply rural character of the human settlements in Fizeş Plain, represented mostly by villages, there is contiguity between the three habitat types, which confers a generally semi natural aspect of the landscape so that the similarity between their bird fauna is a natural. Typical forest species like *Dendrocopos major, Dendrocopos syriacus, Sitta europaea* (Snow, Perrins, 1998) etc. find optimal breeding conditions in the human settlements, just like some typical open area species such as *Sylvia curruca, S. nisoria, S. communis, Saxicola rubetra* or *S. torquatus* (Snow and Perrins, 1998).

Considering the small surface occupied by the forest habitats (9,300 ha) and the present conservation status, mainly favorable, of the bird species identified in open areas, the result of the alpha and beta diversity analysis shows that the ornithological conservation value of these landscape elements is reduced compared to wetlands. It is important to remember that wetlands occupy only 2.19% of the Fizeş Plain, a much smaller surface compared to the other habitat types. This even increases their value for the conservation of bird communities.

From phenologycal point of view, 108 species representing 74% are breeding in the study area. The highest diversity of breeding species were found in the forest (52 species), followed by the human settlements (42 species). On the other hand, in wetlands and open habitats, less than half of the identified species are nesting (Table 3). This phenological composition of bird fauna from the four studied habitats is a consequence of the annual stability and high availability of the trophic resources in forest and human settlements compared to open and wetland areas (Lack, 1969).

As the beta diversity values show, the breeding bird fauna from wetlands is mainly formed by specialists (Table 2, Fig. 2). Only two species *Vanellus vanellus* (identified in meadows and open habitats too) and *Motacilla alba* (in human settlements) are generalists species. In forests, is an equal ratio between specialists and generalists, but in the open habitats and especially in human settlements, the breeding bird fauna is mainly composed of generalists (Fig. 2).

Table 3

Characteristics of bird fauna from studied habitat types

	Habitat types			
	Wetlands	**Forests**	**Open areas**	**Human settlements**
Overall no. of species			**146**	
Overall no. of breeding species			**108**	
Surface (ha)	1209,44	9300,87	41706,64	3934,79
Total no. of noted species	64	57	60	44
Pi index for noted species	**0.9**	**0.41**	**-0.28**	**0.62**
Total no. of breeding species	29	52	27	42
Pi index for breeding species	**0.85**	**0.48**	**-0.49**	**0.67**
No. of breeding habitat specialists	27	26	13	11
No. of breeding habitat generalists	2	26	14	31
(%) breeding specialists within habitats	93.1	50	48.14	26.19
(%) breeding specialists/total specialists	35.06	33.77	16.88	14.29

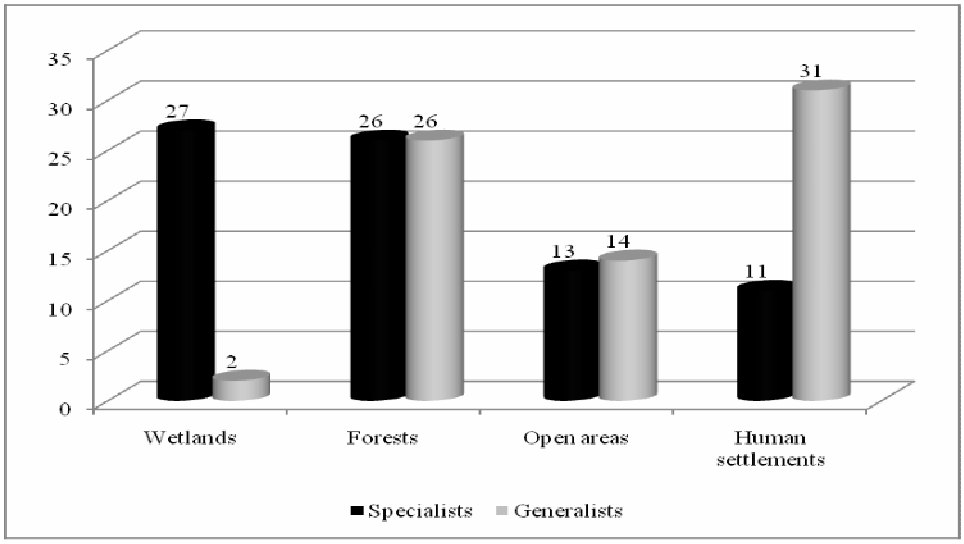

Fig. 2. Number of breeding specialists and generalist species of four studied habitat types

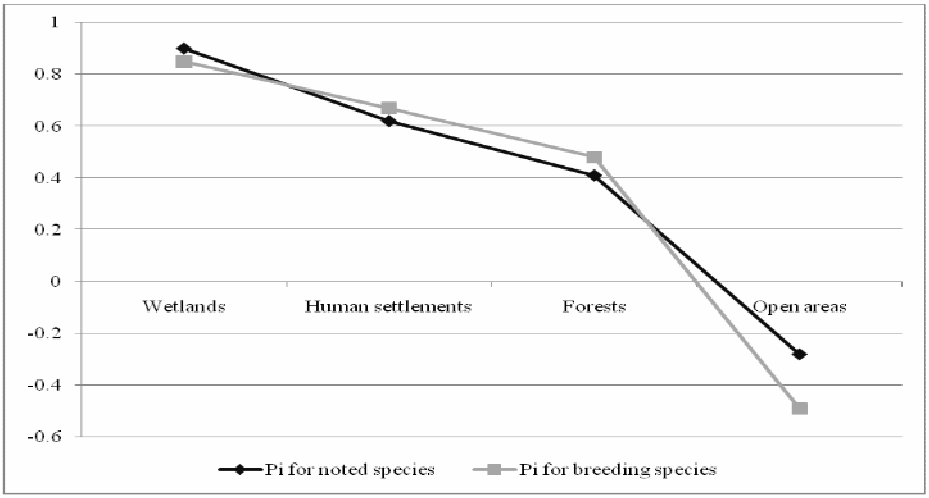

Fig. 3. Preference analysis for overall noted and for breeding species in four studied habitats

Similar results have been obtained in Poland (Skórka *et al*., 2006), where a study of the same four habitat types, as large as 1,400 km^2, has been made. It concluded that the breeding bird fauna from the wetlands is mainly composed of specialists, while the generalists are dominant in the other habitat types.

The numerical dominance of the specialists in wetlands is imposed by the necessary morpho-anatomo-physiological adaptations and also because of the microhabitat requirements of the wetland species that cannot be meet in the terrestrial habitats (Weller, 1999).

Overall, 77 breeding bird specialists (71.3%) and 31 breeding generalists (28.7%) have been identified in the four studied habitats. The numbers of species breeding in two, three and four habitats were 25 (23.15%), 5 (4.62%) and 1 (0.93%).

From 25 generalist species breeding in two habitats, a higher number was found in forests and human settlements (17 species), human settlements and open areas (4 species) and a lower number for forests and open areas as well as for human settlements and wetlands (2 species both). None of the species is common in forests and wetlands due to the fact that in the Fizeş Plain *Ardea cinerea* and *Nycticorax nycticorax* nest only in forests (Ciuaşului forest heronry). Of course, both species get their food resources especially from wetlands. All four species (*Asio otus, Pica pica, Sylvia communis* and *Turdus merula*) that occurred in three habitat types are common for human settlements, forests and open areas. Only *Cuculus canorus* was found breeding as brood parasite in all the four habitat types.

The P_i index values calculated both for overall species richness (including passage and winter visitors) and for breeding species richness in each of the four habitats reached its highest value in wetlands (0.9 for all identified species and 0.85 for the breeding ones) and human settlements (0.62 and 0.67). Relatively high values of the P_i index were also recorded in forests, and had a negative value only in open habitats (Table 3, Fig. 3).

The P_i values are correlated to the phenological status of the bird species. Thus, wetlands and open areas, due to their abundant trophic offer, sustain a number of species of over two times larger than in passage periods and/or in the winter, as compared to the breeding season.

All obtained results indicate that the wetlands are of high importance for the species richness at landscape (Fizeş Plain) scale. More than 35% of all habitat specialists nest here (Table 3), even though the wetland surface of only 2.15% is the smallest of all the habitat types. The high alpha diversity of the wetlands, the high number of specialists and the P_i index values are the consequence of more factors, but mainly because of the abundant trophic offer, for which the number of non breeding passage species are larger than that of the breeding ones (Elmberg *et al*., 1994; Weller, 1999).

Also, the diversity of available microhabitats, a consequence of the advanced succesional stage of Fizeş Plain's wetlands play an important role for a high level of the alpha diversity values and make possible the coexistence of some species with similar ecological requirements, which explains the high number of recorded specialists.

The negative value of the P_i in open areas is due to the simplified spatial structure of this habitat type. Natural meadows cover small surface, and almost all of the shrubs have been cut or burned in order to be transformed into pastures. It is well known that habitat structure is one of the most important factor that controls the species richness (MacArthur and MacArthur, 1961; Lévêque, Mounolou, 2003).

Although positive the P_i index values in forests are lower than in wetlands and/or human settlements. This is mainly because of the small surface occupied by this habitat type in the Fizeş Plain (only 16.56%) and of the intense fragmentation that induces the growth of edge effect (Sisk *et al*., 1997; Maldonado-Coelho and Marini, 2000), but certainly the early successional stage of majority of forest patches is also important (Winkler, 2005). As a consequence of strong edge effect, the number of generalist species (that usually inhabit the ecotone) (Baldi, 1996; Gregory *et al*., 2007) is equal in the Fizeş Plain forest to the number of specialists.

Human settlements are generally not suitable for use by "area-sensitive" forest birds as a breeding habitat and urbanization is one of the most important worldwide threats to biodiversity (Ricketts and Imhoff, 2003). But, because of the traditional land use in Fizeş Plain, there is a contiguity between human settlements and forests, open areas and even wetlands, thus explaining the large number of breeding species and the

high values of the P_i index. Our results support the fact that these habitats are mainly inhabited by generalist species (Kubes and Fuhs, 1998) and some ornithologists (Clergeau *et al*., 2005; Hedblom, 2007) argue that this kind of habitat may play a truly important role in conserving bird species diversity at landscape scale.

Conclusions

Overall, 146 species have been identified (gamma diversity), and 74% of them breed in study area. The alpha diversity has its highest value in wetlands (64 species) and open areas (60 species). The beta diversity analysis shows that the specific composition of wetland bird fauna is a lot different from the terrestrial habitats.

Specialists birds are more abundant in wetlands (27 species) and forests (26 species), but orerall the most of the breeding species inhabit forests (56 species) and human settlements (42 species).

The P_i index record high values in wetlands and human settlements and negative ones only in open areas.

Human settlements are inhabited mostly by habitat generalist species, but high values alpha diversity and P_i index suggest that these habitats may play an important role in conserving bird diversity at Fizeş Plain scale.

Acknowledgements

We are indebted to miss Ruxandra Coroiu for english correction, to dr. Hartel Tibor, and to drd. Cosmin Ioan Moga for their critical reviw of the manuscript. Financial support was provided by the CNCSIS (Grant for young scientist TD–343, no. 75/1.10.2007) and by the British Ornithologists Union (Small Research Grant).

R E F E R E N C E S

Báldi, A. (1996). Edge effect in tropical versus temperate forest bird communities: three alternative hypotheses for the explanation of differences. *Acta Zool. Acad. Sci. Hun.*, **42**, 3, 163 – 172.

Bibby, C. J., Burges, N. D., Hill, D. A., Mustoe, S. H. (2000). Bird census techniques, 2nd Ed., Academic Press, London, 302 pp.

Bleahu, M. D. (2004). Arca lui Noe în secolul XXI. Ariile protejate şi protecţia naturii. Naţional, Bucureşti, 508 pp.

Burley, W. F. (1988). Monitoring biological diversity for setting priorities in conservation. In Wilson, E. O., Peter, F. M., (eds.) *Biodiversity*. National Acad. Press, Washington D. C., 516 pp: 227 – 231.

Clergeau, P., Jokimaki, J., Savard, J. P. L. (2005) Are urban bird communities influenced by the bird diversity of adjacent landscapes? *J. App. Ecol.*, **38**, 1122 – 1134.

Cristea, V., Başnou, C., Puşcaş, M., Caracterizarea florei şi vegetaţiei. În Mârza, I., (ed.), Monografia Comunei Ţaga (jud. Cluj, România), unpublished data.

Elmberg, J., Nummi, P., Pöysä, H., Sjöberg, K. (1994). Relationship between species number, lake size and resource diversity in assemblages of breeding waterfowl. *J. Biogeogr*., **21**, 75 – 84.

Filipaşcu, Al. (1968). Fauna ornitologică a lacurilor de la Geaca (Câmpia Transilvaniei) dupa 100 ani. *Ocrot. Nat.*, **12**, 1, 21 – 43.

Fodorean, I. (2007). Lacurile dulci din Podişul Transilvaniei. Studiu Limnologic. Teză Doc. Univ. „Babeş-Bolyai" Cluj-Napoca.

Gache, C. (1998). Dinamica ornitofaunei în bazinul superior şi mijlociu al Prutului. Teza Doc. Univ. "Al. I. Cuza", Iasi.

Gregory, R, D., Noble, D., Field, R., Marchant, J., Raven, M., Gibbons, D. W., 2003. Using birds as indicators of biodiversity. *Ornis Hungarica*, 12-13, 11 - 24.

Gregory, R. D., Vorisek, P., Van Strienm A., Meyling, A. W. G., Jiguet, F., FornasariI, L., Reif, J., Chylarecki., Burfield, I. J. (2007). Population trend of widespread woodland birds in Europe. *Ibis*, 149,2, 78 – 97.

Hartel, T., Moga, C. I., Öllerer, K., Demeter, L., Sas, I., Ruşti, D. M., Balog, A. (2008). A proposal towards the incorporation of spatial heterogeneity into animal distribution studies in Romanian landscapes. *NWJZ*, **4**, 1, 173 – 183.

Hedblom, M. (2007). Birds and butterflies in Swedish urban and peri – urban habitats: a landscape perspectives. PhD Thesis, Faculty of Natural Resources and Agricultural Sciences, Department of Ecology, Uppsala.

Herman, O. (1871). A Mezöség. I. A Hodos Vagy Szarvasto és Környeke. Természetrajzi, jelesen állatani syempontból táryalva. *Az Erdély Muzeum - Egylet Evkönyvei*, V, 8 – 21.

Hess, G. R., Bartel, R. A., Leidner, A. K., Rosenfeld, K. M., Rubino, M. J., Snider, S. B., Ricketts, T. H. (2006). Effectiveness of biodiversity indicators varies with extent, grain, and region. Biol. Cons., 132, 448 – 457.

Kiss, A., (1999). Avifauna din zonele umede ale Banatului, Ed. Marton, Timişoara, 259 pp.

Koskimies P., R. A. Vaisanen. 1991. Monitoring bird populations. A manual of methods applied in Finland. Helsinki Zool. Mus., Finnish Mus. Nat. Hist., 144pp.

Kovats, L., (1974). Studiu faunistic şi ecologic al populaţiilor de păsări din Bazinul Crişului Repede. Teza Doc. Univ. "Babes-Bolyai", Cluj.

Kubes, J., Fuhs, R., 1998. Village as bird refuge in cultural landscapes (largely agricultural landscape, Czech Republic). *Ekologia*, **17**, 208 – 220.

Lack, D. (1966). Population studies of birds. Oxford Univ. Press, London, 346 pp.

Landres, P., Verner, J., Thomas, J. (1988). Ecological uses of vertebrate indicator species – a critique. *Cons. Biol.* **2**, 316–328.

Lévêque, C., Mounolou, J-C. (2003). Biodiversity. John Wiley and Sons, Chichester, 284 pp.

Mac, I., Idu, P. D., Maier, A., Ciangă, N., Sorocovschi, V., (1987). Câmpia Transilvaniei. În Oancea, D., Velcea, V., Caloianu, N., Dragomirescu, Ş., Dragu, G., Mihai, E., Niculescu, G., Sencu, V., Velcea, I., (ed.) Geografia României III. Carpaţii Româneşti şi Depresiunea Transilvaniei, 541 – 558.

Mac Arthur, R. H., Mac Arthur, J. W. (1961). On bird species diversity. *Ecology*, **42**, 594 – 598.

Maldonado-Coelho, M., Marini, M. (2000). Effects of forest fragment size and succesional stage on mixed-species bird flocks in southeastern Brayil. *Condor*, **102**, 585 – 594.

Munteanu, D., Măties, M. (1968). Aperçu sur l-avifaune des etangs de Geaca. *Lucr. Stat. Cercet. Biol.*, Geol., *Geogr. "Stejarul"*, 2, 185 – 200.

Nuttle, T., Leidof, A., Burger, L, W. jr. (2003). Assessing conservation value of bird communities with partners in flight – based ranks. *Auk*, **120**, 541 – 550.

Pop, G. (2001). Depresiunea Transilvaniei. Edit. Pres. Univ. Clujeană, Cluj-Napoca, 274 pp.

Pop, I., Cristea, V., 2002. Studiu comparativ asupra comunităților silvice din România, edificate de *Quercus cerris* și *Q. farnetto*. *Contribuții Botanice*, XXXV(2)(1999 – 2000): 255 - 285

Pop, I., Cristea, V., Hodișan, I. (2002). Vegetația județului Cluj (studiu fitosociologic, ecologic, bioeconomic și eco-protectiv). *Contribuții Botanice*, XXXV, 2(1999 – 2000), 5 – 255.

Primack, R. B., Pătroescu, M., Rozylowicz, L., Iojă, C. (2004). Conservarea Diversității Biologice. Ed. Tehnică, Bucureşti, 292 pp.

Ricketts, T., Imhoff, M. (2003). Biodiversity, urban areas and agriculture: locating priority ecoregions for conservation. *Cons. Ecol.*, **8**, 2, 1.

Sisk, T. D., Haddad, N. M., Ehrlich, P. (1997). Bird assemblages in patchy woodlands> modeling the effects of edge and matrix habitats. *Ecol. App*., **7**, 4, 1170 – 1180.

Skórka, P., Martyka, R., Wójcik, J. D. (2006). Species richness of breeding birds at a landscape scale: which habitat type is the most important? *Acta Ornithol.*,41, 1, 49 – 54.

Snow, D. W., Perrins, C. M. (1998). Birds of western palearctic. Vol. II. Passerines, Oxford Univ. Press, 686 pp.

Sutherland, W. J. (2000). The conservation handbook. Research, management and policy. Blackwell Science, Cambridge, 278 pp.

Sutherland, W. J., Newton, I., Green, R, E. (eds.) (2004). Bird ecology and conservation. A handbook of Techniques. Oxford Univ. Press, London, 386 pp.

Trelea, S. (1998). Dinamica ornitofaunei in Depresiunea Radauti. Teza Doc., Univ. Al. I. Cuza, Iasi.

Weller, M. (1999). Wetland Birds. Habitat resources and Conservation Implications. Cambridge Univ. Press., Cambridge, UK, 271 pp.

Winkler, D. (2005). Ecological succession of breeding bird communities in deciduous and coniferous forests in the Sopron Mountains, Hungary. *Acta Silv. Lign. Hung.*, **I**, 49 – 58.

Whittaker, R. H. (1972). Evolution and measurement of species diversity. *Taxon*, **21**, 213-251.

*** Hotărâre a Guvernului României nr. 2151 privind instituirea regimului de arie naturală protejată pentru noi zone, *Monitorul Oficial*, I, **38**, 12 ianuarie 2005.

*** Hotărâre a Guvernului României nr. 1284 privind declararea ariilor de protecție specială avifaunistică ca parte integrantă a rețelei ecologice europene Natura 2000 în România, *Monitorul Oficial*, XIX **739**, 31 octombrie 2007.

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 63-74

THE ENZYMATIC ACTIVITY OF THE ANTIOXIDATIVE SYSTEM IN GREEN ALGA *MOUGEOTIA SP*. DURING THE *STATE 2* TRANSITION, IN THE PRESENCE OF ASCORBATE AND HYDROGEN PEROXIDE

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SUMMARY. The activity of the antioxidative enzymes under light stress was studied, in the presence of various ascorbate concentrations and throughout the intensification of the oxidative factor (H_2O_2) during *state 2* transition. The SOD activity has been enhanced under intense light and in the presence of H_2O_2. During *state 2*, in the presence of DCMU, the electrons are used in the PS I for the deactivation of the reactive oxygen species. The APX activity was intensified with and with ascorbate 20 mM, 30 mM and with H_2O_2. The MDAR activity was reduced excepting the samples with ascorbate 20 mM and 30 mM. The DHAR activity was considerably reduced, while the GR activity was intensified in the presence of ascorbate and H_2O_2. The concentration of 20 mM ascorbate has enhanced the NADPH-dehydrogenase activity, while H_2O_2 has produced a slight inhibition. The intensification of lipid peroxidation was emphasized. During *state 2*, in the presence of 10 mM ascorbate there was observed a very high accumulation of TBARS reactive species. The activity of the antioxidative enzymes induced during *state 2* certifies the high accumulation of the photosynthetic reducing equivalents of the reactive oxygen species through the Mehler reaction.

KEYWORDS: lipid peroxidation, light stress, reactive oxygen species

Introduction

The reactive oxygen species (ROS) function as regulatory and signaling elements in many cellular processes (Foyer and Noctor, 2005). ROS are continually produced in photosynthesis and respiration. The measurement of the lipid peroxidation final products is the most employed method for the emphasizing of the oxidative damages. ROS cause the peroxidation of the polyunsaturated fatty acids producing α, β-unsaturated aldehydes.

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The proteins oxidation is a characteristic of the oxidative stress that confers certain conclusions based on the forming of the lipid oxidation products (Shulaev and Oliver, 2006).

The ascorbic acid is the main antioxidant which detoxifies the reactive oxygen species. The expression of dehydroascorbate reductase (DHAR) which is responsible for the ascorbate regeneration adjusts the redox state of the cellular ascorbate which affects one by one the cellular response and the tolerance to ROS (Chen and Gallie, 2006). The ascorbate is involved in the detoxification of the oxygen reactive species such are the superoxide, the singlet oxygen, the ozone and the hydrogen peroxide that are produced during the aerobic metabolic processes of photosynthesis and respiration. The ascorbate is oxidized to the monodehydroascorbate radical which may be reduced to ascorbate in the chloroplast or in the cytosol through MDHA reductase in the NAD(P)H dependent reaction. In chloroplasts, the MDHA radical can be reduced to ascorbate through the reduced ferredoxin associated to the thylakoids, this reaction being much more efficient than the reduction through MDHAR (Miyake and Asada, 1994). The monodehydroascorbate which is produced in the thylakoid lumen by violaxanthin de-epoxidase or by following the electron donation from ascorbate to PS II and PS I is not available as a substrate for reduction through ferredoxin or MDHAR, but is rapidly disproportioned to ascorbate and dehydroascorbate when the lumen pH is decreasing (Asada, 1999). The ascorbate reduces the photoinhibition by promoting the conversion of violaxanthin to zeaxanthin in the xanthophyll cycle to the dissipation of the excitation energy excess as a part of the nonphotochemical quenching (Chen and Gallie, 2006).

The photosynthesis provides powerful reductants and it generates reactive oxygen species in various environmental conditions (Foyer *et al.*, 1994). The unfavorable conditions make the photosynthetic electron flow towards O_2 to enhance leading to the increase of superoxide, H_2O_2 and hydroxyl radical production, these leading to the limitation of the $NADP^+$ concentration during the electrons acceptation from PS I (Asada, 1999). The ROS accumulation in cytoplasm is opposed to superoxide dismutase, ascorbate peroxidase bound by thylakoids and from the chloroplasts stroma, monodehydroascorbate reductase and dehydroascorbate reductase, glutathione reductase and peroxiredoxins (Asada, 2000; Dietz *et al.*, 2006). Under unfavorable conditions, the biosynthesis and the activation of these antioxidants enhances and stabilizes the redox equilibrium of the chloroplasts (Foyer *et al.*, 1994; Asada, 2000). The increase of these enzymes' concentration may limit the photodamages (Hodges *et al*, 1997). In anaerobiosis, the ATP synthesis takes place without affecting the NADP/NADPH ratio due to the cyclic electron transport around PS I (Forti *et al.*, 2003). All the antioxidative enzymes of the chloroplast which are involved in the biosynthesis and in the regeneration of the antioxidants are codified by the nucleus. The regulation of the genes expression depends by the signaling from the chloroplast to the nucleus.

The aim of this study was the analysis of the antioxidative enzymes under light stress in the presence of various concentrations of the enzymatic substrate (ascorbate) and during the intensification of the oxidative factor (H_2O_2) in the *state 2* transition.

Material and Methods

The green alga *Mougeotia sp.* Agardh (AICB 560) derives from the Collection of Algae Cultures of the Institute of Biological Research from Cluj-Napoca (AICB) (Dragoş *et al*., 1997). The strain AICB 560 was grown in Bold's basal medium (BBM), under continuous air stirring, continuous illumination with 443 $\mu mol.m^{-2}.s^{-1}$, at 22^0C. The growth period was of 24 days.

Light treatment, cofactors, generator agents for reactive oxygen and inhibitors. The photosynthetic active radiation (PAR) of 4000 $\mu mol.m^{-2}.s^{-1}$ was applied for 60 minutes in *state 2* induced in aerobiosis with certain specific inhibitors: 300 μM DCMU, 2 μM FCCP and 5 mM iodoacetamide. *State 2* was also induced by using the light 2 specific for PS II emitted by a red filter at 620 nm with an intensity of 2500 $\mu mol.m^{-2}.s^{-1}$. As an enzymatic cofactor there was used 10 mM, 20 mM and 30 mM sodium ascorbate, while 10 mM H_2O_2 was used as an agent for the generation of the reactive oxygen.

The analysis of the antioxidative enzymes. The activity of the analyzed enzymes was measured at room temperature. The extraction medium contains 50 mM potassium phosphate buffer pH= 7,8; 1 mM EDTA; 10 mM mercaptoethanol and 2% PVP (polivinilpirrolidone).

Ascorbate peroxidase (APX) was measured according to Leipner (1998). The extraction medium was added with 1 mM sodium ascorbate. The reaction medium contains: 80 mM potassium phosphate buffer pH = 7.0; 200 μM DTPA; 1 mM ascorbate and 250 μM H_2O_2 to which the cellular extract was added. The enzyme activity was calculated by decreasing the absorption to 290 nm using the extinction coefficient $\varepsilon = 2.8\ mM^{-1}.cm^{-1}$.

*Monodehydroascorbate reductase (*MDAR) was estimated by modifying the Leipner method (1998). The reaction medium contains: 80 mM potassium phosphate buffer, pH = 7.8; 200 μM DTPA; 1 mM ascorbate; 0.12 mM NADPH, one unit of ascorbate oxidase and cell extract. The reaction takes place after adding ascorbate oxidase, and the NADPH oxidation was measured by decreasing the absorption to 340 nm. The enzymatic activity was calculated with the extinction coefficient $\varepsilon = 6.2\ mM^{-1}.cm^{-1}$.

Dehydroascorbate reductase (DHAR) was measured by modifying the Leipner method (1998). The reaction medium contains: 80 mM potassium phosphate buffer, pH = 7.8; 200 μM DTPA; 2.5 mM GSH; 1 mM dehydroascorbate and cell extract. The enzymatic activity was estimated by monitoring the absorption enhancement at 265 nm using the extinction coefficient $\varepsilon = 14\ mM^{-1}.cm^{-1}$.

Superoxid-dismutase (SOD) was measured by modifying the Leipner method (1998). The reaction medium contains: 80 mM potassium phosphate buffer, pH = 7.8; 200 μM DTPA; 13 mM methionine; 1 mM NTB (nitroblue tetrazolium); 4 mM

riboflavin. The mixture is illuminated for 5 minutes with intense light, then the cell extract is added and the absorption decrease at 560 nm is monitored. The control does not contain cell extract and it is not illuminated. The quantity of enzymatic extract which causes the absorption decrease with 50% defines a SOD unit.

Glutathione reductase (GR) was measured by modifying the Leipner method (1998). The reaction medium contains: 80 mM potassium phosphate buffer, pH = 7.8; 200 μM DTPA; 1 mM oxidized glutathione (GSSG) and 0.12 mM NADPH. The enzymatic activity was estimated by monitoring the absorption decrease at 340 nm using the extinction coefficient $\varepsilon = 6.2\ mM^{-1}.cm^{-1}$.

The *NAD(P)H-dehydrogenase measurement.* The reaction medium contained 3 ml of cell extract in Tris-HCl buffer, 0,12 mM NAD(P)H and 120 μM duroquinone. The NAD(P)H-dehydrogenase activity in dark was spectrophotometric calculated by decreasing the absorption at 340 nm. The enzymatic activity was expressed in μmol/minute/mg chlorophyll a using the extinction coefficient $\varepsilon = 6.23\ mM^{-1}.cm^{-1}$.

Lipid peroxidation was measured according to Venisse *et al.* (2001). The algal material was extracted with 10% trichloroacetic acid. The reaction medium containing vegetal extract, 10% trichloroacetic acid and 0,2% thiobarbituric acid is boiled for 30 minutes at 90^0 C and then it is centrifuged. The TBARS content (thiobarbituric acid reactive species) was measured on the basis of the absorption at 532 nm from which the absorption at 600 nm is excluded, using the extinction coefficient $\varepsilon = 155\ mM^{-1}.\ cm^{-1}$.

Results and discussions

In photosynthetic organisms the superoxide is generated through the univalent photoreduction of O_2 on the reducing site of PS I from the thylakoid membrane when the photosynthesis is saturated by light (Miyake and Yokota, 2000; Schreiber and Neubauer, 1990).

Superoxide dismutase (SOD) catalyses the dismutation of the $O_2^{\cdot-}$ superoxide radicals by producing molecular oxygen and H_2O_2, thus decreasing the $O_2^{\cdot-}$ concentration, this being the first level in the enzymatic system for the removal of the active oxygen. Referring to the control values, the SOD activity has been enhanced in the best part of the variants (Fig. 1). The high levels of the SOD activity were observed under light stress (V_1) and in the presence of the hydrogen peroxide concentrations (V_5, V_7). In *state 2*, under light 2 favorable for PS II and in the presence of DCMU which blocks the photosynthetic electrons transport from PS II lead to the use of the electrons in PS I for the deactivation of the reactive oxygen species. SOD and the enzymes which remove the H_2O_2 intensify their activity in proportion with light intensity which plays a regulator role of the antioxidative enzymes in chloroplasts (Cakmak and Marschner, 1992).

By blocking the PS II, the cyclic electron flow around PS I operates at the highest level of photosynthesis inducing the ATP synthesis, where the cytochrome b_6f complex is also involved (Joliot and Joliot, 2002). The photogeneration of the reactive oxygen species is intensified when the plants are exposed to stress factors that keep down the use of the absorbed light energy for the CO_2 fixation (Asada, 2006).

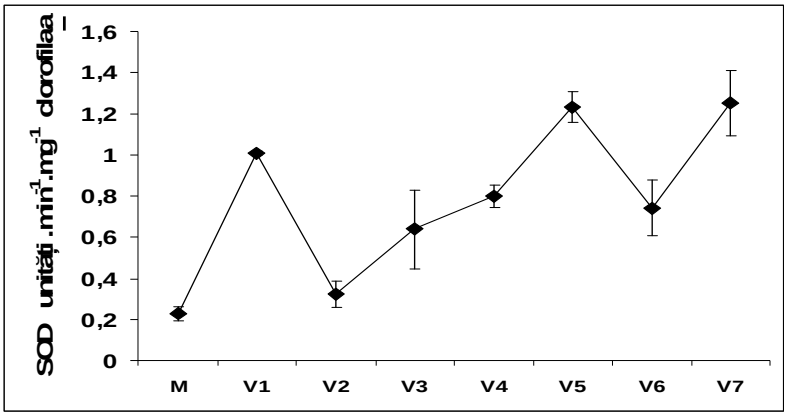

Fig. 1. The activity of the superoxide dismutase (SOD): M – control; V_1 - 4000 μmol.m^{-2}.s^{-1} light; V_2-light + 10 mM sodium ascorbate; V_3 - light + 20 mM sodium ascorbate; V_4 - light + 30 mM sodium ascorbate; V_5 - light + 10 mM H_2O_2; V_6 – red light + 20 mM sodium ascorbate; V_7 – red light + 10 mM H_2O_2 (300 μM DCMU, 2 μM FCCP and 5 mM iodoacetamide were added to V_1 – V_7)

The hydrogen peroxide which is formed from superoxide through disproportionation catalyzed by superoxide dismutase is reduced to water through ascorbate peroxidase by using the ascorbate as an electron donor (Mittler and Zilinskas, 1991). The oxidized ascorbate is reduced to ascorbate by ferredoxin, monodehydroascorbate reductase and dehydroascorbate reductase. The activity of the ascorbate peroxidase (APX) is localized in cytosol and in the chloroplasts (Asada, 1999). The chloroplast and cytosolic isoenzymes differ each other because of the high instability of the chloroplastic form in the absence of ascorbate.

The activity of ascorbate peroxidase has been intensified under light stress (V_1), in the presence of 20 mM, 30 mM ascorbate (V_3, V_4) and hydrogen peroxide (V_5) (Fig.2). Light 2 together with ascorbate and hydrogen peroxide have inhibited the activity of ascorbate peroxidase.

The catalytic removal of the hydrogen peroxide through APX produces monodehydroascorbate (MDA) which is nonenzymatic decomposed through spontaneous disproportination to ascorbate and dehydroascorbate or through ascorbate univalent oxidation in the enzymatic reactions. In the chloroplastic stroma MDA is enzymatically reduced to ascorbate by monodehydroascorbate reductase (MDAR) which uses NADH and NADH as electron donor. MDA can also be reduced by PS I with ferredoxin (Miyake and Asada,1994). Monodehydroascorbate reductase is distinguished by NADH-dehydrogenase and other enzymes through its molecular weight, amino acid composition and through its specificity regarding the electron acceptors and donors. MDAR is located in the chloroplast stroma (Hossain and Asada, 1985).

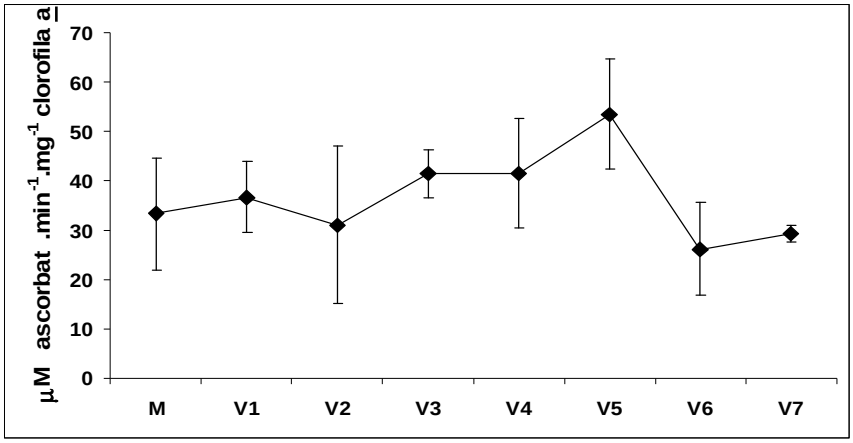

Fig. 2. The activity of ascorbate peroxidase (APX): M – control; V_1 - 4000 µmol.m^{-2}.s^{-1} light; V_2-light + 10 mM sodium ascorbate; V_3 - light + 20 mM sodium ascorbate; V_4 - light + 30 mM sodium ascorbate; V_5 - light + 10 mM H_2O_2; V_6 – red light + 20 mM sodium ascorbate; V_7 – red light + 10 mM H_2O_2 (300 µM DCMU, 2 µM FCCP and 5 mM iodoacetamide were added to V_1 – V_7)

The MDAR activity has been reduced in the best part of the samples, excepting the 20 mM and 30 mM de ascorbate concentrations comparatively with the control (Fig. 3). Concomitantly, the MDA radical will be spontaneously disproportioned to ascorbate and DHA, in this case DHA is reduced through DHA reductase and GSH reductase using GSH and NADPH as electron donors. Thus, the presence of this ascorbate-glutathione regenerative system has been more studied in chloroplasts, even this kind of enzymes also exists in the cytosol.

The dehydroascorbate reductase activity has been significantly reduced both under light stress and in the presence of ascorbate, comparatively to the control values (Fig.3). The low activity of dehydroascorbate reductase points out the existence of a low amount of dehydroascorbate which has been consumed in other metabolic pathways. In the presence of hydrogen peroxide the dehydroascorbate reductase activity was significantly intensified and it has reached the control values.

The suppression of dehydroascorbate reductase expression produces the loss of chlorophyll a, a reduced state of Rubisco and a low rate of CO_2 assimilation. The increasing of dehydroascorbate reductase expression maintains the high level of chlorophyll, Rubisco, LHC II and that of photosynthetic function. DHAR protects against damages mediated by ROS. Through the circular motion of the ascorbate, DHAR affects the ROS level and the photosynthetic activity, influencing the growth rate (Chen and Gallie, 2006).

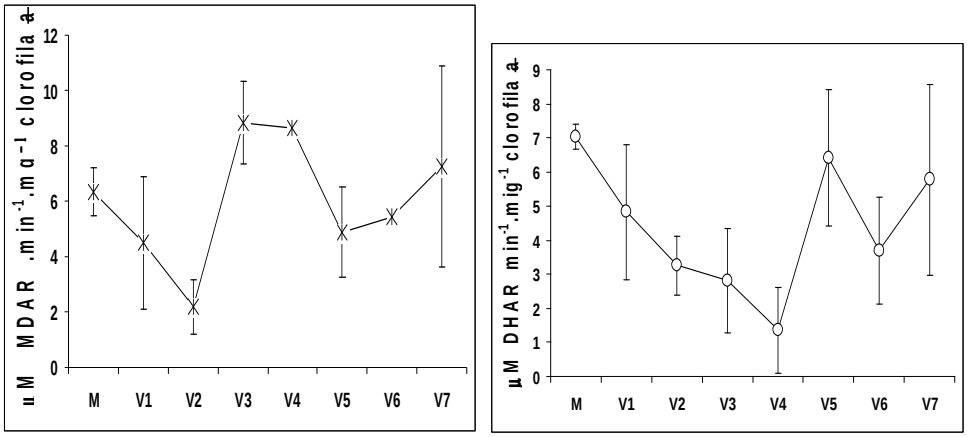

Fig. 3. The monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) activity. M – control; V_1 - 4000 µmol.m^{-2}.s^{-1} light; V_2-light + 10 mM sodium ascorbate; V_3 - light + 20 mM sodium ascorbate; V_4 - light + 30 mM sodium ascorbate; V_5 - light + 10 mM H_2O_2; V_6 – red light + 20 mM sodium ascorbate; V_7 – red light + 10 mM H_2O_2 (300 µM DCMU, 2 µM FCCP and 5 mM iodoacetamide were added to V_1 – V_7).

Glutathione reductase (GR) is a flavoprotein, which catalyzes the reduction of glutathione (GSSG to GSH) in the presence of electrons donor, NADPH. GR together with APX, DHAR, constitute the chloroplast system for removing the H_2O_2. The GR activity is localized mainly in chloroplasts and it is involved in the glutathione regeneration (Karpinski *et al.*,1993; Foyer and Halliwell, 1976).

Against the control values, it was observed an intensification of the GR activity, in the presence of intense light associated with ascorbate (Fig. 4). In the presence of the H_2O_2 concentrations, the enzyme activity reached higher values comparative with control.

NADPH-dehydrogenase is an oxidoreductase, which acts over the NADPH, the substrate involved in CO_2 fixation. The stress factors produce oxygen reactive speciesbased on the intensification of NADPH-oxidase activity (Rao *et al.*, 1996), fact recognized in many plant species (Moller and Lim, 1986). The CO_2 assimilation in photosynthesis depends on the NADPH and ATP generation through the electrons transfer controlled by light, from the water to $NADP^+$. In anaerobic conditions, ATP was synthesized without changes in NADP/NADPH ratio, due to the functioning of cyclic current of electrons. ATP increased and NADPH decreased in anaerobiosis. The inhibitors adding stimulated the O_2 evolution. These observations suggest that photosynthetic generation of reduction equivalents limits the photosynthetic assimilation (Forti *et al.*, 2003).

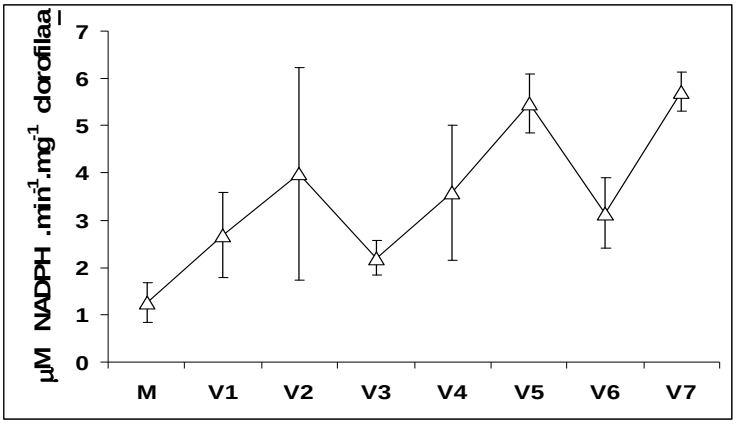

Fig. 4. The glutathione reductase activity (GR): M – control; V_1- light 4000 µmol.m^{-2}.s^{-1}; V_2- light + 10 mM sodium ascorbate; V_3- light + 20 mM sodium ascorbate; V_4- light + 30 mM sodium ascorbate; V_5- light + 10 mM H_2O_2; V_6- red light + 20 mM sodium ascorbate; V_7- red light + 10 mM H_2O_2 (300 µM DCMU, 2 µM FCCP and 5 mM iodoacetamide added to $V_1 – V_7$)

In chloroplasts NAD(P)H-dehydrogenase complex (NDH) mediates the cyclic electrons transport of PS I and that of chlororespiration, coded in chloroplast genome and by the nuclear *ndh* genes. The reduction of plastoquinone in the absence of light depends on the NDH activity and can be observed due to the transitory raising of chlorophyll fluorescence, after the illumination was interrupt (Muraoka et al., 2006).

The obtained results meaning the NADPH-dehydrogenase activity showed a slightly inhibition in its activity in the presence of high light (V_1) and that of hydrogen peroxide (V_5 and V_7) (Fig. 5). The ascorbate concentrations mainly that of 20 mM, intensified the enzyme activity, contributing to the rising of the photosynthetic ATP/NADPH ratio. The NADPH-dehydrogenase complex is involved in the nonphotochemical reduction of plastoquinone in the dark, after a period of illumination (Cournac *et al*., 2000).

The measuring of the final product of lipid peroxidation is the method used to distinguish the oxidative damages. ROS causes the peroxidation of polyunsaturated fatty acids, producing α,β-polyunsaturated aldehydes. The protein oxidation is an independent method of oxidative stress, which offers conclusions based on the determination of formed lipid oxidation products (Shulaev and Oliver, 2006). The raising of lipoxygenase activity corroborates with the high content of MDA, which is a product of lipid peroxidation. The increasing of lipid peroxidation is shown in the rising of lipoxygenase activity (Fryer *et al*., 1998). Lipid peroxidation produced the loss of the membranes integrity and theirs alteration (Venisse *et al*., 2001).

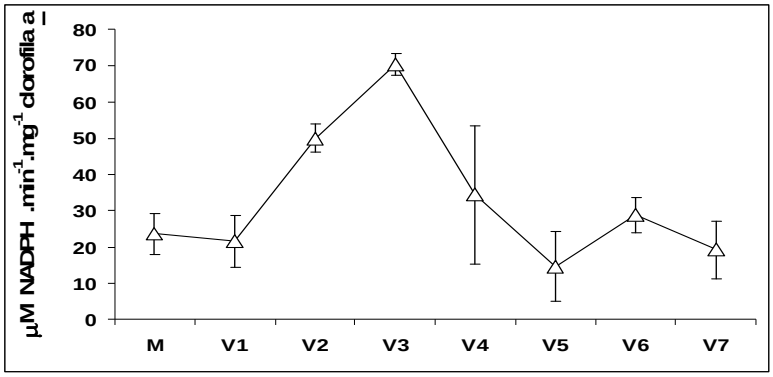

Fig. 5. The NADPH-dehydrogenase activity: M – control; V_1- light 4000 µmol.m^{-2}.s^{-1}; V_2- light + 10 mM sodium ascorbate; V_3- light + 20 mM sodium ascorbate; V_4- light + 30 mM sodium ascorbate; V_5- light + 10 mM H_2O_2; V_6- red light + 20 mM sodium ascorbate; V_7- red light + 10 mM H_2O_2 (300 µM DCMU, 2 µM FCCP and 5 mM iodoacetamide added to $V_1 – V_7$)

The inducement of lipid peroxidation was observed through the accumulation of thiobarbituric acid reactive species (TBARS) due to the reaction between thiobarbituric acid and trichloroacetic acid (Fig. 6). The obtained results showed in the majority of variants, the intensification of lipid peroxidation, probably due to the oxidative damages. In *state 2*, in the presence of 10 mM ascorbate, the highest content of TBARS reactive species accumulated (V_2).

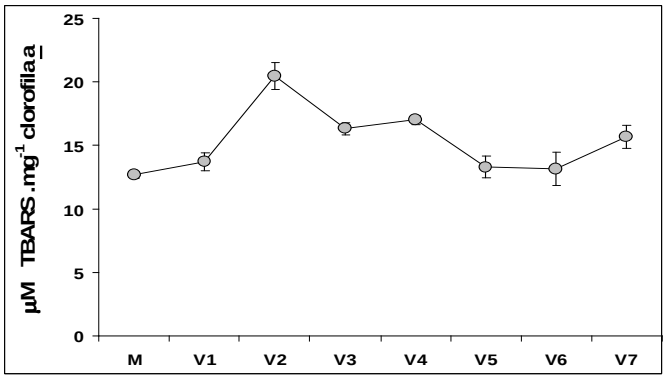

Fig. 6. The lipid peroxidation activity: M – control; V_1- light 4000 µmol.m^{-2}.s^{-1}; V_2- light + 10 mM sodium ascorbate; V_3- light + 20 mM sodium ascorbate; V_4- light + 30 mM sodium ascorbate; V_5- light + 10 mM H_2O_2; V_6- red light + 20 mM sodium ascorbate; V_7- red light + 10 mM H_2O_2 (300 µM DCMU, 2 µM FCCP and 5 mM iodoacetamide added to $V_1 – V_7$).

The activity of antioxidative enzymes induced by the photochemical *state 2* certifies that the relative flux of photosynthetic reductive equivalents of oxygen reactive species through the Mahler pathway is enough high. The maintenance of the electrons current at thylakoid level in the presence of enough amounts of $NADP^+$ as an electrons acceptor is essential for chloroplasts protection against photooxidative stress. In this process participates the cyclic current of electrons and the water-water cycle around PS I (Rizhsky *et al*., 2003). The photogeneration of oxygen reactive species may intensify when plants are exposed to intense light together with the environmental stress factors, fact that inhibits the usage of absorbed light energy for photosynthetic CO_2 fixation. These sorts of conditions produce the limitation of disposable $NADP^+$ for the electrons acceptance from PS I and so, the O_2 is activated (Cakmak and Marschner, 1992).

Conclusions

1. Referring to the control values, the SOD activity was greater in the best part of the samples. The high levels of SOD activity were observed under high light and in the presence of hydrogen peroxide. In *state 2*, the PS II favorable *light 2* and the presence of DCMU which blocks the photosynthetic electrons transport from PS II, facilitate the use of electrons in PS I towards the deactivation of the reactive oxygen species.

2. The ascorbate peroxidase activity was intensified under intense light, in the presence of 20 mM, 30 mM ascorbate and hydrogen peroxide. Light 2 together with the ascorbate and the hydrogen peroxide has inhibited the ascorbate peroxidase activity. The activity of the monodehydroascorbate reductase has decrease in the best part of the samples, excepting the concentrations of 20 mM and 30 mM de ascorbate. The activity of the dehydroascorbate reductase was significantly reduced both under intense light and in the presence of the ascorbate. The low activity of the dehydroascorbate reductase points out the existence of a low amount of dehydroascorbate which has been consumed in other metabolic pathways. In the presence of the hydrogen peroxide the dehydroascorbate reductase activity has been intensified and it has reached the control values.

3. There was observed an intensification of the GR activity in the presence of high light and in the case of its association with the ascorbate. In the presence of the H_2O_2 concentrations the enzymatic activity has reached the highest levels comparatively to the control.

4. The results achieved regarding the NADPH-dehydrogenase activity have shown a slight inhibition under light stress and in the presence of the hydrogen peroxide. The ascorbate concentrations, in particular the 20 mM, have intensified the enzyme activity, contributing to the enhancement of the ATP/photosynthetic NADPH ratio.

5. The inducement of the lipid peroxidation has been measured by monitoring the accumulation of the thiobarbituric acid reactive species (TBARS) subsequently to the thiobarbituric acid and trichloroacetic acid reaction. The

obtained results have emphasized the intensification of the lipid peroxidation in the best part of the variants, most likely because of the accentuation of the oxidative damages. In *state 2*, in the presence of 10 mM ascorbate there was obtained the greater amount of TBARS reactive species.

The activity of the antioxidative enzymes which is induced in photochemical *state 2* certifies the high accumulation of the photosynthetic reducing equivalents of the reactive oxygen species through the Mehler reaction. The maintenance of the electrons flow in thylakoids in the presence of a proper amount of $NADP^+$ as an electron acceptor is essential for the protection of the chloroplasts against the photooxidative stress.

R E F E R E N C E S

Asada, K. 1999. The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annu.Rev.Plant Physiol.Plant Mol.Biol.,* **50**, 601-639.

Asada, K. (2000). The water-water cycle as alternative photon and electron sink. *Philos.Trans.R.Soc. London B, Biol.Sci.,* **355**, 1419-1431

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiol.,***141**, 391-396.

Cakmak, I., Marschner, H. (1992). Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbat peroxidase and glutathione reductase in bean leaves. *Plant Physiol.*, **98**, 1222-1227

Chen, Z., Gallie, D.R. (2006). Dehydroascorbate reductase affects leaf growth, development and function. *Plant Physiol.*, **142**, 775-787.

Cournac, L., Josse, E.M., Joët, T., Rumeau, D., Redding, K., Kuntz, M., Peltier, G. (2000). Flexibility in photosynthetic electron transport: a newly identified chloroplast oxidase involved in chlororespiration. *Phil.Trans.R.Soc.London.B,Biol.Sci*., **355**, 1447-1454.

Dietz, K.J., Jacob, S., Oelze, M.L., Laxa, M., Tognetti, V., Miranda, S.M.N., Baier, M., Finkemeler, I.(2006). The function of peroxiredoxins in plant organelle redox metabolism. *J.Exp.Bot.*, **57**, 1697-1709.

Dragoş, N., Péterfi, L.Şt., Momeu, L., Popescu, C. (1997). An introduction to the algae and the culture collection of algae at the Institute of the Biological Research Cluj-Napoca. Cluj Univ.Press, Cluj-Napoca.

Forti, G., Furia, A., Bombelli, P., Finazzi, G. (2003). In vivo changes of the oxidation-reducţion state of NADP and of the ATP/ADP cellular ratio linked to the photosynthetic activity in *Chlamydomonas reinhardtii*. *Plant Physiol.*, **132**, 1464-1474.

Foyer, C.H., Halliwel, B. (1976). Presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta*, **133**, 21-25

Foyer, C.H., Lelandals, M., Kunert, K.J. (1994). Photooxidative stress in plants. *Physiol.Plant*, **92**, 696-717.

Fryer, M.J., Andrews, J.R., Oxborough, K., Blowers, D.A., Baker, N.R. (1998). Relationship between CO_2 assimilation, photosynthetic electron transport and active O_2 metabolism in leaves of maize in the field during periods of low temperature. *Plant Physiol.,* **116**, 571-580

Hodges, D.M., Andrews, C.J., Johnson, D.A., Hamilton, R.I. (1997). Antioxidant enzyme responses to chiling stress in differentially sensitive inbred maize lines. *J.Exp.Bot.*, **48**, 1105-1113

Joliot, P., Joliot, A. (2002). Cyclic electron transfer in plant leaf. *Proc.Natl.Acad.Sci.USA*, **99**, 10209-10214.

Karpinski, S., Wingsle, G., Karpinska, B., Hällgren, J.E. (1993). Molecular responses to photooxidative stress in *Pinus sylvestris L.* II. Differential expression of CuZn-superoxide dismutases and glutathione reductase. *Plant Physiol.*, **103**, 1385-1391.

Leipner, J. (1998). Chiling-induced photooxidative stress and adaptation of defense systems in maize (*Zea mays* L.) leaves. PhD Thesis. Zürich.

Miyake, C., Asada, K. (1994). Ferredoxin-dependent photoreduction of the monodehydro-ascorbate radical in spinach thylakoids. *Plant Cell Physiol.*, **35**, 539-549

Miyake, C., Yokota, A. (2000). Cyclic flow of electrons within PSII in thylakoid membranes. *Plant Cell Physiol.*, **42**, 508-515.

Mittler, R., Zilinskas, B.A. (1991). Purification and characterization of pea cytosolic ascorbate peroxidase. *Plant Physiol.,* **97**, 962-968.

Moller, J.M., Lim, W. (1986). Membrane bound NAD(P)H dehydrogenase in higher plants. *Annu.Rev.Plant.Physiol.*, **37**, 309-334

Muraoka, R., Okuda, K., Kobayashi, Y., Shikanai, T. (2006). A eukariotic factor required for accumulation of the chloroplast NAD(P)H dehydrogenase complex in *Arabidopsis. Plant Physiol.*, **142**, 1683-1689.

Rao, M.V., Paliyath, G., Ormrod, D.P. (1996). Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana. Plant Physiol.*, **110**, 125-136.

Rizhsky, L., Liang, H., Mittler, R. (2003). The water-water cycle is essential for chloroplast protection in the absence of stress. *J.Biol.Chem.*, **278**, 38921-38925.

Schreiber, U., Neubauer, C. (1990). O_2 dependent elctron flow, membrane energization and the mechanism of non-photochemical quenching of chlorophyll fluorescence. *Photosynth.Res.*,**25**,279-293

Shulaev, V., Oliver, D.J. (2006). Metabolic and proteomic markers for oxidative stress. New tools for reactive oxygen species research. *Plant Physiol.*,**141**,367-372.

Venisse, J.S., Gullner, G., Brisset, M.N. (2001). Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora*. *Plant Physiol.*, **125**, 2164-2172

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 75-89

THE PHOTOCHEMICAL ACTIVITY OF PS II AND PS I PHOTOSYSTEMS IN GREEN ALGA *MOUGEOTIA SP.* IN THE PRESENCE OF ASCORBATE AND HYDROGEN PEROXIDE, IN *STATE 2* TRANSITION

VICTOR BERCEA[1], CRISTIAN COMAN[1]

SUMMARY. The photochemical activity in the photosystems as a response to the photoinhibition inducement through intense light in the presence of different concentrations of enzymatic substrate (ascorbate) and under the intensification of the oxidative factor (H_2O_2) in *state 2* transition was studied. F_0 had risen to 20 mM sodium ascorbate and decreased in the presence of H_2O_2. DCMU led to an increase of F_0 which reflects the reduction of the opened reaction centers. F_m has been stimulated under actinic light at 20 mM and 30 mM ascorbate and in red light. The F_m diminish occurred in H_2O_2. F_v decreased in the presence of 10 mM ascorbate and H_2O_2. High ascorbate concentration led to an increase of F_v proportionally with the exposure period. F_v/F_m as well as the yield have decreased, more pronounced in the presence of H_2O_2. The photochemical efficiency remission as well as F_m's point out the photoinhibition installment in the photosystems. q_p has increased so that the reduction state in the PS II is high leading to an increase of the excitation pressure. The excitation energy dissipation rate has risen also in the photosystems' antenna. Generally, the cytochrome *b_6f* has been in a reduced state. In *state 2*, the reduction of the cytochrome *b_6f* gets dominant by exposure to light which induces a specific activity in PS I therefore the quantity of the electrons which flow towards PS I has also increased.

KEYWORDS: chlorophyll fluorescence, down regulation, photoinhibition, plastoquinone, quantum yield

Introduction

The excessive light exposure in regard to the photosynthetic demand leads to an energetic disequilibrium resulting the photoinhibition which manifests through structural modifications in the photosystems. The photoinhibition conditions result in the formation of reactive oxygen forms either by reduction of O_2 to superoxide anionic radical ($O_2^{\cdot-}$)

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or by energy transfer from the chlorophyll to O_2 in the third excitation state leading to singlet oxygen formation ($^1O_2$).

The ascorbic acid is an important antioxidant and a redox buffer (cell reductant) (Matamoros *et al*., 2006) being involved also in the cells' protection against peroxidic damages, scattering directly the reactive oxygen forms and also in the α-tocopherol recovery (Smirnoff, 2000) as well as a substrate for cytosolic ascorbate peroxidase and for other enzymatic isoforms of the ascorbate-glutation process for hydrogen peroxide detoxification (Dalton *et al.*, 1986). The ascorbate is a cofactor for the violaxanthin de-epoxidase, prolylhidroxilase and for 9-cis-epoxicarothenoid dioxigenase (Smirnoff, 2000; Conklin, 2001). More recently the ascorbate proved to play an important role in modulating the expression of the genes involved in the plants' defensive answer regarding the biotic stress (Pastori *et al*., 2003).

The photosynthetic organisms which develop O_2 can redistribute evenly the light energy between the PS II and PS I photosystems through the mechanism known as *state transitions* thought to be an adaptation for light energy's efficient usage in restrictive conditions (Bonaventura and Myers,1969; Williams and Allen,1987; Veeranjaneyulu and Leblanc, 1994). The state transitions involve a reversible redistribution of the light harvesting antenna between PS I and PS II and optimize the light energy usage in photosynthesis through the cyclic electron current (Canaani *et al*., 1984; Weis, 1985; Finazzi *et al*., 2002).

In green plants *state 1* and *state 2* are induced by exposing the leaves to light of different wavelenghts: light 1 far-red which is above 715 nm or light 2 between 400-600 nm. The light absorbed by PS II (light 2) induces the phosphorylation and the decoupling of LHC II from PS II which migrates towards stroma lamellae rich in PS I and couples to PS I resulting the *state 2*. *State 1* transition involves the light absorbed by PS I (light 1) which generates the dephosphorylation and dissociation of LHC II from PS I and their migration to grana lamellae rich in PS II (Canaani *et al*.,1984).

By Finazzi *et al.* (2001), the *state 1* is induced by incubating the cells in the dark under continous stirring , and the *state 2* is generated by dark incubation in anaerobic conditions by argon pumping. The incubation of the algae in conditions which facilitate the *state 2*, anaerobiosis or aerobiosis and FCCP, generates an electron source through cytochrome b_6f. The reactivation of the liniary electron current between PS II and PS I demands the switch from *state 2* to *state 1*, indicated by the simultaneous fluorescence production (F_m).

The cytochrome b_6f complex plays a key role in the state transitions (Wollman, 2001). The LHC II complexes increase the PS I performance and can represent a mechanism which allows the switch between the liniary and cyclic electron current around PS I (Vallon *et al*., 1991). The *state 2* represents the structural condition when

most of the excitation energy is used in the PS I's photochemistry so that the cyclic electron transport around PS I uses the liniary electron current which involves the both photosystems (Finazzi *et al*., 2001).

In this study photochemical activity of the photosystems has been studied as a response to photoinhibition inducement by intense light in the presence of different concentrations of enzymatic substrate (ascorbate) and under the intensification of the oxidative factor (H_2O_2) in *state 2* transition.

Material and Methods

The green alga *Mougeotia sp*. Agardh (AICB 560), comes from the Algae Culture Collection in the Institute of Biological Research Cluj-Napoca (AICB) (Dragoş *et al*., 1997). The AICB 560 strain was grown on Bold nutrient solution (BBM), under continous air agitation, continous illumination with 443 $\mu mol.m^{-2}.s^{-1}$ at 22^0C. The growth period was 24 days.

The treatement with light, cofactors, agents which generate reactive oxygen and inhibitors. The PAR light intensity (the photosynthetically active radiation) of 4000 $\mu mol.m^{-2}.s^{-1}$ was applied for 60 minutes in *state 2* induced in aerobic conditions by using specific inhibitors: 300 μM DCMU, 2 μM FCCP and 5 mM iodoacetamide. The *state 2* was also induced by using light 2 particular to PS II, sent out by the red filter at 620 nm wavelenght with 2500 $\mu mol.m^{-2}.s^{-1}$ intensity. The sodium ascorbate was used as an enzymatic cofactor in 10 mM, 20 mM, 30 mM concentrations, and 10 mM H_2O_2 was used as a reactiv oxygen generating agent.

The redox state of the P_{700} reaction center in PS I was analyzed on the basis of the changes in the 702 nm absorbance (Melis, 1989). The redox state of the quinonic acceptor in PS II was determined using the changes in the 320 nm absorbance. The cytochrome b_6f redox state was determined after Joliot and Joliot (2002).

Chlorophyll's fluorescence analysis. The chlorophyll's fluorescence was measured with PAM-210 fluorometer according to Schreiber *et al*. (1986). The fluorescence parameters and the quencing analysis were done by applying the saturation puls method. The quantum yield of the photochemical energy conversion was determined using the expression Yield = $\Delta F/F'_m$, and the relation F_v/F_m ($F_v/F_m = F_m-F_0/F_m$) represents the photochemical quantum yield of the closed PS II reaction centers.

Results and discussions

The chlorophyll's fluorescence was the parameter measured during the alga suspension's 60 minutes exposure to regular light with 4000 $\mu mol.m^{-2}.s^{-1}$ intensity. The *state 2* transition was induced by exciting the photosystems with actinic light in

aerobic conditions in the presence of FCCP, DCMU and iodoacetamide or by excitement with light 2 which activates the PS II photosystem, being sent out by the red filter with 2300 $\mu mol.m^{-2}.s^{-1}$ intensity. The transmission spectrum for light 2 presented a maximum in blue at 355 nm, another maximum at 545 nm and the dominant maximum at 620 nm (Bercea *et al.*, 2007). FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) is a decoupling agent which causes the transition to *state 2* by diminishing the ATP and the membrane potential. The iodoacetamide inhibits the Rubisco enzyme's activity (Calvin cycle), and DCMU [3-(3,4-diclorophenyl)-1,1-dimethylurea] inhibits the Q_B reduction inside PS II so that the plastoquinone is oxidized by light.

The ascorbate's involment in photosynthesis is a long-time acknowledgment, especially being emphasized it's photoprotection function (Smirnoff, 2000). The ascorbate's concentration is proportionally light dependent (Grace and Logan, 1996).

At blank, the alga suspension incubated for 1 hour in dark presented normal values for chlorophyll's fluorescence parametrers. High quantum efficiency and quantum yield were observed along side with a slight unradiative dissipation of the excitation energy.

Under the performed treatments, the F_0 values have risen significantly under high intensity light (Fig. 1, V_1). This increase was more pronounced at 20 mM sodium ascorbate (V_3) and in association with light 2 (V_6). F_0 decreased durind the 60 minutes of intense light exposure iin the presence of hydrogen peroxide (H_2O_2) considered to be a reactive oxygen generator. The FCCP ionophor facilitates the pH equilibration among cell components (Finazzi *et al*., 2002), can be an electron transport decoupling agent (Goyal *et al*., 1996) or inhibits the ATP synthesis without involving the proton dissipation inside chloroplast's lumen (Zang and Melis, 2002). The light 2 in *state 2* in the presence of DCMU, which is an electron transport inhibitor, leads to the increase of F_0 which in these experimental conditions reflects the state of the reaction centers inside PS I. The increase of F_0 represents the reduction of the opened reaction centers, respectively the decrease of plastoquinone in oxidized state. In anaerobic conditions, the increase of F_0 shows the partial decrease of the plastoquinone volume, and F_m diminishes progressively (Joët *et al*., 2002).

The maximal fluorescence's evolution (F_m), which is a measure of the closed reaction centers, respectively of Q_A in reduced state has been stimulated under high intensity light (Fig. 1, V_1). Significant growths took place at 20 mM and 30 mM ascorbate at the end of light exposure (V_3, V_4), as well as in red light in the the presence of ascorbate (V_6). The decrease of F_m appeared at 10 mM ascorbate (V_2) and in hydrogen peroxide variants (V_5, V_7).

In *state 2* the F_m fluorescence increased under red light particular to PS II and in the presence of ascorbate which shows that teh PS I's proteic quantity increased due to the

attachement of the LHC II antennary proteins. The controled redox phosphorylation of the thylakoidal proteins represents a unic system for the adjustement of the balanced light energy usage in photosynthesis system in which the plastoquinone concentration is directly involved (Vener *et al*., 1997).

The variable fluorescence (F_v) decreased under intense light action (V_1), in the presence of 10 mM ascorbate (V_2) and different hydrogen peroxide concentrations (V_5, V_7) regarding the period of light exposure, process due primary to the F_m decrease. High ascorbate concentrations led to the rise of F_v proportionally with the exposure period (V_3, V_4). Under red light action at 620 nm and in the presence of ascorbate F_v was at the control level (V_6).

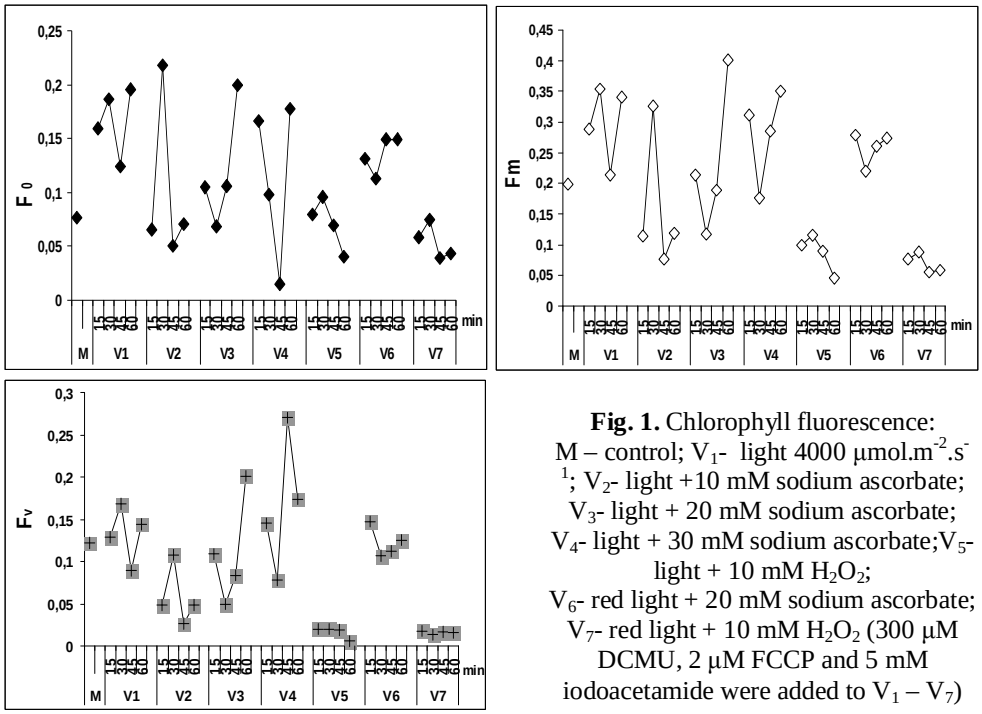

Fig. 1. Chlorophyll fluorescence: M – control; V_1- light 4000 μmol.m^{-2}.s^{-1}; V_2- light +10 mM sodium ascorbate; V_3- light + 20 mM sodium ascorbate; V_4- light + 30 mM sodium ascorbate;V_5- light + 10 mM H_2O_2; V_6- red light + 20 mM sodium ascorbate; V_7- red light + 10 mM H_2O_2 (300 μM DCMU, 2 μM FCCP and 5 mM iodoacetamide were added to $V_1 – V_7$)

The photochemical efficiency of the PS II's closed reaction centers (F_v/F_m) as well the quantum yield of the photosynthetical electron transport chain have reduced dramatically in all experimental variants (Fig. 2). These decreases were proportional with the increase of the light exposure period, 60 minutes respectively. The photochemical efficiency and the quantum yield decreased 2-3 times in the presence of

hydrogen peroxide compared to control values (V_5, V_7), which states that the fraction of the absorbed light used in the electron transport reduced. The low F_v/F_m values are due to F_m decrease and to the disturbance of the liniar electron transport, and on the other hand the conversion efficiency of the photochemical quantum, respectively the PS I reaction centers are closing due to a lack of energized state in the thylakoidal mambrane. The photochemical efficiency decrease was accompanied by the F_v diminish which indicates the photoinhibition installment in the photosystems. The slight high values in the presence of ascorbate concentrations are attributed to photosynthesis *down regulation* process. The down regulation mechanism creates the energy dissipation centers leading to down regulation homogeneity. Not all mechanims work simultaneously: the mechanism of the photosystems' antenna plays a role in the dissipation of the down regulated centers, and the D_1 protein turnover is involved in the formation and function of the down regulation (Critchley and Russell, 1994).

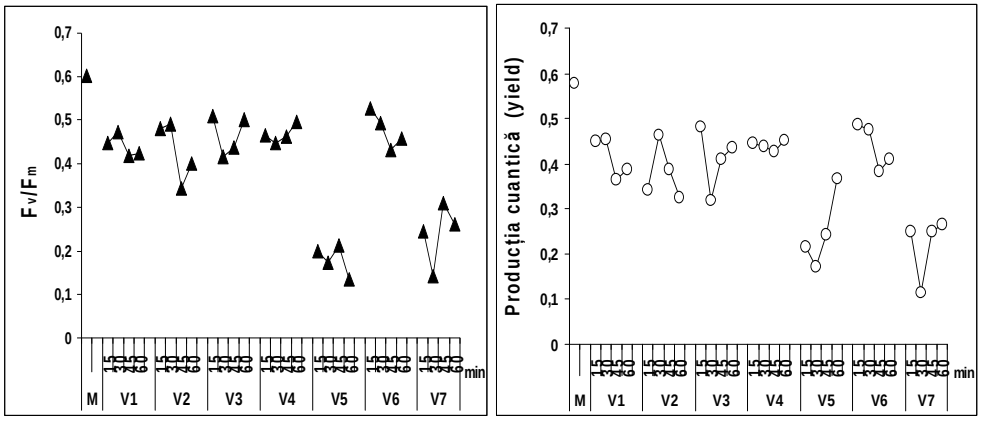

Fig. 2. PS II photochemical efficiency and the photosynthesis quantum yield: M – control; V_1- light 4000 μmol.m^{-2}.s^{-1}; V_2- light + 10 mM sodium ascorbate; V_3- light + 20 mM sodium ascorbate; V_4- light + 30 mM sodium ascorbate; V_5- light + 10 mM H_2O_2; V_6- red light + 20 mM sodium ascorbate; V_7- red light + 10 mM H_2O_2 (300 μM DCMU, 2 μM FCCP and 5 mM iodoacetamide were added to $V_1 – V_7$)

The photochemical coefficient (q_P) increased in the majority of the variants, existing the tendency of decrease at 10 mM, 20 mM ascorbate concentrations (V_2, V_3) and in the combination of red light and H_2O_2 (V_7) (Fig. 3). The high q_P values show the rise of the opened Q_A proportion, respectively the effective excitation energy conversion to photochemistry in the reaction centers, without existing a saturation through light. In

these conditions the PS II reduced state is high without influencing the redox state of the plastoquinone due to electron transfer obstruction between Q_A and Q_B due to DCMU. The growth of the photochemical coefficient, as well as the photochemical activity increase have led to the rise of the excitation energy dissipation rate in the photosystems' antenna (Fig. 3, NPQ).

Nonphotochemical de-excitation of the energy rises wanting to disperse the excessive light energy consequently to the external decline of F_v due to structural damages. The growth of the excitation energy dissipation was done proportionally with the period of exposure to different treatments, more significantly in intense light (V_1), at 20 mM and 30 mM ascorbate concentrations (V_3, V_4) and under the effect of red light in combination with ascorbate (V_6) (Fig. 3).

The photoinhibition is produced by generating an intermediary state in which PS II is inactiv, but the D_1 protein is still intact. The acumulation of this condition is amplified in *state 2* because now only the cyclic photosynthetical electron trasnport is active, while none of the electrons are flowing between PS II and cytochrome b_6f. This allows the repair of the damages occured in PS II due to the cell's capacity to mentain a high rate of ATP synthesis on the way of electron current led by PS I. This capacity can represent an important physiological property in protecting the photosynthetical apparatus from excessive light as well as in abiotic stress conditions (Finazzi *et al*., 2001).

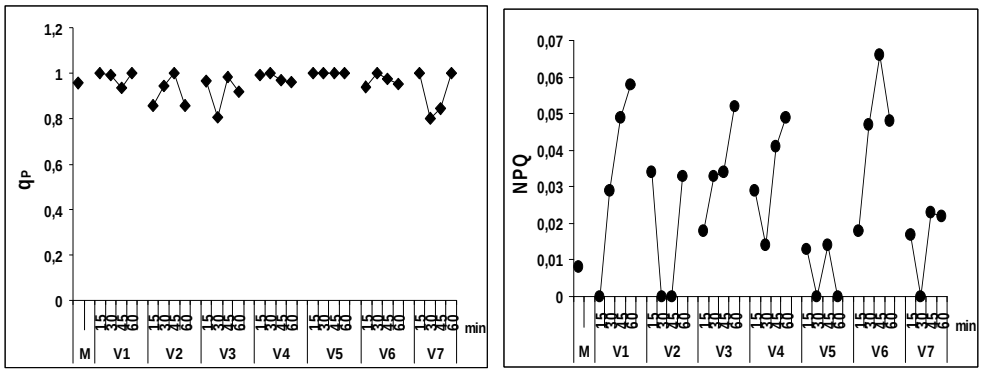

Fig. 3. Evolution of the photochemical coefficient (qP) and of the nonphotochemical coefficient (NPQ): M – control; V_1- light 4000 μmol.m^{-2}.s^{-1}; V_2- light + 10 mM sodium ascorbate; V_3- light + 20 mM sodium ascorbate; V_4- light + 30 mM sodium ascorbate; V_5- light + 10 mM H_2O_2; V_6- red light + 20 mM sodium ascorbate; V_7- red light + 10 mM H_2O_2 (300 μM DCMU, 2 μM FCCP and 5 mM iodoacetamide were added to V_1 – V_7)

Energetic desequilibrium among photochemistry, electron transport and metabolism is high leading to an increased excitation pressure in PS II. On short periods of time, exposure to the high excitation pressure in PS II leads to a reduction of the photosystem's energetic efficiency due to the change in energy direction from PS II to PS I through state transitions or through excessive energy dissipation by nonphotochemical quencing (Huner *et al*., 1998). On longer periods of time, photosynthetiocal acclimatization to high excitation pressure in PS II determines the lessen of the PS II antenna's size, in other words the adjustment of PS II's functional absorbtion area's size reducing the photochemical efficiency expressed by the quantum yield of either CO_2 assimilation, either O_2 evolution.

The excitation pressure increased under 10 mM, 20 mM ascorbate concentrations and under the action of H_2O_2 in association with red light (Fig. 4). The excitation pressure is an indicator of the Q_A primary acceptor's redox state, which in the illustrated variants shows a significant increase of the proportion of Q_A in reduced state.

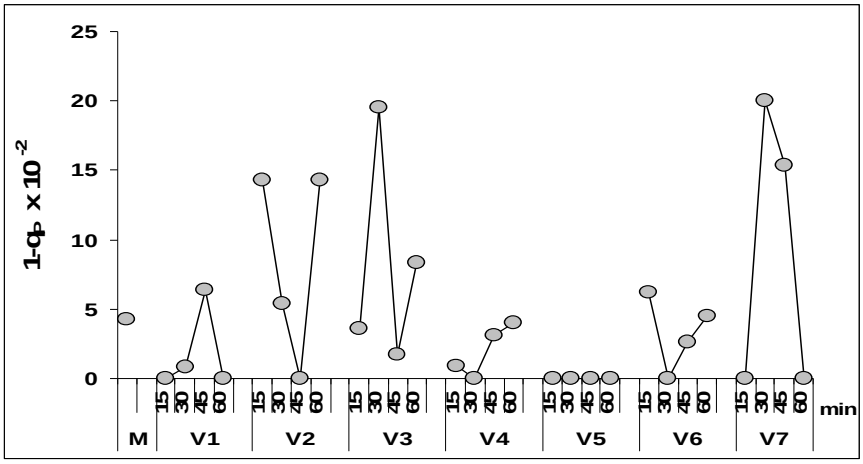

Fig. 4. Evolution of the photosystems' excitation pressure (1 - qP): M – control; V_1- light 4000 μmol.m^{-2}.s^{-1}; V_2- light + 10 mM sodium ascorbate; V_3- light + 20 mM sodium ascorbate; V_4- light + 30 mM sodium ascorbate; V_5- light + 10 mM H_2O_2; V_6- red light + 20 mM sodium ascorbate; V_7- red light + 10 mM H_2O_2 (300 μM DCMU, 2 μM FCCP and 5 mM iodoacetamide were added to V_1 – V_7)

High excitation pressure determines increased quantities of xanthophyls aiming very clearly the zeaxanthin, the growth of the a/b proportion and the decrease of the LHC antennary proteins (Maxwell *et al*., 1995).

Generally speaking, PS II photoinhibition is characterized by the decline of the quantum efficiency, by the diminish in the number of centers participant to the liniary photosynthetic electron transport and by the increase of the centers involved in excitation energy dissipation (Critchley and Russell, 1994).

The PS II's redox state was monitored also by observing the changes in the 320 nm absorbtion (Fig. 5). After intense light action at 4000 μmol.m^{-2}.s^{-1} the oxidation state of the nucleus represented by plastoquinone prevails, respectively the electron loss in the opened Q_A centers (V_1). In the presence of 10 mM ascorbate the oxidation state in the first 15 minutes of exposure is replaced by the reduced state (V_2), situation which repeats oscillatory with every 20 mM ascorbate (V_3) and in the presence of hydrogen peroxide (V_5). High ascorbate concentration as well as the red light combinations maintain the volume of plastoquinone in a permanent oxidative state.

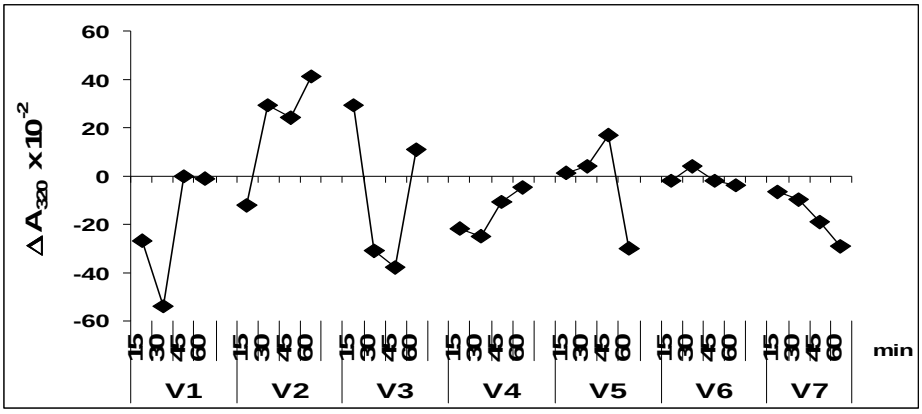

Fig. 5. Changes in absorbtion at 320 nm regarding the redox state of PS II. M – control; V_1- light 4000 μmol.m^{-2}.s^{-1}; V_2- light + 10 mM sodium ascorbate; V_3- light + 20 mM sodium ascorbate; V_4- light + 30 mM sodium ascorbate; V_5- light + 10 mM H_2O_2; V_6- red light + 20 mM sodium ascorbate; V_7- red light + 10 mM H_2O_2 (300 μM DCMU, 2 μM FCCP and 5 mM iodoacetamide were added to $V_1 – V_7$)

The amplitude of the changes induced by LHC II coupling was more lessened in PS I unlike PS II, which explains the reduced photochemical activity in *state 2* up against *state 1*. This can be attributed to a low energy transfer from the carotenoids to chlorophylls in LHC II when they are associated to PS I (Veeranjaneyulu and Leblanc 1994).

The redox state of the P_{700} reaction center inside PS I was analyzed by determining the changes in absorbtion at 702 nm (Fig. 6). Under intense light in *state 2*, the

reaction center worked under reductive state (V_1). In the presence of ascorbate in *state 2* the reductive state of the reaction center was kept in the first moments of light exposure in order that the oxidative state to be reached in the end (V_2, V_3, V_4). Under the effect of hydrogen peroxide the system tends towards the oxidative state of the reaction center after light exposure (V_5, V_7). The association between ascorbate and red light determines a slightly reduced redox state of the P_{700} reaction center. P_{700} was oxidized by far-red light and reduced at dark by stromatic reductants (Joët *et al*., 2002).

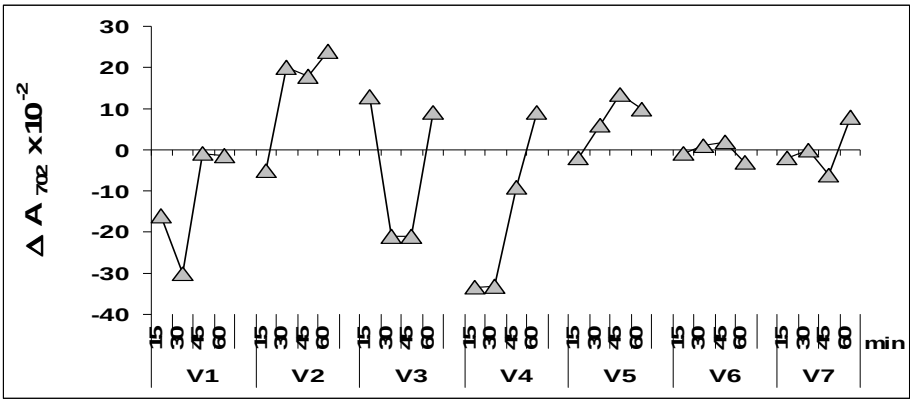

Fig. 6. Changes in absorbtion at 702 nm regarding the redox state of PS I. M – control; V_1- light 4000 μmol.m^{-2}.s^{-1}; V_2- light + 10 mM sodium ascorbate; V_3- light + 20 mM sodium ascorbate; V_4- light + 30 mM sodium ascorbate; V_5- light + 10 mM H_2O_2; V_6- red light + 20 mM sodium ascorbate; V_7- red light + 10 mM H_2O_2 (300 μM DCMU, 2 μM FCCP and 5 mM iodoacetamide were added to $V_1 – V_7$)

The redox state of the cytochrome b_6f is presented in Fig. 7. In general, the cytochrome b_6f was kept in a reduced state in variants with light and ascorbate due to electron accepting as a result of the intensification of the cyclic electron transport around PS I. The idea is sustained by the dominance of the reductive state after 60 minutes of light exposure. In the presence of hydrogen peroxide the cytochrome b_6f was activated in the ozidized state (V_5, V_7). In *state 2*, the reduction of cytochrome b_6f becomes dominant in light exposure which induces a specific activity of PS I thus increasing the quantity of electrons flowing towards PS I. The state transitions describes the revesible association of the LHC antennary complex with each of the photosystems: with PS II in *state 1* and with PS I in *state 2*. The cytochrome b_6f complex interferes with the state transition regulation and establishes the link between the liniary and cyclic electron current which follows the state transition (Finazzi, 2005).

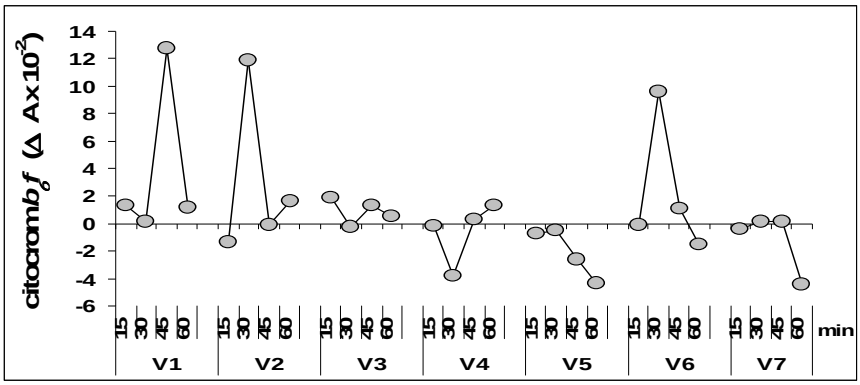

Fig. 7. The redox state of the cytochrome b_6f. M – control; V_1- light 4000 μmol.m^{-2}.s^{-1}; V_2- light + 10 mM sodium ascorbate; V_3- light + 20 mM sodium ascorbate; V_4- light + 30 mM sodium ascorbate; V_5- light + 10 mM H_2O_2; V_6- red light + 20 mM sodium ascorbate; V_7- red light + 10 mM H_2O_2 (300 μM DCMU, 2 μM FCCP and 5 mM iodoacetamide were added to $V_1 – V_7$)

The cytochrome b_6f is the sensor of the PS II's redox state (Wollman *et al.*, 2001) and it's function is to couple the quinol oxidation to transmembranary proton translocation leading to stabilization of the electrochemical gradient which is necessary in coordinating the ATP synthesis (Hamel *et al.*, 2000).

Conclusions

1. The minimal fluorescence (F_0) increased more significantly in the presence of 20 mM sodium ascorbate and in assocation with light 2. F_0 decreased significantly during the 60 minutes of intense light exposure in the presence of hydrogen peroxide (H_2O_2) thought to be a reactive oxygen generator. Light 2 in *state 2* in the presence of DCMU leads to the increase of F_0 this reflecting the reduction of the opened reaction centers, respectively the diminish of plastoquinone in oxidized state.

2. The maximal fluorescence (F_m) was stimulated by high intensity light, at 20 mM and 30 mM ascorbate concentrations as well as in red light in the presence of ascorbate. The decrease of F_m appeared at 10 mM ascorbate concentration and in the variants with hydrogen peroxide. In *state 2*, F_m has increased under red light particular to PS II and in the presence of ascorbate showing that the quantity of proteins inside PS I rose due to LHC II antennary proteins attachement.

3. The variable fluorescence (F_v) decreased significantly in intense light, in the presence of 10 mM ascorbate and different hydrogen peroxide concentrations in relation with the light exposure period, situation assigned especially to the decrease of F_m. High ascorbate concentrations led to to increase of F_v proportional to exposure period. Under 620 nm red light action and in the presence of ascorbate, F_v was at the blank level.

4. Photochemical efficiency (F_v/F_m) as well as the quantum yield of the photosynthetic electrons transport chain decreased in all variants, the decrease being proportional to the increase of the period of light exposure. The photochemical efficiency and the quantum yield decreased 2-3 times in the presence of hydrogen peroxide, compared to the control values, which states that the absorbed light fraction used in the electron transport diminished. The low F_v/F_m values are due to F_m decrease and to the disturbance of the liniar electron transport, and on the other hand the conversion efficiency of the photochemical quantum, respectively the PS I reaction centers are closing due to a lack of energized state in the thylakoidal mambrane. The photochemical efficiency decrease was accompanied by the F_v diminish which indicates the photoinhibition installment in the photosystems. The slight high values iin the presence of ascorbate concentrations are attributed to photosynthesis down regulation process. The down regulation mechanism creates the energy dissipation centers leading to down regulation homogeneity.

5. The photochemical coefficient (q_P) increased in the majority of the variants, existing the tendency of decrease at 10 mM, 20 mM ascorbate concentrations and in the combination of red light and H_2O_2.The q_P shows the rise of the opened Q_A proportion, respectively the effective excitation energy conversion to photochemistry in the reaction centers, without existing a saturation through light. In these conditions the PS II reduced state is high without influencing the redox state of the plastoquinone due to electron transfer obstruction between Q_A and Q_B due to DCMU.

6. The growth of the photochemical coefficient has led to the rise of the excitation energy dissipation rate in the photosystems' antenna. Nonphotochemical de-excitation of the energy rises wanting to disperse the excessive light energy consequently to the external decline of F_v due to structural damages. The growth of the excitation energy dissipation was done proportionally with the period of exposure to different treatments, more significantly in intense light, at 20 mM and 30 mM ascorbate concentrations and under the effect of red light in combination with ascorbate.

7. Energetic desequilibrium in PS II led to an increased excitation pressure. The excitation pressure increased under 10 mM, 20 mM ascorbate concentrations and under the action of H_2O_2 in association with red light. The excitation pressure is an indicator of the Q_A primary acceptor's redox state, which in the illustrated variants shows a significant increase of the proportion of Q_A in reduced state.

8. After intense light action at 4000 μmol.m^{-2}.s^{-1} the oxidation state of the nucleus represented by plastoquinone prevails, respectively the electron loss in the opened Q_A centers.In the presence of 10 mM ascorbate the oxidation state in the first 15 minutes of exposure is replaced by the reduced state (V_2), situation which repeats with every 20 mM ascorbate and in the presence of hydrogen peroxide. High ascorbate concentration as well as the combinations with red light maintain the plastoquinone volume in a permanent oxidation state.

The P_{700} reaction center inside PS I under intense light in *state 2* worked under reductive state. In the presence of ascorbate in *state 2* the reductive state of the reaction center was kept in the first moments of light exposure in order that the oxidative state to be reached in the end. Under the effect of hydrogen peroxide the system tends towards the oxidative state of the reaction center after light exposure.

In general, the cytochrome b_6f was kept in a reduced state in variants with light and ascorbate due to electron accepting as a result of the intensification of the cyclic electron transport around PS I. In the presence of hydrogen peroxide the cytochrome b_6f was activated in the ozidized state. In *state 2,* the reduction of cytochrome b_6f becomes dominant in light exposure which induces a specific activity of PS I thus increasing the quantity of electrons flowing towards PS I.

REFERENCES

Bercea, V., Muntean, E., Vasilescu, C., Drugă, B., Dragoş, N. (2007). The relation between the photochemical activity of PS I, PS II and the state transitions in the green alga *Mougeotia*, strain AICB 560. *Contrib.Bot.*, **XLII**, 73-85.

Bonaventura, C., Myers, T. (1969). Fluorescence and oxygen evolution from *Chlorella pyrenoidosa*. *Biochim.Biophys.Acta,* **189**, 366-383.

Canaani, O., Barber, J., Malkin, S. (1984). Evidence that phosphorylation and dephosphorylation regulate the distribution of excitation energy between the two photosystems of photosynthesis *in vivo*: photoacustic and fluorimetric study of an intact leaf. *Proc.Natl.Acad.Sci.USA*,**81**,1614-1618.

Conklin, P.L. (2001). Recent advances in the role and biosynthesis of ascorbic acid in plants. *Plant Cell Environ.* ,**24**, 383-394.

Critchley, C., Russell, A.W. (1994). Photoinhibition of photosynthesis in vivo: The role of protein turnover in photosystem II. *Physiol. Plant.*, **92**, 188-196.

Dalton, D.A., Russell, S.A., Hanua, F.J., Pascoe, G.A., Evans, H.J.(1986). Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. *Proc.Nat.Acad.Sci.USA*, **83**, 3811-3815.

Dragoş, N., Péterfi, L.Şt., Momeu, L., Popescu, C. (1997). An introduction to the algae and the culture collection of algae at the Institute of the Biological Research Cluj-Napoca. Cluj Univ.Press, Cluj-Napoca.

Finazzi, G., Barbagallo, R.P., Bergo, E., Barbato, R., Forti, G. (2001). Photoinhibition of C*hlamydomonas reinhardtii* in state 1 and state 2. Damages to the photosynthetic apparatus under linear and cyclic electron flow. *J.Biol.Chem.*, **276**, 22251-22257.

Finazzi, G., Rappaport, F., Furia, A., Fleischmann, M., Rochaix, J.D., Zito, F., Forti, G. (2002). Involvement of state transitions in the switch between linear and cyclic elctron flow in *Chlamydomonas reinhardtii. EMBO J.*, **31**, 280-285.

Finazzi, G. (2005). The central role of the green alga *Chlamydomonas reinhardtii* in revealing the mechanism of state transitions. *J.Exp.Bot.*, **56**, 383-388.

Goyal, A., Tolbert, N.E. (1996). Association of glycolate oxidation with photosynthetic electron transport in plant and algal chloroplasts. *Proc.Natl.Acad.Sci.USA*, **93**, 3319-3324.

Grace, S.C., Logan, B.A. (1996). Acclimation of foliar antioxidant systems to growth irradiance in three broad-leaved evergreen species. *Plant Physiol.*, **112**, 1631-1640.

Hamel, P., Olive, J., Pierre, Y., Wollman, F.A., Vitry, C. (2000). A new subunit of cytochrome b_6f complex undergoes reversible phosphorylation upon state transition. *J.Biol.Chem.*, **275**, 17072-17079.

Huner, N.P.A., Öquist, G., Sarhan, F. (1998). Energy balance and acclimation to light and cold. *Trends Plant Sci.*, **3**, 224-230.

Joët, T., Cournac, L., Peltier, G., Havaux, M. (2002). Cyclic electron flow around photosystem I in C_3 plants. In vivo control by the redox state of chloroplasts and involvement of the NADH-dehydrogenase complex. *Plant Physiol.*,**128**,760-769.

Joliot, P., Joliot, A. (2002). Cyclic electron transfer in plant leaf. *Proc.Natl.Acad.Sci.USA*, **99**, 10209-10214.

Matamoros, M.A., Loscos, J., Coronado, M.J., Ramos, J., Sato, S., Testillano, P.S., Tabata, S., Becana, M. (2006). Biosynthesis of ascorbic acid in legume root nodules. *Plant Physiol.*, **141**, 1068-1077.

Maxwell, D.P., Falk, S., Huner, N.P.A. (1995). Photosystem II excitation pressure and development of resistance to photoinhibition.I. Light-harvesting complex II abundance and zeaxanthin content in *Chlorella vulgaris*. *Plant Physiol.*,**107**,687-694.

Melis, A. (1989). Spectroscopic methods in photosynthesis: photosystem stoichiometry and chlorophyll antenna size. *Phil.Trans.R.Soc.Lond.B*, **323**, 397-409.

Pastori, G., Kiddle, G., Antoniw, J., Bernard, S., Veljovic-Jovanovic, S., Verrier, P.J., Noctor, G., Foyer, C.H. (2003). Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. *Plant Cell Physiol.*, **22**, 867-880.

Schreiber, U., Schliwa, U., Bilger, W. (1986). Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer, *Photosynth.Res.*, **10**, 51-62.

Smirnoff, N. (2000). Ascorbate biosynthesis and function in photoprotection. *Philos.Trans R. Soc. London B Biol.Sci.*, **355**, 1455-1464

Vallon, O., Bulte, L., Dainese, P., Olive, J., Bassi, R., Wollman, F.A. (1991). Lateral redistribution of cytochrome b_6/f complexes along thylakoid membranes upon state transitions. *Proc.Natl.Acad.Sci.USA*, **88**, 8262-8266.

Veeranjaneyulu, K., Leblanc, R.M. (1994). Action spectra of photosystem I and II in state 1 and state 2 in intact sugar maple leaves. *Plant Physiol.*, **104**, 1209-1214.

Vener, A.V., Kan, P.J.M., Rich, P.R., Ohad, I., Andersson, B. (1997). Plastoquinol at the quinol oxidation site of reduced cytochrome *bf* mediates signal transduction between light and protein phosphorylation: thylakoid protein kinase deactivation by a single-turnover flash. *Proc.Natl.Acad.Sci.USA*, **94**, 1585-1590.

Weis, E. (1985). Light- and temperature-induced changes in the distribution of excitation energy between photosystem I and photosystem II in spinach leaves. *Biochim.Biophys.Acta*, **807**, 118-126.

Williams, W.P., Allen, J.F. (1987). State 1/state 2 changes in higer plants and algae. *Photosynth Res.*, **13**, 19-45.

Wollman, F.A. (2001). State transitions reveal the dynamics and flexibility of the photosynthetic apparatus. *EMBO J.*, **20**, 3623-3630.

Zhang, L., Melis, A. (2002). Probing green algal hydrogen production.*Phil.Trans.R.Soc.London B*, **357,** 1499-1509.

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 91-99

INFLUENCE OF HEAVY METALS CONTAINED IN SIMULATED RAINFALL ON WATER STATUS AND YIELD COMPONENTS OF MAIZE PLANTS (CV. TURDA 200)

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SUMMARY. The influence of heavy metal contained in simulated rainfall on metal toxicity was investigated using plant water status and yield components as stress indicators. A 2-yr field experiment was designed to study the effects of lead, copper and zinc on the water status and yield components of maize plants. For simulating the action of heavy metals from the ambient air, solutions containing Pb, Cu and Zn were applied on the leaves at 10 days after germination in the following concentrations: 0.2, 2 and $4x10^4$ ppm Cu and Zn, and 0.32, 3.2, and 6.4 $x10^4$ ppm Pb respectively. The analysis were made at 8-10 leaves stage corresponding at 18 days after the treatment. Tissue water content, dry matter, ash content and stomatal conductance of the leaves, were established. At harvest, the dry matter, grain weight, harvest index (HI), and crude protein (CP) were determined. Our results concerning stomatal conductance suggest a lower plant resistance to metal toxicity when the source is the polluted air, and the metal enter the plant through the leaves. The heavy metals are modifying the water content of the leaves with a percentage between 0.4 to 2.3 % in the wet year and between 0.8 to 6.2% in the dry year compared to control. There is no a direct relationship between the water content of the leaves and the concentration of the copper, but the relationship is relevant for the concentration of zinc and lead. The accumulation of the minerals and organic matter is higher with 31% for control, and around 33% for treated plants in the dry year. No significant differences were obtained concerning the effect of each metal on ash content.

KEYWORDS: heavy metals, plant water status, simulated rainfall

Introduction

Both, natural and anthropogenic processes and sources emit metals and their compounds into the air. The processing of minerals, incineration of metals, motor vehicle combustion of fuel containing metal additives result in the emission of metals associated with particulate matter. Many industries have reported the discharge into air

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of heavy metals and their compounds from power stations using fossils fuel such as coal, and from mines, mineral processing industries and refineries.

Heavy metals upon inhalation or ingestion, can be responsible for a range of health effects such as cancer, neurotoxicity, immunotoxicity, cardiotoxicity, reproductive toxicity, teratogenesis and genotoxicity (US EPA, 1999a). Plants and animals depend on some metals as nutrients, however certain forms of some metals can be toxic even at trace levels.

Metals occur in air in different phases, as solids, gases or adsorbed to particles having aerodynamic sizes ranging from below 0.01 micrometres (μm) to 100 micrometres and larger. Heavy metals such as copper (Cu), exists in both, coarse and fine fractions in ambient air (VIC EPA, 1998a). Metals such as arsenic, lead (Pb), and zinc (Zn) enrich the fine fraction of particulate matter (Finlayson-Pitts and Pitts, 1986).

Researches concerning the influence of the atmospheric pollutants such as copper, zinc and lead on the water status of plants may improve the knowledge in the evaluation of short time effects of the heavy metals, related to other environmental conditions. Plant water imbalance influence membrane permeability and enzyme activities causing a reduction of the photosynthesis and of the respiration (Báthory *et al*., 2000; Ciscato *et al*., 1997; De Vos *et al*., 1991; Gupta *et al*.,1999; Greger, 2004).

The goal of this paper was to consolidate available data on heavy metals from the ambient air on plant water status, simulating the real conditions and, to identify a physiological indicator for the evaluation of the phytotoxic effect on vegetation in order to establish new criteria for the threshold values and new practical methods to complete the Order no. 592/2002 of the Ministry of Waters and Environmental Protection.

Materials and Methods

A 2-yr field experiment was designed to study the effects of lead, copper and zinc on the water status of maize plants (cv. Turda 200).

Field experiments were conducted at Livada Research and Development Station, Northern Romania. The study took place over a 2-yr period (2006 and 2007). Sixteen experimental plots, each of 21 m^2, were installed and submitted to different treatments. All types of plots were fertilized with 300 g NPK 1:1:1 (300 kg × ha^{-1}) and 200 g NH_4NO_3 (200 kg × ha^{-1}). All the plots were surrounded by a border of 1m. Maize was sown directly into permanent raised beds at a distance of 60cm between rows and 35-40 cm between plants.

For simulating the action of heavy metals from the ambient air, solutions containing Pb, Cu and Zn were applied on the leaves at 10 days after germination in the following concentrations: 0.2, 2 and $4x10^4$ ppm Cu and Zn, and 0.32, 3.2, and 6.4 $x10^4$ ppm Pb respectively. Simulated rainfall was applied in amounts totaling 76 mm. The rainfall simulation system consisted of six TeeJet nozzles.

(Model 1/2HH-SS50WSQ; Spraying Systems Co., Wheaton, IL) that were threaded directly into the body of an electrically operated solenoid valve. Solenoids were connected directly to a water supply pipe and were controlled by a custom-built electronic timing system. Solenoids operated on a rapid cycle in which they remained open for 1.0 s and were closed for 0.7 s, resulting in an intermittent rainfall pattern that delivered rainfall at an intensity of 76 mm h^{-1}.

Environmental characteristic were recorded for the entire period of the experiment. The analysis was made at 4-6 leaves stage, and 8-10 leaves stage corresponding at 14 and 18 days after the treatment.

The tissue water content of leaves that have been air-dried was determined and calculated according to the following formula: $U_1 = \{[(A-B)] /A\} \times 100$; where U_1 is the humidity in excess, A is the fresh weight of the sample; B is the weight of the air-dried leaves. Next, the plant material was oven-dried at 60°C and the hygroscopic humidity was established. Based on these two determination, the free water content of the leaves was calculated. The dry matter content was established based on the gravimetric loss of free water associated with heating to 105°C for a period of three hours.

Ash content was determined incinerating the samples in an electric oven at 200-400°C and after that at 600°C for six hours.

For chemical analysis plant species were dried at 80°C for 24 h before grinding. Ground plant samples were treated according the following protocols.

Chemical analysis of leaves. After drying to a constant weight at 80°C, all samples were ground through a Wiley mill (Arthur H. Thomas, Philadelphia, PA) equipped with a 1-mm screen. Nitrogen content of grain was determined using the Dumas combustion method (Leco FP-428 analyzer), and crude protein (CP) was calculated by multiplying the concentration of N in the leaves by 6.25.

Leaf conductance was determined with a diffusion porometer AP4 (Delta-T, UK), and was expressed in mmol $m^{-2} \cdot s^{-1}$.

Yield and yield components. At harvest, a 0.5-m^2 (0.18 by 3 m) portion at the center of each experimental plot was sampled. From this sample, the biomass and harvest index (HI), were measured. Dry matter and grain weight were determined by drying the sampled plants at 80°C to constant weight.

Statistical analyses. Experimental design was a randomized complete block with four replications. Annual data for each parameter over the whole 2-yr period were subjected to analysis of variance (ANOVA), using a year-combined randomized complete block design according to Mc Intosh (1983). Treatment means were compared using Fisher's protected least significant difference (LSD) test at $P \leq 0.05$. The LSDs for different main effect and interaction comparisons were calculated using the appropriate standard error terms following Gomez and Gomez (1984). The Statistix v. 7.0 (Analytical Software, 2000) package was used for this purpose.

Results and discussion

Figure 1 shows monthly temperatures and rainfall at the experimental site over the 2-yr study. Differences in temperature between the two study years were relatively modest. During the first period (April–May) the mean temperature was 4.5°C in 2006 and 6.2°C in 2007. The mean maximum temperature during this period was 22°C in 2006 and 25°C in 2007.

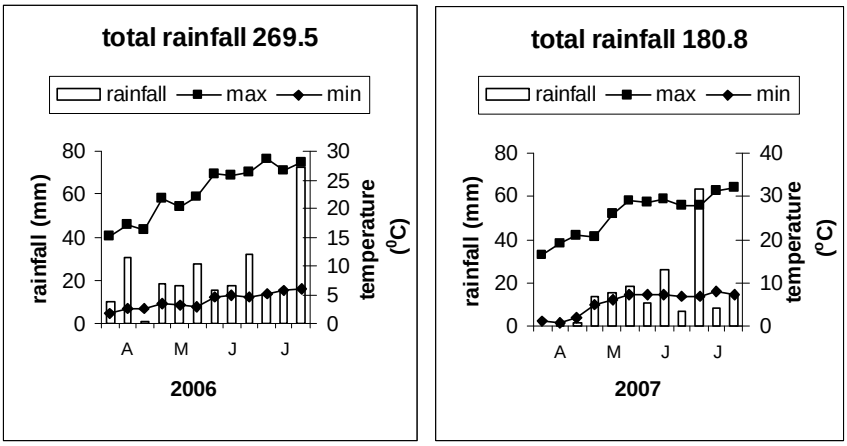

Fig. 1. Monthly (April, May, June, July), annual rainfall and mean maximum and minimum temperature for 2 yr at Livada Research and Development Station.

Rainfall varied considerably between years. Mean annual rainfall varied greatly: 2006 was the wettest year (269.5 mm), followed by 2007 (180.8mm). Mean annual rainfall for the area over the last 20 yr is 255 mm; 2006 was therefore a normal year and 2007 was a dry year (±30 mm of mean figure).

Concerning the effects of the applied treatment on the water balance of the plants it may be seen in fig. 2-4 that the heavy metals are modifying the water content of the leaves with a percentage between 0.4 to 2.3 % in the wet year and between 0.8 to 6.2% in the dry year compared to control. A possible explanation may be that in the 2006, the concentration of the heavy metals was lower due to the better hydration of the cells. Anyway, there are significant differences between control and the applied treatments. Comparing the effects among the heavy metals it may be seen that there is no a direct relationship between the water content of the leaves and the concentration of the copper, but the relationship is relevant for the concentration of zinc and of lead.

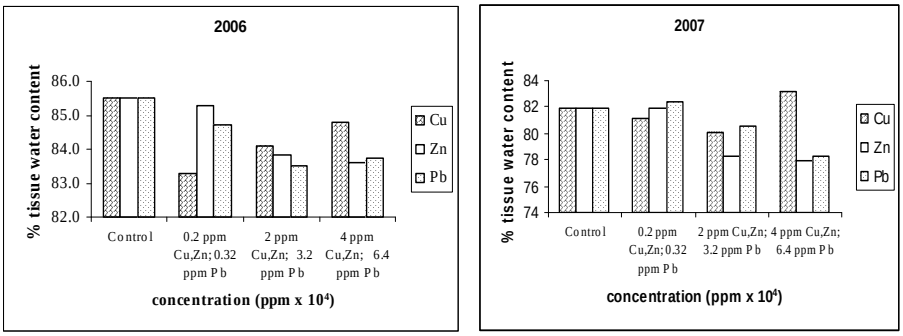

Fig. 2. Water content of maize leaves (cv. Turda 200) related to applied treatment

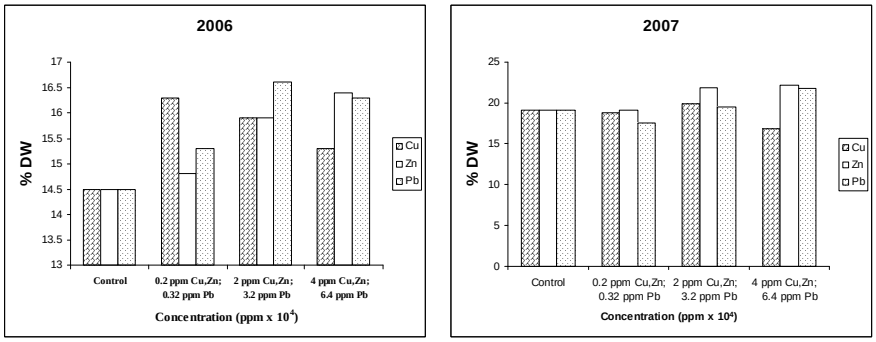

Fig. 3. Dry weight (%) content of maize leaves (cv. Turda 200) related to applied treatment

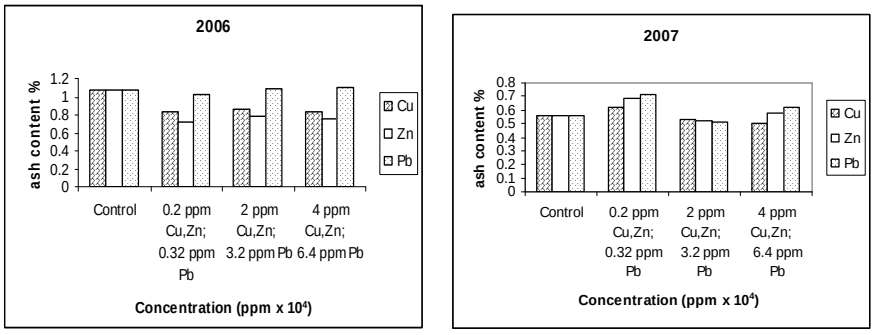

Fig. 4. Ash content (%) of maize leaves (cv. Turda 200) related to applied treatment

Concerning the dry matter content of the leaves, it can be seen in fig. 3 that the accumulation of the minerals and organic matter is higher with 31% for control, and around 33% for treated plants in the dry year. Mineral content expressed by values of ash concentration is higher in 2006 with 84% compared to 2007 for the control. No significant differences were obtained concerning the effect of each metal on this parameter.

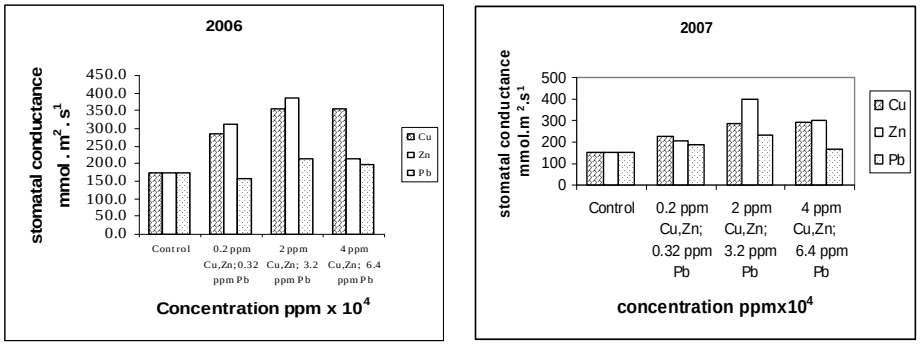

Fig. 5. Stomatal conductance ($mmol \cdot m^{-2} \cdot s^{-1}$) of maize leaves (cv. Turda 200) related to applied treatment

Although the amount of water lost by the plants under experimental conditions differed by a factor 2 to 2.5, as is shown in Fig.5, there was neither a decrease in growth (fresh weight and dry weight) nor in ash content of the plants. Contrary to the results of experiments concerning the effects of heavy metals from the soil, in this experiment stomatal conductance was higher at treated variants compared to control. Stomatal conductance, responds both to the immediate or local environment of the leaf, such as CO_2 partial pressure and irradiance, and to signals of water stress. Stomatal conductance is regulated with respect to changing soil water potential and atmospheric demand to minimize use of available water during CO_2 uptake and maintain soil-to-leaf hydraulic continuity **(**Sperry *et. al*., 2002). Zhank *et al*, in a recent study (2008), found an aquaporin cDNA *BjPIP1* isolated from heavy-metal accumulator Indian mustard (*Brassica juncea* L.) which was more abundantly expressed in roots compared to aerial parts of plant. The expression of *BjPIP1* in leaves was up-regulated by drought, salt, low temperature, and heavy metal stress, suggesting that BjPIP1 was involved in resistance to abiotic stresses. It was also suggested that BjPIP1 might enhance plant drought resistance by decreasing transpiration via reducing stomatal conductance. Furthermore, overexpression of *BjPIP1* in tobacco enhanced Cd resistance of root growth, and lowered transpiration rate and stomatal conductance upon Cd exposure, suggesting that BjPIP1 might increase heavy-metal resistance by maintaining reasonable water status in tobacco. Taken together, these results sustain the idea that BjPIP1 might improve plant heavy-metal resistance through alleviating water deficit and oxidative damage induced by metal ions. Considering these results, a higher stomatal conductance obtained in our experiments, may be explained by a lower plant resistance to metal toxicity when the source is the polluted air, and the metal enter the plant through the leaves.

Yield and Yield Components. The response of plant water status to applied treatment varied depending on the environmental factors. The highest yield occurred in 2007, the driest of 2 yr (Fig. 1), while the lowest yield occurred in 2006, the wettest

year. Total dry matter was low in 2007, and significantly higher in 2006. Harvest index values were significantly higher in the year with the lowest grain yield and biomass (2006) (Table 1).

Table 1

Yield components of the maize plants under experimental treatment

	Total dry matter t ha^{-1}	Grain yield	Harvest index kg kg^{-1}	Grain protein content g
Year				
Control				
2007	10.51b	9.41a	0.41b	138a
2006	12.81a	8.12b	0.56a	109b
Treatment				
1	11.27a	8.33b	0.55a	111b
2	11.11a	7.98b	0.51a	121b
3	9.76b	7.35b	0.42b	105b
4	10.22a	9.89a	0.40b	131a
5	10.14a	9.03a	0.39b	145a
6	9.28b	8.68b	0.40b	129a

Within year means followed by the same letter are not significantly different at P < 0.05 according to LSD

1- 0,2 x10^{4}ppm Cu and Zn and 0,32 x10^4 ppm Pb yr 2006

2- 2 x10^{4}ppm Cu and Zn and 3,2 x10^4 ppm Pb yr 2006

3- 4x10^4 ppm Cu and Zn, and 6,4 x10^4 ppm Pb yr 2006

4- 0,2 x10^{4}ppm Cu and Zn and 0,32 x10^4 ppm Pb yr 2007

5- 2 x10^{4}ppm Cu and Zn and 3,2 x10^4 ppm Pb yr 2007

6- 4x10^4 ppm Cu and Zn, and 6,4 x10^4 ppm Pb yr 2007

Under heavy metal stress maize plants showed reduced grain yield and accumulated little dry matter. At the same time, the evapotranspiration rate enhanced significantly. Their water use efficiency, an indicator to stress tolerance, increased but as the heavy metal stress intensified the water use potential also started to decline. Grain yield did not show a clear response to the applied heavy metals; some treatments exhibited no significant differences with respect to the control. Similarly, applied treatments had no clear effect on grain protein content. The behavior of biomass was similar to that of grain yield, although values were markedly lower in treatments involving metal application at high concentration. Harvest index exhibited a similar response, although the highest mean value was recorded for the control.

The lack of any clear response by maize plants to heavy metals contained in simulated rainfall reflects the plant capacity to recover along the vegetation cycle influenced by the annual variations in the amount and distribution of rainfall and the inner water balance.

Conclusions

Study of changes in the plant water status is an important component of eco-physiological analyses. It contributes significantly to evaluation of the physiological and, consequently, health status of maize plants.

The results of analyses of water status of the assimilatory tissues allow us to conclude that the heavy metals are modifying the water content of the leaves with a percentage between 0.4 to 2.3 % in a wet year and between 0.8 to 6.2% in a dry year compared to control. Comparing the effects among the heavy metals it may be seen that there is no a direct relationship between the water content of the leaves and the concentration of the copper, but the relationship is relevant for the concentration of zinc and lead. The accumulation of the minerals and organic matter is higher with 31% for control, and around 33% for treated plants in the dry year. No significant differences were obtained concerning the effect of each metal on ash content.

Our results concerning stomatal conductance suggest a lower plant resistance to metal toxicity when the source is the polluted air, and the metal enter the plant through the leaves.

Total dry matter was significantly correlated to the total amount of rainfall. The highest yield occurred in the driest of 2 yr while the lowest yield occurred in the wettest year. Harvest index values were significantly higher in the year with the lowest grain yield and biomass.

The lack of any clear response by maize plants concerning yield parameters to heavy metals contained in simulated rainfall reflects that the longterm impacts of airborne pollutants generated by different sources are strictly related to exposure frequency, and duration, the plant having the capacity to recover along the vegetation cycle.

REFERENCES

Bathory, D., Keul, M., Bercea, V. (2000) Reacții fiziologice ale plantulelor de porumb *Zea mays* L. sub influența Pb, *Contrib. botanice*, **I,** 111-121

Ciscato, M., Valke, R., Van Loven, K., Clijsters, H. (1997) Effects of in vivo treatment on the photosynthesis apparatus of *Triticum durum* two cultivars with different stress sensitivity, *Physiol.Plant.,* **100**, 901-908

De Vos, R.C.H., Schat, H., De Waal, M.A.M., Vooijs, R., Who, E. (1991) Increase resistance to cooper–induced damage of the root cell plasmalemma in cooper-tolerant Silene cucubalus, *Physiol. Plant*, **82,** 520-528

Greger, M. (2004) *Metal availability, uptake, transport and acumulation in plants,* In: Heavy metals stress on plants. From biomolecules to ecosystems, Prasad MNV (Ed), Springer Verlag, 1-27

Gupta, M., Cuypers, A., Vangronsveld, J., Clijters, H. (1999) Cooper affects the enzimes of the ascorbate-glutathione cycle and its related metabolites in the roots of *Phaseolus vulgaris*, *Physiol. Plant*., **106**, 262-267

Finlayson-Pitts, B., Pitts, J. N., (1986). *Atmospheric chemistry: fundamentals and experimental techniques.* John Wiley & Sons, New York.

Gómez, K. A., and Gómez, A. A. (1984) *Statistical procedures for agricultural research.* John Wiley & Sons, New York.

McIntosh, M.S. (1983) Analysis of combined experiments. *Agron. J*. **75**, 153–155

Sperry, J. S., Hacke, U. G., Oren, R., Comstock, J. P. (2002) Water deficits and hydraulic limits to leaf water supply. *Plant Cell Environ.,* **25:** 251–263

Zhang, Y., Wang, Z., Chai, T., Wen, Z., Zhang, H. (2008) Indian Mustard Aquaporin Improves Drought and Heavy-metal Resistance in Tobacco, *Mol Biotechnol*. **12**, 18622723

***1999a US EPA Data Collection for the Hazardous Waste Identification Rule. Office of Solid Waste, Washington DC. *(http://www.epa.gov/epaoswer/hazwaste/id/hwirwste/risk.htm)*

***1998a VIC EPA Review of data on heavy metals in ambient air in Australia, Technical Report No. 3, *Environment Australia, May 2002, ISBN 0 6425 4781 5*

*** (2000) *Analytical Software. Statistix 7.0.* Analytical Software, Tallahassee, FL

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 101-114

GLUCOCORTICOIDS- A POTENTIAL ANTI-ANGIOGENIC CANCER THERAPY

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SUMMARY. The glucocorticoids (GC) are among the most widely prescribed anti-inflammatory and immunosupressive drugs. In addition to these well-known effects, GC exert regulatory actions on tumor angiogenesis at both genomic and non-genomic level. Consequently, GC can be exploited as anti-angiogenic agents in cancer therapy.

KEYWORDS: glucocorticoids, angiogenesis, cancer, therapy

Introduction

In tumor therapy, glucocorticoids (GC) have been used for their anti-inflammatory and anti-emetic effects, in the treatment of hematological malignancies based on their efficient cytolytic activity on cells of lymphoid origin. Reports in the last two decades demonstrated that GC could also inhibit solid tumor growth in experimental tumor models. However, these pre-clinical studies further show that high and frequent dosing of GC is a prerequisite in obtaining antitumor effects. These doses resulted in considerable morbidity and mortality as a result of severe immune suppression (Schiffelers, *et al*., 2005). In mice, doses of 100 to 200 mg/kg per day need to be administered for prolonged periods of time to obtain significant tumor growth inhibition (Folkman, *et al*., 1983, Lee, *et al*., 1987, Penhaligon and Camplejohn,1985, Pucci, *et al*., 1988). Due to rapid elimination, GC can poorly accumulate at target sites while large amounts are wasted or unintendedly localize at healthy tissue sites. Therefore, an attractive strategy to increase intratumoral drug concentration and to reduce the probability of side effects of GC might be targeted delivery of GC to tumor tissue. Recently, it was reported that polyethylene glycol (PEG)-coated liposomes encapsulating prednisolone phosphate (PLP) adminstered intravenously (iv) inhibit tumor growth with 80% to 90% in two murine tumor models: subcutaneous B16.F10 melanoma and C26 colon carcinoma at a dose of 20 mg/kg (Schiffelers, *et al*., 2005). The antitumor activity of liposomal GC formulation was primarily exerted through the inhibition of tumor angiogenesis ([a]Banciu, *et al*., 2008, [b]Banciu, *et al*., 2008). Based on these studies, the goal of this review is to offer an overview of anti-angiogenic actions of GC involved in tumor growth inhibition.

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Glucocorticoid mechanisms of action

GC can exert other effects on mammalian cells including apoptotic, necrotic and anti-angiogenic effects (Banciu, *et al*., 2008). All these GC actions are mediated by genomic and non-genomic mechanisms. The genomic mechanisms are determined by the interaction of GC with their cytosolic receptors (cGCR) followed by cGCR activation and translocation into the nucleus. Once in the nucleus, GC/cGCR complexes regulate the expression of genes for many immunoregulatory and inflammatory cytokines (TNF α, GM-CSF, IL-1, IL-2, IL-3, IL-6, IL-8, IL-11), for apoptotic proteins (members of the Bcl-2 family such as Bcl-x_s, Bad, Bax, Bid, FasL) as well as for the angiogenic proteins (bFGF,VEGF, eotaxin, IL-8, MMP-1, 3, 9, TIMP 1, 2) (Burnett, *et al*., 1986, Amsterdam, *et al*., 2002, Smoak and Cidlowski,2004, Schmidt, *et al*., 2004, Stellato,2004, Yang, *et al*., 2002, Reichenstein, *et al*., 2004). The **genomic** actions of GC consist of: 1) **transcription mechanisms**, 2) **posttranscriptional and translational mechanisms**. Higher dosages increase cGCR occupation, which intensifies the GC effects at the genomic level. If cGCR are saturated, GC additionally induce non-genomic effects. **Non-genomic** actions comprise two different mechanisms: 1) **direct actions** such as binding of GC to specific membrane-bound receptors (mGCR), intercalation of GC molecules into cellular membranes, 2) **indirect actions** like GC interfering with the disposal of norepinephrine at α1-adrenoceptor sites, cGCR-mediated inhibition of arachidonic acid release (Buttgereit, *et al*., 2004, Wanner, *et al*., 2004). The responses induced by non-genomic mechanisms of GC include anti-angiogenic, immunosuppressive and anti-inflammatory effects, induction of necrosis.

In conclusion, GC can regulate production of the majority of the factors involved in all angiogenic steps. Therefore, below a condensed overview of tumor angiogenesis is given.

Tumor angiogenesis

The original hypothesis for the dependence of solid tumors on angiogenesis was presented by Folkman in 1971 (Sato,2003). Angiogenesis, the development of new blood vessels from preexisting microvasculature, is a complex process that is regulated by the balance of pro-angiogenic and anti-angiogenic factors. Imbalance of factors involved in angiogenesis is the cause of pathological angiogenesis inducing either excessive (eg, cancer) or inadequate neovascularization (eg, coronary artery disease) (Rosen,2002, Sato,2003). However, the growth of tumors beyond a size of 2 mm^3 in a body requires the additional step of angiogenesis that consists of two stages. The first stage is called ”angiogenic switch” and is caused by the activation of various oncogenes and/or mutation of tumor supressor genes in tumor cells, which collectively activates the pro-angiogenic genes (Crowther, *et al*., 2001, Sato,2003). However, upregulation of an angiogenic factor is not sufficient in itself for a tumor cell to become angiogenic,

certain negative regulators or inhibitors of vessel growth may need to be down-regulated (Gupta and Qin,2003, Sato,2003). Several studies have been shown that hypoxia is a potent stimulus for the release of pro-angiogenic factors by tumor cells *in vitro* and *in vivo* (Crowther, *et al*., 2001). These processes are regulated by hypoxia inducible factor-1 (HIF)-1 (Sato,2003). The second stage involves coupling the first stage of tumor-regulated angiogenesis to the costimulatory effects of microenvironmental stress factors present in tumor tissue and secreted by tumor cells, inflammatory cells, endothelial cells (ECs) (Crowther, *et al*., 2001, Gupta, *et al*., 1984, Albini, *et al*., 2005). Recent data shows that the inflammatory cells (macrophages, T cells, neutrophils, eosinophils, monocytes that expressed Tie-2 receptors, mast cells) infiltrating the tumor fully participate in the angiogenic process by recruitment, proliferation, migration and activation of the endothelial cells as well as by production of the majority of angiogenesis initiators (Albini, *et al*., 2005, Naldini and Carraro,2005). Consequently, inflammation-dependent angiogenesis seems to be a central force in tumor growth and expansion, a concept supported by the observation that the use of anti-inflammatory drugs leads to angiogenesis inhibition (Albini, *et al*., 2005).

Anti-angiogenic mechanisms of glucocorticoids on tumors

As it was mentioned above, GC exert these effects on many cell types including inflammatory cells, ECs. GC regulate production as well as activity of a broad variety of factors involved in angiogenesis such as, enzymes responsible for synthesis of key mediators of inflammation, enzymes for degradation of basal membranes and reorganization of extracellular matrix of blood vessels, peptide growth factors, mediators of inflammatory reaction, cell adhesion molecules (Table 1).

Table 1.

Involvement of principal pro-angiogenic factors in tumor angiogenesis steps (Gupta and Qin,2003, Lutsenko,2003, Szekanecz,1999, Cohen, *et al*., 1996, Crowther, *et al*., 2001, Jackson, *et al*., 1997, Brizzi, *et al*., 1999, Frederick and Clayman,2001, Lee, *et al*., 2000, Tonini, *et al*., 2003, Bottazzi, *et al*., 1992, Ajuebor, *et al*., 2003, Lebrecht, *et al*., 2004, Marchesi, *et al*., 2004, Salcedo, *et al*., 2001)

Pro-angiogenic factors	Tumor angiogenesis steps			
	Degradation of basement membrane	Migration of ECs into the interstitial space and sprouting	ECs proliferation	Formation of new blood vessels
VEGF	*	*	*	*
bFGF	*	*	*	*
TNF α	*	*		*
PDGF	*	*	*	*
IL-8	*	*	*	
IL-1	*	*	*	
IL-6		*		
IL-9		*		

GM-CSF		*	*	*
M-CSF		*	*	
GM-CSF		*	*	
IGF-II	*	*	*	
MMPs	*	*		
vascular adhesion molecules E-selectin, ICAM, VCAM	*	*	*	*
MCP-1		*		*
Eotaxin		*		
Thrombopoietin			*	

The symbol (*) indicates in which step of angiogenesis, pro-angiogenic factors exert their stimulatory effects on tumor growth.

GC regulation of key enzymes

The inhibitory effects of GC exerted on enzymes involved in production of pro-inflammatory mediators determine a blockage of tumor angiogenesis. The inducible nitric oxide synthase (iNOS) in the immune cells and in the tumor cells may be a target for this GC action. In rat C6 tumor glial cells, induction of iNOS produces high levels of nitric oxide (NO) that is thought to increase tumor growth and induce angiogenesis and increase vascular permeability. Dexamethasone showed a maximum inhibition of iNOS mRNA levels mainly through a posttranscriptional mechanism reaching a maximum of approximately 83% inhibition (Shinoda, *et al*., 2003). GC also inhibit iNOS expression in rat glomerular mesangial cells by reducing iNOS mRNA translation and increasing degradation of iNOS protein, in parallel with actions on gene transcription and mRNA stability (Stellato,2004).

One of the main mechanisms of GC to suppress tumor angiogenesis is the inhibition of prostaglandin E_2 (PGE_2) synthesis. PGE_2 plays a crucial role in the initiation and maintenance of tumor growth by inducing the angiogenesis and metastasis (Masferrer, *et al*., 2000). PGE_2 is produced in large amounts in tumors from arachidonic acid. GC inhibit the key enzyme involved in arachidonic acid release, cytosolic phospholipase A_2 ($cPLA_2$). In the A549 human adenocarcinoma cell line, dexamethasone inhibited epidermal growth factor (EGF)-stimulated $cPLA_2$ activation and arachidonic acid release by blocking the recruitment of Grb2 (adapter protein) to the activated EGF receptor through a cGCR-dependent (RU486-sensitive), transcription-independent (actinomycin-insensitive) mechanism. This effect is a result of dexamethasone-induced phosphorylation status of a N-terminal domain sequence of Grb2 and subcellular localization of lipocortin 1 (LC1) that displaces Grb2 from growth factor receptor signalling complexes and thereby blocks the transducing signal leading to activation of c-Jun N-terminal kinase (JNK)1, mitogen-activated protein kinases (MAPK) and $cPLA_2$ (Croxtall, *et al*., 2000, Alldridge, *et al*., 1999, McLeod and Bolton,1995). This GC action is mediated via upregulation of genes encoding LC1

possibly through glucocorticoid response elements (GREs) of DNA as well as a rapid release of co-chaperone Src from the protein complex after cGCR activation. Src is reponsible by subsequent activation of LC1 and rapid inhibition of arachidonic acid release (McLeod and Bolton,1995, Smoak and Cidlowski,2004, DeFranco,2002). Moreover, certain studies demonstrated that dexamethasone might induce production of PLA_2-inhibitory proteins *in vitro* in calf thymus, responsible for teratogenicity of GC (Gupta, *et al*., 1984).

One of the key enzyme responsible for PGE_2 production is cyclooxygenase 2 (COX-2). This enzyme is induced in infammatory cells and in human tumor cells by cytokines and tumor promoters such as FGF-2. In two tumor models, Lewis lung carcinoma-bearing mice and HT -29 human colon tumor in nude mice, expression of COX-2 was observed in newly tumor blood vessels, whereas under normal physiological conditions the quiescent vasculature expressed only the form of constitutive enzyme COX-1. Acceleration of COX-2 mRNA decay by GC involves primary loss of polyadenylated mRNA. GC might induce the activation of recruitment of RNAse complexes such as exosomes that is critical in regulating the efficiency of adenylate/uridylate-rich elements (ARE)-dependent mRNA turnover (Stellato,2004). Croxtall et al demonstrated three different types of GC mechanisms involved in release of the PGE_2 formation in A549 human adenocarcinoma cells (Croxtall, *et al*., 2002). Firstly, the members of group A comprise the new generation of GCs, mometasone, fluticasone and budesonide and also beclomethasone diproprionate and prednisolone, that do not have significant efect on arachidonic acid release and $cPLA_2$ activity. However, they have a profound inhibitory activity on COX-2 expression via their inhibitory effects on nuclear factor κB (NF-κB). Secondly, group B, comprises methylprednisolone which, in contrast to group A, inhibits only $cPLA_2$ activity without considerably inhibiting COX-2 expression. The mechanisms of these GC are related to inhibition of $cPLA_2$ through a non-genomic mechanism. Finally, group C includes beclomethasone, dexamethasone, hydrocortisone and triamcinolone acetonide which inhibit both cPLA2 activity and COX-2 expression. However, even within this group beclomethasone and dexamethasone express a differential effect upon $cPLA_2$ activity and COX-2 expression that appears to be concentration-dependent. Even in group C where both $cPLA_2$ activity and COX-2 expression are inhibited, these pathways are still mechanistically distinct. These observations appear to support the notion that the genomic versus non-genomic actions of GCs are not simply concentration-dependent, but they are also mediated via distinct cellular pathways following cGCR activation (Croxtall, *et al*., 2002, Buttgereit, *et al*., 1998).

GC also downregulate expression of certain enzymes directly involved in angiogenesis such as, metalloproteinases (MMPs)-1, 3, 9 (Yang, *et al*., 2002). These proteins are reponsible for degradation of basal membranes and reorganization of extracellular matrix of blood vessels. Excepting the GC, however, none of the several compounds now available to reduce MMP transcription are in clinical use. Inhibition of MMPs transcription exerted by cGCR occurs through an indirect mechanism via

conformational change of activator protein 1(AP-1) and physical interaction between cGCR and NF-κB, respectively. In the case of MMP-9, another transcription factor, Ets-1, in combination with c-Jun, are targets for the negative regulation of MMP-9 expression by dexamethasone in mesangial cells (Eberhardt, *et al*., 2002, Bondeson, *et al*., 1999, Vincenti and Brinckerhoff,2002). Second, GC enhance synthesis of the inhibitor of NF- κBα (IκBα), resulting in sequestration of NF-κB in the cytoplasm. The overexpression of IκBα reduces expression of inflammatory cytokines (IL-1, IL-6 and TNFα) and MMP-1 and MMP-13, but does not reduce anti-inflammatory cytokines or tissue inhibitor of metalloproteinases (TIMP) (Vincenti and Brinckerhoff,2002, (Eberhardt, *et al*., 2002, Bondeson, *et al*., 1999).

GC regulation of production of pro-angiogenic/ pro-inflammatory factors

One of the principal inhibitory mechanisms of GC on tumor angiogenesis is reduction of pro-angiogenic factors such as growth factors and mediators of inflammation. Inhibition of VEGF and bFGF expression through the reduction of PGE_2 level might be obtained after GC treatment (Luo, *et al*., 2004, Sakai, *et al*., 2001). For some pro-angiogenic cytokines such as IL-1, 2, 3, 4, 6, 8, GM-CSF, chemokines such as, monocyte chemoattractant proteins (MCP-1, 3, 4), eotaxin, GC supress their transcription by inhibiting trancription factors (Barnes,1998). GC can also exert inhibitory effects on these pro-angiogenic factors via inhibition of HIF-1α mRNA stabilization. There are some reports on dexamethasone inhibition of HIF-1 mRNA stabilization in CD4+ T cells (Gaber, *et al*., 2004, Gaber, *et al*., 2005).

It is known that in neoplastic tissues, such as non-small cell lung cancer, IL-8 is correlated with the extent of neovascularization, tumor progression and survival (Gupta and Qin,2003). Inhibition of IL-8 exerted by GC can occur at either the transcriptional or posttranscriptional level. It was found a transcriptional mechanism of dexamethasone in the inhibition of IL-1-induced IL-8 gene supression in human glioblastoma cell lines through inteference with NF-κB to recognize GREs (Mukaida, *et al*., 1994). Other studies focused on the effects of dexamethasone on basal level IL-8 gene expression in cultured airway epithelial cells, observed a decrease of the protein synthesis due to a rapid degradation of IL-8 mRNA rather than due to a supression of transcription (Chang, *et al*., 2001).

GC also regulate genes methylation. In mouse lung cancer induced by benzo[a]pyrene, budesonide determined methylation of gene encoding IGF-II and c-*myc* gene leading to decrease in mRNA levels of these genes (Tao, *et al*., 2002, Lubet, *et al*., 2004). IGF-II may play a crucial role in the progression of tumorigenesis by promoting angiogenesis (Lee, *et al*., 2000, Toretsky and Helman,1996). *C-myc* gene is associated with cell proliferation and apoptosis and is overexpressed in lung tumors. In normal tissues, approximately 5% of the cytosine in DNA is methylated as 5-methylcytosine, whereas in tumors the level of methylation usually is decreased by 30% or more. In contrast with this global DNA methylation, in tumors there is a hypermethylation of tumor-supressor genes (Tao, *et al*., 2002). GC inhibit expression of these factors through a mechanism of their genes silencing.

Other inhibitory effects of GC on mRNA turnover and translation are known for a substantial number of proteins involved in inflammation and angiogensis such as IL-1α, IL-6, IL-8, IFN-β, FGF 2 and GM-CSF (Stellato,2004). cGCR α enhance transcription of specific ribonucleases that break down mRNA containing constitutive AREs in the untranslated 3'-region, thus shortening the turnover time of mRNA (Barnes,1998, Smoak and Cidlowski,2004). A similar mechanism is responsible for inhibition of GM-CSF synthesis. GM-CSF plays a key role in survival of inflammatory cells, in stimulation of pro-angiogenic protein production such as TNF α, IL-1, IL-8, TGF-α, IL-12p40, proliferation, migration and differentiation of vascular endothelial cells (Broudy, *et al*., 1987, Jackson, *et al*., 1997). In particular, GC can regulate the mRNA turnover of eotaxin, that is a strong and selective chemoattractant for eosinophils, T cells and basophils and an activator of eosinophils to produce TGF-α and β (Frederick and Clayman,2001, Salcedo, *et al*., 2001, Stellato,2004). This protein is extremely suppressed by GC partially by transcriptional regulation as well as by posttranscriptional regulation (Stellato,2004). GC also increases mRNA turnover of the JNK-dependent VEGF gene in keratinocytes. A number of genes are posttranscriptionally repressed by dexamethasone, such as IL-1, VEGF, TNF α, IL-6, IL-8, COX-2 through GC inhibition of p38/SAPK pathway (Stellato,2004).

Inhibitory effects mediated by cGCR on AP-1 in lymphoid cells is through glucocorticoid-induced leucine zipper (GILZ) gene. GILZ contains a leucine zipper domain that confers the possibility to dimerize with the leucine zipper containing AP-1 constituents c-Fos and c-Jun, leading to AP-1 activity inhibition. GILZ is involved in inhibition of expression of Fas ligand (FasL) by inhibition of activation-induced upregulation of the FasL promotor as well as two transcription factors involved in FasL regulation, Egr-2 and Egr-3 in T cells (Mittelstadt and Ashwell, 2001, Mitsiades, *et al*., 1999).

The expression of cell adhesion molecules that play an important role in mediating tumor cell adhesion to vascular endothelial cells and promoting the metastatic process are repressed by GC (Ding, *et al*., 2003). These effects are exerted via inhibition of IL-1β and TNF α production or a direct GC inhibitory effect on the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin at the level of gene transcription (Barnes,1998, Rioja, *et al*., 2004, Bratt and Heimburger,1999). The inhibitory effects of GC on NF- κB are exerted on transcription of vascular cell adhesion molecule 1 (VCAM-1) (Simoncini, *et al*., 2000, Ding, *et al*., 2003).

GC regulation of anti-angiogenic/ anti-inflammatory factor production

Interestingly, the expression of certain anti-angiogenic factors is not or only slightly affected after GC administration. Moreover, production of some anti-angiogenic proteins such as TIMP-1 is associated with attenuated tumor growth, reduced metastasis and suppression of angiogenesis is increased after GC treatment.

TIMP-1 mRNA increases strongly after ACTH and dexamethasone treatment of adrenal cortex cells (Reichenstein *et al*., 2004). Similar studies indicate an increase of sputum TIMP concentrations after oral prednisolone, 40 mg daily at patients with chronic bronchitis (Burnett, *et al*., 1986).

Several studies have reported that IL-12p70 is an anti-angiogenic/anti-inflammatory cytokine that is mainly produced by macrophages and neutrophils (Kalinski, *et al*., 2001, Latsi, *et al*., 2003, Ethuin, *et al*., 2003). IL-12p70 suppresses the expression of VEGF, bFGF and MMP-9 mRNAs. Additionally, IL-12p70 was found to stimulate mRNA expression of IFN-γ and its inducible anti-angiogenic chemokine IP-10 in ECs cultured with IL-12. IL-12p70 significantly promotes apoptosis and inhibits proliferation rate of human tumors and extensive necrosis in the murine, thereby reducing tumor vessel density (Gupta and Qin,2003). GC can stimulate indirectly production of IL12p70 by inhibition of PGE_2 release in monocite-derived dendritic cells (Vassiliou, *et al*., 2004, van der Pouw Kraan, *et al*., 1995). Banciu *et al.* (2006) also demonstrated that prednisolone phosphate incorporated in long-circulating liposomes did not affected production of IL-12p70 in B16F10 tumor –bearing mice (Banciu, *et al*., 2006). Several studies have shown an increase of IL-12p70 in peripheral blood monocytes after hydrocortisone administration in septic shock (Keh, *et al*., 2003). IL-10, a anti-inflammatory cytokine downregulates a number of macrophage functions, including the production of IL-1α, IL-6, TNF α. After dexamethasone pulse therapy, an increase of IL-10 mRNA expression was observed (Verhoef, *et al*., 1999).

Perspectives

The present article deals with GC actions on tumor angiogenesis. Complexity of GC actions at both genomic and non-genomic level offers promising anti-angiogenic agents in cancer therapy. Therefore one of the future issues in pharmaceutical research might be to identify GC types which show the strongest anti-angiogenic activity as well as to design optimal GC delivery systems to ensure a high antitumor activity without the occurrence of adverse effects.

REFERENCES

Ajuebor, M. N., Hogaboam, C. M., Le, T., Swain, M. G. (2003). C-C chemokine ligand 2/monocyte chemoattractant protein-1 directly inhibits NKT cell IL-4 production and is hepatoprotective in T cell-mediated hepatitis in the mouse. *J Immunol* **170**: 5252-5259.

Albini, A., Tosetti, F., Benelli, R., Noonan, D. M. (2005). Tumor inflammatory angiogenesis and its chemoprevention. *Cancer Res* **65**: 10637-10641.

Alldridge, L. C., Harris, H. J., Plevin, R., Hannon, R., Bryant, C. E. (1999). The annexin protein lipocortin 1 regulates the MAPK/ERK pathway. *J Biol Chem* **274**: 37620-37628.

Amsterdam, A., Tajima, K., Sasson, R. (2002). Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. *Biochem Pharmacol* **64**: 843-850.

Banciu, M., Metselaar, J. M., Schiffelers, R. M., Storm, G. (2008). Antitumor activity of liposomal prednisolone phosphate depends on the presence of functional tumor-associated macrophages in tumor tissue. *Neoplasia* **10**: 108-117.

Banciu, M., Schiffelers, R. M., Fens, M. H., Metselaar, J. M., Storm, G. (2006). Anti-angiogenic effects of liposomal prednisolone phosphate on B16 melanoma in mice. *J Control Release* **113**: 1-8.

Banciu, M., Schiffelers, R. M., Metselaar, J. M., Storm, G. (2008). Utility of targeted glucocorticoids in cancer therapy. *J Liposome Res* **18**: 47-57.

Barnes, P. J. (1998). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci (Lond)* **94**: 557-572.

Bondeson, J., Foxwell, B., Brennan, F., Feldmann, M. (1999). Defining therapeutic targets by using adenovirus: blocking NF-kappaB inhibits both inflammatory and destructive mechanisms in rheumatoid synovium but spares anti-inflammatory mediators. *Proc Natl Acad Sci U S A* **96**: 5668-5673.

Bottazzi, B., Walter, S., Govoni, D., Colotta, F., Mantovani, A. (1992). Monocyte chemotactic cytokine gene transfer modulates macrophage infiltration, growth, susceptibility to IL-2 therapy of a murine melanoma. *J Immunol* **148**: 1280-1285.

Bratt, J., Heimburger, M. (1999). Prednisolone interferes with neutrophil adhesion and neutrophil mediated endothelial injury. *Scand J Rheumatol* **28**: 308-313.

Brizzi, M. F., Battaglia, E., Montrucchio, G., Dentelli, P., Del Sorbo, L., Garbarino, G., Pegoraro, L., Camussi, G. (1999). Thrombopoietin stimulates endothelial cell motility and neoangiogenesis by a platelet-activating factor-dependent mechanism. *Circ Res* **84**: 785-796.

Broudy, V. C., Kaushansky, K., Harlan, J. M., Adamson, J. W. (1987). Interleukin 1 stimulates human endothelial cells to produce granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor. *J Immunol* **139**: 464-468.

Burnett, D., Reynolds, J. J., Ward, R. V., Afford, S. C., Stockley, R. A. (1986). Tissue inhibitor of metalloproteinases and collagenase inhibitory activity in lung secretions from patients with chronic obstructive bronchitis: effect of corticosteroid treatment. *Thorax* **41**: 740-745.

Buttgereit, F., Straub, R. H., Wehling, M., Burmester, G. R. (2004). Glucocorticoids in the treatment of rheumatic diseases: an update on the mechanisms of action. *Arthritis Rheum* **50**: 3408-3417.

Buttgereit, F., Wehling, M., Burmester, G. R. (1998). A new hypothesis of modular glucocorticoid actions: steroid treatment of rheumatic diseases revisited. *Arthritis Rheum* **41**: 761-767.

Chang, M. M., Juarez, M., Hyde, D. M., Wu, R. (2001). Mechanism of dexamethasone-mediated interleukin-8 gene suppression in cultured airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **280**: L107-115.

Cohen, T., Nahari, D., Cerem, L. W., Neufeld, G., Levi, B. Z. (1996). Interleukin 6 induces the expression of vascular endothelial growth factor. *J Biol Chem* **271**: 736-741.

Crowther, M., Brown, N. J., Bishop, E. T., Lewis, C. E. (2001). Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. *J Leukoc Biol* **70**: 478-490.

Croxtall, J. D., Choudhury, Q., Flower, R. J. (2000). Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism. *Br J Pharmacol* **130**: 289-298.

Croxtall, J. D., van Hal, P. T., Choudhury, Q., Gilroy, D. W., Flower, R. J. (2002). Different glucocorticoids vary in their genomic and non-genomic mechanism of action in A549 cells. *Br J Pharmacol* **135**: 511-519.

DeFranco, D. B. (2002). Navigating steroid hormone receptors through the nuclear compartment. *Mol Endocrinol* **16**: 1449-1455.

Ding, Y. B., Chen, G. Y., Xia, J. G., Zang, X. W., Yang, H. Y., Yang, L. (2003). Association of VCAM-1 overexpression with oncogenesis, tumor angiogenesis and metastasis of gastric carcinoma. *World J Gastroenterol* **9**: 1409-1414.

Eberhardt, W., Schulze, M., Engels, C., Klasmeier, E., Pfeilschifter, J. (2002). Glucocorticoid-mediated suppression of cytokine-induced matrix metalloproteinase-9 expression in rat mesangial cells: involvement of nuclear factor-kappaB and Ets transcription factors. *Mol Endocrinol* **16**: 1752-1766.

Ethuin, F., Delarche, C., Gougerot-Pocidalo, M. A., Eurin, B., Jacob, L., Chollet-Martin, S. (2003). Regulation of interleukin 12 p40 and p70 production by blood and alveolar phagocytes during severe sepsis. *Lab Invest* **83**: 1353-1360.

Folkman, J., Langer, R., Linhardt, R. J., Haudenschild, C., Taylor, S. (1983). Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. *Science* **221**: 719-725.

Frederick, M. J., Clayman, G. L. (2001). Chemokines in cancer. *Expert Rev Mol Med* **2001**: 1-18.

Gaber, T., Dziurla, R., Tripmacher, R., Burmester, G. R., Buttgereit, F. (2005). Hypoxia inducible factor (HIF) in rheumatology: low O2! See what HIF can do! *Ann Rheum Dis* **64**: 971-980.

Gaber, T., Spies, C. M., Jakstadt, M., Mayer, K., Tripmacher, R., Dziurla, R., Naumann, L., Lauster, R., Buttgereit, F. (2004). Impact of Glucocorticoids on HIF-1a expression in human CD4+T lymphocytes.

Gupta, C., Katsumata, M., Goldman, A. S., Herold, R., Piddington, R. (1984). Glucocorticoid-induced phospholipase A2-inhibitory proteins mediate glucocorticoid teratogenicity in vitro. *Proc Natl Acad Sci U S A* **81**: 1140-1143.

Gupta, M. K., Qin, R. Y. (2003). Mechanism and its regulation of tumor-induced angiogenesis. *World J Gastroenterol* **9**: 1144-1155.

Jackson, J. R., Seed, M. P., Kircher, C. H., Willoughby, D. A., Winkler, J. D. (1997). The codependence of angiogenesis and chronic inflammation. *Faseb J* **11**: 457-465.

Kalinski, P., Vieira, P. L., Schuitemaker, J. H., de Jong, E. C., Kapsenberg, M. L. (2001). Prostaglandin E(2) is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer. *Blood* **97**: 3466-3469.

Keh, D., Boehnke, T., Weber-Cartens, S., Schulz, C., Ahlers, O., Bercker, S., Volk, H. D., Doecke, W. D., Falke, K. J., Gerlach, H. (2003). Immunologic and hemodynamic effects of "low-dose" hydrocortisone in septic shock: a double-blind, randomized, placebo-controlled, crossover study. *Am J Respir Crit Care Med* **167**: 512-520.

Latsi, P., Pantelidis, P., Vassilakis, D., Sato, H., Welsh, K. I., du Bois, R. M. (2003). Analysis of IL-12 p40 subunit gene and IFN-gamma G5644A polymorphisms in Idiopathic Pulmonary Fibrosis. *Respir Res* **4**: 6.

Lebrecht, A., Grimm, C., Lantzsch, T., Ludwig, E., Hefler, L., Ulbrich, E., Koelbl, H. (2004). Monocyte chemoattractant protein-1 serum levels in patients with breast cancer. *Tumour Biol* **25**: 14-17.

Lee, K., Erturk, E., Mayer, R., Cockett, A. T. (1987). Efficacy of antitumor chemotherapy in C3H mice enhanced by the antiangiogenesis steroid, cortisone acetate. *Cancer Res* **47**: 5021-5024.

Lee, O. H., Bae, S. K., Bae, M. H., Lee, Y. M., Moon, E. J., Cha, H. J., Kwon, Y. G., Kim, K. W. (2000). Identification of angiogenic properties of insulin-like growth factor II in in vitro angiogenesis models. *Br J Cancer* **82**: 385-391.

Lubet, R., Tao, L., Wang, W., Kramer, P., Perreira, M. (2004). Effects of budesonide on overall 5-methylcytosine levels and specific methylation and messenger RNA expression of the insulin-like growth factor II gene in mouse lung tumors. *Chest* **125**: 157S.

Luo, J. C., Shin, V. Y., Liu, E. S., Ye, Y. N., Wu, W. K., So, W. H., Chang, F. Y., Cho, C. H. (2004). Dexamethasone delays ulcer healing by inhibition of angiogenesis in rat stomachs. *Eur J Pharmacol* **485**: 275-281.

Lutsenko, S., Kiselev SM, Severin SE. (2003). Molecular mechanisms of tumor angiogenesis. *Biochemistry* **68**: 349-365.

Marchesi, F., Monti, P., Leone, B. E., Zerbi, A., Vecchi, A., Piemonti, L., Mantovani, A., Allavena, P. (2004). Increased survival, proliferation, migration in metastatic human pancreatic tumor cells expressing functional CXCR4. *Cancer Res* **64**: 8420-8427.

Masferrer, J. L., Leahy, K. M., Koki, A. T., Zweifel, B. S., Settle, S. L., Woerner, B. M., Edwards, D. A., Flickinger, A. G., Moore, R. J., Seibert, K. (2000). Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. *Cancer Res* **60**: 1306-1311.

McLeod, J. D., Bolton, C. (1995). Dexamethasone induces an increase in intracellular and membrane-associated lipocortin-1 (annexin-1) in rat astrocyte primary cultures. *Cell Mol Neurobiol* **15**: 193-205.

Mitsiades, N., Poulaki, V., Mastorakos, G., Tseleni-Balafouta, S. T., Kotoula, V., Koutras, D. A., Tsokos, M. (1999). Fas ligand expression in thyroid carcinomas: a potential mechanism of immune evasion. *J Clin Endocrinol Metab* **84**: 2924-2932.

Mittelstadt, P. R., Ashwell, J. D. (2001). Inhibition of AP-1 by the glucocorticoid-inducible protein GILZ. *J Biol Chem* **276**: 29603-29610.

Mukaida, N., Morita, M., Ishikawa, Y., Rice, N., Okamoto, S., Kasahara, T., Matsushima, K. (1994). Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression. *J Biol Chem* **269**: 13289-13295.

Naldini, A., Carraro, F. (2005). Role of inflammatory mediators in angiogenesis. *Curr Drug Targets Inflamm Allergy* **4**: 3-8.

Penhaligon, M., Camplejohn, R. S. (1985). Combination heparin plus cortisone treatment of two transplanted tumors in C3H/He mice. *J Natl Cancer Inst* **74**: 869-873.

Pucci, M., Lotti, T., Tuci, F., Brunetti, L., Rindi, L., Fibbi, G., Pasquali, F., Chiarugi, V. P. (1988). Modulation of growth of melanoma. *Int J Dermatol* **27**: 167-169.

Reichenstein, M., Reich, R., LeHoux, J. G., Hanukoglu, I. (2004). ACTH induces TIMP-1 expression and inhibits collagenase in adrenal cortex cells. *Mol Cell Endocrinol* **215**: 109-114.

Rioja, I., Bush, K. A., Buckton, J. B., Dickson, M. C., Life, P. F. (2004). Joint cytokine quantification in two rodent arthritis models: kinetics of expression, correlation of mRNA and protein levels and response to prednisolone treatment. *Clin Exp Immunol* **137**: 65-73.

Rosen, L. S. (2002). Clinical experience with angiogenesis signaling inhibitors: focus on vascular endothelial growth factor (VEGF) blockers. *Cancer Control* **9**: 36-44.

Sakai, Y., Fujita, K., Sakai, H., Mizuno, K. (2001). Prostaglandin E2 regulates the expression of basic fibroblast growth factor messenger RNA in normal human fibroblasts. *Kobe J Med Sci* **47**: 35-45.

Salcedo, R., Young, H. A., Ponce, M. L., Ward, J. M., Kleinman, H. K., Murphy, W. J., Oppenheim, J. J. (2001). Eotaxin (CCL11) induces in vivo angiogenic responses by human CCR3+ endothelial cells. *J Immunol* **166**: 7571-7578.

Sato, Y. (2003). Molecular diagnosis of tumor angiogenesis and anti-angiogenic cancer therapy. *Int J Clin Oncol* **8**: 200-206.

Schiffelers, R. M., Metselaar, J. M., Fens, M. H., Janssen, A. P., Molema, G., Storm, G. (2005). Liposome-encapsulated prednisolone phosphate inhibits growth of established tumors in mice. *Neoplasia* **7**: 118-127.

Schmidt, S., Rainer, J., Ploner, C., Presul, E., Riml, S., Kofler, R. (2004). Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. *Cell Death Differ* **11 Suppl 1**: S45-55.

Shinoda, J., McLaughlin, K. E., Bell, H. S., Swaroop, G. R., Yamaguchi, S., Holmes, M. C., Whittle, I. R. (2003). Molecular mechanisms underlying dexamethasone inhibition of iNOS expression and activity in C6 glioma cells. *Glia* **42**: 68-76.

Simoncini, T., Maffei, S., Basta, G., Barsacchi, G., Genazzani, A. R., Liao, J. K., De Caterina, R. (2000). Estrogens and glucocorticoids inhibit endothelial vascular cell adhesion molecule-1 expression by different transcriptional mechanisms. *Circ Res* **87**: 19-25.

Smoak, K. A., Cidlowski, J. A. (2004). Mechanisms of glucocorticoid receptor signaling during inflammation. *Mech Ageing Dev* **125**: 697-706.

Stellato, C. (2004). Post-transcriptional and nongenomic effects of glucocorticoids. *Proc Am Thorac Soc* **1**: 255-263.

Szekanecz, Z., Halloran,MM, Haskell,CJ, Shah,MR, Polverini,PJ, Koch,AE. (1999). Mediators of Angiogenesis: The Role of Cellular Adhesion Molecules. *Trends in Glycoscience and Glycotechnology* **11**: 73-93.

Tao, L., Li, Y., Wang, W., Kramer, P. M., Gunning, W. T., Lubet, R. A., Steele, V. E., Pereira, M. A. (2002). Effect of budesonide on the methylation and mRNA expression of the insulin-like growth factor 2 and c-myc genes in mouse lung tumors. *Mol Carcinog* **35**: 93-102.

Tonini, T., Rossi, F., Claudio, P. P. (2003). Molecular basis of angiogenesis and cancer. *Oncogene* **22**: 6549-6556.

Toretsky, J. A., Helman, L. J. (1996). Involvement of IGF-II in human cancer. *J Endocrinol* **149**: 367-372.

van der Pouw Kraan, T. C., Boeije, L. C., Smeenk, R. J., Wijdenes, J., Aarden, L. A. (1995). Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. *J Exp Med* **181**: 775-779.

Vassiliou, E., Sharma, V., Jing, H., Sheibanie, F., Ganea, D. (2004). Prostaglandin E2 promotes the survival of bone marrow-derived dendritic cells. *J Immunol* **173**: 6955-6964.

Verhoef, C. M., van Roon, J. A., Vianen, M. E., Lafeber, F. P., Bijlsma, J. W. (1999). The immune suppressive effect of dexamethasone in rheumatoid arthritis is accompanied by upregulation of interleukin 10 and by differential changes in interferon gamma and interleukin 4 production. *Ann Rheum Dis* **58**: 49-54.

Vincenti, M. P., Brinckerhoff, C. E. (2002). Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. *Arthritis Res* **4**: 157-164.

Wanner, A., Horvath, G., Brieva, J. L., Kumar, S. D., Mendes, E. S. (2004). Nongenomic actions of glucocorticosteroids on the airway vasculature in asthma. *Proc Am Thorac Soc* **1**: 235-238.

Yang, E. V., Bane, C. M., MacCallum, R. C., Kiecolt-Glaser, J. K., Malarkey, W. B., Glaser, R. (2002). Stress-related modulation of matrix metalloproteinase expression. *J Neuroimmunol* **133**: 144-150.

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 115-127

PHYSIOLOGICAL STUDIES ON THE NITROGEN FIXING BACTERIA ISOLATED FROM MOUNTAINOUS SOILS

RAHELA CARPA[1], MIHAIL DRĂGAN-BULARDA[1]

SUMMARY. Studies were carried out upon some nitrogen fixing bacteria strains isolated from five altitudinal vegetation zones of Parâng Mountains. These physiological analyses consisted of studying the influence of four different temperatures on the growth and development of *Azotobacter* strains isolated from mountainous soils. These strains were cultivated on mannitol medium and on sucrose medium at pH 7 and continous shaking to 150 rpm. The nitrogen fixing capacity of the strains originating from Parâng at the chosen temperatures was determined indirectly. Through these indirect methods the products resulted from nitrogen fixation were measured, namely extracellular proteins (by Lowry modified method) and produced ammonia secretion in culture medium (by Nessler method).

KEYWORDS: *Azotobacter*, physiological analysis, nitrogen fixation

Introduction

In the natural systems, nitrogen for plant growth comes from the soil, from rainfall or other atmospheric deposition or from other biological nitrogen fixation. Among these, biological nitrogen fixation is reported to have the largest contribution. While much of this is through symbiotic N_2 fixation, nonsymbiotic and associative fixation are of some signifiance in crops and in specific ecosystems where nitrogen for plant growth is a limiting factor (Burris and Roberts, 1993). Nitrogen is an essential nutrient for all life on earth. Among the nitrogen cycle, biological nitrogen fixation takes the role of biological conversion of atmospheric dinitrogen to forms available for plant and microbial growth by a variety of prokaryotic microbes (Rekosz-Burlaga and Garbolinska, 2006).

Diazotrophs while fixing nitrogen with the aid of their enzyme complex nitrogenase are affected by several factors such as acidity or alkalinity, oxygen, temperature, some inorganic salts, source of energy and fixed nitrogen.

It is well known that the temperature at which microbial cells grow has a considerable influence on the physiological conditions of the microbes, resulting in the alteration of the growth rate and the degradation activity upon organic substances (Yamano and Takahashi, 1982). *Azotobacter sp*. can fix nitrogen and

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it's known that are mesophilic microorganism with growth temperature from 25-32 ºC (Mishustin and Shilnikova, 1969; Santek and Maric, 1995). Since the significance of biological nitrogen fixation have been recognized people tried to find ways to prove that the organism in concern can fix nitrogen. There are both direct and indirect methods for measuring the fixed nitrogen.

Material and Methods

Growth studies in liquid culture. The strains used in this study were obtained from soils of Parâng Mountains. The strains were cultivated on different carbon sources media: on mannitol medium (modified Burk) and on sucrose medium (Atlas, 2004). The culture media were ajusted to the growth optimal pH (7-7.5) of the *Azotobacter* strains (Garrity *et al*., 2004,). In this study besides the influence of the carbon sources the different temperature was also tested (25 ºC, 30 ºC, 35 ºC and 40 ºC). Optical density was measured spectrophotometrically at 600 nm

Methods of nitrogen fixing quantification. In this study nitrogen fixation capacity of diazotrophic strains was quantified indirectly by measuring the products of nitrogen fixation activity; extracellular protein and ammonia concentrations.

Extracellular protein concentration was measured with modified Lowry method (Hartree, 1972). The samples taken from growth medium were centrifuged at 15000 rpm for 15 min at 0 ºC and the supernatant was analyzed with Lowry method. Absorbance of the samples was read at 650 nm and the blank as the uninoculated medium itself, since the salts contained in the medium interact with the reagents causing overestimation.

The ammonia concentration was measured colorimetrically with the Nessler's reagent (Nesslerization method) (Burris and Wilson, 1972). Absorbance of the samples was measured at 400 nm against the blank solution prepared as the uninoculated initial medium, since the salts contained in the medium interact with the reagent causing overestimation.

Statistical analysis. The ANOVA test (analysis of variance) was introduced by the statistician R. A. Fischer and has the role to verify how much the real values of a characteristic deviate from the teoretic values, calculated as means or regression equations, as well as how much these variations depend or not on the grouping factor (Snedecor and Cochram 1978). The statistical calculation was done with the free version One-Way ANOVA GraphPad. A value of $p<0.05$ was considered statistically significant. One-Way ANOVA test was used for a general comparison of different samples.

Results and Discussions

In these growth experiments the maximum cell density was obtained at 35 ºC after 30-34 hours of incubation. The samples grown at 30 ºC and 40 ºC presented similar growth profiles. There were differences in the moment when the maximum was attained, after 24-34 hours of incubation respectively after 12-24 hours of incubation. Those grown at 25 ºC presented a very slow but steady grows.

It is visible that for the strains coming from the alpine zone the greatest growth was registered at 35° C on both culture media (Fig. 1). An explanation could be the greater number of cysts present in the soil from these altitude, cysts which were more intensely brought to activity at this high temperature. Another explanation would be that the very large temperature variations from the mountains determined the selection of strains with a larger temperature optimum. Another surprising result is that the maximum value of optical density in the case of mannitol is higher then the one from sucrose medium. This maximum is however registered at the sample grown at 35 ºC and after only 34 hours of incubation. This shows that the preference of *Azotobacter* for the carbon source is relative, the bacteria living in a less hospitable environment like this having to use efficiently any source of nutrition.

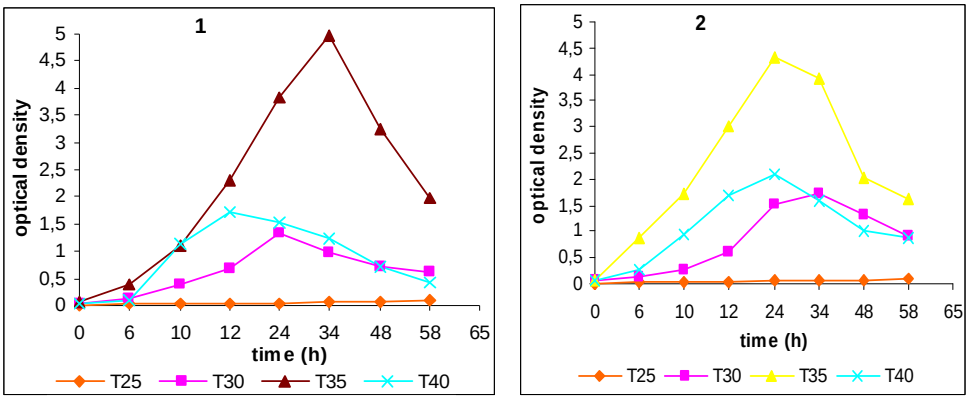

Fig. 1. Effect of temperature on growth of nitrogen fixing consortium isolated from alpine zone (1 = mannitol, 2 = sucrose)

In subalpine zone there are less cysts so, as the Fig. 2 shows, on both culture media the maximum is attained after 24 hours of incubation. The preference for sucrose seen at the bacteria coming from this zone can be due to the big quantity of fruits from *Vaccinium myrtillus*, *Vaccinium vitis-idaea* and other species which fall on the ground and decompose resulting sucrose, glucose, etc.

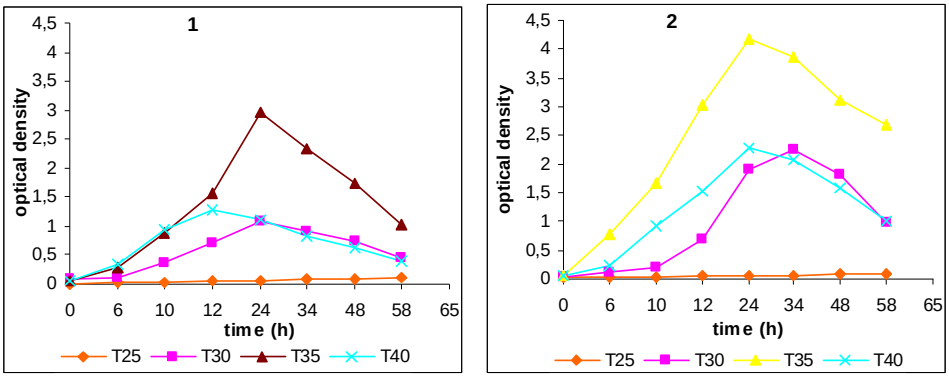

Fig. 2. Effect of temperature on growth of nitrogen fixing consortium isolated from subalpine zone (1 = mannitol, 2 = sucrose)

In the coniferous zone the maximum values are more reduced than in other vegetation zones, due to the smaller number of nitrogen fixing bacteria wich live in this acid environment. The hemicellulose contained by coniferous has a high mannose content which is reduces resulting a big quantity of mannitol in the soil of these forests (Volesky and Szczesny, 1983). The strains of *Azotobacter* from here developed thus a preference for mannitol, as shown in Fig. 3.

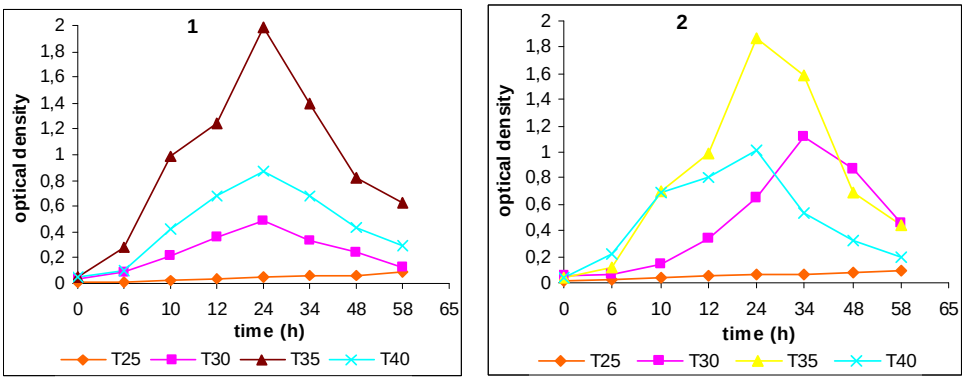

Fig. 3. Effect of temperature on growth of nitrogen fixing consortium isolated from coniferous zone (1 = mannitol, 2 = sucrose)

In the beech forest the maximum is attained on sucrose medium after 24 hours of incubation at 35 ºC (Fig. 4). Sucrose is first split into glucose and fructose, which are then catabolized.

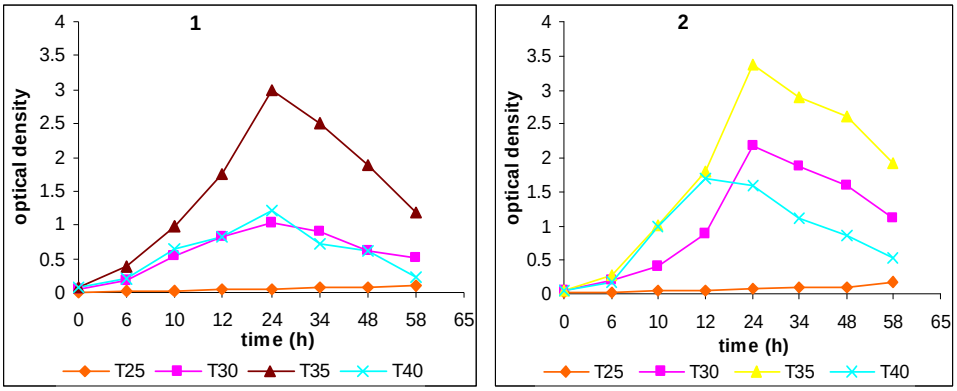

Fig. 4. Effect of temperature on growth of nitrogen fixing consortium isolated from beech zone (1 = mannitol, 2 = sucrose)

The strains isolated from beech zone can have a preference for glucose and implicitly for sucrose due to the relative large quantity of glucose. This comes from the degradation of cellulose from the thick litter. More, due to high content of tannin, the beech leaves decompose slowly and the glucose influxe in soil from the litter is not seasonal but represents a permanent nutritive source.

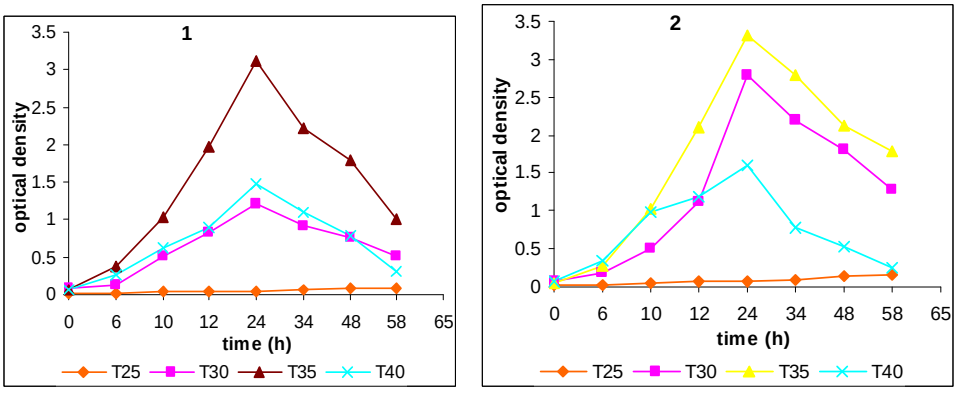

Fig. 5. Effect of temperature on growth of nitrogen fixing consortium isolated from Maleia flood plain (1 = mannitol, 2 = sucrose).

In the flood plain the water exces is the main impediment to the developement of *Azotobacter* sp., these bacteria being aerobes. At high temperatures the water evaporates and the environment becomes propitious. Therefore the strains isolated from the flood plain reach a maximum at 35° C on both media after 24 hours of incubation (Fig. 5).

All above data represent the mean three independent measurements. Regarding the growth of the strains isolated from the mountainous soils there is between the two culture media a statistically significant difference of the results obtained ($p<0.0001$).

The influence of temperature on the level of extracellular proteins. The effect of temperature on the concentration of extracellular proteins is presented in the Figures 6-10. An increasing tendency of the extracellular proteins level can be observed during the incubation at the temperature values of 25 ºC, 30 ºC and 35 ºC. During incubation even after 48 hours, when the culture was already in the stationary faze, the level of extracellular proteins appears to be increasing. Only at 40 ºC is observed a decreasing of the extracellular proteins level after 24-34 hours of incubation. The extracellular proteins levels varied substantially on the two culture media, at diferent temperature values.

At the strains coming from the alpine zone the maximum concentration of the extracellular proteins was obtained at 35 ºC after 58 hours of incubation. The extracellular proteins concentration values were 4.9112 mg/l on mannitol and 3.3728 mg/l on sucrose. As Fig. 6 shows the proteins concentration is also increasing when grown at 30 ºC. On the cultures at 40 ºC a maximum concentration is attained after 24-30 hours of incubation and then it decreases.

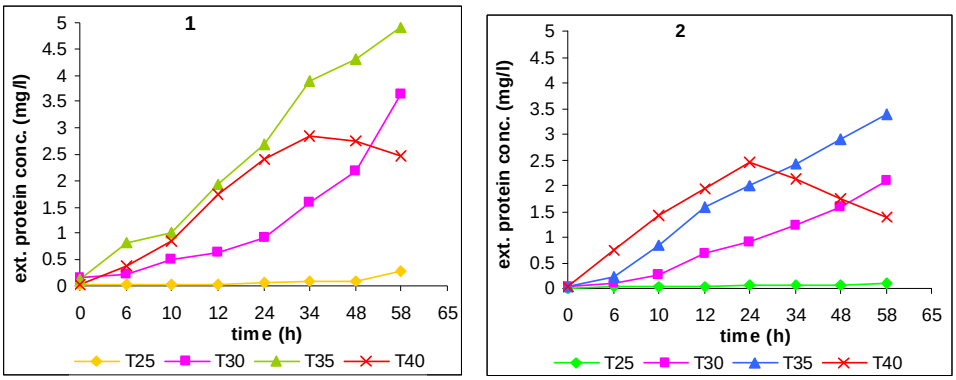

Fig. 6. Effect of temperature on extracellular protein levels at strains isolated from alpine zone (1 = mannitol, 2 = sucrose).

At the strains coming from the subalpine zone the maximum level of the extracellular proteins was obtained at 35 ºC after 58 hours of incubation. The extracellular proteins concentration values reached 3.4412 mg/l on mannitol medium and 3.3362 mg/l on sucrose medium. In the Fig. 7 is visible that the proteins concentration growth at 30 ºC is parallel and close to the one at 35 ºC.

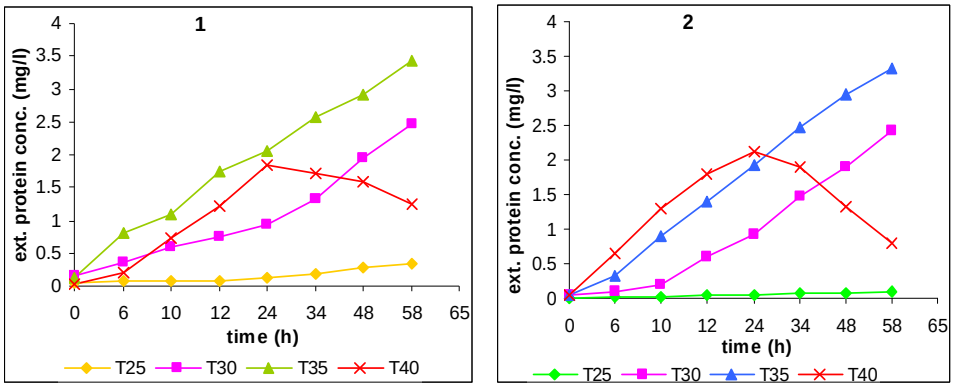

Fig. 7. Effect of temperature on extracellular protein levels at strains isolated from subalpine zone (1 = mannitol, 2 = sucrose).

The lowest values of extracellular proteins in the culture medium were recorded at the strains from coniferous zone. In this case the maximum concentration was recorded on sucrose at 35 ºC (2.1152 mg/l) (Fig. 8).

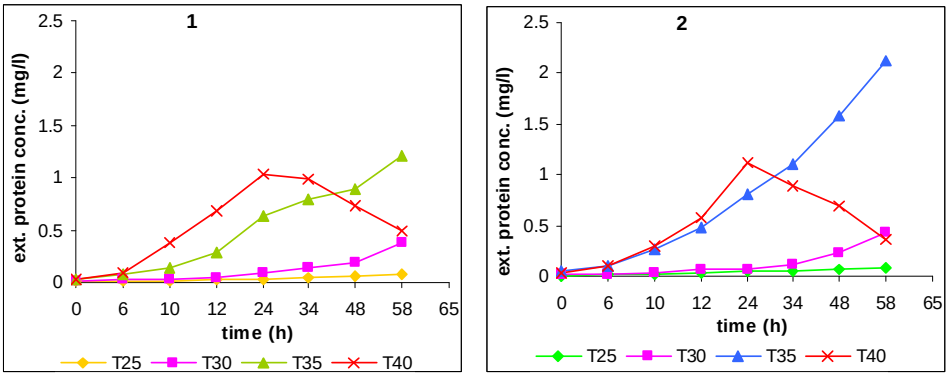

Fig. 8. Effect of temperature on extracellular protein levels at strains isolated from coniferous zone (1 = mannitol, 2 = sucrose).

The temperature influenced the extracellular proteins concentration also in the beech zone. In the Figure 9 a growth of extracellular proteins is visible, greater values of their concentration being recorded on sucrose medium (the maximum is 4.9233 mg/l).

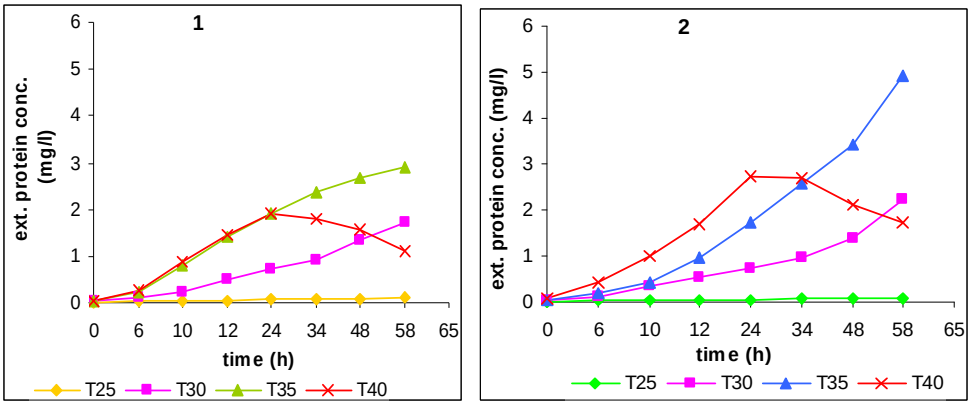

Fig. 9. Effect of temperature on extracellular protein levels at strains isolated from beech zone (1 = mannitol, 2 = sucrose).

The highest level of extracellular proteins in the culture media was recorded at the samples from Maleia flood plain (Fig. 10). The maximum value of the protein concentration was obtained on sucrose medium and it reached 5.6344 mg/l.

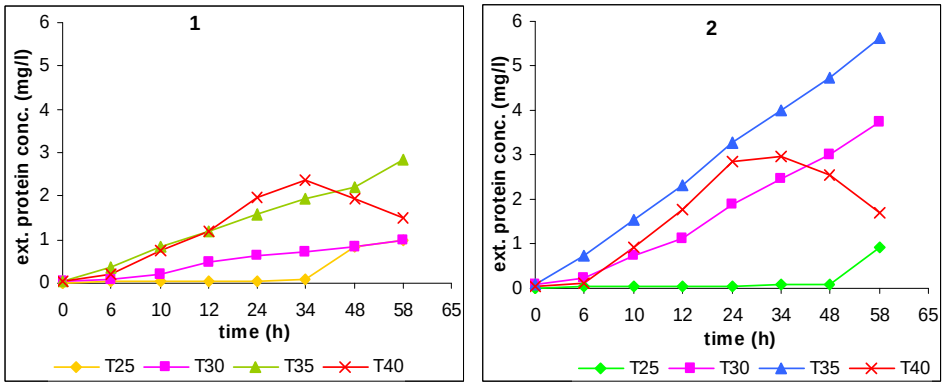

Fig. 10. Effect of temperature on extracellular protein levels at strains isolated from Maleia flood plain (1 = mannitol, 2 = sucrose).

For all the samples studied at 40 ºC after reaching a maximum after 24 hours of incubation a decrease of proteins concentration in the culture medium happens. An explanation of this diminishing is given by the fact that at higher temperatures the biochemical reactions occur much quicker and with a lower number of effectors and the excess of proteins is metabolized.

At the temperature of 25 ºC the proteins concentration was very low, conforming with the slow development of these bacteria at this temperature.

The maximum extracellular proteins concentration reached 4.9112 mg/l with the samples from the alpine zone (Fig. 6) on mannitol medium (1) and 5.6344 mg/l with the samples from Maleia flood plain on sucrose medium (2) (Fig. 10). From these results is noticeable that the maximum extracellular proteins level was attained when the strains were incubated at 35°C.

In the extracellular proteins case a statistically significant difference between the two media culture was also found for every single altitudinal vegetation zone ($p<0.0001$).

The influence of temperature on ammonia secretion. Effect of temperature on the ammonia secretion, as a fixation product, can be observed in the Figures 11-15. In the Fig. 11 is visible that at 35°C the ammonia secretion attains a maximum of 2.968 mg/l on mannitol medium and 3.227 mg/l on sucrose medium.

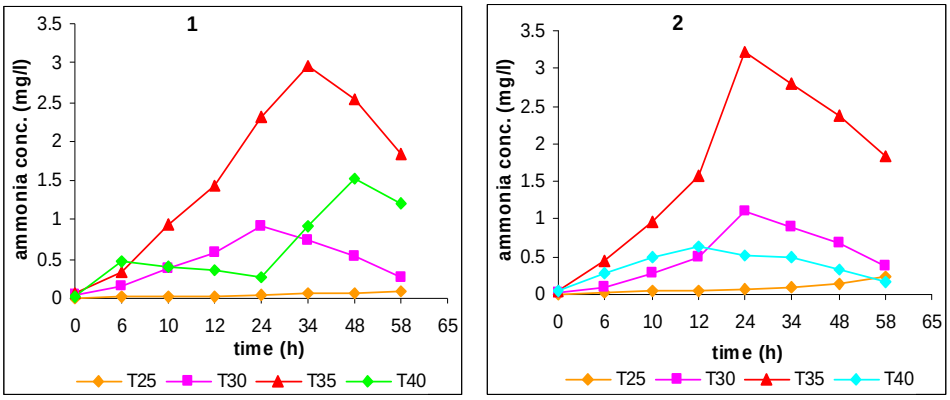

Fig. 11. Effect of temperature on ammonia secretion at strains isolated from alpine zone (1 = mannitol, 2 = sucrose).

At the samples from the subalpine zone the maximum of the produced ammonia concentration was attained at 35 °C on both culture media and the recorded values were close: 4.393 mg/l (2) and 4.185 mg/l (1) respectively. The low level of produced ammonia on the two culture media can be observed both at low temperatures (25 °C) and at high temperatures (40 °C) (Fig.12).

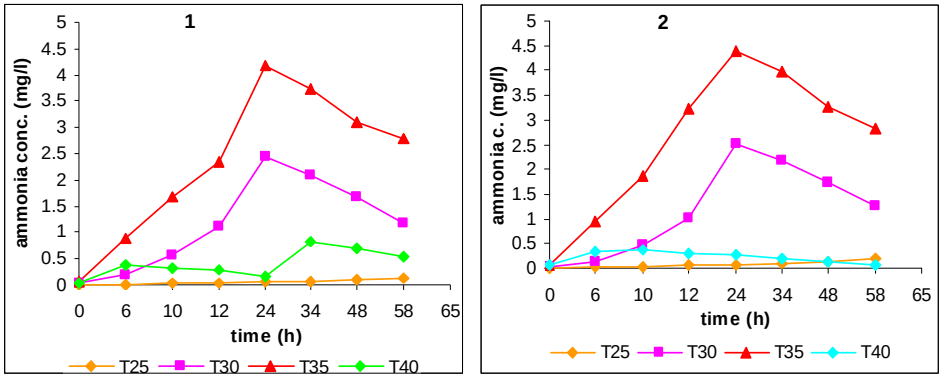

Fig. 12. Effect of temperature on ammonia secretion at strains isolated from subalpine zone (1 = mannitol, 2 = sucrose).

The lowest level of ammonia produced on both culture media was registered at the samples from the coniferous zone, where it did not pass above 2.5 mg/l (Fig. 13).

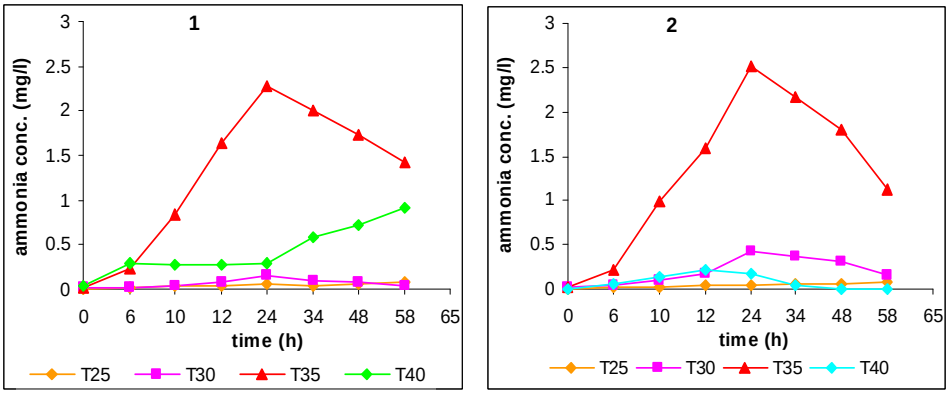

Fig. 13. Effect of temperature on ammonia secretion at strains isolated from coniferous zone (1 = mannitol, 2 = sucrose).

At the samples from the beech zone the maximum level of ammonia secretion was also reached at 35 ºC. A parallel growth with this one can be observed at 30 ºC (Fig. 14).

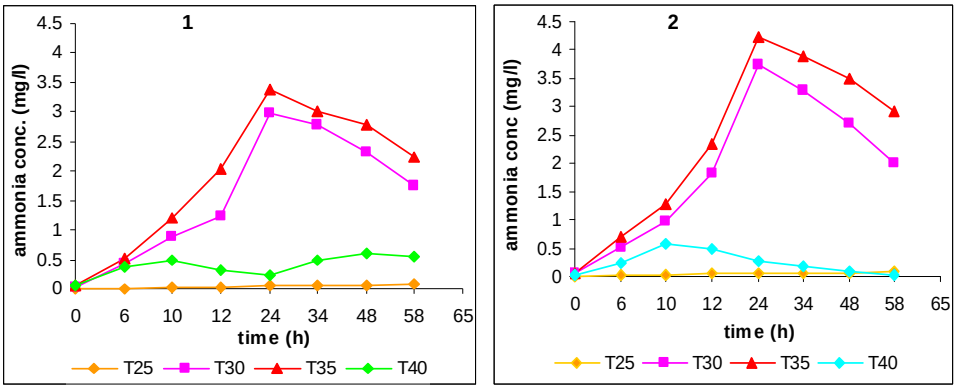

Fig. 14. Effect of temperature on ammonia secretion at strains isolated from beech zone (1 = mannitol, 2 = sucrose).

The highest level of ammonia secretion in the culture media was also recorded at the samples from the Maleia flood plain (Fig. 15). The maximum value of the produced ammonia concentration was 6.230 mg/l on the sucrose medium and 3.882 mg/l on the mannitol medium.

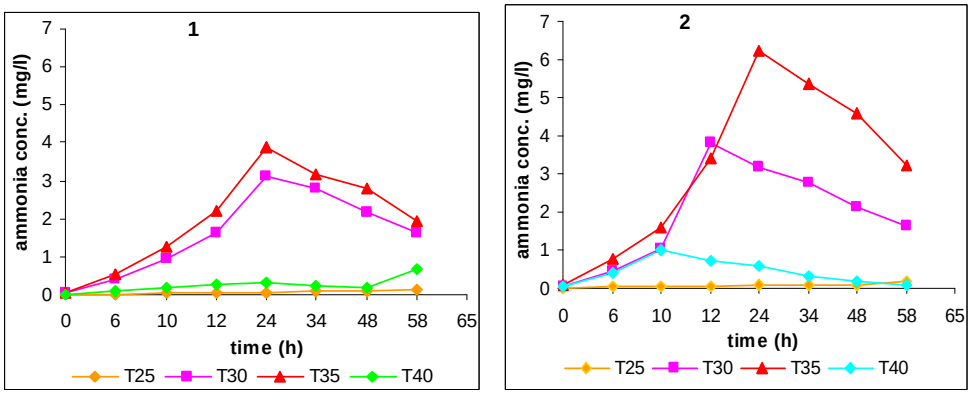

Fig. 15. Effect of temperature on ammonia secretion at strains isolated from Maleia flood plain (1 = mannitol, 2 = sucrose).

When the strains were grown at 30 ºC and 35 ºC the level of the ammonia secretion reached the maximum between the 20-th and the 40-th hour of incubation but it diminished at 40 ºC. Very low values of ammonia secretion in both culture media were registered at 25 ºC.

The maximum ammonia concentrations where obtained in the zone where the growth is in the stationary faze, afterwards it decreases abruptly even to exhaustion. This happens because ammonia is volatile and it is quickly released in the atmosphere.

The results from above show that the grows of temperature diminishes both bacteria growth and nitrogen fixation products, extracellular proteins and ammonia. This can be due to the diminishing of the nitrogenase activity (Sylvia *et al*., 1999). In other words, the sensibility of the nitrogenase to warmth, the enzyme responsible for nitrogen fixation, determined the diminishing of nitrogen fixation and therefore the reduction of growth. Besides, the decrease of nitrogen and oxygen solubility as temperature grows may be the cause of the reduction of growth and of nitrogen fixing activity at higher temperatures, of 40 ºC (Martyniuk and Martyniuk, 2003).

Conclusions

The dynamics of growth and nitrogen fixation at different physiological conditions and nutrient requirements of *A. chroococcum* in chemically defined N-free medium was determined.

The maximum cell concentration was obtained when bacteria strains (*A. chroococcum*) were grown at neutral pH, 35 ºC, 150 rpm and in medium with sucrose.

The maximum nitrogen fixation products were attained when bacteria fixing strains (*A. chroococcum*) were grown at pH 7, 35 ºC, 150 rpm and in medium with sucrose concentration as 5.6344 mg/l extracellular protein and 6.2309 mg/l ammonia.

REFERENCES

Atlas, R.M. (2004). Handbook of Microbiological Media. 3rd ed., Ed. Burgess, Minneapolis.

Burris, R.H., Wilson, P.W. (1972). Methods for measurement of nitrogen fixation. Colowick, S.P., Kaplan, N.O, Methods in Enzymology, Acad. New York, 355-367.

Burris, R.H., Roberts, G.P. (1993). Biological Nitrogen Fixation. *Annu. Revue Nutr.*, **13**, 317-335.

Garrity, G.M., Bell, J.A., Lilbum, T.G. (2004). Taxonomic Outline of the Prokaryotes. Bergey's Manual of Systematic Bacteriology. 2nd Edition, Release 5.0, Springer Verlag, New York.

Hartree E. F. (1972). Determination of Protein: A Modification of the Lowry Method that Gives a Linear Photometric Response. *Anal. Biochem.*, **48**, 422-427.

Martyniuk, S., Martyniuk, M. (2003). Occurance of *Azotobacter spp*. in some Polish Soils. *P. J. Environmental Studies*, **12** (3), 371-374.

Mishustin, E.N., Shilnikova, V.K. (1969). The biological fixation of atmospheric nitrogen by free-living bacteria. *Soil Biol. Rev. Res.*, Unesco, Belgium, 65-109.

Rekosz-Burlaga, H., Garbolinska, M. (2006). Characterization of selected groups of microorganisms occuring in soil rhizosphere and phyllosphere of oats, *Polish J. Microbiol*., **55** (3), 227-235.

Santek, B., Maric, V. (1995). Temperature and dissolved oxygen concentration as parameters of *Azotobacter chroococcum* cultivation for use in biofertilizer, *Biotechnol. Lett*., **17** (4), 453-458.

Snedecor, G., Cochram, W. (1978). Statistical methods, 6th ed., Ed. Iowa State Univ. Press, Ames Iowa

Sylvia, D.M., Fuhrman, J.J., Hartel, P.G., Zuberer, D.A. (1999). Principles and Applications of Soil Microbiology, Prentice Hall, N.J., 259-321.

Voleskz, B., Szczesny, T. (1983). Bacterial conversion of pentose sugars to acetone and butanol. Springer Berlin/Heidelberg.

Yamano, H., Takahashi, K. (1983). Temperature effect on the activity of soil microbes measured from heat evolution during the degradation of several carbon sources, *Agric. Biol. Chem.*, **47** (7), 1493-1499.

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 129-137

THE ENZYMATIC ACTIVITY FROM THE SEDIMENTS OF THE ARIEŞ RIVER FROM UPSTREAM TO DOWNSTREAM

ANDREEA BODOCZI[1], MIHAIL DRĂGAN-BULARDA[1]

SUMMARY. The aim of our study was to distinguish the activity of some enzymes along the course of the Arieş River to understanding the complex processes that happen in these habitats of special significance. The sediment samples from different points on the Arieş River (Abrud, Baia de Arieş, Sălciua, Turda, Luncani) have been taken seasonally during the year 2007. In the sediment samples the following enzymatic activities have been quantitatively determined: phosphatase, actual and potential dehydrogenase and catalase. Based on the relative values for the enzymatic activities, the enzymatic indicator of the sediment quality (EIQS) was also calculated.

KEYWORDS: sediment, enzymatic activity, quality indicator

Introduction

The sediments constitute a key link in the biogeochemical cycle of the elements in the aquatic systems. Here are finalized the mineralization processes of the organic substances that were not degraded in the water column (Muntean *et. al.*, 2001).

The sediments are very heterogeneous systems where the different physical phases (solid, liquid and gases) and numerous biotic microorganisms, the small organisms, enzymes) and a biotic (minerals, humus materials, organo-mineral[2] aggregate) components are involved in physical, chemical and biological processes. All the biochemical transformation depends on the enzymes presence (Gianfreda and Bollag, 1996).

The action of the microorganisms on the environmental substrates takes the enzymatic way through oxidoreduction and hydrolysis, respectively through the action of some final products of the microbial metabolism (Muntean *et. al.*, 2004).

Thus, the enzymatic activity determination offers, in a shorter time than the microbiological analysis, the suggestive data regarding the processes that are taking place in sediments or in other natural habitats.

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The growing enzymatic activity in sediments indicates pollution, but in the same time have the ability to reduce pollution semnificatively.

The sediments are medium in which vary different factors participate to realize some complex functions. These different factors are considered to be the major mineral matrix, the texture, quantity of organic carbon, geographical localization and conditions.

According to Malcom and Stanley (1982), the sediments have three major components: detrital material, derived from the erosion of the continents, biogenic material, formed by biological productivity, and autogenic material, formed *in situ*. The final character of sediment is related to the relative proportion of these components.

The determination of the enzymatic activities in the aquatic sediments constitutes a research instrument to evaluate the functional diversity of the micro biota

The hydrographic basin of Aries River it is situated in the north-western part of the country, in the middle of the Apuseni Mountains. The Arieş River basin has an S form, a length of 131 Km and a medium width of 17, 5 Km. From the spring to the confluence with Mures river, the Arieş covers almost 167 Km, with this length entering in the middle rivers category.

Arieş River water quality establishment is a major present interest, for human health, as well as for the flora and fauna. Pollution sources of the Arieş River are various, starting with mining activity, the atmosphere pollution, house holding activity, as well as the anthropic, uncontrolled factors etc. Wastewater releasing in different effluents of the Arieş River affects the pH of water and the chemical composition. The presence of heavy metals in aquatic systems offers a continuous monitoring of these waters which is difficult to achieve according to the fact that heavy metals are rapidly concentrated in living organisms (Gallo *et al.*, 2006; Kobayashi *et. al.*, 2001; Tao *et. al.*, 2005; Tudor *et. al.*, 2006).

The complex problems of the Arieş River pollution determined us to observe this process, trying to establish in what measure this water is affected.

Material and Methods

The enzymological studies from the Arieş river sediment performed in 2007 consisted in determination of quantitative enzymatic activity. Sediments were taken from the river bed at 50 cm from the bank, after the first 5-10 cm of sediments was moving off.

Sediments were taken from the upstream and downstream of five major sampling sites in the following order: Abrud, Baia de Arieş, Sălciua, Turda and Luncani.

The following enzymatic activities were quantitatively established for the sediment samples: phosphatase (Pha), catalase (CA) and dehydrogenase: actual dehydrogenase (ADA) – reducing of the 2,3,5-triphenyltetrazolium chloride – TTC in samples without glucose; potential dehydrogenase (PDA) – with glucose, and non-enzymatic catalytic activity (NCA) – non-enzymatic decomposition of H_2O_2. The humidity of each sample was established during their preparation for the

analysis, because of different sediment categories that may have variable water content (which can influence the expression of the microbial charge and enzymatic activities reported to the sediment weight)

Phosphorus is one of the essential nutrients for the plants` growth. In the phosphorus cycle, the inorganic and organic forms are associated by mineralization processes, mediated by biotic and a biotic activities. In the mineralization processes, the organic phosphorus fractions, represent a great quantity of phosphorus in soil and sediments, and thus are transformed in inorganic forms which are usually utilized by plants, as a result of the phosphatase actions (Gianfreda and Bollag, 1996).

For the phosphatase activity determination the reaction mixtures consisted of 2.5 g sediment + 2 ml toluene (antiseptic agent) + 10 ml enzymatic substrate (disodium phenylphosphate 0.5%) + 50 ml ammonium alum 0.3% + 5 ml borax buffer (pH = 9.4) + 0.8 ml Gibbs reagent (2,6-dibromoquinone-4-chloroimide). The incubation was carried out at 37° C for 24 h. The PhA was expressed in mg phenol/g.d.s. (gram dry sediment) by spectrofotometricaly measured absorbance at 597 nm.

The catalase activity is performed expressing the decomposition intensity of H_2O_2 which is formed in the aerobic microorganism breathing process. Catalases are found almost in every animal cell and in a smaller quantity in plant cells. In the case of microorganisms, catalases are found only in aerobic microorganisms (cianobacteria) (Regelsberger *et al*., 2002). For a long time, the decomposition role of the H_2O_2 has been associated with the aim to protect the living cell by its hurting effect. Lately it was demonstrated that these enzymes could play the peroxide's role as well, and in this way they can degrade or embed xenobiotic substances (fenolic substances and aninilic derivates resulted by industrial processes and agriculture) in soil organic substances and probably in those of sediments as well (Gianfreda and Bollag, 1996).

The reaction mixture for CA consisted of 3 g sediment + 10 ml phosphate buffer (pH=6.8) + 2 ml solution 3% H_2O_2 + 10 ml N H_2SO_4 + distilled water + 0.05 N $KMnO_4$ (Drăgan-Bularda, 2000). The incubation was carried out at room temperature for 1 h. The same technique was used for the determination of NCA, but the sediment samples have been thermicaly inactivated by autoclaving. The CA and NCA were expressed as mg H_2O_2/g.d.s.

The dehidrogenase activity may be considered as a major indicator of biologic activity of the organisms, but it was been used as an ecotoxicological test to evaluate the pollutants` effect over the microbiota of the soil or sediments.

The ADA and PDA were determined from the autoclaved and non-autoclaved sediment samples by the Drăgan-Bularda method (2000). The composition of the reaction mixtures were the following: 3 g sediment + 0.5 ml 3% TTC + 1 ml distilled water, respectively 1 ml 3% glucose solution + 10 ml acetone. The incubation was carried out at 37° C for 24 h. The ADA and PDA were represented as mg triphenylformazan nitrogen/g.d.s., by measuring absorbance at 485 nm.

All the enzymatic activities determination was performed using an UNICAM UV-VIS spectrophotometer.

The quality of the studied sediment was enzymologically characterized by the intensity of the enzymatic activities defined by the values of the enzymatic indicator of the sediment quality (EIQS). The higher enzymatic indicator quality, the higher the enzymatic potential of sediment. EIQS offers a wide image on its enzymatic potential, being calculated based on the formula elaborated by Munteanu *et. al.*, in 1996.

Theoretically, the enzymatic indicator may exhibit values between 0 (where no activity exist in the studied samples) and 1 9where are the real individual values are equal to the maximal theorethical individual values of all activities). Two parameters characterize the enzymological quality of the studied sediments: the intensity of the enzymatic activities defined by the value of the enzymatic indicator of the sediment quality (EIQS) and the stability of those activities defined by the value of the enzymatic variability coefficient of (EVCSQ). The enzymatic potential of the sediments rises with the increase of EIQS. The stability of the enzymatic activity rises with the decrease of EVCSQ. Therefore, the quality of some sediments are higher when EIQS is higher respectively EVCSQ is lower (Muntean, *et. al.*, 2001).

We aimed to determine the values of the enzymatic indicator for Arieş River to appreciate the sediment quality, respectively to establish some protection measures when anthropogenic influences appeared unlikely.

The enzymatic indicator for the sediment quality (EIQS) offers an ample image on its enzymatic potential being calculated on the formula issued by Muntean et al., in 1996. Based on the absolute values of the enzymatic and non-enzymatic catalytic activities for all analyzed samples, the enzymatic indicators for the sediment quality were calculated. In addition, for each enzymatic activity, the enzymatic variability coefficient for the sediment quality (EVCSQ) was calculated, representing the ratio between the standard deviation and average (Sach, 1968).

Results and Discussions

The quantitative enzymatic activities of actual and potential dehydrogenase, phosphatase and catalase

The dehydrogenase activity is expressed as mg formazan/g.d.s.

Seasonally, it has been determined that higher values of the dehydrogenase activities were observed in autumn. The potential dehydrogenase activity is more intense than the actual one. This fact reflects the stimulative action of the carbon easy assimilation on the enzyme synthesis by the living organisms (Crişan, *et. al.*, 1999, 2001)

Actual and potential dehydrogenase activities were been detected in all analysed samples. The reduced values of dehydrogenase activities upstream of Arieş River may be occurred by sediment with a sandy consistence which doesn't allow the microorganism proliferation.

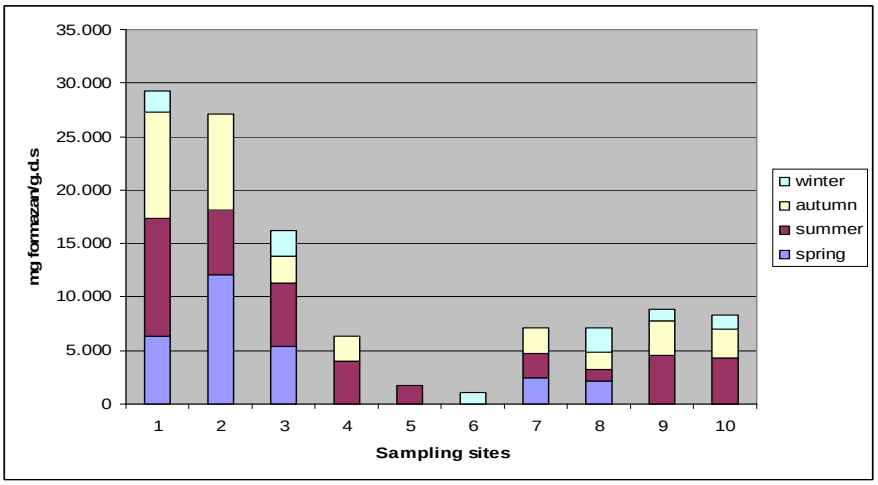

Fig. 1. The intensities of actual dehydrogenase activities from the Arieş River sediment in 2007: 1 – Abrud upstream; 2 – Abrud downstream; 3 –Baia de Arieş upstream; 4 – Baia de Arieş downstream; 5 – Sălciua upstream; 6 - Sălciua downstream; 7 – Turda upstream; 8 – Turda downstream; 9 – Luncani upstream; 10 - Luncani downstream

The highest values of the dehydrogenase activities were established in autumn, when the vegetal origin organic matter from the end of the vegetation period favourize the microorganism's developments and intensify those enzymatic activities.

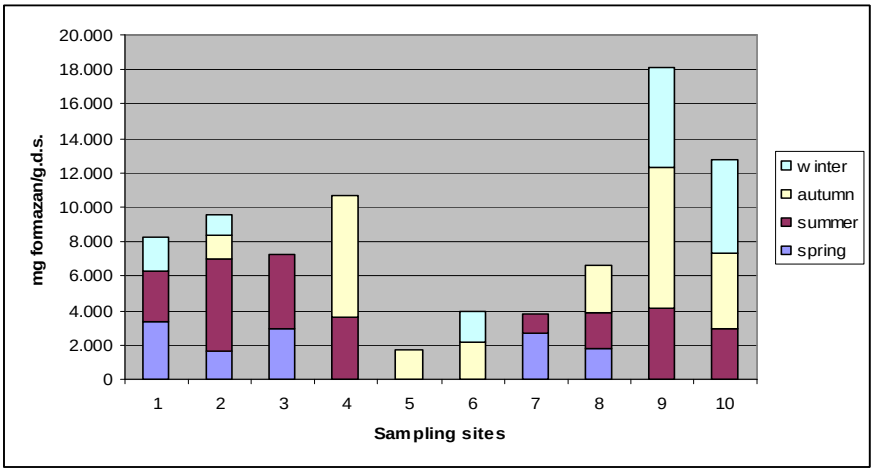

Fig. 2. The intensities of potential dehydrogenase activities from the Arieş River sediment in 2007: 1 – Abrud upstream; 2 – Abrud downstream; 3 –Baia de Arieş upstream; 4 – Baia de Arieş downstream; 5 – Sălciua upstream; 6 - Sălciua downstream; 7 – Turda upstream; 8 – Turda downstream; 9 – Luncani upstream; 10 - Luncani downstream

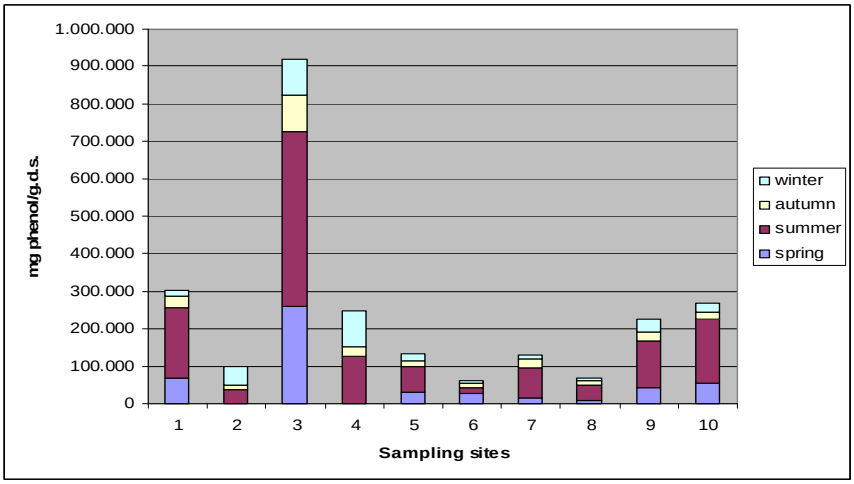

Fig. 3. The intensities of phosphatase activities from the Arieş River sediment in 2007: 1 – Abrud upstream; 2 – Abrud downstream; 3 –Baia de Arieş upstream; 4 – Baia de Arieş downstream; 5 – Sălciua upstream; 6 - Sălciua downstream; 7 – Turda upstream; 8 – Turda downstream; 9 – Luncani upstream; 10 - Luncani downstream

For the phosphatase activity, the highest value was determined during the summer.

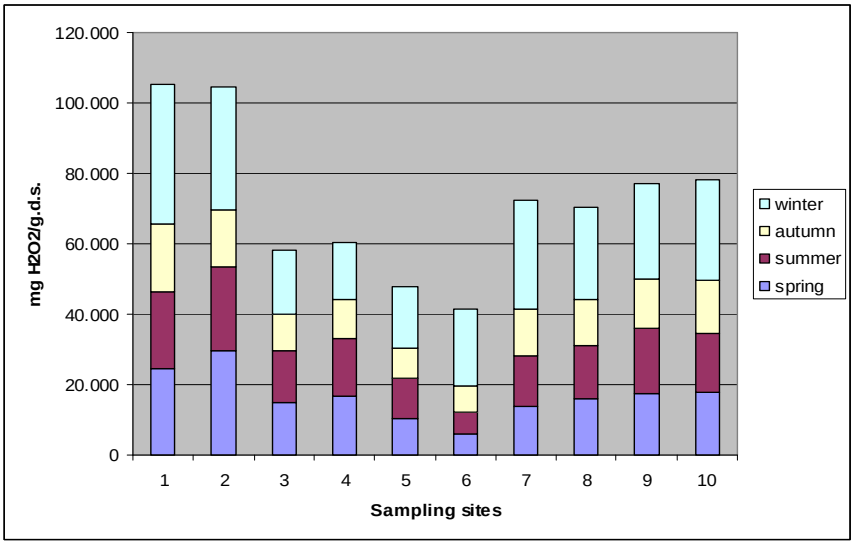

Fig. 4. The intensities of catalase activities from the Arieş River sediment in 2007: 1 – Abrud upstream; 2 – Abrud downstream; 3 –Baia de Arieş upstream; 4 – Baia de Arieş downstream; 5 – Sălciua upstream; 6 - Sălciua downstream; 7 – Turda upstream; 8 – Turda downstream; 9 – Luncani upstream; 10 - Luncani downstream

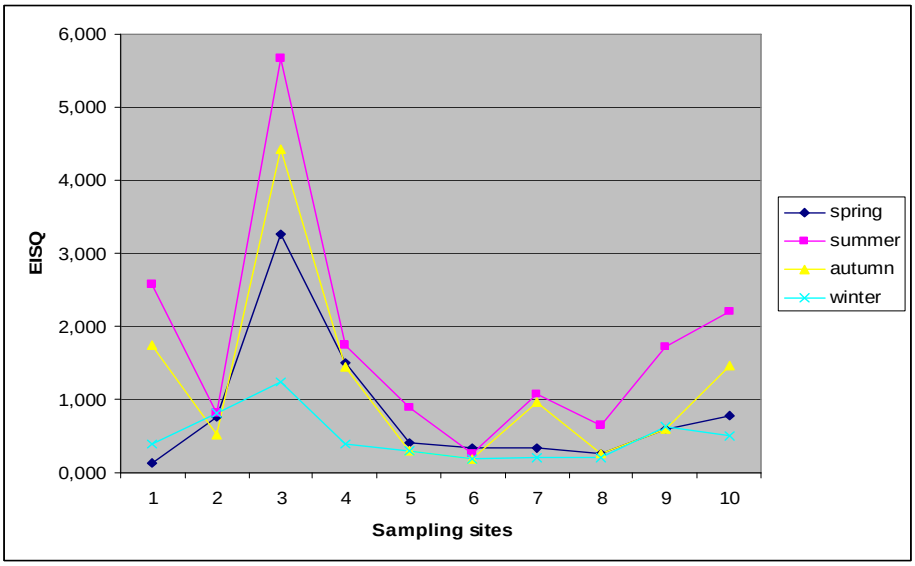

Fig. 5. The enzymatic indicator of the sediment quality from the Arieş River sediment in 2007: 1 – Abrud upstream; 2 – Abrud downstream; 3 –Baia de Arieş upstream; 4 – Baia de Arieş downstream; 5 – Sălciua upstream; 6 - Sălciua downstream; 7 – Turda upstream; 8 – Turda downstream; 9 – Luncani upstream; 10 - Luncani downstream

According that the catalase activity is determined by expressing the intensity of the H_2O_2 decomposed, that appeared in the respiration processes of the aerobe organisms, we may affirm that at the Arieş River sediments levels there are a lower numbers of microorganisms which have a reduced enzymatic activity.

The higher values of catalytic activities were observed in autumn and summer, even there are no major differences from one season to another.

The most reduced intensities of the catalytic activities were registered in downstream of Sălciua sampling point.

The enzymatic indicator of the sediment quality (EIQS)

The investigated sediments of the Arieş River have a decreased enzymatic activity in majority of the sampling sections. The highest levels of EISQ were reported in summer and autumn when the enzymatic and microbial activity in the sediments are intensified by the accumulation of the organic substances deposited by the end of the vegetation period.

Conclusions

All the enzymatic activities chosen to be determined from the Arieş River sediments constitutes a research tool to evaluate the functional diversity of the micro biota involved in the biogeochemical cycles.

All four studied quantitative enzymatic activities have shown variations of their intensities depending on the seasons, on the sampling place and on the necessary substrate from the enzymes synthesis by the microorganisms.

Knowledge of the intensity of the enzymatic activity represent a new and important direction for investigation of water protection activity, by the characterization of the aquatic ecosystems impurity degree, the possibilities of the evolution prognosis and water quality maintenance.

REFERENCES

Cuşa, V., (1996). The methodological instruction for microbiological analysis of the aquatic sediments, Bucharest: Research and Environmental Engineering Institute, vol. 4, pp. 2-7.

Crişan, R., Muntean, V., Paşca, D., Coldea, G., (1999). The enzymatic activity of the peat placed under different vegetal associations of the Căpânța tinove. *Stud. Cercet. Biol (Bistrița)*, **5**, 25-33.

Crişan, R., Muntean, V., Paşca, D., (2001). The enzymatic activity of the therapeutical peat from Băile Someşeni-Cluj, *Stud. Cercet. Biol. (Bistrița)*, **6**, 13-17.

Drăgan-Bularda, M., (2000). Microbiologie Generală - Lucrări Practice, Universitatea Babeş-Bolyai, Cluj-Napoca, Ediția a III-a

Drăgan-Bularda, M., Grigore, C., Țura, D., (2004). The protection and utilization of the salt lakes using the enzymatic indicator of the mud quality. *Stud. Univ. Babeş-Bolyai, Biol.*, **49** (1), 129-140.

Gallo, M., Trento, A., Alvarez, A., Beldoménico, H., Campagnoli, D., (2006), Dissolved and particulate heavy metals in the Salado River, *J. Water, Air Soil Poll*, **171** (1-4), 367-384.

Gianfreda, L., Bollag, J-M., (1996). Influence of natural and anthropogenic factors on enzyme acivity in soil, In: Stozky, G., Bollag, J-M, (Eds.), *Soil Biochemistry*, Vol. 9, Marcel Dekker Inc., New York, pp. 123-129.

Kiss, S., Drăgan-Bularda, M., Paşca, D., (1986). Environmental enzymology, *Stud. Univ. Babes-Bolyai, Biol*, **31** (1), 3-13.

Kobayashi, J., Kizu*b*, R., (2001). Evaluation of Metal Contents in River Water Using a Simple Fractionation Method, *J. Health Sci.*, **47** (5), 460-463.

Malcolm, S.J., Stanley, S.D., (1982). The sediment environment, In: Nedwell D.B. (Ed.) *Sediment microbiology*, Acad. Press, London, pp. 1-14.

Muntean, V., Crişan, R., Drăgan-Bularda, M., (1996). Enzimological classification of salt lakes in Romania, Netherlands: Kluwer Academic Publishers, *Inernat J. Salt Lake Res*, vol. **5**, pp. 35-44.

Muntean, V., Crişan, R., Paşca, D., (2001). Cercetări enzimologice asupra nămolului din balta sărată Alunei-Târnăveni, *Stud. Certcet. Biol., (Bistriţa)*, **6**, 7-12.

Muntean, V., Stef, L.C., Drăgan-Bularda, M., (2004). Cercetări enzimologice asupra unor sedimente din râul Mureş, Romanian Biological Sciences, I (2-4), 107-114.

Reglsberger, G., Jakopitch, ., Plasser, L., Schwaiger, H., Furtműller, P.G., Peschek, G.A., Zámocky, M., Obinger, Z.C., (2002). Occurrence and biochemistry of hydroperoxidases in oxygenic phototophic prokaryotes (Cyanobacteria), *Plant Physiol. Biochem.*, **40**, 479-490.

Sach, L., (1968), Statistische Auswertungesmethoden, Springer, Berlin, p. 485.

Tao, F.T., Jiantong, L., Banding, X., Xiaoguo, Xu., (2005). Mobilization potential of heavy metals: A comparision between river and lake sediments, *J. Water, Air Soil Poll*., **161** (1-4), 209-225.

Tudor, H., Gryte, C., (2006). Detoxifying agents for metal-contamined waters, *J. Water, Air Soil Poll*., **173** (1-4), 209-242.

STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 139-150

SENSITIVITY OF BACTERIAL, YEAST BIOFILMS AND PLANKTONIC CELLS TO SEVERAL ANTIMICROBIAL AGENTS

ILDIKÓ-IRÉN RÁCKÖVY[1], MIHAIL DRĂGAN-BULARDA[2]

SUMMARY. Microorganisms often colonize a substratum and form microcolonies or biofilms where they are enclosed in exopolymer matrices. Biofilms are commonly resistant to a broad range of antimicrobial agents, and resistance mechanisms involve exopolymer matrices, changes in gene expression and metabolic alterations. Due to these different resistance mechanisms, it is difficult to select and titrate antimicrobial agents to be effective against biofilms. In this context the effects of the 5 sanitizing agents (Microzid AF, Omnicide, Terralin, P3 Topax 66 and Domestos) on *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus mirabilis*, *Candida albicans* and a MRSA strains adhering to stainless steel were compared using epifluorescence microscopy. These biocides were tested on planktonic bacteria on suspension tests and surface test. Data showed that the antimicrobial efficacy measured on planktonic bacteria is not a reliable indicator of performance when biofilm is present. When biofilms were exposed to these sanitizing agents, these were not destroyed by the concentration affective on surface and suspension tests.

KEYWORDS: biofilms, sanitizing agents, pathogen bacteria, pathogen yeast, epifluorescence microscopy, planktonic cells.

Introduction

Microbial cells attach to inert surfaces and produce exopolymers that facilitate their adhesion. Following attachment, cells grow, divide, colonize the surface and form microcolonies. The size and number of microcolonies increases and results in the formation of biofilm (Surdeau *et al*., 2006). Numerous studies have demonstrated that micro-organisms in biofilm are more resistant to antimicrobial agents than their planktonic counterparts, and this lower susceptibility is due to the structural organization of biofilm and to cell physiology and metabolism (Palmer and White, 1997; Sauer *et al.*, 2002).

The structure of a microbial biofilm is characterized by the bacterial cells embedded in a thick, highly hydrated anionic matrix. The physiological status of cells found in biofilms is heterogeneous (Johansen et al., 1997).

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Cells present in the upper part of a biofilm (surface biofilm cells) have properties similar to planktonic cells. They have easy access to nutrients and oxygen, and few problems with the discharge of metabolic waste products because they are constantly being removed from the medium and they are metabolically active. They are large in size and very accessible to antimicrobial agents (Anwar et al., 1992).

Cells embedded in the thick exopolymer matrix exist in a sheltered, encapsulated community and are protected from antimicrobial agents (Stewart et al., 1998; Stewart, 2003). These innermost cells are probably less metabolically active because of poor access to essential nutrients and oxygen, and they are smaller than other cells (Marshall, 1994). The efficacy of disinfectants is regulated by a complex system of interactions between the test microorganisms, the practical conditions and specific disinfectant under evaluation (Bessems, 1998).

The aim of this study was to investigate the microbiocidal activity of 5 sanitizing agents against the norms EN 1276 (dilution-neutralization methods against planktonic bacteria and yeast) and against formed biofilms.

The effective concentration of biocide corresponds to a 10^5 -fold decrease of viable bacterial counts after 5 min. The efficacy of these 5 sanitizing agents against a bacterial and mold biofilm formed on an inert surface such as stainless steel was determined for a 15-60 min. contact period without neutralization of the biocide.

Materials and Methods

Test organisms. The micro-organisms used in this study were*, Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Proteus mirabilis* ATCC 43071 and *, Candida albicans* ATCC 10231 (Culti-Loops-ready-to use cultures for direct planting, UK), and a methicillin-resistant *S. aureus* (MRSA) (University of Târgu Mureş).

Working cultures were incubated aerobically for 24 h at 37^{o}C in 10 ml Tryptone soya Broth (TSB; Oxoid), Stock cultures were made on Tryptone Soya Agar (TSA; Oxoid) plates, stored at 4°C.

Chemicals. The antimicrobial activity of the following biocides was investigated (see Table 1).

Stainless steel. The solid surface selected for this study was stainless steel, which is commonly found in the hospital environment, especially in operating rooms and in the food industry. The stainless steel was cut into 2.5x 6 cm plates, cleaned with 70% ethanol, were then rinsed in distilled water and sterilized by autoclaving. Samples were stored in Petri dishes.

Biofilm culture. Each strain was grown in fresh medium. Each microbial suspension was adjusted to approximately 10^8 cells/ml using the Thoma (Helber) counting chamber. Biofilms were produced by sedimentation of the bacterial suspension of stainless steel plates for 24 h at 37°. Plates were rinsed with sodium chloride solution to remove non-adherent bacteria. From each strain we made a control sample. Then the sample surfaces were rinsed with sterile distilled water.

Table 1

The types and composition of the studied disinfectants

Biocides	Used concentration	Active agents
P3-Topax66	5 %	Potassium-hydroxide, Sodium-hypochlorite, Non-ionic surfactant
Terralin	0.5 %	-Quaternary ammonium compounds, benzyl-C12-18-alkyldimethyl, chlorides
		-2-Phenoxyethanol
		-Amines
Domestos	2 %	Sodium-hypochlorite, Non-ionic surfactant, Sodium-hydroxide
Microzid AF	Undiluted	Ethanol , Propanol
Omnicide	0.5 %	Glutaraldehyde, Dimethylcocobenzil-ammonium chloride

Disinfectants activity assay on planktonic bacteria. The bactericidal activity of disinfectants was determined using the dilution-neutralization method against planktonic cells (Wilson, 1986; Borneff, 1988; EN1276, 1997; Jelenikné Nikolics and Lévai, 2000). The same concentrations (see Table 1) were applied for 15-60 min. at 20°C on control microbial suspensions in clean conditions (low amounts of protein load). After the contact time, 1.0 ml of this mixture was added to 8 ml of the appropriate neutralizer for 5 min. at 20°C. We take immediately a sample of 1.0 ml of neutralized mixture and transfer into separate Petri dishes and immediately add 15 ml melted TSA (Tryptic Soy Agar).Colony – forming units (CFU) were counted after 48 h incubation at 37°C. The neutralizer solution we used in this study was sodium thiosulphate pentahydrate 1%(m/v) , associated with polysorbate 80 3%(v/v) and lecithin 0.3%(m/v).

Disinfectant activity assay on microbial biofilms. The strains-infected surfaces treatment with studied disinfectants and the examination of the surfaces.

We poured the above mentioned disinfectants on the formed biofilm so as to cover the surfaces in Petri-dishes (Pap, 2002). The disinfectants were applied for 15-60 minutes. After that the samples were colored with acridin orange (acridin orange - 0.02 g, distilled water- 100ml).

The samples were examined using epifluorescence microscope and we took pictures of them.

The control surfaces were colored similarly as the samples. We wanted to be sure about the existence of the biofilm and the presents of the living and dead cells in it.

Results and Discussion

Bactericidal activity is characterized by the concentration of the tested products for which a 10^5 reduction in viability is demonstrated under the required test conditions. The initial bacterial counts decreased by 10^6 , suggesting that the studied biocides were sufficiently active to achieve a 10^5 –fold reduction of the initial bacterial count in suspensions (Table 2).

Table 2

The efficiency of biocides against planktonic bacteria

Bacterial strains	Bacterial test suspension		Omnicide 0.5%	Terralin 0.5%	Microzid undiluted
Pseudomonas aeruginosa ATCC 27853	10^{-6}: 185; 160 10^{-7}: 30; 25 N: 1.9 x 10^8	Vc: Na: R:	0; 6 < 1.5 x10^2 > 10^5	0; 0 <1.5x 10^2 > 10^5	0;0 < 1.5x 10^2 > 10^5
Escherichia coli ATCC 25922	10^{-6}: 194; 209 10^{-7}: 27; 32 N: 2.1x 10^8	Vc: Na: R:	1 ; 1 < 1.5 x10^2 > 10^5	0; 0 <1.5x 10^2 > 10^5	0;0 <1.5x10^2 > 10^5
Staphylococcus aureus ATCC 25923	10^{-6}: 237; 210 10^{-7}: 28; 24 N: 2.3x 10^8	Vc: Na: R:	0 ; 5 < 1.5 x10^2 > 10^5	0; 0 <1.5x 10^2 > 10^5	0; 0 <1.5x10^2 >10^5

Vc = viable count
Na = the number of cfu/ml in the test mixture
R = reduction in viability
N = the number of cfu/ml of the bacterial test suspension

Efficiency of studied biocides against *Candida albicans* was presented in Table3.

Table 3.

Yeasticidal activity of the biocides Omnicide, Terralin and P3-Topax 66

Test organism	Omnicide, %	V_{C1}	V_{C2}	N_a = x x 10	Ig N_a / Ig R
Candida albicans ATTC 10231	0.2	35	25	300	2.40 3.70
10^{-6} suspension	0.5	1	7	40	1.60 **4.50**
	Terralin, %				
Candida albicans ATTC 10231	0.2	60	50	550	2.60 3.60
10^{-6} suspension	0.5	2	8	50	1.69 **4.48**
	P3-Topax 66, %	**V_{C1}**	**V_{C2}**	**N_a = x x 10**	**Ig N_a / Ig R**
Candida albicans ATTC 10231	2.0	20	26	230	2.30 3.90
10^{-6} suspension	5.0	0	4	20	1.30 4.87

V_c = number of colonies count per ml(one plate or more)
N_a = number of survivors per ml in the test mixture, x = average of V_{c1} and V_{c2}
R = reduction(expressed in logaritm)
(Ig R= Lg N_o – Lg N_a)

The product Omnicide shall demonstrate a decimal log(Ig) reduction in counts of 4 in 30 minutes. Terralin was applied for 60min at 20^{o}. Data of Table 3 demonstrates the activity of Omnicide and Terralin on yeast suspensions allso with dillution-neutralization method. The effective concentrations of Omnicide and Terralin were 0.5% but the smaller concentration of the product as 0.2% was not effective enough to reduse the *Candida albicans* cell`s viability with lg 4.

One can see that P3-Topax 66 possesses biocide activity in thirty minutes in both concentrations using dilution-neutralization method.

All biocides tested in the suspension test achieved > 4 $\log_{10}$ reduction in viable microbial concentrations.

The disinfectants were tested on 24 h biofilms formed on stainless steel surfaces. The data showed that the biofilm presents a certain resistance. We also examined whether without mechanical influence the biocides are capable of destroying the microbes from the stainless steel surfaces. We also would like to mention that during our investigations the used microorganisms presented different biofilm formation. *C. albicans, E. coli, P. aeruginosa, P. mirabilis, S. aureus* and MRSA strains showed a good attachment and growth on the surface. The orange colored cells died during the treatment.

The examined biocides were effective on the formed biofilms as follows in Fig.1-12.

One can see in Fig.1-2, *P. mirabilis, E. coli* and *S. aureus* remained adhered to the surface even after the treatment with Microzid AF. Biofilm formed with *P. aeruginosa* and *C. albicans* were removed in high percent. The *C. albicans* biofilm cells can be observed on Figure 1 there are also living green-colored cells and the dead cells are orange.

The disinfectant Terralin, practically, destroyed completely from the surface the *C. albicans* cells. *P. mirabilis* cells remained on the surface in lower percent but *E. coli, P. aeruginosa* and *S. aureus* dead cells were present in high rate. This result can be observed on the Fig. 3-6.

Omnicide disinfectant was not as effective in killing or removing adherent *E. coli* cells as the *P. aeruginosa* and *S. aureus* were. It could not remove the *C. albicans* biofilm cells.

Figure 7 shows the *Staphylococcus* cells remained on stainless steel surface after treatment.

In all cases Domestos was more effective than the used disinfectants so far in killing and removing the adhered cells. Domestos destroyed completely every formed biofilm from the surface except *C. albicans*. In this case there remained only a few cells (see Fig. 10).

We had good results in using the Topax disinfectant. This disinfectant proved to have a good biofilm removing effect. It removed completely the *E. coli, P. mirabilis* and *P. aeruginosa* cells. It remained only some *S. aureus* adhered cells on the surfaces. Figure 11 shows the remained *E. coli* cells and fig.12 shows the remained *C. albicans* cells.

Analyzing the results from the point of view of the investigated microorganisms, we can say that most of the disinfectants were not able to decompose effectively the adhered *E. coli* biofilm from the surface except P3- Topax 66 which removed the cells completely.

S. aureus biofilm was removed by the P3-Topax 66 and Domestos but Omnicid reduced considerably the adhered biofilm cell numbers from the stainless surface. The MRSA clinical strains biofilm did not show considerable sensibility comparing to the *S. aureus* biofilm. A similar result has Luppens et al. (2002).

P. aeruginosa biofilm was removed only by two disinfectants: Domestos and P3-Topax 66 but the Omnicide reduced considerably the cell numbers. The aderation on the surface of the *P. mirabilis* biofilm was removed effectively by Terralin disinfectant.

The Marshall-experience (1994) proved that 30% of the *P. aeruginosa* cells in two hours irreversibly adhere on the polystyrene surface and present a risk of contaminating swimming pools (Bobichhon et al., 2003).

During our investigation it turned out that *C.albicans* was the most resistant as none of the disinfectants removed it completely from the surface although Domestos and P3-Topax 66 decreased the adhered cell numbers on the surface. Like Hawser et al (1994), Hawser and Douglas (1994) and Beczner (2002) demonstrated a high level resistance of the *C. albicans* biofilm, our investigations showed similar results as well. A large variety of disinfectants is available all over the world. The active components of biocide formulations are generally halogens (e.g. sodium hypochlorite), oxidizing agent (e.g. hydrogen peroxide), aldehydes (e.g. formaldehyde), surface-active agents (e.g. quaternary ammonium compounds) and phenols. However, the use of such antimicrobial agents does not completely prevent contamination in medicine and the food industry (Donlan and Costerton, 2002). Our study evaluated: certain disinfectants have destroying effect on the biofilms.

A range of bacterial strains like: *C. albicans, P. aeruginosa, S. aureus, E. coli* were tested to determine the most suitable effective concentration of these disinfectants.

The biofilm formation of the studied microbes was different. *C. albicans* cells strongly attached to the surface and formed biofilm like *S. aureus*. The removal of the *E. coli* cells was quite difficult. There was no significant difference in activity when the biocides were tested against *S. aureus* or MRSA.

The strongest removal effect on the biofilm had Domestos, which except *C. albicans*, removed all the biofilms. From the point of view of the effectively follow P3-Topax 66, Omnicide and in some cases Terralin. The weakest efficiency had Microzid AF.

Comparing the results of both experiments we have observed that the effect of the disinfectants on plankton cells and biofilm cells are different, bacteria included in biofilm are more resistant than their plankton counterparts.

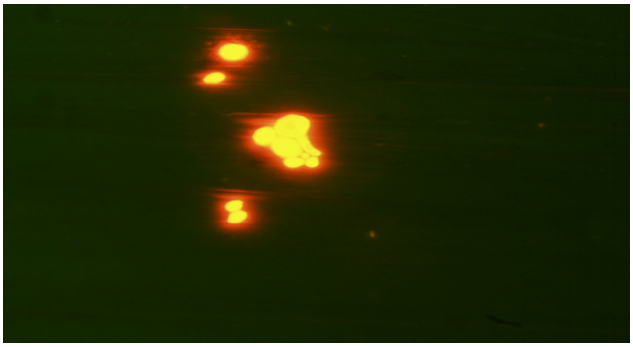

Fig. 1. *C. albicans* biofilm cells after they were treated with Microzid AF (without dilution - time applied 15 minutes)

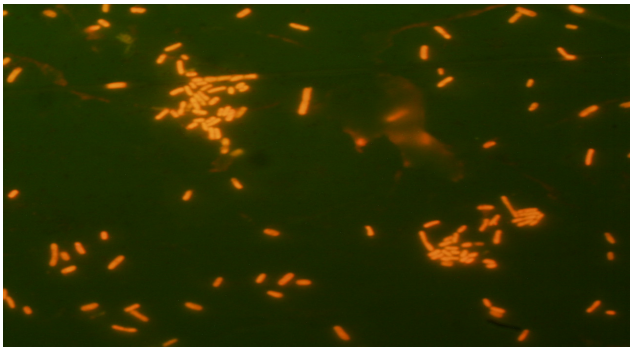

Fig. 2. *E. coli* biofilm cells on stainless steel surface after they were treated with Microzid AF (time applied 15 minutes)

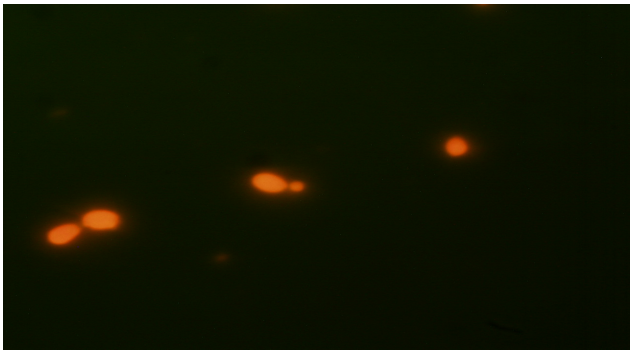

Fig. 3. Cells remained on the surface: *C. albicans* biofilm after a treatment with 0.5% Terralin concentration (time applied: 60 minutes)

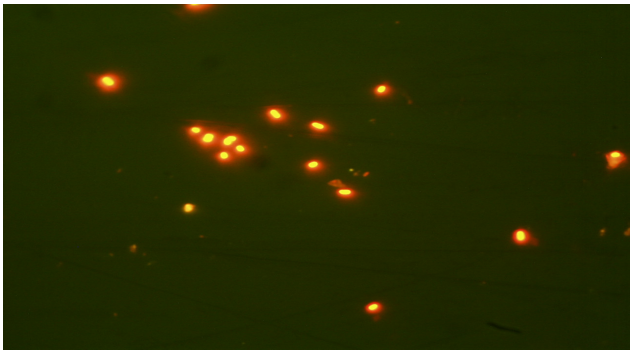

Fig. 4. Cells remained on the surface: *P. mirabilis* biofilm after a treatment with 0.5% Terralin concentration (time applied: 60 minutes)

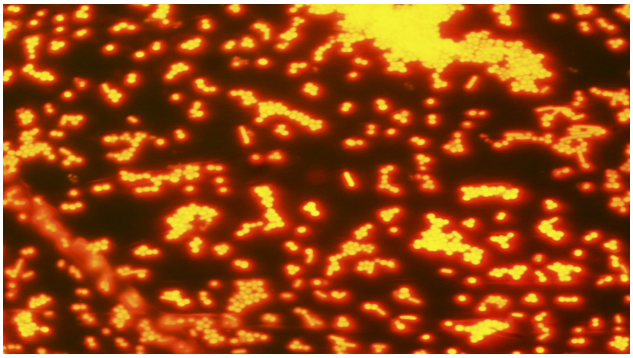

Fig. 5. Cells remained on the surface: *S. aureus* biofilm after a treatment with 0.5% Terralin concentration (time applied: 60 minutes)

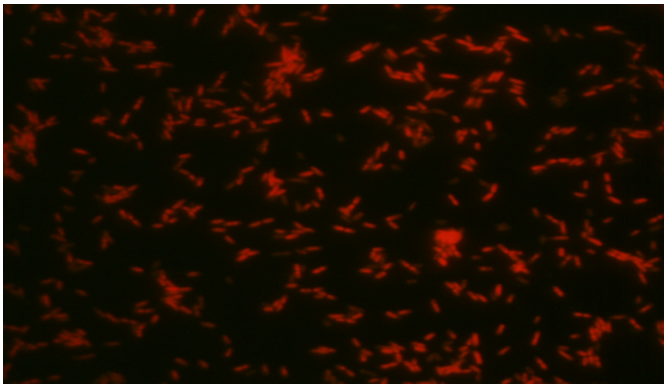

Fig. 6. Cells remained on the surface: *P. aeruginosa* biofilm after a treatment with 0.5% Terralin concentration (time applied: 60 minutes)

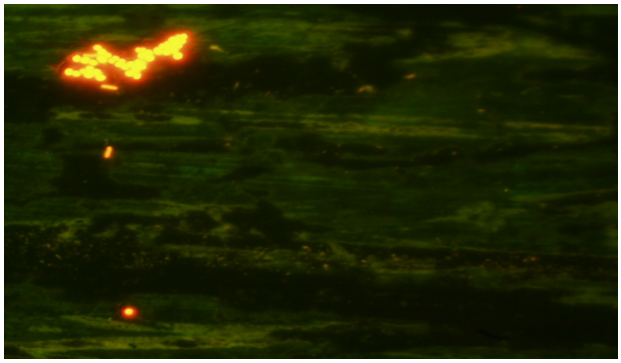

Fig. 7. *S. aureus* biofilm treated with 0.5% Omnicide (time applied:15 minutes)

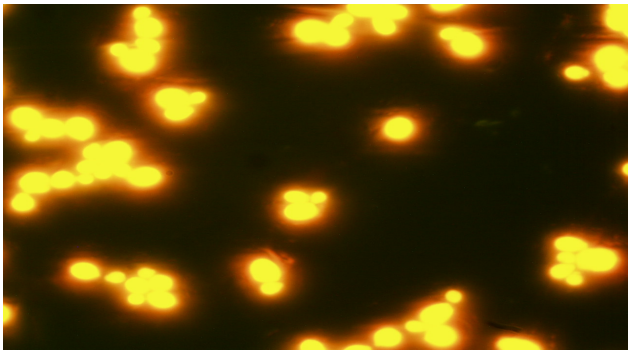

Fig. 8. *C. albicans* biofilm treated with 0, 5% Omnicide (time applied:15 minutes)

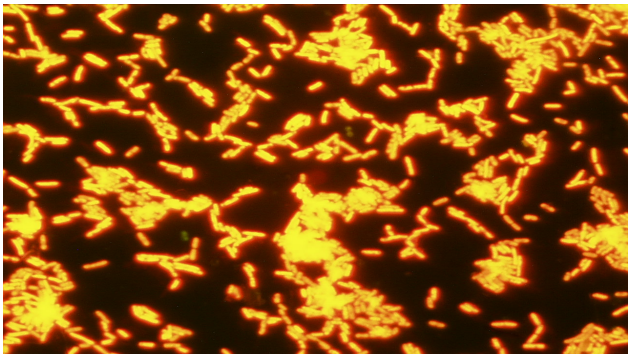

Fig. 9. *E. coli* biofilm treated with 0.5% Omnicide (time applied: 15 minutes)

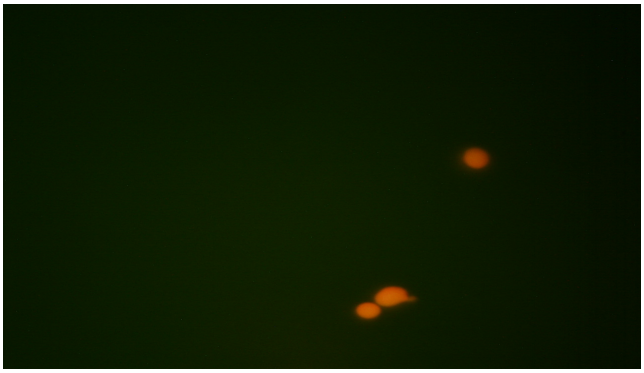

Fig. 10. *C. albicans* biofilm after a treatment with 2% Domestos

Fig. 11. *E. coli* biofilm after a 0.5% P3-Topax treatment

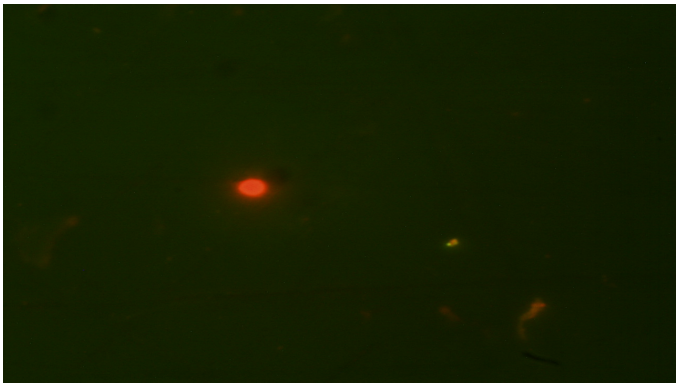

Fig. 12. *C. albicans* biofilm after a 0.5% P3-Topax treatment

The 24 h – biofilm modeled in our experiment has not got a very high resistance, but even in this short time bacteria cells can attach to the surface and form biofilm. The removal of the microbes was not a problem but the remaining organic substance could offer a good culture medium for forming a new biofilm layer. Normally, micro-organisms grow in close association with surfaces and generate microbial biofilms. According to Donlan and Costerton (2002), Cole (2006) one hypothesis explaining the enhanced resistance of bacteria forming biofilm is the presence of an exopolymer matrix. Similarly, LeChevallier et al. (1988) and Stephens (2002) suggested that exopolymers exclude and/or hinder the access of antimicrobial agents to organisms within a biofilm. These experiments suggest that the efficiency of the disinfectants on removing biofilm needs further study.

Conclusions

The effective concentration of these 5 disinfectants varied greatly in vitro, depending on the method of cultures (planktonic/biofilm), and the presence or absence of exopolymers.

Under the study conditions, the concentration needed to destroy the biofilms was increased compared with that for planktonic cells, confirming that bacteria or yeast included in biofilm are more resistant than their planktonic counterparts.

The efficacy of disinfectant is regulated by a complex system of interactions between the test organisms, the practical conditions and the specific disinfectant under evaluation.

REFERENCES

Anonymus (2002) Disinfectants and antiseptics. *Pharmacopeal Forum,* **28** (1), 143-152.

Anwar, H., Strap, J.L., Costerton, J.W. (1992) Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy. *Antimicrob. Agents Chemoter.*, **36,** 1347-1351.

Beczner, J. (2000) Biofilm-A Challenge to the food industry, *Acta Alimentaria*, **30** (4), 329-331.

Bessems, E. (1998) The effect of practical conditions on the efficacy of disinfectants. *J. Biodeterior.*, **41,** 177-183.

Bobichhon, H., Dufour, M.F., Pitor, U. (2003) In vitro susceptibility of public indoor swimming pool fungi to three disinfectants. *Mycoses*, **36** (9-10), 305-311.

Borneff, J. (1988) Untersuchungen zur Keimverbreitung in Haushaltkuechen. I. Problemstellung, Versuche, Ergebnisse. *Zentralblatt Bakteriol. Mikrobiol. Hygienie,* **186** (1), 1-29.

Cole, K., Reipa, V. (2006) Long-term monitoring of biofilm growth and disinfection using a quarty crystal microbalance and reflectance measurements. *J.Microbiol. Methods*, **66,** 449-459.

Donlan, R.M., Costerton, J.W. (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.,* **15,** 167-193.

Hawser, S.P., Douglas, L.J. (1994) Biofilm formation by *Candida* species on the surface of catheter materials in vitro. *J. Medical Microbiol.,* **62** (3), 915-921.

Hawser, S.P., Baillie, G.S., Douglas, L.J. (1994) Production of extracellular matrix by *Candida albicans* biofilms. *J. Medical Microbiol.,* **47** (3), 253-256.

Johansen, C., Falholt, P., Gram, L. (1997) Enzymatic removal and disinfection of bacterial biofilms. *Appl. Environ. Microbiol.,* **63** (9), 3724-3728.

Jelenikné Nikolics, M., Lévai, T. (2000) Biofilmek vízellátó rendszerekben. *Gyógyszerészet*, **44,** 621-624.

LeChevallier, M.W., Cawthon, C.D., Lee, R. (1988) Inactivation of biofilm bacteria. *Appl. Environ. Microbiol.*, **54** (10), 2492-2499.

Luppens, S.B., Reij, M, W., van der Heijden, R.W. (2002) Development of a standard test to assess the resistance of *Staphylococcus aureus* biofilm cells to disinfectants. *Appl. Environ. Microbiol.,* **68,** 4194-4200.

Marshall, K.C. (1994) Biofilms: An overview of bacterial adhesion. Activity, and control at surfaces. *ASM News*, **58** (4), 202-207.

Palmer, Jr.R.J., White, D.C. (1997) Developmental biology of biofilms: implications for treatment and control. *Trends Microbiol., **5,*** 435-440.

Pap, K. (2002) Fertőtlenítő és szanitálószerek mikrobiológiai vizsgálata és validálása, Szent István Egyetem Élelmiszertudományi Kar, diploma munka, 25-35.

Sauer, K., Camper, A.K., Ehrlich, G.D. (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J. Bacteriol.*, **184,** 1140-1154.

Stephens, C. (2002) Microbiology: breaking down biofilms. *Current Biol.,* **12**, R132-134.

Stewart, P.S., Grab L., Diemer J.A. (1998) Analysis of biocide transport limitation in an artificial biofilm system. *J. Appl.Microbiol.,* **85**, 495-500.

Stewart, P.S. (2003) Diffusion in biofilms. *J.Bacteriol.,* **185,** 1485-1491.

Surdeau, N., Bauthors, S., Gelle M.P. (2006) Sensitivity of bacterial biofilms to a new antimicrobial agent, Oxil 320N. *J. Hospital Infection,* **62,** 487-493.

Wilson, J.D. (1986) Validation of Sanitization. In Carleton F.J., Agalloco, J.P. 1986. Validation of Aseptic Pharmaceutical Process. New York, Marcel Dekker, Inc.

*** EN 1276- (1997) Chemical disinfectants and antiseptics-Test method and requirements (phase2, step1). Brussels, European Committee for Standardization.

STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, LIII, 2, 2008, p. 151-153

IN MEMORIAM

Prof. Univ. Dr. *DEACIUC ION*

(17 iunie 1938 – 25 iunie 2008)

Profesorul Ion V. DEACIUC, biochimist, eminent cercetător în domeniul hepatologiei, cadru didactic de excepţie, s-a stins din viaţă la vârsta de 70 de ani.

A văzut lumina zilei în comuna Săcel, judeţul Maramureş, la 17 iunie 1938. După şcoala primară din comuna natală, a urmat cursurile liceale în Sighetu Marmaţiei. Pitorescul Munţilor Maramureşului, natura în forma ei pură, mărginind Izvorul Abastru al Izei, i-au hrănit enorma sete de cunoaştere şi pasiunea pentru tot ce poartă flacăra vieţii. Aşa se face că paşii l-au purtat spre Facultatea de Biologie-Geografie a Universităţii Babeş-Bolyai din Cluj, pe care a absolvit-o în mod strălucit în 1960.

Remarcat fiind de profesorii săi, îndeosebi de academicianul Eugen Pora, a fost angajat după absolvire la Academia Română, filiala Cluj, ca cercetător asociat, în Laboratorul de Fiziologie animală. La scurtă vreme a fost trimis la Institutul de Biochimie din Kiev, Ucraina pentru studii doctorale, susținând teza de doctorat cu titlul *Rolul ciclului acidului citric în biosinteza acizilor graşi la animale*, în 1967.

După întoarcerea în țară, a lucrat, până în 1974, ca cercetător şi apoi ca cercetător principal la Centrul de Cercetări Biologice Cluj, în cadrul Laboratorului de Fiziologie animală.

În anul 1974 a beneficiat de o bursă postdoctorală la University of Pennsylvania, Philadelphia, S.U.A., în cadrul Departamentului de Biochimie şi Biofizică.

Perioada 1975-1982 este marcată de o prodigioasă activitate didactică, în calitate de şef de lucrări, apoi de conferențiar la Facultatea de Biologie, Geografie şi Geologie a Universității Babeş-Bolyai din Cluj-Napoca. În acelaşi timp, a continuat o intensă activitate de cercetare, în laboratoarele de biochimie ale facultății, de a căror dotare şi modernizare a fost în permanență preocupat, aducându-le la standarde internaționale. Ion Deaciuc a fost un adevărat dascăl, de un desăvârşit profesionalism în pregătirea şi predarea cursurilor; informația ştiințifică, mereu actualizată, era prezentată într-o formă accesibilă studenților şi în acelaşi timp cu pasiunea pe care profesorul a pus-o în întreaga sa activitate. A fost foarte apropiat de studenți, un om care ştia să asculte, să ajute, să descrețească frunțile cu o glumă, dar şi să îndrume. În laboratoarele de biochimie era o permanentă forfotă de tineri, veniți să învețe meserie, să-şi facă lucrările de licență, să ceară un sfat sau pur şi simplu să respire câteva minute atmosfera aceea optimistă, de lucru, de neastâmpăr creator. Pentru mulți dintre cei care au avut norocul să-l aibă professor, Ion Deaciuc a fost un model, a cărui imagine s-a păstrat peste ani.

În această perioadă, a publicat un manual de biochimie şi un caiet de lucrări practice pentru studenți, precum şi o carte intitulată *Reglarea celulară a metabolismului glucozei şi acizilor graşi* (Editura Academiei, 1973). În activitatea de cercetare, a studiat mecanismele de reglare a gluconeogenezei şi cetogenezei în ficatul păsărilor şi mamiferelor; a lucrat în colaborare cu cercetători de la Centrul de Cercetări Biologice Cluj, Universitatea de Medicină şi Farmacie Cluj, Universitatea de Ştiințe Agricole şi Medicină Veterinară Cluj, Institutul de Chimie şi Facultatea de Chimie Cluj. De asemenea a activat ca şi consultant al Departamentului de Metabolism de la Boehringer Mannheim GmbH, R.F.G., în probleme legate de agenți hipoglicemici şi inhibitori ai gluconeogenezei. Activitatea ştiințifică desfăşurată între anii 1965-1982 s-a concretizat în 23 de lucrări, publicate în reviste prestigioase.

În anul 1983 a lucrat ca cercetător invitat în cadrul Departamentului de Metabolism de la Boehringer Mannheim GmbH, R.F.G., după care s-a stabilit în Statele Unite, ocupând pe rînd pozițiile de Assistant Professor şi Associate Professor of Physiology la Louisiana State University Medical Center, Department of Physiology, New Orleans, apoi de Associate Professor şi Professor of Medicine la University of

Kentucky, Lexington şi University of Louisville, School of Medicine, Dept. of Medicine, Gastroenterology/Hepatology, Louisville, Kentucky. Deşi provenea din sisteme de învățământ şi de cercetare în multe privinţe diferite, s-a impus repede în noul mediu, prin acelaşi profesionalism, seriozitate şi pasiune pentru ştiinţă. Principalul domeniu de cercetare abordat a fost patologia ficatului alcoolic, aducând contribuţii valoroase la elucidarea mecanismelor steatohepatitei alcoolice şi non-alcoolice, privind participarea apoptozei la debutul şi evoluţia suferinţei hepatice induse de alcool, rolul celulelor endoteliale ale sinusoidelor în comunicarea intercelulară din ficatul alcoolic, sistemul de citokine şi receptori ai acestora în alterarea hepatică determinată de alcool.

Activitatea didactică în universităţile americane a constat din cursuri de Biologie celulară şi moleculară, Biochimie, Bioenergetică şi metabolism, predate studenţilor de nivel licenţă, master şi doctorat. Profesorul a condus numeroase teze doctorale şi a îndrumat în activitate bursieri post-doc. A susţinut peste 25 de conferinţe invitate în universităţi din Statele Unite, India, Japonia şi România şi a publicat, după anul 1983, peste 90 de lucrări ştiinţifice, cărţi şi capitole de carte. Lumea ştiinţifică internaţională l-a perceput ca pe o autoritate de prim rang în domeniul său de cercetare, prezent cu contribuţii valoroase la nenumărate manifestări ştiinţifice ale RSA (*Research Society on Alcoholism*), ISBRA (*International Society for Biomedical Research on Alcoholism*), APS (*American Physiological Society*), AAAS (*American Association for the Advancement of Science*), AASLD (*American Association for the Study of Liver Diseases*).

Cu toate acestea, Profesorul Deaciuc a rămas profund legat de meleagurile natale, revenind periodic în satul copilăriei, dar şi la prietenii, colegii din *Alma Mater Napocensis.* Aducea cu sine de fiecare dată câte o conferinţă – "dare de seamă" despre ultimele rezultate obţinute, dar şi sfaturi competente pentru colegii mai tineri, dorinţa de a-i sprijini în evoluţia profesională. Trecerea anilor nu i-a ştirbit vitalitatea, puterea de muncă, optimismul şi entuziasmul. Se temea doar că ar putea veni o zi în care să nu mai poată intra în laborator, să-şi pună halatul şi să mai caute printre secretele celulelor hepatice.

Deşi plecat de multă vreme din ţară, Profesorul Deaciuc s-a simţit mereu integrat în colectivul de cadre didactice al Facultăţii de Biologie şi Geologie. Personalitatea sa puternică, activitatea ştiinţifică şi didactică de excepţie au marcat fără îndoială evoluţia acestei facultăţi. Colegii, prietenii, foştii studenţi îl vor păstra în memorie aşa cum a fost – împătimit de munca lui, vesel, activ, comunicativ şi în acelaşi timp de o mare sensibilitate, aplecându-se cu sfioasă mirare către toate minunile vii ale lumii acesteia.

Conf. Univ. Dr. Corina Roşioru