ANALELE ŞTIINŢIFICE
ALE
UNIVERSITĂŢII „ALEXANDRU IOAN CUZA”
DIN IAŞI

(SERIE NOUĂ)

SECŢIUNEA II

a. GENETICĂ ŞI BIOLOGIE MOLECULARĂ

TOMUL X, fascicula 3 **2009**

Editura Universităţii „ALEXANDRU IOAN CUZA” Iaşi

EDITORIAL OFFICE

Universitatea "Alexandru Ioan Cuza", Facultatea de BIOLOGIE
Laboratorul de Biochimie şi Biologie Moleculară
Bulevardul Carol I, Nr. 20A, 700506
Iaşi, România
gbmpapers@yahoo.com

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PRELIMINARY STUDIES CONCERNING CHROMOSOME CONSTITUTION OF SOME *CORNUS MAS* L. GENOTYPES (BACAU DISTRICT)

ELENA TRUŢĂ[1*], GABRIELA CĂPRARU[1], CRĂIŢA ROŞU[1], STEFANIA SURDU[1], GOGU GHIORGHIŢĂ[2], ION VIOREL RAŢI[2]

Keywords: Cornelian cherry, chromosomes, karyotype, symmetry

Abstract: The cytogenetic characterization is necessary to decipher the controversies on biogeography, taxonomy and evolution of the genus, evolution of chromosome number. Some of *Cornus* species have the following diploid chromosome numbers: *Cornus suecica* L. 2n=22, *Cornus mas* L. 2n=18, 54, *Cornus sanguinea* L. 2n=22, *Cornus sericea* L. 2n=22, *Cornus alba* 2n=22. Our preliminary observations performed on *Cornus mas* genotypes (Bacau district) confirmed the existence of 2n=18 chromosomes. Karyotypic formula is $2n = 18 = 14m + 4sm$, the centromeres having median and submedian position. Chromosome size ranged between 2.92 – 4.63 microns, with variability limits from 2.86 to 4.71 microns. The length of haploid complement is 33.62 microns. Centromeric index varies from 36.14 to 44.83, while the arm ratio values are comprised in interval 1.23 - 1.77.

INTRODUCTION

The *Cornaceae* family comprises 15 genera, *Cornus* being the most important from these. This genus includes approximately 65 species of great alimentary and pharmacological value, due to the presence of anthocyans with antioxidative and anti-inflammatory properties (Seeram et al., 2002), due to edible oil, ascorbic acid (amount double than in oranges, reported to the fresh weight unit), saccharides, pigments, tannins etc. The cornelian cherry seeds are dried and roasted to obtain a powder that is consumed as coffee substitute. The fruits are utilized to prepare soft drinks, jams, jellies, syrups. Also, they can be used as antidiarrheic and in enteritis treatment, while the bark, stem and roots of Cornelian cherry have antifever effect. The wood, very hard and resistant, but homogenous and elastic, is used in turning works or even in construction of some car components. An old legend says the Cornelian cherry plants have been used to build the Troian Horse. Also, in *Aeneid*, Vergilius noted the utilization of Cornelius cherry wood in spear making. Even the common name of plant comes from its wood hardness (*http://membres.lycos.fr/bouainepa3moine/arbres/arbres.htm*).

This family constituted the object of a big number of studies resorting to modern molecular and cladistic methods in view to evaluate the genetic diversity (Murrell, 1993, Xiang et al., 1996, Caetano-Anolles et al., 1999; Fan and Xiang, 2001, 2003; Xiang et al., 2002; Zhang et al., 2008). The establishment of phylogenetic relationships in *Cornus* genus was possible by methods of molecular genetics allowing the study of DNA profile. For example, the analysis of restriction sites from chloroplast DNA and of some specific regions from DNA (rbcL, matK, 26S rDNA, ITS) led to the elucidation of several controversial aspects concerning the biogeography, taxonomy and genus evolution, number chromosome evolution, morphology and phytochemistry of the reprsentants of *Cornus* genus, and to the characterization and grouping of Cornelian cherry genotypes (Trigiano et al., 2001; Xiang et al., 1996, 2005; Ercisli et al., 2008).

This study is dedicated to the knowledge of diploid chromosome number and of morphological characteristics of mitotic chromosomes in some Romanian provenances of Cornelian cherry, and also to the karyotype construction for the studied genotypes.

MATERIAL AND METHODS

The seedlings whose root meristematic tips were used for chromosome analysis have been obtained by seed germination of respective genotypes. To obtain maximum volume of data on chromosome number and their morphology, several stages must be carried out. As mitotic inhibitor 8-hydroxyquinoline was used. The slides with metaphase somatic chromosomes were prepared by squash method, and analyzed at Nikon Eclipse 600 microscope, the photos being performed with a Cool Pix Nikon digital camera (100x objective, 1600 x 1200 dpi resolution). The images were processed in Adobe Photoshop program. Our measurements included the total length of chromosomes, length of long arm, length of short arm, arm ratio (r = long arm/short arm), centromeric index (Ci = 100 x short arm/total length), arm difference (d = long arm – short arm), relative length of each chromosome (expressed as a percentage of the absolute length of each chromosome pair out of the total length of the chromosome complement).

To establish the chromosome morphological type and for the karyotype construction we resorted to Levan’s nomenclature (Levan et al., 1964). The chromosome pairs were placed in karyotype in descending order of their size.

RESULTS AND DISCUSSIONS

Cornelian cherry is considered as a species with medium genetic variability, which now is not subjected to high risks of genetic erosion. Cultivated varieties do not exist, but few selected wild seedlings or clonally propagated are cultivated in yards, fences of farms or as ornamentals in gardens. The *Cornus* species, like other small trees, have a complex and deep dormancy and do not exist infallible method to surpass this state and to promote the germination even the favourable conditions are assured. In nature, the germination oh this species becomes in the spring of the second of even third year. The seeds must be very soon separated by fruit pulp, because this contains substances inhibiting the germination. Another factor that makes difficult the germination is the "stony" consistency of the seeds. The results dedicated to the attempts to surpass the dormancy state are more or less satisfactory. Řezníček (2007) used the cold stratification of Cornelian cherry seeds, followed by a stage of 5 intervals of cold-warm alternation and an auxin treatment, but well results were obtained only after two years after seed harvesting. Although we immediately made the separation of seeds from pulp fruits and we tried a large scale of treatments to break the seed dormancy (mechanical scarification, chemical scarification with H_2SO_4, on variable time intervals, warm and/or cold stratification, GA3 treatments applied for variable periods), the germination percentage was very low.

According to literature, some of *Cornus* species have the following diploid chromosome numbers (STACE, 1997, cf. *www.floranordica.org*): *Cornus suecica* L. 2n=22, *Cornus mas* L. 2n=18, 54, *Cornus sanguinea* L. 2n=22, *Cornus sericea* L. 2n=22, *Cornus alba* 2n=22. The DNA content is 6.80 pg/2C (Plant DNA C-values database, Royal Botanic Gardens, Kew).

In P6 – Bălăneasa genotype originated in Bacău district, the metaphases displayed 2n=18 chromosomes (fig. 1), number corresponding to that discovered by DUDUKAL, 1984.

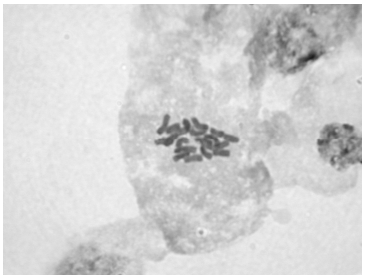

Fig. 1. *Cornus mas* L. – metaphase 2n=18

As shown in Table 1, the chromosomes exhibit average lengths varying from 2.92 to 4.63 μm, the length of haploid complement being 33.62 μm. The values obtained for the average chromosome lengths are very close to those published by Dudukal (1984), respectively 2.9 – 4.6 μm. If this author established the existence of 7 metacentric pairs and 2 acrocentric pairs, our measurement allowed us to classify the chromosomes in 7 metacentric pairs, with median placed centromeres, and 2 submetacentric pairs, with chromosomes having submedian placed centromeres. Therefore, the haploid complement of P6 – Bălăneasa genotype have 7m + 2 sm chromosome formula, respectively the karyotypic formula is 2n = 18 = 14m + 4sm.

Table 1. Average values for somatic chromosome features P6 – Bălăneasa genotype of *Cornus mas* L. (Bacău district) (Length of haploid complement = 33.62 microns)

Chromosome pair	Chromosome type	Total length		Long arm		Short arm		Arm ratio	Arm difference (µm)	Centromeric index	Relative length (%)
		µm	Limits of variability	µm	Limits of variability	µm	Limits of variability				
I	m	4.63	4.55-4.71	2.76	2.72-2.81	1.87	1.83-1.91	1.48	0.89	40.38	13.78
II	m	4.45	4.43-4.47	2.53	2.52-2.54	1.92	1.89-1.95	1.32	0.62	43.08	13.23
III	m	3.96	3.92-4.01	2.22	2.20-2.24	1.74	1.71-1.76	1.27	0.48	43.97	11.78
IV	m	3.91	3.89-3.93	2.41	2.40-2.42	1.50	1.48-1.51	1.61	0.91	38.35	11.62
V	sm	3.71	3.69-3.73	2.37	2.37-2.37	1.84	1.33-1.36	1.77	1.03	36.14	11.04
VI	m	3.47	3.43-3.51	2.17	2.16-2.18	1.30	1.27-1.32	1.67	0.87	37.43	10.32
VII	m	3.44	3.41-3.47	1.90	1.88-1.92	1.54	1.54-1.55	1.23	0.36	44.83	10.23
VIII	m	3.13	3.09-3.17	1.97	1.95-1.98	1.17	1.14-1.19	1.23	0.80	37.21	9.32
IX	sm	2.92	2.86-2.92	1.85	1.83-1.87	1.07	1.03-1.11	1.73	0.78	36.61	8.68

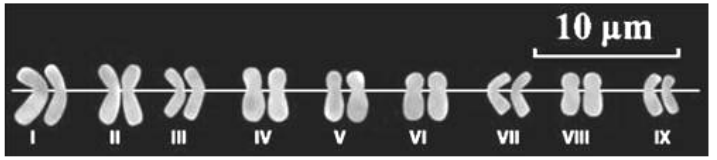

Fig. 2. Karyotype for P6 – Bălăneasa genotype of *Cornus mas* L. (Bacău district)

It must also note that other two chromosome pairs – IV and VI – have arm ratios very close to 1.7 (1.61, respectively 1.67); over this limit, the chromosomes could be classified as submetacentric. No secondary constrictions were found, fact also different by the data published by above mentioned author that evidenced satellite like structures. Generally, the karyotypes with small chromosomes (under 4 microns) and preponderantly metacentric and submetacentric are considered to be symmetrical. A symmetrical karyotype is characterized by the predominance of metacentric and submetacentric chromosomes of approximately the same size. Increasing asymmetry can occur either through the shift of centromere position from median/submedian to terminal/subterminal, or through the accumulation of differences in the relative size between the chromosomes of the complement, thus making the karyotype more heterogeneous (Acosta et al., 2005; Paszko, 2006). These symmetrical karyotypes are probably primitive and show relative chromosome stability, being little evolved – they have not been supported significant rearrangements (Stebbins, 1971). The karyotype increased asymmetry suggests the intervention – during species evolution - of some chromosome recombinational and restructuration events.

CONCLUSIONS

The diploid chromosome number in P6 – Bălăneasa genotype of *Cornus mas* L. (Bacău district) is 2n=18.
The average size of chromosomes ranged between 2.2 – 4.63 µm, and the haploid complement length is 33.62 µm.
The haploid complement of P6 – Bălăneasa genotype have n = 7 m + 2 sm chromosome formula, respectively the karyotypic formula is $2n = 18 = 14$ m + 4 sm.
The karyotype has a high symmetry degree.

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1 – Biological Research Institute Iaşi
2 – University of Sciences, Bacău
* - email: *trutaelena@yahoo.com*

Acknowledgements. The research was supported by project 52-142/15.09.2008 from PNCD II national research program

CYTOGENETIC DAMAGE INDUCED BY MAGNESIUM IN WHEAT ROOT MERISTEMS

ELENA TRUŢĂ[1*], MARIA MAGDALENA ZAMFIRACHE[2], ZENOVIA OLTEANU[2], LĂCRĂMIOARA OPRICĂ[2], RAMONA GALEŞ[2]

Keywords: magnesium, chromosome aberrations, genotoxicity, wheat

Abstract: Like other metals, magnesium can be genotoxic for plants by generation of oxygen free radicals. This metal can induce mitotic alterations – chromosome breaks, achromatic lesions, chromosome aggregations, lagging chromosomes, micronuclei. Mitotic spindle modifications leading to poliploidy and aneuploidy were also evidenced. In this experiment, the effects of magnesium on wheat chromosome material were evaluated. Magnesium was administrated as magnesium sulphate ($MgSO_4.7H_2O$), for 3 hours, in four concentration variants - 1 mM, 25 mM, 50 mM şi 100 mM. The cell division was not significantly modified, comparatively to control, but the ana-telophase aberration frequency surpassed the control in all treated variants (especially in 1mM $MgSO_4.7H_2O$ (26.72%) and 100 mM $MgSO_4.7H_2O$ (22.44%). The most numerous abnormal metaphases were registered in 50 mM treated variant.

INTRODUCTION

Magnesium, like other metals such as cadmium, chromium, zinc, and manganese, may be genotoxic through generation of reactive oxygen species (ROS) (AMORIM et al., 2000). MgSO4 is found in nature as kieserite, MgSO4.H2O, which often accompanies the potassium salts. Some plant studies proved that magnesium sulphate induced mitotic abnormalities such as chromosome breakage, achromatic lesions, chromosome clumping, lagging chromosomes, micronuclei. Spindle abnormalities leading to the formation of polyploidy and aneuploidy were also observed in *Vicia faba* L. (ABRAHAM and RAJALAKSHMY, 1989). Also, in animal experiments, msignificantly more chromosomal abnormalities (terminal deletions, fragments, stickiness) than the situation encountered in respective controls (BELL et al., 1975). In this experiment, magnesium - considered as an essential anti-oxidant macromineral (AL-SHABANAH, 1998), was evaluated for its effects on genetic material of *Triticum aestivum*, in conditions of the seed treatment with 1, 25, 50, and 100 mM $MgSO_4.7H_2O$, for 3 hours.

MATERIAL AND METHOD

Wheat caryopses were maintained for three hours in solutions of 1 mM, 25 mM, 50 mM, and 100 mM $MgSO_4.7H_2O$. The amounts of magnesium in these solutions are: 0.0243 mg Mg/ml, for 1mM, 0.6076 mg Mg/ml, in 25 mM solution, 1.2152 mg Mg/ml, for 50 mM, respectively 2.4305 mg Mg/ml, for the most concentrated tested solution of magnesium sulphate. After treatment, the seeds were placed in Petri dishes, in dark, for germination. The rootlets were fixed in a mixture of absolute ethyl alcohol and glacial acetic acid, in a 3:1 ratio, and then stored in 70% ethyl alcohol, at refrigerator. A modified solution of carbol fuchsin was used for chromosome staining. Five preparations obtained by squash method/variant have been analyzed and 10 microscopic fields / slide were scored, in view of calculus of mitotic index and chromosomal aberrations.

RESULTS AND DISCUSSIONS

Mg^{2+} is the most abundant free divalent cation in the plant cytosol. The functions of Mg^{2+} in plants (as well as in other organisms) are mainly related to its capacity to interact with nucleophilic ligands. Mg^{2+} is essential for the function of many cellular enzymes (RNA polymerases, ATP-ases, protein kinases, phosphatases, glutathione synthase, and carboxylases) and for the aggregation of ribosomes, this metal playing an important role in reactions involved in replication, transcription, translation (Shaul, 2002). The magnesium ions maintain the tertiary structure of transfer RNA and assure the stability of double helicated DNA against thermal denaturation (Watanabe and Iso, 1984). The significance of Mg^{2+} homeostasis has been particularly established with regard to Mg^{2+} role in photosynthesis. It is known the fact that the

magnesium is the central atom of the chlorophyll molecule. At physiologically relevant concentrations, magnesium itself is not genotoxic, but is highly required to maintain genomic stability (Hartwig, 2001).

Concerning influence on wheat cell division (Table 1; Fig. 1), the magnesium treatments not determined significant differences of mitosis intensity comparatively to control. The mean values of mitotic index, expressed in %, ranged between 6.38±0.44 (1mM) and 7.93±0.41 (100 mM), the mean of control being 6.81±0.41. Very slight decreases of mitotic index appeared in 1mM, 25mM, and 50 mM $MgSO_4.7H_2O$ treated variants, while the maximum tested concentration (100 mM $MgSO_4.7H_2O$) exerted a stimulant effect (the number of dividing cells surpassed with 15% the control). It is visible a direct relation between the concentration increase of magnesium sulphate and behaviour of mitotic index. Some authors evidenced mitodepressive effect of magnesium sulphate.

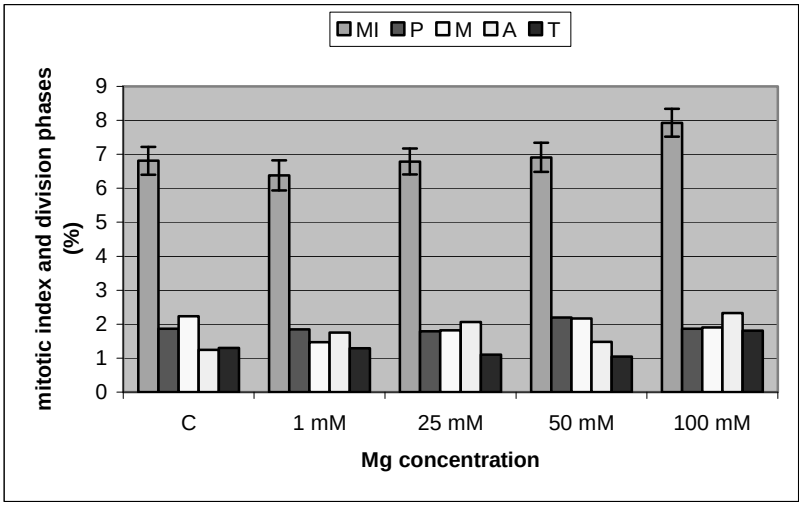

Fig. 1. The behaviour of mitotic index (MI) and preponderance (%) of mitotic phases (P=prophase; M=metaphase; A=anaphase; T=telophase), in wheat root meristems after sulphate magnesium treatment

In 100 mM $MgSO_4.7H_2O$ variant, the increase of mitotic index was realized by ana-telophase (A-T) number increase. In 1 mM $MgSO_4.7H_2O$ treated variant, with the lowest value of this cytogenetic parameter, a depression of metaphase percentage was registered.

Table 1. Variation of mitotic index and preponderance of each mitotic phase after sulphate magnesium treatment of wheat seeds

Variant	Total analyzed cells	Dividing cells		Mitotic index %	Prophases		Metaphases			Anaphases			Telophases		
		Nr.	x±SE		Total	x±SE	%	Total	x±SE	%	Total	x±SE	%	Total	x±SE	%
Control	3295	225	45±4.82	6.81±0.41	67	13.4±1.96	1.87	74	14.8±2.87	2.23	41	8.2±1.24	1.24	43	8.6±1.28	1.30
1 mM	3943	247	49.4±4.09	6.38±0.44	72	14.4±2.95	1.85	57	11.4±1.50	1.47	68	13.6±1.36	1.75	50	10±1.70	1.29
25 mM	2891	197	39.4±3.2	6.79±0.38	52	10.4±0.97	1.79	53	10.6±0.74	1.82	60	12±0.94	2.06	32	6.4±1.5	1.10
50 mM	3083	210	42±3.36	6.91±0.43	67	13.4±1.16	2.20	66	13.2±1.28	2.17	45	9±1.81	1.48	32	6.4±1.28	1.05
100mM	3024	245	51±4.15	7.93±0.41	58	11.6±2.20	1.87	59	11.8±1.90	1.90	72	14.4±0.52	2.33	56	11.2±1.15	1.81

Table 2. Frequency and types of ana-telophase (A-T) chromosome aberrations and of metaphase abnormalities in wheat root meristems, after sulphate magnesium treatment

Variant	A-T	Aberrant A-T		Simple ana-telophase aberrations (%)			Complexe aberrations (%)	Total metaph ases	Abnormal metaphases		Fragmented metaphases (%)	C-metaphases (%)	M with expulsed chromosomes (%)
		% from total A-T	% from dividing cells	bridges	expulsed chromosomes	lagging chromosone s	multipolar ana-telophases			% from total metaphases	% from dividing cells			
Control	84	27.38	10.22	5.77	0.00	2.22	0.44	1.77	74	12.16	4.00	1.33	0.44	2.22
1 mM	118	55.93	26.72	14.57	0.40	4.85	0.80	5.66	57	15.78	3.64	1.21	0.00	2.42
25 mM	92	40.21	18.78	10.15	0.00	5.07	0.00	3.55	53	7.54	2.03	0.00	0.50	1.52
50 mM	77	44.15	16.19	10.47	0.00	1.90	0.47	3.33	66	18.18	5.71	0.00	0.00	5.71
100mM	128	42.96	22.44	11.01	0.40	4.48	0.40	5.71	59	15.25	3.67	0.40	0.00	3.26

The frequency of ana-telophases with chromosome aberrations, expressed in % from total dividing cells, was higher in all experimental variants, comparatively to control (Table 2, Fig. 2). The most numerous aberrations were encountered in 1mM $MgSO_4.7H_2O$ (26.72%) and in 100 mM $MgSO_4.7H_2O$ (22.44%). In literature, some of published data confirm that the lower Mg concentrations led to increased noxious effects (STILWELL and CORUM, 1982). Both in control and in the four magnesium treated variants, the simple chromosome aberrations with the highest incidence were the bridges (Fig. 5), followed by lagging chromosomes (Fig. 3, 6) and multipolar ana-telophases. A high number of complex chromosome aberrations (bridges + expulsed chromosomes, multipolar ana-telophases + bridges, multipolar ana-telophases + bridges + expulsed chromosomes etc.) (Figures 3, 4) were registered in ana-telophases of variants treated with 1mM and 100 mM $MgSO_4.7H_2O$.

The chromosome bridges - connections containing chromosome material remained between the two nuclei – often cause tetraploidizations. The undivided chromosome parts appear to impede the final separation of the two daughter cells. However, not every chromosome bridge leads to a tetraploid cell. Observations over long periods of time showed that many of the partially divided cells with chromosome bridges later completed cell division, although the process was slower compared to cells without chromosome bridges. Concerning the lagging chromosomes at anaphase, they represent a potential source of aneuploidy. After cytokinesis occurs, a lagging chromosome may give rise to a monosomic daughter cell and a trisomic one.

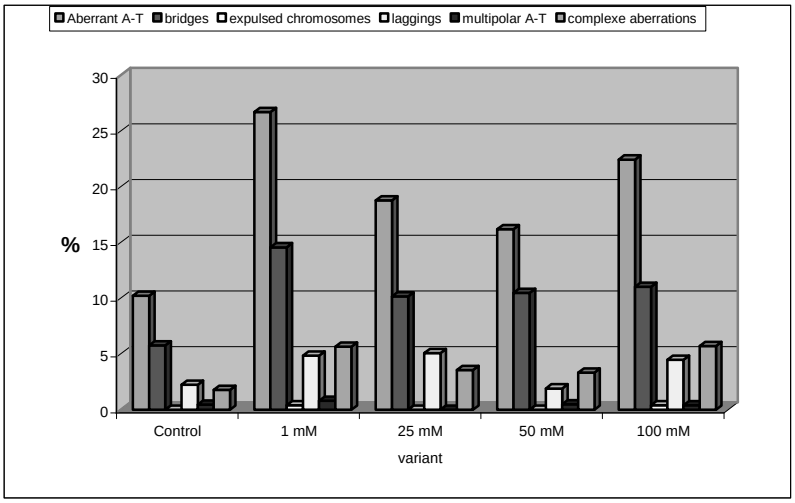

Fig. 2. Frequency and types of ana-telophase (A-T) chromosome aberrations (in %) in wheat root meristems, after sulphate magnesium treatment

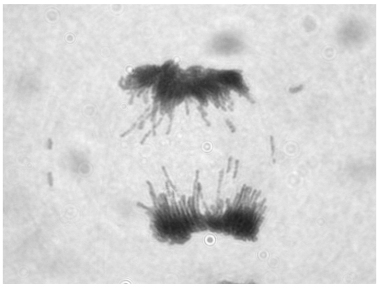

Fig. 3. Anaphase with lagging chromosomes and expulsed chromosomes – 50 mM

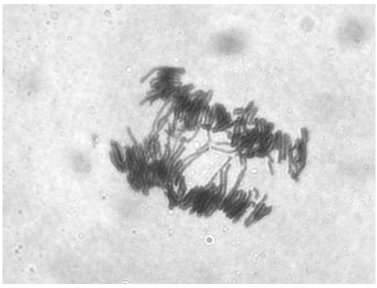

Fig. 4. Multipolar anaphase with bridges and expulsed chromosomes – 100 mM

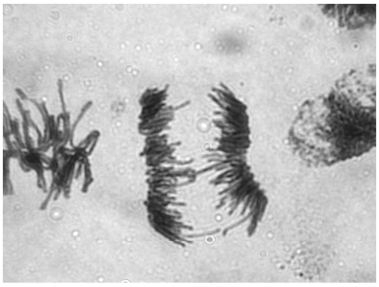

Fig 5. Anaphase with bridges -100 mM

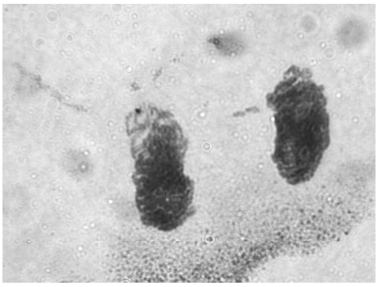

Fig. 6. Telophase with laggard – 100 mM

The metaphases were also influenced by magnesium treatments. The most important number of abnormal metaphases (5.71%) was registered in 50 mM variant. In this case, they consisted only in expulsed chromosomes – potential source for aneuploidy (Fig. 7).

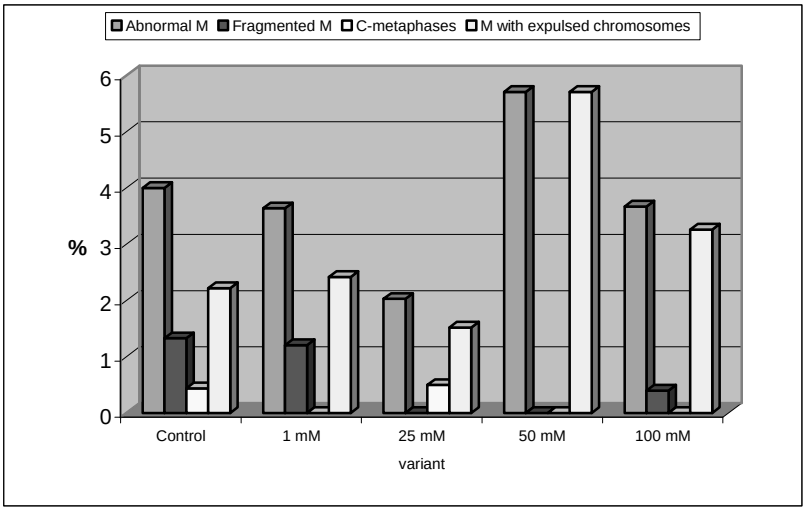

Fig. 7. The incidence (%) of abnormal metaphases (M) and of types of metaphase abnormalities, after sulphate magnesium treatment

The metaphase abnormalities were represented by fragmented metaphases, colchicine-like metaphases (C-metaphases) and metaphases with expulsed chromosomes (Fig. 7, 8).

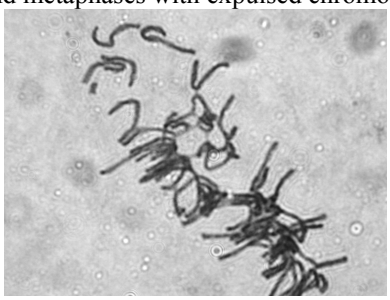

Fig. 8. Metaphase with expulsed chromosomes – 100 mM

Therefore, the results obtained in this experiment confirm the potential of magnesium to damage the genetic material when this metal is administrated at critical concentrations.

CONCLUSIONS

After the treatment of wheat caryopses with sulphate magnesium solutions of different concentrations, they were not evidenced significant differences between treated variants and control concerning the intensity of cell division; only in 100 mM sulphate magnesium treated variant, a stimulation of mitoses with 15% was observed. Very slight decreases of mitotic index appeared in 1mM, 25mM, and 50 mM $MgSO_4.7H_2O$ treated variants

The frequency of ana-telophase chromosome aberrations surpassed that of control in 1mM $MgSO_4.7H_2O$ (26.72%) and 100 mM $MgSO_4.7H_2O$ (22.44%) variants. The chromosome bridges were the most numerous.

The highest number of abnormal metaphases (5.71%) was encountered in 50 mM variant.

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1 – Biological Research Institute Iaşi
2 – University "Alexandru Ioan Cuza", Iaşi, Faculty of Biology
* - *trutaelena@yahoo.com*

SOME CYTOGENETIC EFFECTS INDUCED IN BARLEY BY THE TREATMENTS WITH HYDROALCOHOLIC ROSEMARY EXTRACT

IONELA DACIANA MIERLICI[1*], GOGU GHIORGHITA[2], GABRIELA CAPRARU[3]

Keywords: barley, hydroalcoholic rosemary extract, mitotic index, chromosome aberrations
Abstract: The paper presents some results regarding the cytogenetic effects induced in barley by the treatments with rosemary extract (*Rosmarinus officinalis)*. It was proved that the treatment with this extract upon the barley caryopses had as effect the stimulation of the mitotic division in the radicle apex of the species and did not lead to important cytogenetic changes (chromosome aberrations) in the ana-thelophase of the radicle mitoses.

INTRODUCTION

Rosemary (*Rosmarinus officinalis)*, an evergreen shrub, is one of the herb spices of the family *Labiatae*. It is used for flavoring food and as a beverage, as well as in cosmetics. In folk medicine it is used as an antispasmodic in renal colic and dysmenorrhea, in relieving respiratoty disorders and in stimulating the growth of hair.

The composition of rosemary extracts is quite complex, and many components have been identified, including phenolic acids, diterpenes such as rosmanol and carnosol derivates, and flavonoids, in particular, flavones (Gerhardt et al., 1983; Aeschbach et al., 1986, Brieskorn et al., 1973; Cuvelier et al., 1994; Fieski et al., 1989; Nakatani, 1989).

Rosemary extract relaxes the smooth muscles of the trachea and intestine and has choleretic, hepatoprotective and antitumerogenic activity (Al Sereiti et. al., 1999; Newal et al., 2002; Albu et al., 2004). These effects can be related to its high content of phenolic compounds like caffeic acid derivates such as rosmarinic acid. Phenolic compounds are secondary plant metabolites that have long been associated with flavor and colour characteristics of fruits and vegetables. These compounds attract great interest due to their postulated health-protecting properties, foremost to their antioxidative effect, manifested by the ability to scavenge free radicals or to prevent oxidation of low density lipoprotein (Newal et al., 2002, Miliauskas et al., 2004; Albu et al., 2004).

The antioxidant activity of rosemary extracts depends on their composition. There are many reports that determined their antioxidant capacity by various methods using lipid and aqueous systems. In lipid systems, extracts with higher diterpene content were the most effective (Hopia *et al.*, 1996), while in aqueous systems rosmarinic acid exhibited the highest antioxidant activity (Frankel *et al.*, 1996; Cuvelier *et al.*, 2000). Several reports have been published about the distribution of rosmarinic and/or carnosic acids during growth and vegetative development of rosemary leaves (Hidalgo *et al.*, 1998; Munné-Bosch *et al.*, 1999; Munné-Bosch and Alegre, 2000).

The use of the extract from rosemary leaves as an antioxidant was first reported by Ostric-Matijasevic in 1955 (Rac & Ostric-Matijasevic, 1955). There are several data evince the rosemary extracts action on the delay of the fats oxidation (Berner et al., 1973; Chang et al., 1977, Braco et al., 1981; Tateo et al., 1988; Wu J.W. et al., 1982). Several phenolic compounds with antioxidant activities have been isolated and identified from rosemary leaves: carnosol, rosemanol, carnosic acid and rosemaridiphenol (Brieskorn et al., 1969; Löliger et al., 1989; Schuler et al., 1990; Lamaison et al., 1991).

Starting from the aboved informations, we investigated some cytogenetic effects induced by the treatments with hydroalcoholic rosemary extracts in barley.

MATERIAL AND METHODS

As biological materials, barley caryopses have been used (*Hordeum vulgare* L, 2n=14), *Madalin* cultivar, from S.C.A. Podu Iloaie, 2006 . The seeds have been treated with hydroalcoholic rosemary extract (HRE) of 39%. Various dillutions of this extracts were used (0,10%, 0,25%; 0,50% and 1%) for 6 and 12 hours treatment of the seeds. At each experimental variant 25 caryopses have been treated. The control variant consisted in seed immersion, for the same period, in distilled water. After the treatment, the seeds were washed in sewerage water, and germinated in Petri dishes (covered by filter paper imbued in distilled water) and kept in the thermostate at 22ºC.

Some cytogenetic investigations have been made on radicular meristemes collected from the germinated seeds. These consisted of observations upon the development of the mitotic cycle and of the identification of possible chromosome aberrations. For the cytogenetic observations, roots (lenght: 10-15mm) were detached of the caryopses and fixed by immersion for 20 hours in absolute ethilic alcohol: icy acetic acid (3:1), at the room temperature. Roots were then hydrolized and colored by Feulgen method , (Tudose, 1993).

For the completion of the microscopic preparations the squash technique was used. Microscopic semipermanent (by assembly into glicerine) and permanent preparations (by icluding them into Canada balsam) were used.

The results obtained are presented in Tables 1-4.

RESULTS AND DISCUSSIONS

1. Effects of the 6 hour treatment with hydroalcoholic rosemary extract at barley

1.1. Mitotic index and the frequency of the mitotic division phases

Treating barley caryopses (*Mădălin* cultivar), with alcoholic rosemary extract, for 6 hours, led to changes in frequency of the cells in mitosis. At small concentrations (0.10% and 0.25% HRE) a stimulation of the division took place as compared to the control plants, emphasized by the increase in the mitotic index (MI). According as the increase in the extract concentration, the total amount of the cells in division decreased, the lowest value of the MI being registered at the 0.50% variant, (Table 1).

It seems that the values of the MI mainly influenced by the frequency of the cells in prophase, existing an obvious relationship between these 2 parameters. This way, at the variants (0.10% and 0.20% EHR) wherein MI has high values (7.03 and 8.23%) there is also a higher frequency of the cells in prophase (4.53 and 4.77%), as well as for higher concentrations of HRE (0.50 and 1.0%) where the MI has values of 5.49 and 6.55%, and the frequency of the cells in prophase is lower, of 2.99 and 3.76%. The action of the rosemary extract seems to have been especially on the cells in initial phases of the mitotic division. Though there are some changes in the frequency of the other phases of division too, these are less important. We might observe though only the effect of the 0.25% HRE solution which led to an increase in the frequency of the cells in metaphase (1.41%, as compared to 1.02% at the control) and in the telophase (1.34%, as compared to 0.63% at the control), (Table 1).

Table no. 1. Frequency of the cells in mitotic division from the barley radicle apex after the 6 hour treatment with hydroalcoholic rosemary extract

Variant	Total no of analyzed cells	Total cells in division		Total cells in prophase		Total cells în metaphase		Total cells in anaphase		Total cells in telophase	
	Nr.	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%
M	6363	395	6.21	250	3.93	65	1.02	40	0.63	40	0.63
0.10%	7037	495	7.03	319	4.53	67	0.95	32	0.45	77	1.09
0.25%	5891	485	8.23	281	4.77	93	1.41	42	0.71	79	1.34
0.50%	6916	380	5.49	207	2.99	57	0.82	42	0.61	74	1.07
1.00%	6962	456	6.55	262	3.76	82	1.18	45	0.65	67	0.96

1.2. Frequency of the cells with chromosome aberrations

Treatments with hydroalcoholic rosemary extracts, regardles the concentration, led to the increase in the frequency of the cells with chromosome aberrations (tab. 2). There hasn't been an obvious relationship between the EHR concentration and the frequency of the aberrant cells. This way, while at the 0,10% EHR soncentration there was the highest percentage of aberrant cells (4,24%), at a higher concentration (of 0,25%) we see an obvious decrease of this percentage (only 1,86%). Treatments with 0,50 and 1,0% solutions caused a level of the chromosome aberrations between the two already mentioned (being of 3,16 and 3,51%). As for the spectrum of these chromosome aberrations, the majority was formed of the multiple pole anathelophases (AT), metaphases with expelled chromosomes and C-metaphases (tab. 2).

Table no.2. Frequency and types of aberrant cells in the mytosis of the barley radicle meristemes after the 6 hour treatment with hydroalcoholic rosemary extract

Variant	Cells in division	Cells with aberrations		Aberrant cells with: Multiple bridges		AT with delayed chromosomes		AT with expelled chromosomes		Multiple pole AT		Polar deviation		Metaphases with expelled chromosomes		C-metaphases		Multiple pole AT and multiple bridges	
		Nr.	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	%
M	395	0	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0.00
0.10%	495	21	0	0.00	0	0.00	0	0.00	7	1.41	2	0.40	5	1.01	7	1.41	0	0.00	1.41
0.25%	485	9	2	0.41	0	0.00	0	0.00	1	0.21	3	0.62	0	0.00	2	0.41	1	0.21	0.41
0.50%	380	12	1	0.26	0	0.00	0	0.00	8	2.11	0	0.00	2	0.53	0	0.00	1	0.26	0.00
1.00%	456	16	1	0.22	0	0.00	0	0.00	10	2.19	1	0.22	1	0.22	2	0.44	1	0.22	0.44

2. Effects of the 12 hour treatment with hydroalcoholic rosemary extract at barley

2.1. Mitotic index and the frequency of the mitotic division phases

In the case of prolonging the treatment period with hydroalcoholic rosemary extracts to 12 hours there could also be observed a stimulation of the cell division at the level of the radicle meristemes, and this led to the increase in the MI values. Up to the 0.50% HRE level, this effect was pro ratio to the HRE concentration, and after this, it had a lower influence on the cell division. This way, between 0.10 and 0.50% HRE the MI values were between 8.86 and 10.45%, as compared to 7.62% at the control plants, and then they came back to a value similar to the control one (7.99%), at the concentration of 1.0% HRE. Together with the increase in the treatment period it takes place an intensification of the division process in the barley radicle apex cells, pro ratio to the increase in the extract concentration, except of the 1.0% variant, where this parameter has lower values.

The maximum mythogen effect took place at 0.50% EHR concentrations, (10.45% as compared to 7.62% at the control), (Table 3). The increase in the MI values was completed, at least in the case of two of the HRE concentrations used by the increase of the cell frequency from the prophase (5.39% and 7.10% at the 0.10 and 0.50% EHR concentrations, as compared to 4.47% at the control). On the other hand, at the 0.25% HRE treatment, the increase of the MI value was completed based on the cells from metaphase, whose frequency was of 2.97%, as compared to 1.35% at the control. As for the frequency of the cells from various phases of division, the highest percentage was of the cells in prophase, followed by the ones in metaphase, telophase and then in anaphase (Table 3).

Table no. 3. Frequency of the cells in mitotic division from the barley radicle apex after the 12 hour treatment with alcoholic rosemary extract

Version	**Total no of analyzed cells**	**Total cells in division**		**Total cells in prophase**		**Total cells in metaphase**		**Total cells in anaphase**		**Total cells in telophase**	
	Nr.	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**
M	6312	481	7.62	282	4.47	85	1.35	49	0.78	65	1.03
0.10%	6322	560	8.86	341	5.39	81	1.28	45	0.71	93	1.47
0.25%	6365	627	9.85	274	4.30	198	2.97	70	1.10	94	1.48
0.50%	5933	620	10.45	421	7.10	100	1.61	37	0.62	62	1.05
1.00%	6573	525	7.99	309	4.70	96	1.46	52	0.79	68	1.03

2.2. The frequency of the cells with chromosome aberrations

What is interesting is the fact that the prolongation of the treatment period with EHR solutions did not led to the intensification of the cytogenetic disorders too. As it comes out of Table 4, though at the variants subject to HRE treatment - 12 hours ore a higher frequency of the chromosome aberrations in the mitosis of the radicle meristemes than that of the tests was registered (0,89 – 1,52%, as compared to 0,00% at the test), this frequency is though lower than at the plants coming from seed treated for 6 hours. At the same time, there wasn't observed a certain relationship between the EHR concentration used and the frequency of the aberrant cells.

The main types of aberrant cells observed were: the cells with simple and multiple bridges, anathelophase with expelled chromosomes and/or delayed chromosomes, as well as cells with complex aberrations (with lower frequency), represented by the multiple polar anathelophases and with multiple bridges, (tab. 4).

Table no.4. Frequency and types of aberrant cells in the mytosis of the barley radicle meristemes after the 12 hour treatment with alcoholic rosemary extract

Variant	Cells in division	Cells with aberrations		Aberrant cells with:															
				Simple bridges		Multiple bridges		AT with delayed chromosomes		AT with expelled chromosomes		Multiple pole AT		Polar deviation		C-mitosis		Multiple pole A-T and multiple bridges	
		Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%
M	481	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
0.10%	560	5	0.89	0	0.00	0	0.00	0	0.00	0	0.00	4	0.71	1	0.18	0	0.00	0	0.00
0.25%	627	9	1.44	2	0.32	0	0.00	1	0.16	2	0.32	0	0.00	1	0.16	0	0.00	3	0.48
0.50%	620	6	0.97	1	0.16	2	0.32	1	0.16	0	0.00	2	0.32	0	0.00	0	0.00	0	0.00
1.00%	525	8	1.52	1	0.19	3	0.57	0	0.00	0	0.00	4	0.76	0	0.00	0	0.00	0	0.00

CONCLUSIONS

Our investigations show that treatments with hydroalcoholic rosemary extracts (EHR) treatments, at concentrations of 0,10 – 0,50%, had a contribution to the intensification of the mitotic activity in radicle barley meristemes (*Mădălin* cultivar), regardless the treatment period (6 or 12 hours). The increase of the mitotic index values was especially completed based on the cells in prophase. This effect was expressed best at the 0,50% EHR treatment, for 12 hours. Generally speaking, EHR treatments did not obviously influence the percentage distribution of the cells in various phases of mitosis, the highest frequency having cells in prophase, and the lowest in anaphase.

Treatments with hydroalcoholic rosemary extract did not lead to an important increase of the cells with chromosome aberrations in the mitosis of the barley radicle meristemes. Between aberrations, the most frequent were the anathelophases with delayed and expelled chromosomes, multiple pole anathelophases. The results obteind determine to apreciated that the HRE treatment has mytogen , but non-mutagenic effect.

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1. SC CCPPM PLANTAVOREL SA, Piatra Neamt
2. University of Bacău, Dept. of Biology. Academy of Romanian Scientist
3. University “Alexandru Ioan Cuza”, Iasi, Faculty of Biology
* ionela_daciana@yahoo.com

CYTOGENETIC EFFECTS INDUCED BY DEPOSIT MYCOFLORA IN *VICIA FABA* BEANS FROM THE COLLECTION OF SUCEAVA GENEBANK

DIANA BATÎR-RUSU[1], CRISTIAN TUDOSE [2*]

Keywords: *Vicia faba* beans, deposit mycoflora, cytogenetics
Abstract: The purpose of our work was to study the cytogenetic effects induced by deposit mycoflora in *Vicia faba* (2n = 12) beans from the collection of Suceava Genebank. Cytogenetic effects were studied by means of classical plant chromosomes methods of study. We have observed that the values of the mitotic indexes are decreasing in accordance with the increasing of the storage age and also with the number of mycoflora species that are infesting the beans. Infestation with specific mycoflora produced a relatively large number, statistically significant by comparison to the controls, of interphasic aberrations and also of chromosomal aberrations in mitosis ana-telophase in all studied probes. Comparing our results with similar published data, we can strongly state that the cytogenetic effects induced by deposit mycoflora in *Vicia faba* seeds are similar with those produced by the action of a weak mutagenic agent

INTRODUCTION

The purpose of our work is to study the cytogenetic effects induced by deposit mycoflora in various species seeds from the collection of Suceava Genebank. This paper shows the cytogenetic effects induced by deposit mycoflora in *Vicia faba* (2n = 12) beans from the above mentioned collection. Cytogenetic effects were studied by means of: calculation of the mitotic index and frequency of mitosis phases, registration of the frequency and types of abnormal interphases and identification of chromosomal aberrations in mitotic ana-telophases, in accordance with classical plant cytogenetics methods.

MATERIALS AND METHODS

The biological material consisted of five probes of beans selected from the collection of Suceava Genebank:

- The control: SVGB-273, 23 years old, germination ratio 100%, not infested by mycoflora,
- SVGB – 272, 18 years old, germination ratio 92%, infested by *Penicillium* sp.
- SVGB – 212, 20 years old, germination ratio 92%, infested by *Penicillium* sp.
- SVGB – 300, 20 years old, germination ratio 83%, infested by *Rhizopus nigricans* and *Colletotrichum lindemuthianum*
- SVGB – 271, 23 years old, germination ratio 87%, infested by *Penicillium* sp. and *Aspergillus flavus*

The beans germinated in Petri dishes, on filter paper, wetted with distilled water for all variants till the roots reached 10-15 mm in length. We calculated the germination percent and the duration of germination for all experimental variants. When the roots reached the length of 10-15 mm they were fixed in Battaglia for 30 minutes. We have performed fast cytological slides according to Feulgen method (Cimpeanu at al., 2002).
We have studied:

1. The mitotic index and frequency of mitosis phases
2. The frequency and types of aberrant interphases
3. The frequency and types of chromosomal aberrations in mitotic ana-telophases (A-T).

We have analysed this 3 steps on 5 fresh preparations. For each slide we have studied: 10 microscopic fields (objective 40x) on which we have counted all the cells in interphase, prophase, metaphase, anaphase and telophase; 10 microscopic fields on which were counted all the cells in normal and abnormal interphases and the type of interphasic aberrations and 50 ana-telophases were analysed, counting the normal, aberrant ana-telophases and the type of chromosomal aberrations. All data were statistically processed (Fowler and Cohen, 1990). Microphotographs were performed with the digital camera of Nikon research microscope with the 100X objective.

RESULTS AND DISCUSSIONS

The infestation with fungi generally determined a significant decrease of the mitotic index (Table 1). Only for the SVGB-272 probe the frequency of dividing cells is 6,45%, a very close value to

the mitotic index registered for the control (6,47%) (fig. 1). The lowest value of the mitotic index (1,69%) was observed for the SVGB-271 probe .

The values of the mitotic index are proportionally decreasing with the increase of the number of infesting species of fungi and with the storage age of beans.

For the majority of probes, and also for the controls, we have recorded a predominance of prophases, followed by metaphases and telophases. Anaphases were recorded only for controls, but in a very small percentage (Table 1 and Figure 2). An exception was noted for the probe SVGB-271 where the frequency of prophases was low and metaphases were predominant. The frequency of prophases is decreasing proportionally with the increase of infestation and of the storage age of the beans (Fig. 2). The frequency of telophases is decreased for all probes in comparison to the controls. An exception was noted for the SVGB-272 probes in which we have registered a high frequency of telophases in comparison to the controls.

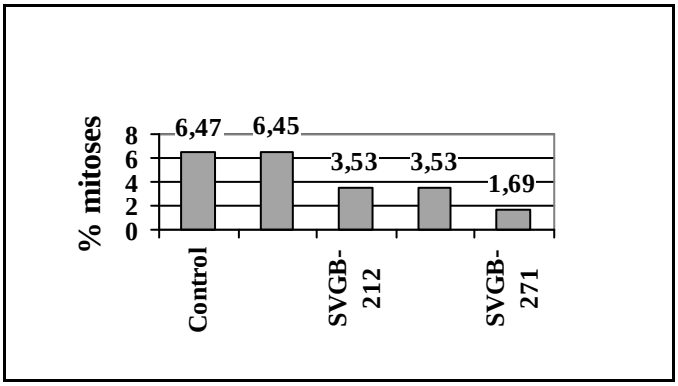

Fig. 1: Mitotic index in *Vicia faba* studied probes

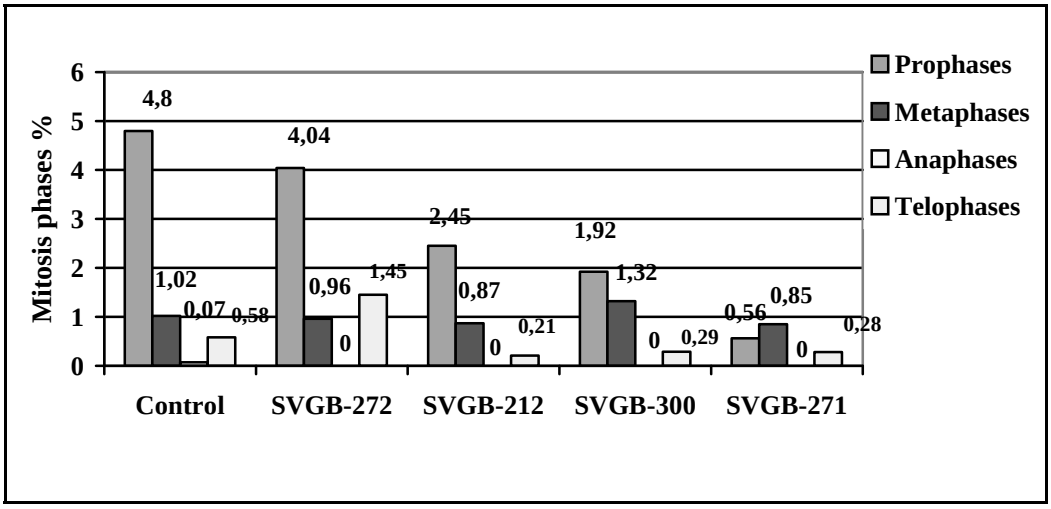

Fig. 2: The frequency of mitosis phases in *Vicia faba* studied probes

Infestation with fungi induced the increase of interphasic aberrations in all the studied probes (Table 2 and Fig. 3).

The highest frequency of aberrant interphases (1,71%) was registered for SVGB-272 probe. For the other probes the registered values are similar and quite low, but, in comparison to the very small value of aberrant interphases counted in the control probes (0,03%), we can state that the infestation with mycoflora induced a statistically significant increase of anaphase aberrations, without regard to the type of fungi or to the age of probes.

The most frequent interphasic aberration is represented by the binucleated cells, which frequency is increasing proportionally with the age of probes and with the number of infesting mycoflora species (Fig. 4).

In SVGB-272 probe the infesting mycoflora induced some interphases with one micronucleus (Fig. 5 and 6) and, in a very low percentage, interphases with two micronuclei. Analysing all the mentioned data in comparison with data from literature (Maniu et al. 2002, 2005) we can strongly state that the cytogenetic effects induced by deposit mycoflora in *Vicia faba* beans are similar with those produced by the action of a weak mutagenic agent.

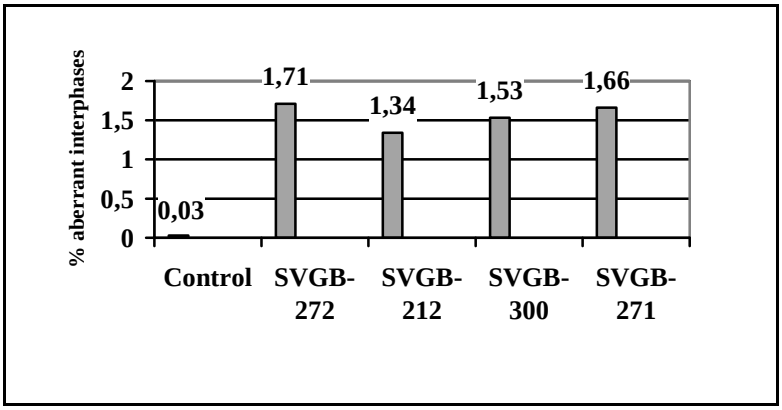

Fig. 3: Total frequencies of aberrant interphases in *Vicia faba* studied probes

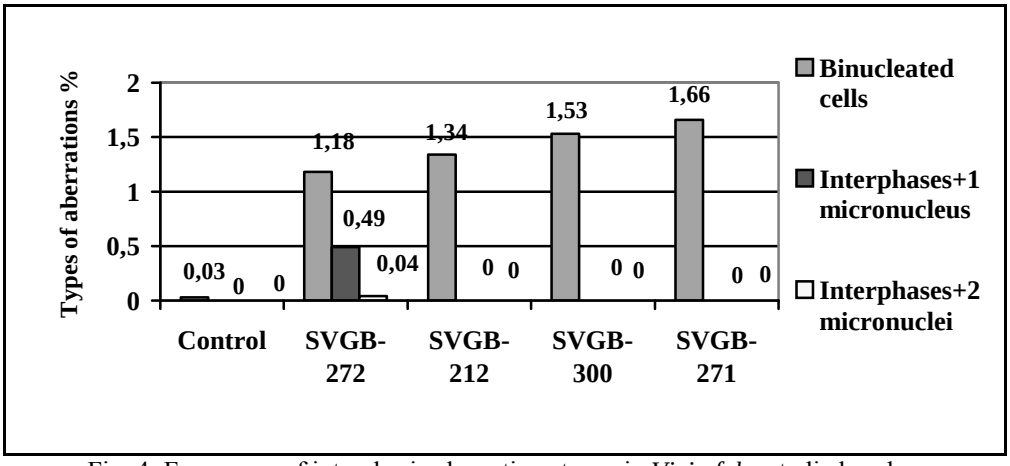

Fig. 4: Frequency of interphasic aberrations types in *Vicia faba* studied probes

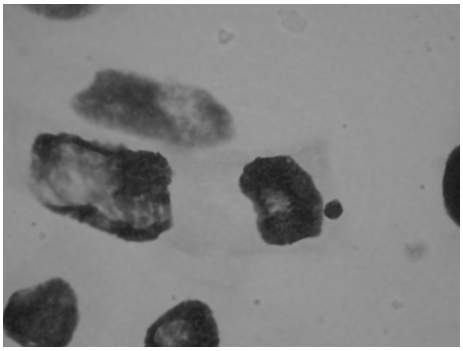

Fig. 5: Interphase with one micronucleus (SGVB-300 probe)

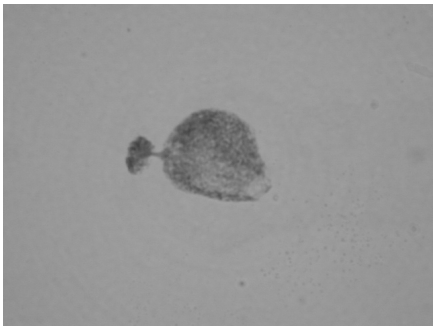

Fig.6: Chromatic continuity between nucleus and micronucleus in interphase (SVGB-271 probe)

Analysing the data from table 3 one can observe that both for the control, SVGB-272 and SVGB-271 probes were registered chromosomal aberrations in mitosis ana-telophase. In the rest of two probes the number of observed ana-telophases was too small to perform statistic analysis.
The highest percentage of aberrant ana-telophases (5,67%) was observed for the SVGB-271probe (Fig. 7). The chromosomal aberrations types show a great variety, being distributed randomly (Fig.8).

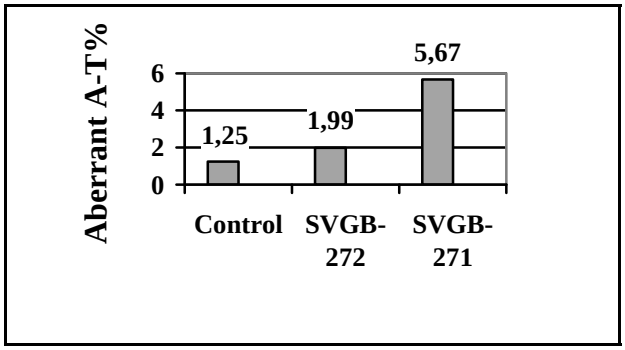

Fig. 7: Total frequency of aberrant ana-telophases in *Vicia faba* studied probes

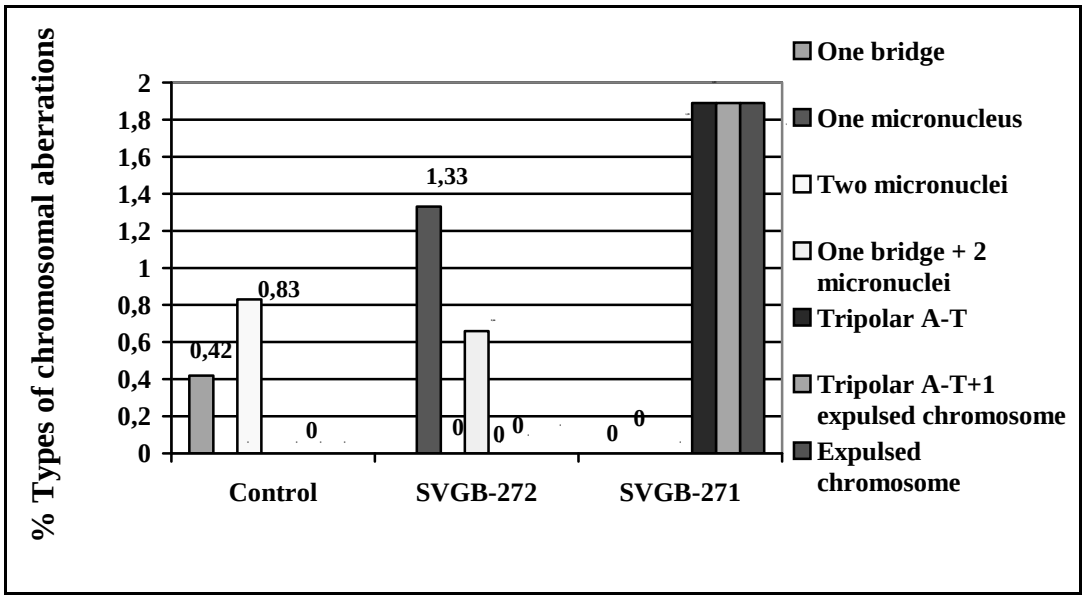

Fig. 8: Frequency of chromosomal A-T aberrations in *Vicia faba* studied probes

We have observed and counted many simple chromosomal aberrations such as: one bridge, one micronucleus, two micronuclei, tripolar ana-telophases, expulsed chromosomes and some complex chromosomal aberrations such as: one bridge with two micronuclei, tripolar A-T with expulsed chromosomes, etc. (fig. 9-14). The highest frequency was registered for tripolar ana-telophases and expulsed chromosomes.

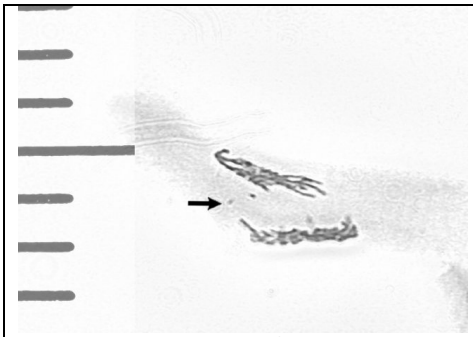

Fig.9: Ana-telophase with 2 fragments (SVGB-272)

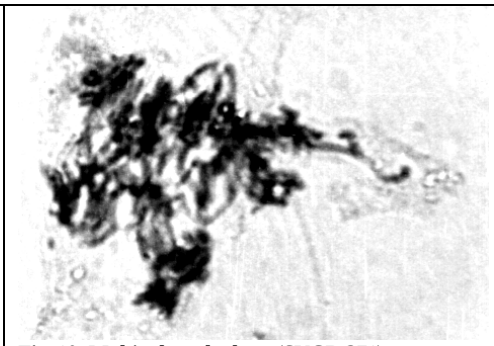

Fig. 10: Multipolar telophase (SVGB-271)

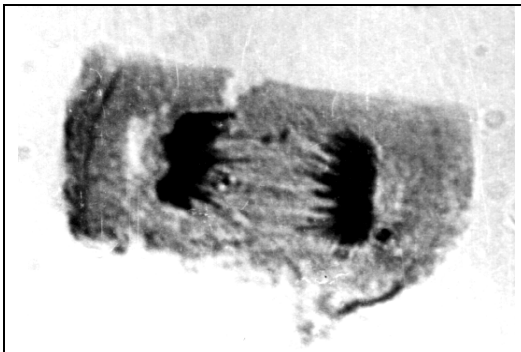

Fig.11:Ana-telophase with fragments (SVGB-300)

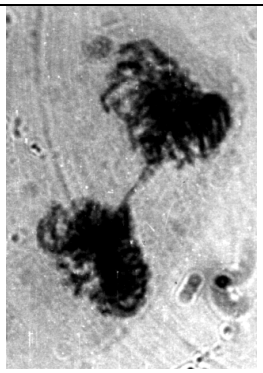

Fig.12: Ana-telophase with a bridge (SVGB-271)

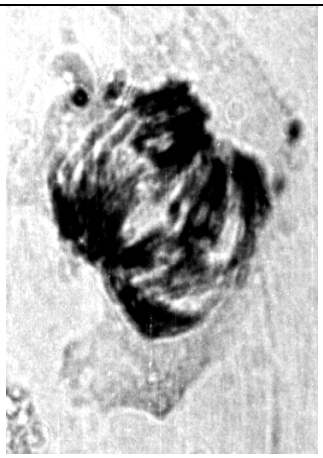

Fig. 13: Hexapolar ana-telophase (SVGB-272)

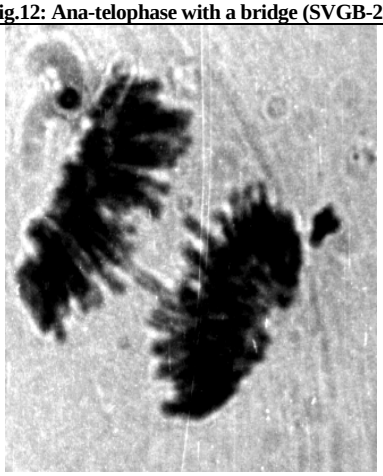

Fig. 14: Pentapolar ana-telophase with a bridge and expulsed chromosomes (SVGB-300)

The increase of the number of aberrations in ana-telophase is statistically significant reported to the controls; comparing our results with similar published data (Maniu et al. 2002, 2005) we can strongly state that the cytogenetic effects induced by deposit mycoflora in *Vicia faba* beans are similar with those produced by the action of a weak mutagenic agent.

CONCLUSIONS

The values of the mitotic indexes are decreasing in accordance with the increasing of the storage age and also with the number of mycoflora species that are infesting the beans.

Infestation with specific mycoflora produced a relatively large number, statistically significant by comparison to the controls, of interphasic aberrations and also of chromosomal aberrations in mitosis ana-telophase in all studied probes.

Comparing our results with similar published data, we can strongly state that the cytogenetic effects induced by deposit mycoflora in *Vicia faba* seeds are similar with those produced by the action of a weak mutagenic agent.

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1 – Suceava Genebank, Collecting Department
2 – University “Al.I.Cuza” Iaşi, Faculty of Biology, Discipline of Genetics
* cristian.tudose@uaic.ro

Table 1: Frequency of mitoses and of mitosis phases in *Vicia faba* studied probes

Probes	Total studied cells	Total interphases		Total mitoses		Total prophases		Total metaphases		Total anaphases		Total telophases	
	Nr.	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%
Control	2753	2575	93,53	178	6,47	132	4,8	28	1,02	2	0,07	16	0,58
SVGB-272	3318	3104	93,55	214	6,45	134	4,04	32	0,96	-	-	48	1,45
SVGB-212	2859	2758	96,47	101	3,53	70	2,45	25	0,87	-	-	6	0,21
SVGB-300	3029	2922	96,47	107	3,53	58	1,92	40	1,32	-	-	9	0,29
SVGB-271	2842	2794	98,31	48	1,69	16	0,56	24	0,85	-	-	8	0,28

Table 2: Frequency of interphasic aberrations in *Vicia faba* studied probes

Probes	Total studied cells	Total normal interphases		Total aberrant cells			*Aberration types*					
							Binucleated cells		Interphases with one micronucleus		Interphases with two micronuclei	
	Nr.	Nr.	%	Nr.	%	$\pm s\overline{x}$	Nr.	%	Nr.	%	Nr.	%
Control	2941	2940	99,97	1	0,03	0,0006	1	0,03	-	-	-	-
SVGB-272	3045	2993	98,29	52	1,71	0,032	36	1,18	15	0,49	1	0,04
SVGB-212	3511	3464	98,66	47	1,34	0,003	47	1,34	-	-	-	-
SVGB-300	3270	3220	98,47	50	1,53	0,004	50	1,53	-	-	-	-
SVGB-271	3133	3081	98,34	52	1,66	0,004	52	1,66	-	-	-	-

Table 3: Frequency of chromosomal aberrations in mitosis ana-telophase (A-T) in *Vicia faba* studied probes

Probes	Studied A-T	Total normal A-T		Total abnormal A-T			Types of chromosomal aberrations in mitosis A-T													
							One bridge		One micro-nucleus		Two micro-nuclei		One bridge + 2 micro-nuclei		Tri-polar A-T		Tripolar A-T with expulsed chromosome		Expulsed chromosome	
	Nr	Nr	%	Nr	%	$\pm s\overline{x}$	Nr	%	Nr	%	Nr	%	Nr	%	Nr	%	Nr	%	Nr	%
Control	239	236	98,75	3	1,25	0,046	1	0,42	-	-	2	0,83	-	-	-	-	-	-	-	-
SVGB-272	151	148	98,01	3	1,99	0,092	-	-	2	1,33	-	-	1	0,66	-	-	-	-	-	-
SVGB-271	53	50	94,33	3	5,67	0,44	-	-	-	-	-	-	-	-	1	1,89	1	1,89	1	1,89

THE INFLUENCE OF THE ATTACK OF THE FUNGUS *MELAMPSORELLA CARYOPHYLLACEARUM* (DC.) J. SCHRÖT. (“WITCH BROOMS” ON FIR) ON THE PEROXIDASE AND CATALASE ACTIVITY IN HOST PLANT

ALEXANDRU MANOLIU[1*], ROMEO IRIMIA[2], PETRONELA GRĂDINARIU[1], EUGEN UNGUREANU [3]

Keywords: *Melampsorella caryophyllacearum*, fir, peroxidase, catalase activity
Abstract: In this paper are presented the results concerning the influence of the attack of the fungus *Melampsorella caryophyllacearum* (“witch brooms“ on fir) on the peroxidase and catalase activity in host plant. The research were effectued in year 2007 in the stationary Izvorul Muntelui and Cerebuc from Ceahlău massif and the obtained results highlights the fact that those two biochemical parameters were influenced differently by the attack of the parasitic fungus.

INTRODUCTION

Melampsorella caryophyllacearum is an obliged parasitic fungus, belonging to *Basidiomycotina*, *Uredinomycetes*, *Uredinalles*, *Pucciniastraceae*. It is a hetero - macrociclical euforme species (0+I=II+III), with two alternative hosts: 0 – spermogons (pycnidium), I – aecidium on *Abies* and *Picea species*, and II – uredosorus and III - teleutosorus on *Cerastium* and *Stellaria* species. The attack on coniferous caused characteristic formations, named “witch brooms” determinated by the fungus penetrations in the meristematic tissues, causing an active growth of supernumerary gemmae. These gemmae grow and form new and elastic shoots upward, arranged in fascicles as brooms, with reduced leaves, soft and yellow, who are falling slightly; “witch brooms” can resist several years (over to 20) because they have their own cambium, they grow every year and may reach over 1m high. To the starting place of the “witch brooms”, on strain as on the branches grows “broom rust”, formations in the shape of cask, who keeps ticking and after the “brooms” disappearing. In the broom rust, the wood suffers structure modifications, the year rings are bigger, the tracheal route is irregular, the wood is solid, splits up heavily, became breakable and crack irregular; the bark next to the broom rust falls, developing the wood, permitting the penetration of other fungi especially firs tinder, who produces woods rot (Cojocaru D.C.).

In Romania, this fungus is very frequent, a synthesis of dates being published relatively recent by Vera Bontea (Bontea Vera).

Because of damages produced by this species, were made by a lot of researches, especially to a world level, from whom we mention those concerning the structure of the fungus haustorium (Berndt R. et al.), the management of the factors who influence the occurrence of this disease (Solla A. et al.), the damages produced in different countries (Merriee W. et al., Nicolotti G. et al.), biochemical changes produced in the host plant (Solla A. et al., Yamada T. et al.).

In the Biological Research Institute Iasi, carried out somes complex researches about the physiological and biochemical reactivity of plants to the attack of some phytopatogen agents (Antohe Anca et al., Jurca Valentina et al., Manoliu Al. et al., Merriee W. et al., Pisică - Donose Alice et al., Roşu Crăiţa – Maria). In this paper it presents the influence of the attack of the fungus *Melapsorella caryophyllacearum* on the peroxidase and catalase activity on fir, comparatively with the healthy plant.

MATERIALS AND METHODS

The research were performed during the year 2007 and they have permited the monitoring of the biochemical parameters (peroxidase and catalase activity) in the host plant healthy and parasited by the *Melampsorella caryophyllacearum*. The samples were gathered from the stationary Izvorul Muntelui and Cerebuc , Ceahlau massif. The working material for the determination of peroxidase and catalase activity it constituted from vegetative organs (diseased and healthy leaves). To determine peroxidase and catalase activity was used the iodometrical method (Cojocaru D.C.).

RESULTS AND DISCUSSIONS

Peroxidase (EC 1.11.1.7) is an enzyme involved in several metabolism processes from plants, being one of the best studied plant enzymes.

Peroxidase participates directly in the resistance mechanism of the plants against the pathogens agents, being considered a biochemical marker wich can be used for preventing the disease resistance, the general tendency found being of enzymatic level growth with the evolution of symptoms (Roşu Crăiţa – Maria).

Our results concerning the influence of *Melampsorella caryophyllacearum* attack on peroxidase activity are presented in figure 1, from which occurs that in both stationary, this biochemical marker presented higher values in the diseased leaves comparatively with healthy leaves.

So, in the Izvorul Muntelui stationary the activity of peroxidase was 0.1232 UP/g min. in diseased leaves and 0.0308 UP/g min. in healthy leaves; in Cerebuc stationary the activity of peroxidase had the following values: 0.0295 UP/g min. in the diseased leaves and 0.0164 UP/g min. in healthy leaves. Those dates confirm the obtained results from other authors concerning the plants reactivity to the actions of phytopatogens agents.

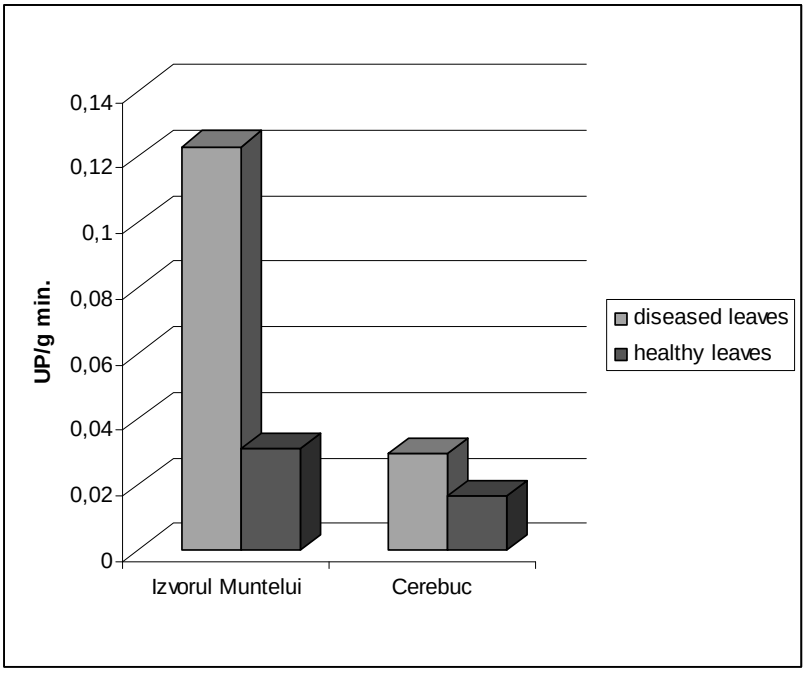

Fig. 1. The influence of *Melampsorella caryophyllacearum* attack on peroxidase activity

Catalase is an enzyme with heteroproteical structure, who's actions is coupled with many other biochemical reactions of the metabolism, which explains the presence of this enzyme in the living cell. The enzyme catalyzes, the degradation of hydrogen peroxide reaction in the living cell, removing the toxic effect of the hydrogen peroxide resulted after redox processes in a cellular level. Until now exists few works concerning the catalases role in the host plant –

parasite relationship. Also the researches made to the patho-system *Beta vulgaris – Cercospora beticola* have emphasized that there are fluctuations depending on the catalase activity with the appearance of the first symptoms of disease (Roşu Crăiţa – Maria).

The data concerning the attack of the *Melampsorella caryoplhyllacearum* on the catalyze activity are presented in figure 2, from which resulted that in the Izvorul Muntelui stationary this enzyme had the value 65.60 UC mg/min. in the diseased leaves and 70.00 UC mg/min. in the healthy leaves; in the Cerebuc stationary the value of this enzyme had been of 60.80 UC mg/min. in the diseased leaves and 85.70 UC mg/min. in the healthy leaves.

These results don’t confirm the obtained dates after the researches made to other species of plants (Antohe Anca et al., Pisică - Donose Alice et al.).

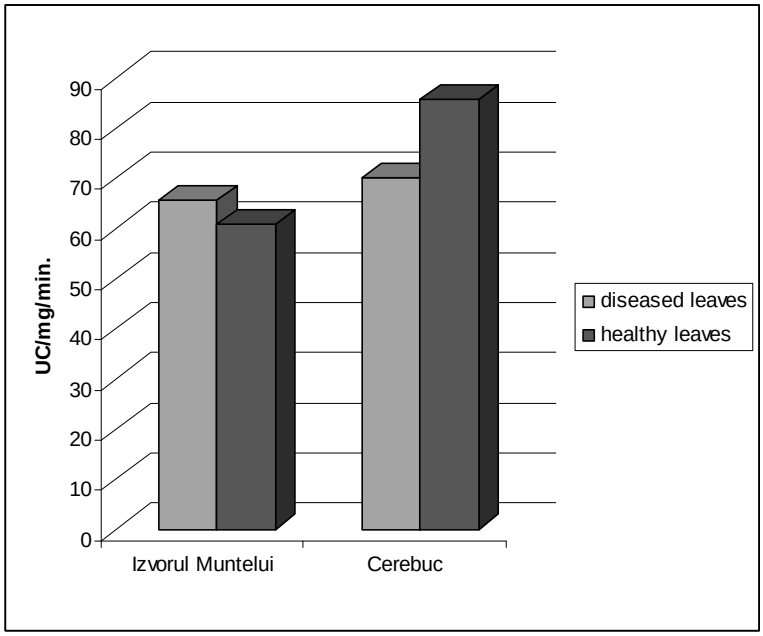

Fig. 2. The influence of *Melampsorella caryophyllacearum* attack on catalase activity

CONCLUSIONS

The attack of *Melampsorella caryoplhyllacearum* determined a growth of peroxidase activity in diseased leaves, comparatively in healthy leaves in both stationeries taken in the study.

Instead the attack of *Melampsorella caryoplhyllacearum* a diminution of ctalase activity in diseased leaves, comparatively in healthy leaves in both stationeries taken in the study.

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1 Biological Research Institute Iasi
2 Ocolul Silvic Oituz, Bacău
3 „Alexandru Ioan Cuza" University of Iasi, Faculty of Biology
* alexandru.manoliu@uaic.ro

PRELIMINARY STUDY CONCERNING THE RADIOFREQUENCY ELECTROMAGNETIC FIELD INFLUENCE ON THE CATALASE ACTIVITY IN THE HIPPOPHAE RHAMNOIDES SEEDS GERMINATION

CĂLIN LUCIAN MANIU[1*], EUGEN UNGUREANU[1], SMARANDA VÂNTU[1], IGOR CREŢESCU[2]

Keywords: electromagnetic field, oxidative stress, reactive oxygen species, catalase.
Abstract. The data accumulated by now shows that the topic of biological effects of electromagnetic radiation is far from being exhausted. Is undoubtedly that a non-ionizing radiation field maintained on a biological entity has some effects on it. To try shaping issues regarding this, this work aims to study the impact of radiation generated by an emission-reception radio station that emits on 462.6875 MHz frequency. For this purpose, were used *Hippophae rhamnoides* L. seeds which germinated in the laboratory, under controlled conditions, concentrically arranged around the radiation source, in which case electromagnetic radiation has a different impact. Seed germination lasted 35 days, while the device has continuously worked, and the seeds were constantly irradiated. The intensity of the magnetic component of the field was precisely measured in all places where the seeds were placed for germination. It was calculated the percentage of germination and it was determined the catalase activity involved in eliminating the oxidative stress effects. Accordingly to the distance from the source, significant variations of the parameters mentioned above in conjunction with the radiation intensity were found.

INTRODUCTION

Accelerated and widespread use of different electric and electronic devices increased the exposure to radio and microwave frequency electromagnetic fields (EMFs). These EMFs are classified as non-ionizing radiation but they can cause damage depending on the power level, frequency, and the properties of exposed tissue. There is some evidence that microwaves (300 MHz–300 GHz) produces changes in the cell membrane's permeability and cell growth rate as well as interference with ions and organic molecules, like proteins (Kwee et al., 1998, 2001; de Pomerai et al., 2003; Repacholi, 2001; Pologea-Moraru et al., 2002; Banik et al., 2003). Plants are essential components of a healthy ecosystem and have important role in the living world as main primary producers of food and oxygen; therefore it would be beneficial to investigate their interaction with today's increased exposure to radio and microwave frequency fields. Additionally, higher plants are useful test organisms for environmental studies because they are eukaryotic multicellular organisms. Many of them are sensitive to different kinds of stresses and are easy to grow in controlled laboratory conditions without too much expense (Wang, 1991). During the years it became more and more interesting to test the effects of EMFs on higher plants (Tkalec et al. 2005, 2007). Considering the increasing interest for the subject, this work focus on the influence of 462.6875 Mhz EMF on the oxidative stress during the *Hippophae rhamnoides* seeds germination. This species was chosen because of the following aspects. Firstly, the period of germination is relatively long, the experiment is held over a period of 35 days, this issue was important because the seeds were irradiated for a long time, unlike other species that germinate very fast (3-5 days). Secondly, sea buckthorn (*Hippophae rhamnoides* L.) is a species which has some interesting biochemical characteristics: vitamins B, C, E, K, carotenoids (the most dominant carotenoid in sea buckthorn, it's admitted to be associated with reduced risk of breast, stomach, esophageal, and pancreatic cancers), flavonoids (it have been found in controlling arteriosclerosis, reducing cholesterol level, turning hyperthyroidism into euthyroidism and eliminating inflammation), tannins, metallothionein (acts as detoxifying agency for heavy metals and as free radical scavenger for most toxic radical) and 5-hydroxytryptamine (5-HT), a chemical neurotransmitter substances (Lian, 2000; Thomas, 2003).

MATERIALS AND METHODS

To seek evidence of the influence of electromagnetic field (EMF) of radio frequency on oxidative stress, during the germination of seeds, was used a source consisting of two Motorola T5725 emission-reception radio stations that have been programmed to automatically call one another throughout experiment. The communication system frequency is set on channel 6 at 462.6875 MHz with 500mW transmit power. Thus, around the two emission-reception radio stations were delimitated four concentrically levels (different distances from the source), with four groups with five Petri Dishes (A1-A5; B1-B5; C1-C5; D1-D5), in each plate with about 20 seeds. The control lots, consisted in six Petri Dishes (M1-M6), were positioned sufficiently far from the EMF source. It was monitored the temperature and the humidity, which were

maintained constant in both irradiated and control lots. Experiment diagram is depicted in the Appendix fig.1 and fig.2. The magnetic induction (B) of the field was measured with a digital teslameter in the indicated points on the drawing. The values are in μT. After germination period (35 days), the plant material was processed to determine the activity of the catalase, enzyme involved in the removal of oxidative stress (Artenie et al., 2008). Also it was determined the total protein synthesis and was calculated the percentage number of the germinated seeds. From each sample was counted the number of germinated seeds and reported to the total number of seeds. Data were represented graphically in the diagrams (Appendix at the end of the paper) which appear after the statistical processing. On the charts, the vertical error bars shows the 95% (0.05) confidence level for mean. Interval estimates are often desirable because the estimate of the mean varies from sample to sample. The interval estimate gives an indication of how much uncertainty there is in our estimate of the true mean. The narrower the interval, the more precise is our estimate (Kotz et al., 1988-2008).

RESULTS AND DISCUSSION

After investigations, it was obtained a number of results regarding the catalytic activity of catalase. Catalase is an enzyme present in large quantity in peroxisomes where neutralize H_2O_2 resulting from redox processes.

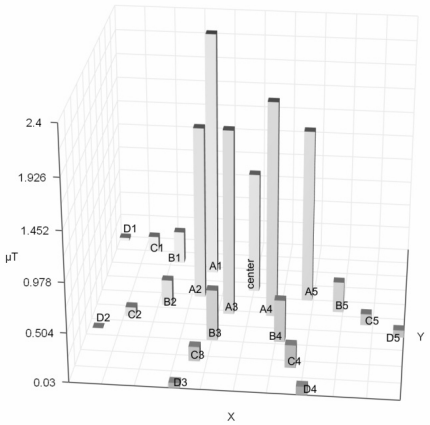

Fig.1. Spatial representation of the intensity of magnetic induction (μT) in relation to the probes arrangement.

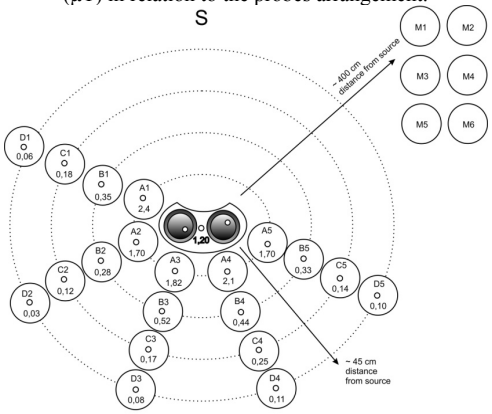

Fig.2. Schematic representation of the experiment. At the center are the two radio stations, around which were arranged the Petri Dishes. In the center of each plate is indicated the magnetic induction in μT.

Peroxisomes are a common constituent of eukaryotic cells. In plants there are two important differentiated forms: the leaf peroxisomes, which participate in photorespiration and the glyoxysomes, which are present in seeds containing oils (triacylglycerols) and play a role in the conversion of triacylglycerols to carbohydrates. They contain all the enzymes for fatty acid β-oxidation (Heldt, 2005). The experiment conducted, shows a significant decrease in the catalase activity in relation to the control. The profile of these decrease is similar in both activity expressed in enzyme units at 100g material and for specific activity (enzyme units per 100 mg protein) as is illustrated by the fig. 3 and fig. 4.

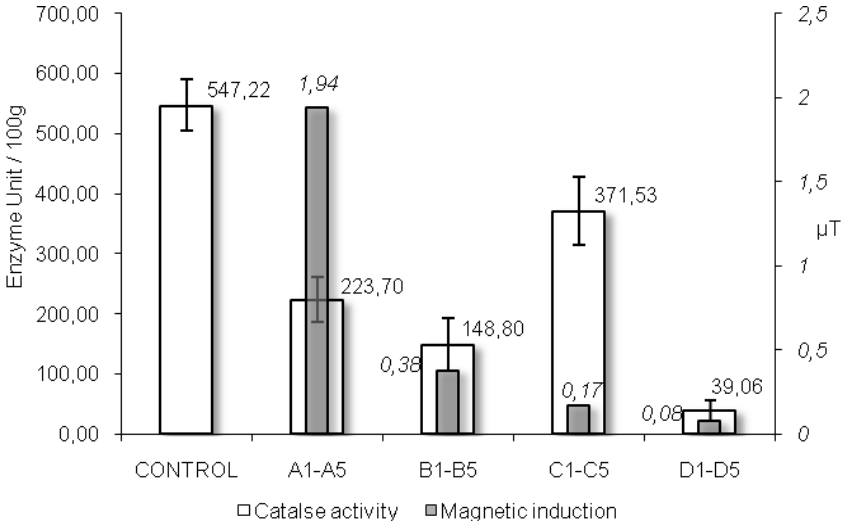

Fig.3. Variation of the catalase activity and magnetic induction.

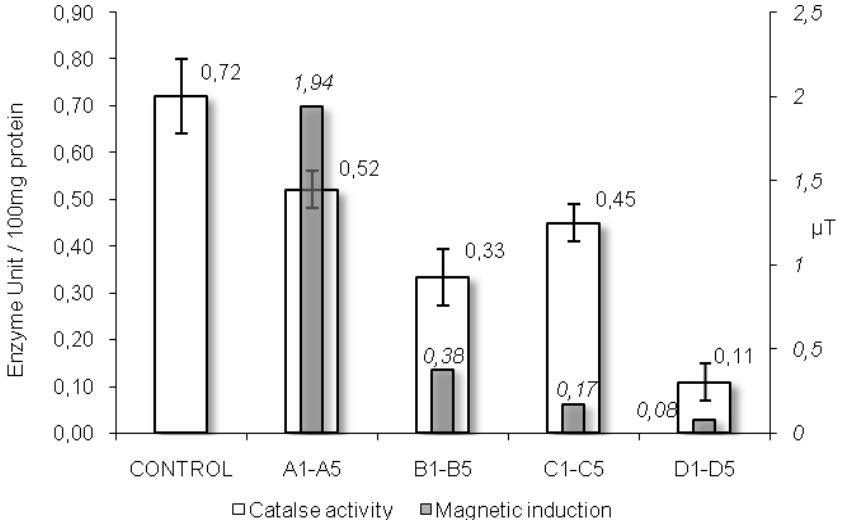

Fig.4. Variation of the catalase specific activity and magnetic induction.

The amount of protein highlighted by Bradford method shows a significant variation for A1-A5, B1-B5 and D1-D5 in relation to the control (fig. 5), in such cases were founded decreases. C1-C5 samples, shows an amount of protein approximately equal to control lots. The proteins highlighted in the experiment comes both from the reserve proteins of seeds and "de novo" synthesis.

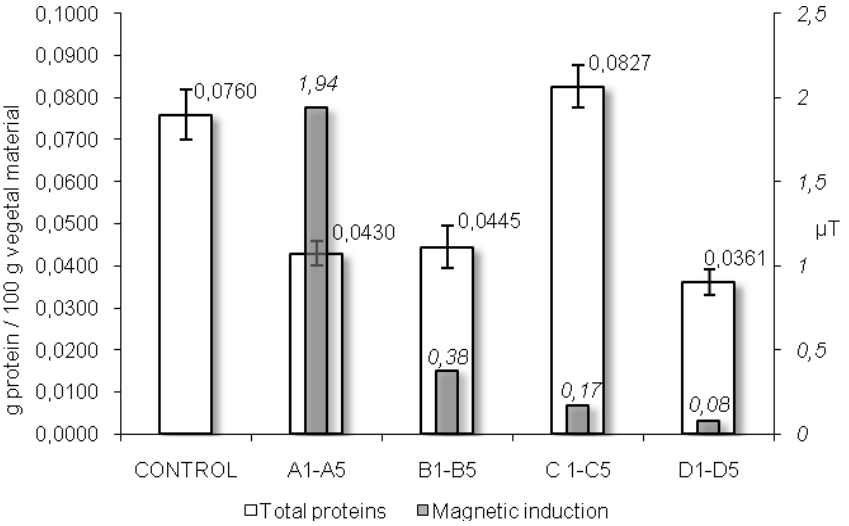

Fig.5. Variation of the total protein quantity and magnetic induction.

Percentage of seeds germinated (fig. 6) during the experiment indicates that low intensity magnetic induction can have a stimulating effect. This is observed for samples B1-B5 and D1-D5, where the percentage of germination reached very high values, 97% respectively 94%.

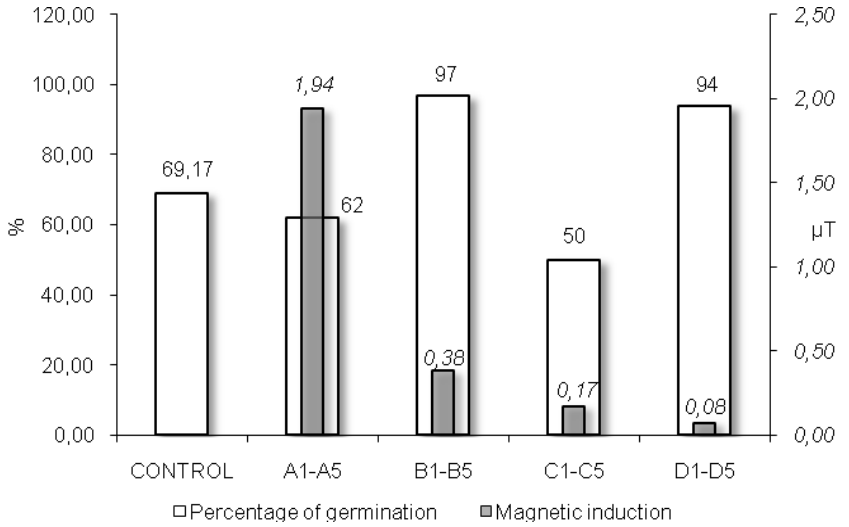

Fig.6. The percentage of seeds germination and the variation of magnetic induction

In the other two cases, compared with the control, there is a negative trend in germination. In case of A1-A5, high intensity magnetic induction does not seem to have affected germination (percentage difference being only 8% compared to control), where C1-C5 can be considered a decrease of 19%. From germination behavior of toward various intensities of magnetic induction, it may find a correlation between enzyme activities involved in removing the effects of oxidative stress. In cases B1-B5 and D1-D5, a high percentage of germination is correlated with a decreased oxidative stress, due to a weak catalase activity, correlate with a low amount of total protein. Low values identified in the total proteins quantity may be due to a greater consumption of resources during germination where samples B1-B5 and D1-D5 had the highest percentage of germination.

CONCLUSIONS

The performed experiment, with 462.6875 MHz electromagnetic radiation frequency, obtained from two emission-reception radio stations, has demonstrated that there is no direct proportionality correlation between the intensity of induction and the effects caused by the different magnetic induction during the seeds germination. Thus, there are cases where electromagnetic radiation may be used as a stimulatory agent since two cases were found with a very high percentage of germination in correlation with a low catalase activity and low total protein amount. In these circumstances, it is required more detailed investigations, particularly targeted on that induction values that caused the stimulation of germination.

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[1] "Alexandru Ioan Cuza" University, Faculty of Biology, Iaşi, Romania
[2] "Gh. Asachi" Technical University, Faculty of Chemical Engineering, Iaşi, Romania
[*] cmaniu@uaic.ro

STUDIES ON CATALASE AND PEROXIDASE ACTIVITY IN *PHANEROCHAETE CHRYSOSPORIUM* BURDS. CULTIVATED ON SPRUCE SAWDUST MEDIA

ALEXANDRU MANOLIU[1]*, MIHAELA BĂLAN[2], LĂCRĂMIOARA OPRICĂ[2]

Key words: ***Phanerochaete chrysosporium,*** catalase, peroxidase, spruce sawdust

Abstract: The aim of this study is to present the results regarding the influence of different spruce sawdust concentrations on catalase and peroxidase activity in ***Phanerochaete chrysosporium***. Determinations were made using mycelium and liquid culture after 7 days and 14 days from seeding. This study showed that the enzymes activity was influenced by spruce sawdust concentration introduced in medium culture and also it was influenced by the fungus age.

INTRODUCTION

Cellulose is the most abundant organic material in nature, always renewing by photosynthesis process and because of its crystalline structure is very resistant to chemical and biological degradation. Also, there are more than 200 species of microorganisms which biodegrades cellulose wastes. The Microbiology laboratory from Biological Research Institute Iasi carried out the research regarding the biology of the cellulolytic fungi for more than 15 years ago. The most important studies were those regarding Krebs cycle's dehydrogenases activity in the cellulolytic species ***Alternaria alternaria*** grown on media containing the deciduous and coniferous sawdust (Manoliu & al., 2002), the analysis of the proteins synthetised by cellulolytic fungi ***Chaetomium globosum*** and ***Alternaria alternaria*** grown on media containing beech and pine sawdust (Oprică & al., 2004), the evolution of cellulase complex in ***Alternaria alternaria*** grown on media containing forestry industry wastes - leafy and coniferous sawdust (Manoliu & al., 2005), the influence of electromagnetic field (EMF) on cellulase activity in cellulolytic fungi ***Trichoderma viridae*** and ***Chaetomium globosum*** grown on media containing leafy sawdust (Manoliu & al., 2007), the influence of magnetic and electromagnetic field on peroxidase activity in ***Chaetomium globosum*** and ***Trichoderma viridae*** grown on media containing leafy and coniferous sawdust (Manoliu & al., 2008).

Phanerochaete chrysosporium Burds. is capable to degrade the lignocellulose, lignin, cellulose and hemicellulose components and that is the reason why he is one of the most intense studied cellulolytic microorganisms (Broda & al., 2006).

In this study we present the influence of different concentration of spruce sawdust on catalase and peroxidase activity in ***Phanerochaete chrysosporium,*** in mycelium and culture liquid.

MATERIAL AND METHODS

The study was preformed using ***Phanerochaete chrysosporium*** (BCCM/IHEM, Culture Collection N^0 5772) from the collection of the Biological Research Institute of Iaşi. In order to investigate the influence of different concentrations of spruce sawdust on catalase and peroxidase activity, the fungus was grown on *Sabouraud* medium with the following composition: peptone 10 g / l, glucose 35g / l, distilled water 1000 ml (Constantinescu, 1974); the carbon source (glucose) was replaced by 3 different concentrations of spruce sawdust resulting 4 work variants: V1 - 20 g / l, V2 - 30 g / l and V3 - 40 g / l, V4 - control (the carbon source from the sample was not replaced). The incubation was made at 28^0C and the activity of catalase and peroxidase was determined at 7 and, respectively, 14 days from inoculation, using mycelium and liquid culture.

In order to determine the catalase activity it was used the spectrophotometric method (Artenie & al., 2008); for the peroxidase activity o-dianisidine method (Cojocaru, 2009); the enzymic activity was reported to soluble protein amount which was determined using Bradford method (Artenie & al. 2008).

RESULTS AND DISCUSSIONS

The results regarding the influence of different concentration of spruce sawdust on catalase and peroxidase activity are presented in figures 1-4. Figure 1 represents the catalase activity in fungus mycelium showing that after 7 days from inoculation only on 2 variants there were obtained values bigger than the control sample: V1 – 765,562 UC/mg/min. and V_3 -708,672 UC/mg/min., compared with V_4 – control – 680,054 UC/mg/min.; the smallest value for the catalase activity was obtained at V_2 - 663,450 UC/mg/min.

At 14 days after inoculation, the biggest value for the activity of catalase was determined at V4 - 921.269 UC / mg / min., followed in descending order by V2 - 885.83 UC / mg / min., V1 - 845.19 UC / mg / min., V3 - 807.153 UC / mg / min. Following the dynamics activity of this enzyme, it was noticed that at 14 days after inoculation the enzyme activity increased compared to the values recorded at 7 days after inoculation: at V1 - from 765.562 UC / mg / min. to 845.190 UC / mg / min., V2 - from 663.450 UC / mg / min. to 885.83 UC / mg / min., V3 - from 708.672 UC / mg / min. to 807.153 UC / mg / min., V4 - from 680.054 UC / mg / min. to 921.269 UC / mg / min.
The intensification of catalase activity of the fungus mycelium at 14 days from inoculation may suggest that there is a defense reaction of it against the spruce sawdust.

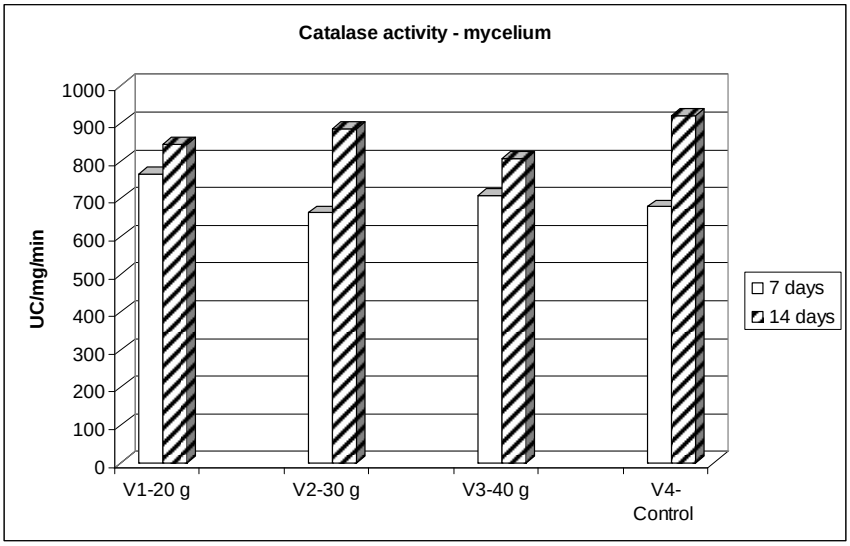

Fig. 1. The influence of different concentration of spruce sawdust on the activity of catalase in *Phanerochaete chrysosporium* - mycelium

The results concerning the influence of different concentrations of spruce sawdust on activity of catalase in liquid culture are presented in figure 2; it can be noticed that at 7 days after inoculation the highest activity of this enzyme was found in V1 - UC 109.79 / ml / min., followed by V3 - 87.89 UC / ml / min., V2 - 45.84 UC / ml / min; in the control (V4) the catalase activity was zero.

At 14 days after inoculation the catalase activity increased significantly but it maintained the same value difference between the variants; the highest activity of this enzyme was noticed at V1 - 516.366 UC / ml / min., followed by V3 - 352.950 UC / ml / min., V2 - 313.950 UC / ml / min., V4 - 0.

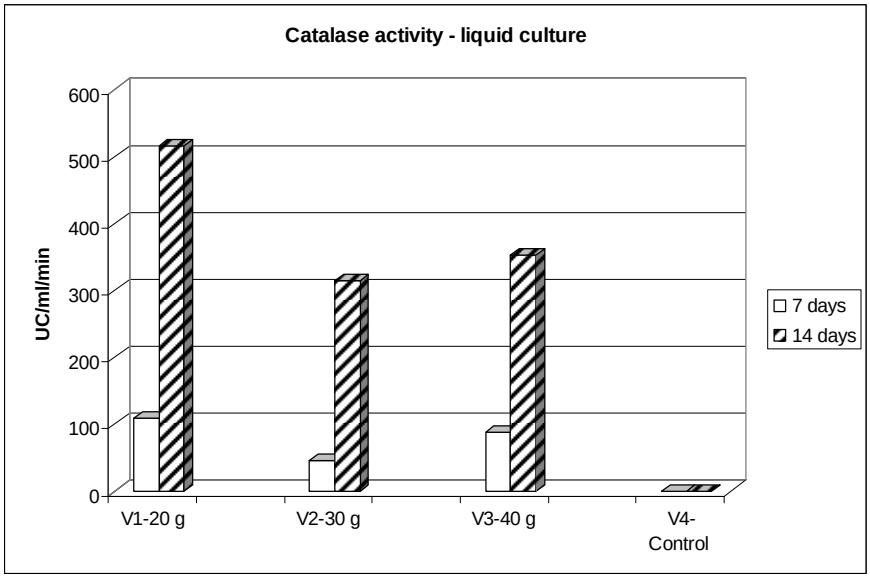

Fig. 2. The influence of different concentration of spruce sawdust on the activity of catalase in *Phanerochaete chrysosporium* – liquid culture

The figure 3 presents the results regarding the influence of different concentrations of spruce sawdust on the peroxidase activity in mycelium, from which results that at 7 days after inoculation the lowest activity of this enzyme was at V2 - 9.5734 x 10^{-3} UP/ mg / min., followed in ascending order by: V1 - 17.64 x 10^{-3} UP / mg / min., V3 - 22.15 x 10^{-3} UP / mg / min., V4 - 31.538 x 10^{-3} UP / mg / min.

At 14 days after inoculation the peroxidase activity in mycelium had the following values: V4 - 26.49 x 10^{-3} UP / mg / min., V3 - 4.0 x 10^{-3} UP / mg / min., V1-3, 36 x 10^{-3} UP / mg / min. and V2 - 0. Following the dynamics of the activity of the catalase it was noticed a decrease of the activity of the peroxidase at 14 days after inoculation, compared with the values obtained at 7 days after inoculation: V1 - from 17.64 x 10^{-3} UP / mg / min. to 36 x 10^{-3} UP / mg / min., V2 – from 9.5734 x 10^{-3} UP / mg / min. to 0, V3 - 22.15 x 10^{-3} UP / mg / min to 4, 0 x 10^{-3} UP / mg / min, V4 – from 31.538 x 10^{-3} UP / mg / min. to 26.49 x 10^{-3} UP / mg / min.

Knowing that 14 days after inoculation there was a progressive increase of catalase activity and a decrease in peroxidase activity, we can concluded that at this time the amount of hydrogen peroxide may be higher and it should be removed by catalase; it is known the fact that in living organisms peroxidase operates on a small quantitie of hydrogen peroxide and catalase removes the excess of peroxide.

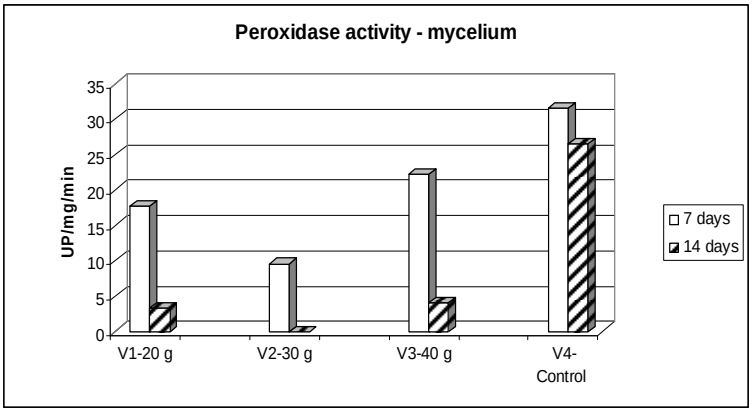

Fig. 3. The influence of different concentration of spruce sawdust on the activity of peroxidase in *Phanerochaete chrysosporium* – mycelium

The data about the influence of spruce sawdust on peroxidase activity in liquid culture are presented in figure 4; we noticed that at 7 days after inoculation, the activity of this enzyme had the following values: V2 - 0.382 UP / ml / min., V1 - 0.322 UP / ml / min., V3 - 0.067 UP / ml / min., V4 - 0.

At 14 days after inoculation the value of peroxidase activity was zero in all variants which contained spruce sawdust in different concentrations; in V4 the enzyme activity was 0.0274 UP / ml / min.

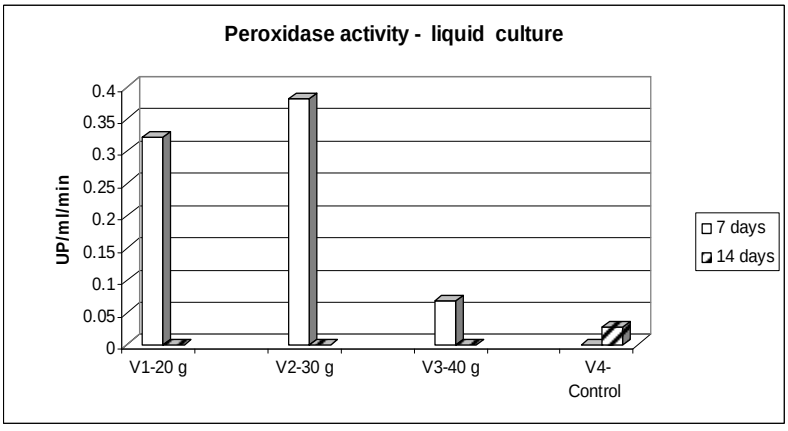

Fig. 4. The influence of different concentration of spruce sawdust on the activity of peroxidase in *Phanerochaete chrysosporium* – liquid culture

CONCLUSIONS

The activity of the catalase in mycelium of ***Phanerochaete chrysosporium*** was stimulated 7 days after inoculation at the variants containing 20 g of spruce sawdust/1.000 ml and

40 g of spruce sawdust /1.000 ml; after 14 days from inoculation the activity of this enzyme was not influenced by any different concentrations of spruce sawdust. The catalase activity in liquid culture was stimulated in all the variants (containing different concentrations of spruce sawdust) both at 7 and 14 days after inoculation.

The activity of the peroxidase in mycelium of ***Phanerochaete chrysosporium*** was inhibited by the presence in the culture medium of various concentrations of spruce sawdust in both intervals of time taken into study; in liquid culture it was stimulated in all variants in different concentrations of sawdust spruce but only after 7 days from inoculation.

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[1] Biological Research Institute, Iasi

[2] “Alexandru Ioan Cuza” University of Iasi, Faculty of Biology

* alexandru.manoliu@uaic.ro

BACTERIAL LIPOPOLYSACCHARIDE ENHANCED IMMUNOLOGICAL RESPONSIVENESS IN EXPOSED RATS

MARIUS MIHASAN[1], LUCIAN HRITCU[1*], VLAD ARTENIE[1], ALIN CIOBICA[1], MARIUS STEFAN[1], LUCIAN GORGAN[1]

Keywords: lipopolysaccharide, brain and serum protein content, immune response
Abstract: In order to investigate the effects of the bacterial lipopolysaccharide (LPS) on the brain and serum protein content, LPS was stereotaxically infused into the substantia nigra (SN) of rats at different dosages (3 µg, 10 µg or 250 µg/kg). The results showed that 7 days after neurosurgery there is no variations in brain protein content, while in serum protein content, the amount of gamma-globulins significantly increased compared to sham-operated control rats. The results suggested an immunologic response in the injected rats exposed to the bacterial lipopolysaccharide.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra (SN) and the presence of Lewy body inclusions in residual neurons. In recent years, increasing evidence has strongly suggested a role for inflammation in the brain in the pathogenesis of PD (Sun et al., 2003). Bacterial endotoxin lipopolysaccharide (LPS) is one of common toxins produced by Gram (-) bacteria, including *Escherichia coli*. This agent can elicit a multitude of pathophysiological effects, including inflammation, macrophage activation, fever, and septic shock (Burrell, 1994). The blood-brain barrier can become leaky as a result of sepsis (Brandtzaeg et al., 1989), allowing LPS to enter the cerebrospinal fluid.

Previous studies have demonstrated that bacterial LPS exposure, that mimics Gram (-) bacterial infections, could cause a significant loss of dopamine (DA) neurons in the substantia nigra (SN) of rat. LPS by inducing DA neurons degeneration serve as an important agent for elaboration of an experimental PD model. Along with DA neuron loss are the α-synuclein positive Lewy body-like inclusion formation and innate immunity dysfunction manifested by increase in number of reactive microglia, increase in pro-inflammatory cytokine levels, and blood-barrier leakage (Wang et al., 2009). LPS administration, upon first exposure, engages the innate immune system leading to the development of the acute phase response (APR; Heumann and Roger, 2002). The APR occurs as a result of immune activation, consisting primarily of the peripheral release of pro-inflammatory cytokines by circulating macrophages and monocytes. After the first exposure to LPS animals develop an adaptive immune response and the activity of the innate immune system is diminished. As a result of this, upon secondary LPS exposure, tolerance develops and a decrease in both physiological and behavioral measures of sickness is often observed (Franklin et al., 2003). In other study (Makela et al., 1983) an injection of bacterial lypopolisaccharide (LPS) into mice caused a considerable increase in the serum concentration of IgM and IgG (total Ig rose three-to four-fold in 7 days) and a corresponding increase in the concentration of antibodies.

The present study was conducted to determine the effects of LPS-induce immune challenge on brain and serum protein content of rats, with relevance for Parkinson’s disease.

MATERIALS AND METHODS

Animals

29 male Wistar rats weighing 300 ± 50 g at the start of the experiment were used. The animals were housed in a temperature- and light-controlled room (22°C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water ad libitum. Rats were treated in accordance with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and all procedures were in compliance with the European Council Directive of 24 November 1986 (86/609/EEC).

Stereotaxic injection of LPS into SN of rats

All surgical procedures were conducted under aseptic conditions, under sodium pentobarbital (45 mg/kg b.w., i.p., Sigma) anesthesia. Rats were mounted in the stereotaxic apparatus with the nose oriented 11° below horizontal zero plane. For injection of LPS into the SN of rats, the following coordinates were used: 5.5 mm posterior to bregma; 2.0 mm lateral to the midline; 7.4 mm ventral to the surface of the cortex (Paxinos and Watson, 2005). Rats were divided into different dosage of LPS (3 µg, 10 µg or 250 µg/kg). LPS was prepared as a stock solution of 1 µg/µl in sterile saline solution. After each injection, the needle was left in situ for an additional 5 min to avoid reflux along the injection track. The sham-operated rats were injected with same volume of sterile saline solution. 7 days after neurosurgery, all rats were anesthetized, rapidly decapitated and whole brain were removed. The temporal lobes were collected. Each of brain tissue samples was weight and homogenized with a Potter Homogenizer coupled with Cole-Parmer Servodyne Mixer in

bidistilled water (1g tissue/10ml bidistilled water). Samples were centrifuged 15 min at 3000rpm. Following centrifugation, the supernatant was separated and pipetted into tubes. Also, whole blood was collected and incubated for 1h at room temperature and than serum was separated, by centrifugation 15 min at 3000 rpm.

SDS-PAGE analysis of soluble proteins

SDS-PAGE was performed using the discontinuous buffers system of Laemlli. Gradient 5-15% or 5-20 % gels were casted fallowing the procedure described by Ausubel, 2002, using a MIDI Gradient gel Mixer, Roth, Germany and a TV400YK (Scie-Plas,UK) electrophoresis apparatus.

The rat serum was diluted 1:10 with distillated water prior of use. 10 µl of diluted serum was mixed with SDS-loading buffer containing 100 mM mercaptoethanol (final concentration) and boiled for 10 min. on a Thermomixer (Eppendorf, Germany).

The brain extract were obtained as described above. Approximately 125 µg of proteins were mixed with SDS-loading buffer, boiled for 5 min. and loaded on each lane.

The gel was run at 55 mA/gel until the blue dye run out (usually overnight) and afterwards was stained using Coomasie Brillant Blue R 250 0,25% in 45% methanol, 10% acetic acid for about 2 hours. The destining was done in 30% methanol, 10% acetic acid until the background was completely de-stained. The gels were kept in 10% acetic acid until photographed.

Gel densitometry and molecular weight determination

The stained gels were photographed and quantified using ImageQuant TL from GE Healthcare. For quantitation a calibration curve was constructed by loading on gel in parallel with the samples 2,5; 5 and 10 µg bovine serum albumine (BSA). In the conditions used, the calibration curve had an regression coefficient of 0, 98. Molecular weight determination was done by running in parallel with the samples a wide range molecular weight protein marker from Sigma. The marker was used to fit a curve from which the molecular weight of the unknown proteins was determined.

Target proteins were recognized according to their molecular weight: an unknown negative control at 78 kDa, serum albumin at around 61 kDa (Peters, 1962), immunoglobulin heavy chain at about 51 kDa and light chain at about 21 kDa (Mathews, 2000). Samples were run in several repetitions (6-8). Each repetition was densitometrated and mean, standard deviation and confidence level for mean was calculated according to common statistical methods.

RESULTS AND DISSCUSIONS

The aqueous brain extracts from LPS treated rats (250 µg) and sham-operated rats control were resolved on 5-20 % gradient gels. About 20 protein fractions could be exponentiated by Commasie staining, but no significant and repetitive differences could be observed (Fig.1, A). All samples were rather uniform regarding their concentration, with the most abundant proteins not exceeding 12 % of total protein in both sample and control (56 kDa – 12% of total protein, 44 kDa – 9% of total protein and 6,6 kDa – 10.5% of total proteins (Fig.1, B).

Regarding the electrophoresis of serum proteins, one aspect must be highlighted. Unlike the older methods using disk electrophoresis or acetate gels, we used SDS-PAGE in both denaturing and reducing conditions. Because of this, the electrophoretic profile of separated proteins is slightly different. More precisely, the gamma-globulins do not migrate as a single high-molecular weight band, but are separated in two distinct fractions (Marshall, 1984).

In both the sample (LPS treated rats, 3 µg and 10 µg) and the control (sham-operated rats) around 20 fractions could be separated and visualized by Commasie staining. Unlike the brain extracts, the electrophoretic profile of serum proteins shows a much greater heterogeneity (Fig. 2), with 4 predominant protein fractions making 60 – 70 % of total protein content. According to their molecular weight, three out of these 4 proteins were recognized as being albumin (61 kDa), immunoglobulin heavy chain (51 kDa) and light chain (21 kDa). The forth band, an unknown protein of 78 kDa was arbitrary considered as a control.

Using bovine serum albumine (BSA) as standard for a calibration curve, the amount of each of these proteins could be measured by gel-densitometry. As it can be seen in table 1, injection of LPS (3µg and 10µg) in rats leads to a small decrease in the amount of serum albumin

and an increase in the amount of gamma-globulins (both the light and the heavy chains). This would indicate an immunologic response in the injected rats.

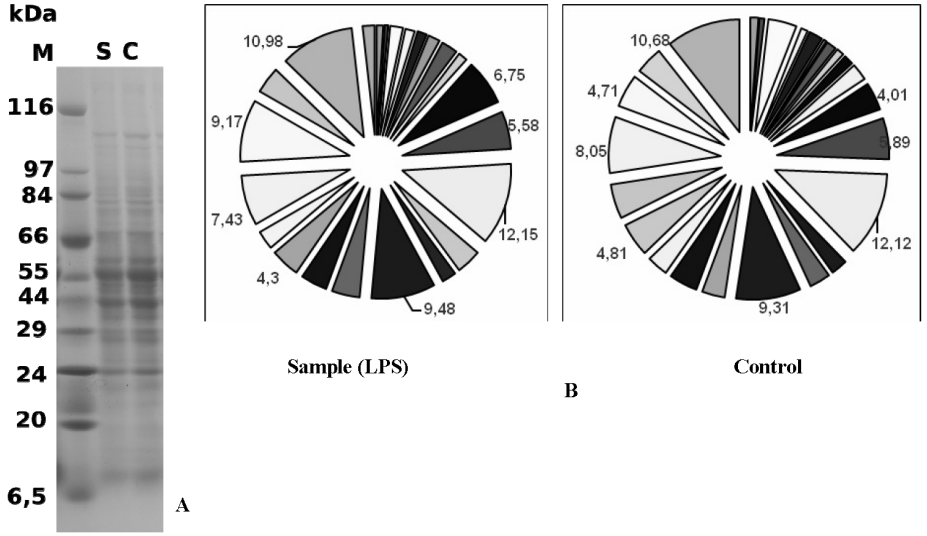

Fig. 1. Effects of LPS administration (250 µg) on rats brain protein content. A - electrophoretic profile of brain proteins; B- diagram of the percentage of total protein content resulted from gel densitometry of the same samples

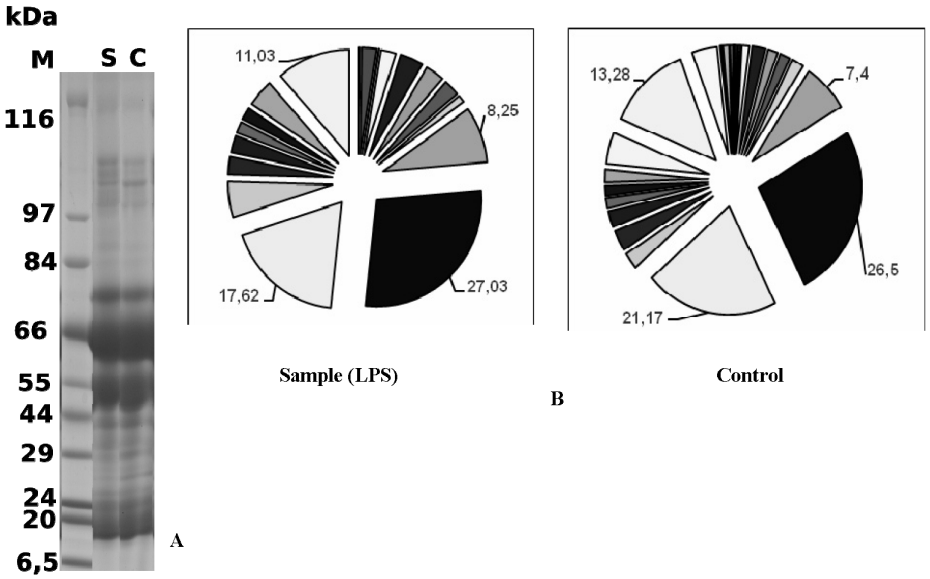

Fig. 2. Effects of LPS (3 µg and 10 µg) administration on rat serum protein content. A - electrophoretic profile of serum proteins; B- diagram of the percentage of total protein content resulted from gel densitometry of the same samples (LPS – 27.03% - albumin; 17.62% –gamma-globulins heavy chain; 11.03% - gamma-globulins light chain; Control – 26.5% - albumin; 21.17% - gamma-globulins heavy chain; 13.28% - gamma-globulins light chain.

Table 1. Effects of LPS administration on serum protein content

	LPS		**Control**		**p**
	Mean	**S.E.M.**	**Mean**	**S.E.M.**	
Unknown 78 kDa control	13.88	0.28	13.1	0.27	0.44
Albumine	46.74	0.32	48.86	0.49	0.0017
Gamma-globulins heavy chain	35.91	0.3	33.6	0.79	0.02
Gamma-globulins light chain	23.6	0.57	20.47	0.41	0,00415

CONCLUSIONS

On the basis of our results obtained by LPS administration, we can conclude that in the rats, stereotaxic administration of LPS induced immune responsiveness in rats.

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Acknowledgements. This research was supported by the National Council of Scientific Research and University Education
(CNCSIS, Grant IDEI, cod 85, no. 1073/2009), Romania.

[1] "Alexandru Ioan Cuza "University of Iasi, B-dul Carol I, Nr. 20A, 700506, Iasi-Romania;
*hritcu@uaic.ro

ON THE ACTIVITY OF SOME INTESTINAL ENZYMES IN THE *CTENOPHARYNGODON IDELLA* SPECIES

GABRIELA VASILE [1*], ELENA CIORNEA [1]

Key words: alanine-aminotransferase, aspartate-aminotransferase, pepsin, trypsin, digestive tube, grass carp
Abstract: The present paper systematizes the results attained on the activity of some transferases and proteases from the median part of the digestive tube, in three summer-old grass carp from the Piscicultural Farm of Ţigănaşi - Iaşi district, the data obtained being statistically processed for each parameter in part. The values recorded support the idea that the digestive tube of *Ctenopharyngodon idella* might be regarded as a potential source of proteolytic and aminotransferasic enzymes.

INTRODUCTION

A correct fish feeding with food containing all necessary nutritive substances, in the amounts required by the organism, for assuring maximum growth, without affecting its general physiological condition, represents one of the essential conditions of a sound growth, under profitable economic conditions.

Digestion is a complex biochemical and mechanical process occurring at the level of the digestive tube, during which the ingested raw aliments are transformed into simpler, easily assimilable substances.

Once swallowed, the food goes - without significant modifications - towards the esophagus. In the mouth of fish, the food suffers no chemical transformation once the salivary glands are absent, chemical digestion beginning only in the intestine (in the case of species without stomach), that is the nutritive substances are completely digested, prior to their absorption (OPREA and GEORGESCU, 2000).

The paper analyzes the activity of some intestinal enzymes, starting from the idea that the digestive tube of cyprinids might represent a possible source of proteolytic and transaminasic enzymes.

MATERIALS AND METHOD

The investigations were performed on three summer-old representatives of the *Ctenopharyngodon idella* species, from the Piscicultural Farm of Ţigănaşi - Iaşi district. Under laboratory conditions, fresh tissue samples have been taken over from the living fish while, after its death, fragments were cut from the median part of the digestive tube (between the esophagus and the duodenum) - from which the nutritive rests had been previously eliminated.

The alanine- and aspartate-aminotransferase activity was determined by the colorimetric method, with 2, 4 - dinitrophenylhydrazyne, while that of pepsin and trypsin - with the Folin - Ciocâlteu reactive, on using caseine and denaturated hemoglobin, respectively, as a substrate (ARTENIE and TĂNASE, 1981; COJOCARU, 2008).

For each parameter and individual in part, parallel determinations have been performed, in view of subsequent statistical processing, involving calculation of the mean standard error and deviation, variance, confidence level, superior and inferior limit, as well as of the variation and precision coefficient of the mean value (ZAMFIRESCU and ZAMFIRESCU, 2008).

RESULTS AND DISCUSSION

Ctenopharyngodon idella, also known as the grass carp or Cteno, performs its active feeding at 20°C, the digestive tube being 2 - 3.5 times longer than the body. Initially, the fry consumes phyto- and zooplankton, after which it gradually passes to the macrophytophagous nutrition. As this species consumes submerse and - especially - emerse vegetation, it came to be called “living mower”. When the aquatic plants are absent, the grass carp consumes Spanish trefoil, clover, maize etc., possibly “attacking” the additional food of the common carp (*Cyprinus carpio*) (GROZEA and BURA, 2002). The grass carp preferably consumes *Potamogeton angustifolius, Ceratophyllum demersum, Ceratophyllum submersum, Elodea canadensis, Lemna sp., Phragmites communis, Phararis arundinacea, Scirpus lacustris, Carex vulpina, Typha sp*., out of the existing aquatic vegetation, and *Trifolium repens, Medicago sativa, Sailix sp.,* leaves of

acacia, vetch as green mass, distributed in certain places of the piscicultural basin, respectively, out of the terrestrial plants (MACOVEI, 2008).

The economic interest manifested for the extended growth of such fish is justified by several reasons: first, it consumes and up grades the macrophyte vegetal biomass, which other fish species do not use; it combats the aquatic vegetation without either technical means or fuel consumption; it contributes to improving the living conditions for the other fish species grown, together with it, in polyculture, especially by improving the oxygen regime and by increasing the biogenic substances, as well as the planktonic and bentonic biomass.

At worldwide level, *Ctenopharyngodon idella* is grown in numerous countries (China, Austria, Germany, Taiwan, Malaysia, Hong Kong, Sweden, Canada, USA, France, Romania, Israel, Egypt, Finland, Great Britain), which is another proof of its economic value (BUD *et al*., 2004).

A first objective of the present study was to determine the activity of alanine-aminotransferase, an enzyme also known as glutamate-piruvate-transaminase (GPT or TGP), which catalyzes the conversion reaction of alanine and α-ketoglutarate into piruvate and glutamate (COJOCARU, 1997).

The results obtained show that the activity of alanine-aminotransferase varies between 18.9 and 24.4 conventional extinction units, the highest coefficient of mean variation - of 1.224 - being registered in individual number two. The data listed in Table I indicate narrow values for all statistical indices.

Table I. The values of the activity of intestinal alanine-aminotransferase and of the main statistical indices in three summer-old *Ctenopharyngodon idella*

Samples	Individual activity *(CEU)	Mean *(CEU)	Standard error	Standard deviation	Variance	Confidence level (95%)	VC%	m%
1	18.9	18.9	0.115	0.2	0.04	0.496	1.058	0.61
	18.7							
	19.1							
2	21.5	21.6	0.152	0.264	0.07	0.657	1.224	0.707
	21.4							
	21.9							
3	22.8	22.7	0.088	0.152	0.023	0.379	0.67	0.387
	22.6							
	22.9							
4	21.2	21.3	0.145	0.251	0.063	0.625	1.185	0.684
	21							
	21.5							
5	24.5	24.4	0.13	0.225	0.05	0.56	0.92	0.531
	24.25							
	24.7							

*(CEU) = conventional extinction units, VC% = mean variation coefficient, m% = mean precision coefficient

The values of mean and standard deviation permitted the subsequent calculation of the (inferior and superior) limits of the confidence intervals characterizing the activity of alanine-aminotransferase in the digestive tube, on using a critical value $t(\alpha, n-1)$, given by $\alpha = 0.05$ (*i.e*., a 95% probability) and $n-1$ degrees of freedom (where n represents the number of values from each sample), *i.e*. $t(0.05, 4) = 2.132$.

As graphically illustrated in Figure 1, the limits of the confidence intervals are extremely narrow for all samples under investigation, the smallest interval varying between 22.61 and 22.91 conventional extinction units.

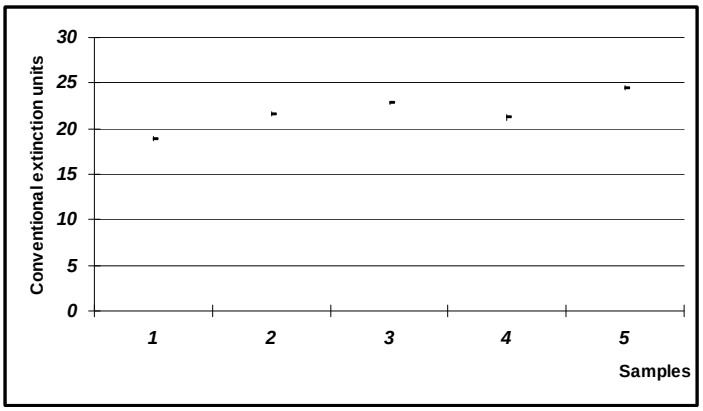

Fig.1. Confidence intervals of the alanine-aminotransferase activity in three summer-old *Ctenopharyngodon idella*

Aspartate-aminotransferase, also known as glutamate-oxaloacetate-transaminase (GOT or TGO), catalyzes the transamination reaction between aspartate and α-ketoglutarate, with formation of glutamic acid and oxalyl-acetic acid.

Table II provides an overall image on the activity of this intestinal aminotransferase, somehow lower values, comparatively with alanine-aminotransferase (representing only 72.81% of its activity) being observed, on one hand while, on the other, somehow more ample variations (oscillating between 12.9 and 18.03 conventional extinction units) were recorded.

Table II. The values of the activity of intestinal aspartate-aminotransferase and of the main statistical indices in three summer-old *Ctenopharyngodon idella*

Samples	Individual activity *(CEU)	Mean *(CEU)	Standard error	Standard deviation	Variance	Confidence level (95%)	VC%	m%
1	14.3	14.3	0.115	0.2	0.04	0.496	1.398	0.807
	14.1							
	14.5							
2	18	18.03	0.317	0.55	0.3	1.368	3.054	1.763
	17.5							
	18.6							
3	12.9	12.9	0.145	0.251	0.06	0.625	1.945	1.123
	12.7							
	13.2							
4	17.9	17.9	0.115	0.2	0.04	0.496	1.117	0.645
	17.7							
	18.1							
5	16.2	16.1	0.158	0.275	0.07	0.684	1.701	0.982
	15.9							
	16.45							

*(CEU) = conventional extinction units, VC% = mean variation coefficient, m% = mean precision coefficient

On analyzing the confidence intervals of the aspartate-aminotransferase activity in the digestive tube of *Ctenopharyngodon idella* representatives, mention should be made of the fact that they are somehow larger, the largest one being registered in individual number two (17.48 - 18.58 conventional extinction units).

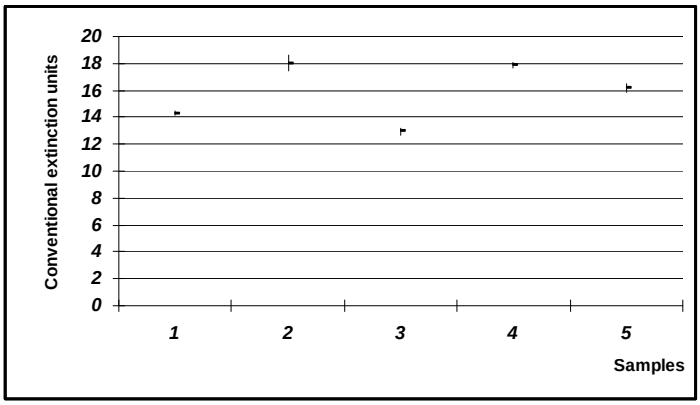

Fig.2. Confidence intervals of the aspartate-aminotransferase activity in three summer-old *Ctenopharyngodon idella*

A comparative graphical representation of the mean activity of the two intestinal transaminases (Fig.3) permits the conclusion that alanine-aminotransferase evidences higher values, comparable with those given in the literature, which suggests that alanine transamination occurs more intensely than that of the aspartic acid (KONOVALOV, 1980).

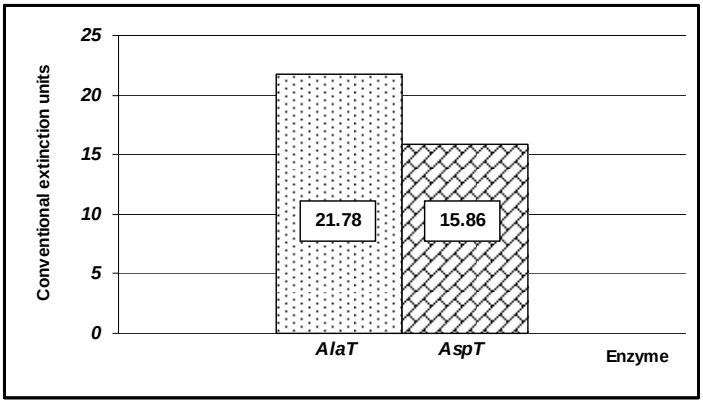

Fig.3. Comparative representation of the alanine- and aspartate-aminotransferase activity in three summer-old *Ctenopharyngodon idella*

The following stage of the present investigation involved the determination of the activity of intestinal pepsin, an endopeptidase which splits preferentially the peptidic links from the substrate, created by the aminic groups of the aromatic aminoacids - phenylalanine and tyrosine.

In fish, the proteasic activity either increases or decreases proportionally with the amount of proteins present in food, as well as with the quality and quantity of the available food,

which actually represents an adaptation of the secretory function of the intestinal tractus to the type of food (VASILE *et al*., 2005).

In the case of vegetarian fish, the stomachal region is not clearly individualized, while the mucous membrane of the anterior intestine, between the oesophagus and the choledoc, may evidence or not a weak peptidic activity (GUILLAUME *et al*., 1999).

In grass carp, the activity of intestinal pepsin varies between 0.26 and 0.65 nmoles Tyr/min. x ml, the highest variation and precision coefficient of the mean being of 5.844 and 3.374, respectively (Table III). The extremely low values recorded for intestinal pepsin agree with the literature data, according to which, in cyprinids, fish having no stomach and, consequently, with a reduced gastric function, gastric digestion, maintained especially through the pepsin, disappears. Such fish species use the buccal cavity and the pharynx for breaking up the food, a mechanical function replacing the stomachal one, the absence of gastric digestion being compensated by a more abundant presence of trypsin in the intestine.

Table III. The values of the activity of intestinal pepsin and of the main statistical indices in three summer-old *Ctenopharyngodon idella*

Samples	Individual activity (nmoles Tyr/ min. x ml)	Mean (nmoles Tyr/ min. x ml)	Standard error	Standard deviation	Variance	Confidence level (95%)	VC%
1	0.3046	0.3	0.005	0.008	0.021	2.907	1.678
	0.2945						
	0.3121						
2	0.3593	0.35	0.002	0.003	0.008	1.01	0.583
	0.3542						
	0.3612						
3	0.2656	0.26	0.001	0.003	0.007	1.13	0.652
	0.2689						
	0.2629						
4	0.6562	0.65	0.012	0.021	0.052	3.253	1.878
	0.6312						
	0.6735						
5	0.3359	0.33	0.011	0.019	0.048	5.844	3.374
	0.3156						
	0.3548						

VC% = mean variation coefficient, m% = mean precision coefficient

Here again, the limits of the confidence intervals of the activity of intestinal pepsin have been calculated and plotted graphically (Fig.4), a more ample variation domain being observed in the last two samples subjected to analysis (0.63 - 0.67 and 0.31 - 0.35 nmoles Tyr/min. x ml).

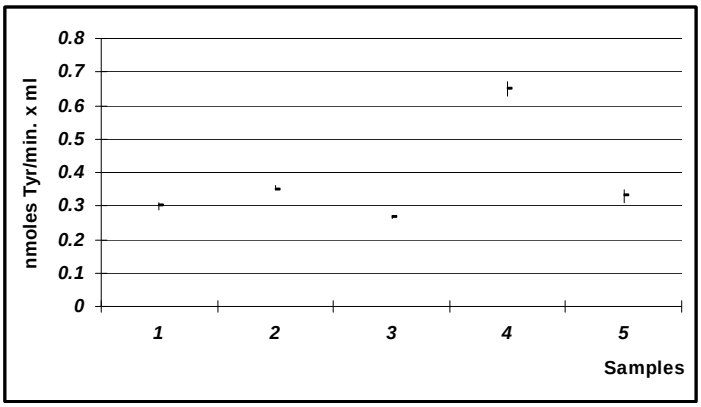

Fig.4. Confidence intervals of the intestinal pepsin activity in three summer-old *Ctenopharyngodon idella*

Trypsin is also an endopeptidase present in fish, synthesized in the pancreas, as its inactive zymogene (trypsinogene), the activation of which occurs, nevertheless, either at intestinal level, under the action of enterokinase, or autocatalitically, which causes a subsequent chain of reactions - through the action of the newly-formed trypsin on chemotrypsinogene, followed by the successive activation of other enzymes, namely elastase, colagenase, carboxypeptidase and phospholypase. Trypsin splits preferentially the peptidic links formed by arginine and lysine, being present and active both in the fish of prey and in the peaceful species (OPREA and GEORGESCU, 2000; COJOCARU, 2008).

In fish, trypsin evidences an optimum activity at pH = 7.5 - 8.5 (APETROAEI, 1995), the existing literature data showing differences in the enzymatic spectrum, in close correlation with the nutrition type, the fish of prey having a higher content of proteolytic enzymes while, in peaceful fish, the glycosidases are prevailing (MĂRGĂRINT, 1982).

In the case of cyprinids, trypsin acts directly upon the albumins, thus finalizing their simplification into aminoacids, which is also the case of the species possessing a stomach; consequently, trypsin finalizes the digestion of proteins.

The results obtained on the activity of intestinal trypsin in three summer-old grass carp evidence somehow more ample oscillations from one individual to another, the minimum value being of 0.356 nmoles Tyr/min. x ml, while the maximum one is of 0.802 nmoles Tyr/min. x ml (Table IV).

As evidenced in Figure 5, the limits of the confidence intervals plotted for the activity of intestinal trypsin are extremely narrow, the values obtained during parallel determinations being only slightly different from the mean value.

Table IV. The values of the activity of intestinal trypsin and of the main statistical indices in three summer-old *Ctenopharyngodon idella*

Samples	Individual activity (nmoles Tyr/ min. x ml)	Mean (nmoles Tyr/ min. x ml)	Standard error	Standard deviation	Variance	Confidence level (95%)	VC%
1	0.4531	0.453	0.002	0.004	0.011	1.014	0.585
	0.4589						

Samples	Individual activity (nmoles Tyr/ min. x ml)	Mean (nmoles Tyr/ min. x ml)	Standard error	Standard deviation	Variance	Confidence level (95%)	VC%
	0.4498						
2	0.6406	0.642	0.004	0.008	0.020	1.294	0.747
	0.6512						
	0.6348						
3	0.3593	0.356	0.004	0.006	0.017	1.949	1.125
	0.3489						
	0.3621						
4	0.7968	0.794	0.004	0.008	0.019	1.012	0.584
	0.8012						
	0.7856						
5	0.8	0.802	0.01	0.017	0.43	2.181	1.259
	0.8215						
	0.7868						

VC% = mean variation coefficient, m% = mean precision coefficient

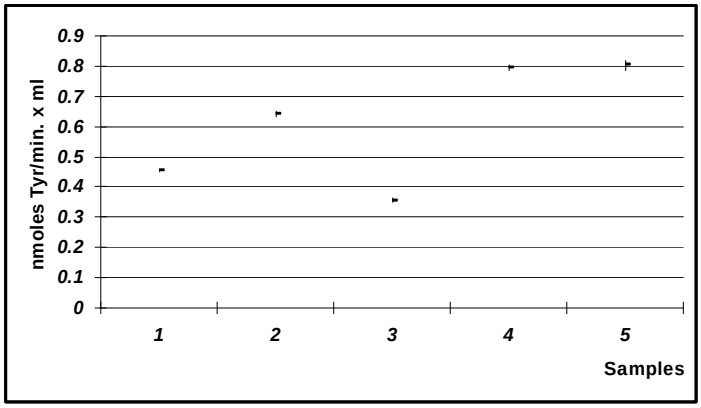

Fig.5. Confidence intervals of the intestinal trypsin activity in three summer-old *Ctenopharyngodon idella*

A comparative analysis of the mean values recorded by the activity of intestinal pepsin and trypsin (Fig. 6) shows that pepsin represents only 63.33% of the triptic activity, which might be explained by the fact that, generally, pepsin acts at stomachal level, in the presence of a slightly acid pH, while trypsin usually acts at intestinal level, where it is present in higher amounts.

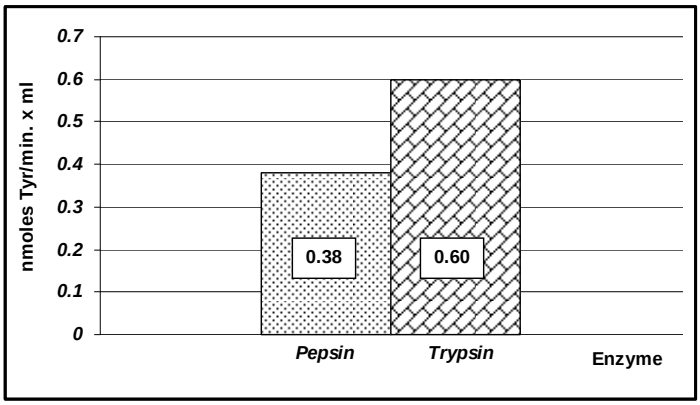

Fig.6. Comparative representation of pepsin and trypsin activity in three summer-old *Ctenopharyngodon idella*

CONCLUSIONS

The results of the present investigation permit the following conclusions:

1. At the level of the median part of the digestive tube, one may observe a quite significant proteolytic and transaminasic activity, which might be correlated with the absence of the stomach in this species, proteins degradation occurring mainly at intestinal level.

2. The enzymatic activity observed was more pronounced in the case of alanine-aminotransferase and, respectively, trypsin, the limits of the confidence intervals being quite narrow for all biochemical parameters taken into study.

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1) "Alexandru Ioan Cuza" University of Iaşi, Faculty of Biology, Bd. Carol I, Nr. 20 A, 700506, Iaşi, România
*) gabriela.vasile@uaic.ro

BIOCHEMICAL INVESTIGATIONS ON *RANA RIDIBUNDA* PALL. AND *RANA ESCULENTA* L.

CIPRIAN RADU MÎNDRESCU[1], GOGU GHIORGHIŢĂ[2], VLAD ARTENIE[3]

Key words: *Rana ridibunda, R. esculenta*, biochemical indices

Abstract: Our investigations pointed out the existence of a high variability of some biochemical indices in the hepatic, muscular and blood tissue at *Rana ridibunda* Pall. and *Rana esculenta* L. Higher amounts of sugars and transaminases were found in the tissues of *Rana esculenta* L. species, while *Rana ridibunda* Pall. was characterised by higher levels of fats (triglycerides and cholesterol). With some exceptions, the highest values of the analysed biochemical indices were found in blood. The electrophoresis of plasma blood proteins of the two species of green frogs showed the presence of 3 – 4 proteic fractions in blood. The albuminic fraction at *Rana esculenta* L. contains two sub-fractions, and this fact sustains the idea of a hybrid nature of this species.

INTRODUCTION

The amphibians have a complex vital cycle, which makes them influenced by a larger range of abiotic factors than other groups of animals. The amphibians are extremely sensitive to the climatic changes, which may interrupt their growth time, the hibernation period, the ability to find food (Donnely and Crump, 1998; Blaustein et al., 2001). The amphibians are key-elements in various ecosystems, thus the changes produced in their populations also affect the other species from the community (Donnely and Crump, 1998). Regarding all theses facts and that there is a global tendency of a decreasing biodiversity, including of the amphibians (approximately 200 species are in this situation, other 32 species are affected by extinction according to (Alford and Richards, 1999; Houlahan et al., 2000) programs were launched to supervise the amphibians, which use varied research methods and techniques to get information on their adaptation potential.

There are also numerous references on the metabolism of different compounds, on the influence of several environmental agents (some ions), on some biochemical parameters at the amphibian species (Matei-Vladescu, 1964; Şuteu and Pora, 1971; Alyousif, 1991). As the study of the plasmatic albumins represents a discriminating technique for the species from the *Rana esculenta* complex, the literature offers many data on this topic (Uzzell and Berger, 1975; Wijnands and VanGelder, 1976; Günther and Lübcke, 1979; Csata, 1998).

Our aim was to investigate the level of some biochemical indices in the hepatic and muscular tissue, and in the blood of two species of frogs: *Rana ridibunda* Pall. and *Rana esculenta* L.

MATERIAL AND METHODS

Ten green frogs were chosen to dose some biochemical compounds from the blood, 6 from the *Rana ridibunda* Pall. species and 4 from the *Rana esculenta* L. species. Finally, a sufficient quantity of blood was drawn for analysis, at three individuals from the first species and three from the second one. We also have done the electrophoresis of the proteins from the blood serum. The analyzed biochemical components were the same at the level of the hepatic and muscular tissue. In this respect cell homogenates were prepared of 0.5 g of tissue (for each), which was mortared with pounded glass up to the consistence of a paste. The final product was then brought to the state of a cell suspension, using 4.5 ml of physiological serum. The obtained homogenates (with a standard dilution of 1:10) were collected in centrifugal test tubes and centrifuged to a 4000 rot/min speed. The supernatants were flowed in dry test-tubes. These samples were processed in the Clinical and Microbiological Lab from the Municipal Hospital Dorohoi, using its technical means: the Cobas Mira automatic system for biochemical analyses and the typical reagents used by this equipment.

The analyzed blood was drawn from the abdominal vein, using a 4% sodium citrate solution as an anti-clotting solution.

The following biochemical indices were analyzed for each investigated individual: carbohydrates, proteins, triglycerides, cholesterol, urea, GOT (Glutamic Oxalacetic Transaminase), GPT (Glutamic Pyruvic Transaminase), creatinine, and amylase. Regarding the fact that the extraction of the carbohydrates from the liver or muscle was made with physiological serum, we may consider that the analysed components are soluble carbohydrates, (especially glucose, probably some fructose, as well). Together with the above mentioned analysis, the blood serum protein electrophoresis was accomplished as well for six male frogs (3 of *R. ridibunda* Pall. and 3 of *R. esculenta* L.). The interpretation of the results was done using the following statistic indices: the minimum and maximum values, the average and standard

deviation (stdev). The Student t-test was used to compare the average of each parameter for the two investigated species. The results are presented in the Tables 1 – 6.

To obtain the blood plasma for electrophoresis, the blood samples were centrifuged for 15 minutes at 4000 rot/min speed. The electrophoresis of the blood proteins was done on a sheet of cellulose acetate, using the BIOTEC Fischer Phaero Stab 0305 F electrophoresis kit. The obtained plasma was taken with a tiny pipette and introduced in the buckets of a special device. The strip of cellulose acetate was dipped in an ATX buffer solution (code 070961) for 2 – 3 minutes using some tweezers, then it was dried by pressing it between two sheets filter paper. The migration strip thus obtained was placed on the electrophoresis deck (after one end had been cut to control the order of the samples). The migration strip was placed in the start position with an applicator which was immersed for 5 seconds in the buckets with plasma. Afterwards it was applied on the cellulose strip from the electrophoresis deck (by a slight press for 5 seconds). This way, approximately 10 µl plasma was transferred on the migration strip in the initial position. After that, the electrophoresis deck was dipped in the ATX buffer solution (code 070965) so that the ends of the strip of cellulose acetate to be sunk into the solution.

The migration took 30 minutes, using a 220 V voltage and an electric current of 30 mA/bath. After that the migration time was over, the cellulose strip was introduced in a Ponceau colouring solution for 7 minutes. The decolouration of the samples was made through two successive baths, three minutes in a standard solution ATX (code 9353210). The samples were then subbmitted for at least 3 minutes to an operation of transparency (to eliminate any trace of colouring substance) using the Phaero-clear solution (code 353310). In order to dry, the strip of cellulose acetate was applied on a glass blade, which was introduced in a thermostat at 80 – 100 °C, for 30 – 45 minutes. After this, the strip sticks closely to the glass blade, thus the handling of the samples being easier. The results are presented in pictures 2 and 3.

All these experiments took place in the Clinical and Microbiological Lab from the Municipal Hospital in Dorohoi, using the Lab's programme for scanning and interpreting the samples: TurboScan (1.2.9) BIOTEC – Fischer GmbH. The processing of the data was made with a programme calibrated for human blood. The diagram for the protein fractions separated through the electrophoresis (albumins, globulins α_1, α_2, β and γ) was automatically drawn by the above mentioned programme.

RESULTS AND DISCUSSIONS

Comparing the results of the biochemical analyses, we may state that there are some quantitative differences, both due to the species and organs which were analysed. Thus, when compared to *Rana esculenta* L., *Rana ridibunda* Pall. shows in the hepatic tissue higher values of cholesterol (which is absent in *R. esculenta*), urea, amylase, triglycerides and creatinine, lower values of carbohydrates and GOT and similar values of GPT and proteins (Table 1). There are also differences between the two species regarding the minimum and maximum values of the analysed parameters. In the case of *Rana ridibunda* Pall., the biggest differences between the extreme values can be noticed with the triglycerides, carbohydrates, cholesterol and total proteins, and the smallest with the creatinine (Table 1).

Table 1 - The values of some biochemical parameters in the hepatic tissue of *Rana ridibunda* Pall. and *Rana esculenta* L.

Parameter	*R. ridibunda* Pall.					*R. esculenta* L.				
	obs. no.	min	max	average	standard error	obs. no.	min	max	average	standard error
GOT (UE/g tissue)	6	0	0	0	0	4	0,01	0,02	0,015	0,002
GPT (UE/g tissue)	5	0,20	0,28	0,23	0,01	4	0,19	0,22	0,21	0,006
Amylase (UE/g tissue)	6	0,25	0,47	0,38	0,04	4	0,11	0,41	0,24	0,06

Carbohydrates (mg% tissue)	6	690	1660	1043,3	151,60	4	850	1800	1332,5	197,41
Cholesterol (mg% tissue)	6	12	295	117,5	57,73	4	0	0	0	0
Triglycerides (mg% tissue)	6	194	1891	979,5	281,50	3	82	263	177,66	52,50
Proteins (mg% tissue)	6	3600	7000	4783,3	564,75	4	2900	10000	5075	1654,48
Urea (mg% tissue)	6	73	114	99,17	5,80	4	63	99	74	8,47
Creatinine (mg% tissue)	5	1,0	3,3	2,10	0,52	3	0,4	1,0	0,63	0,18

In the muscular tissue (Table 2), the species *Rana ridibunda* Pall. registers higher average values of the cholesterol, triglycerides and amylase, lower values for carbohydrates, proteins and close values of the urea and creatinine, when compared to *Rana esculenta* L. It should be noticed that the GOT activity in the muscular tissue is absent at the two investigated species. The cholesterol was absent in the hepatic and muscular tissue of *Rana esculenta* L. At the level of the muscular tissue, the analysed indices registered high individual variations. Thus, at *Rana ridibunda* Pall., GPT ranges between 0,02 and 0,101 UE/g, the quantity of carbohydrates is between 20 and 240 mg/100 g, triglycerides - between 36 and 244 mg/100 g. A similar situation was found at *Rana esculenta* L. for these parameters.

Table 2 - The values of some biochemical parameters in the muscular tissue of *Rana ridibunda* Pall. and *Rana esculenta* L.

Parameter	*R. ridibunda* Pall.					*R. esculenta* L.				
	obs. no.	min	max	average	standard error	obs. no.	min	max	average	standard error
GOT (UE/g tissue)	6	0	0	0	0	4	0	0	0	0
GPT (UE/g tissue)	6	0,02	0,10	0,055	0,01	4	0,04	0,12	0,084	0,02
Amylase (UE/g tissue)	6	0,09	0,16	0,111	0,01	4	0,05	0,11	0,08	0,01
Carbohydrates (mg% tissue)	6	20	240	106,66	38,44	4	110	190	142.5	17,97
Cholesterol (mg% tissue)	6	3	39	15,33	5,22	4	0	0	0	0
Triglycerides (mg% tissue)	6	36	244	128,5	35,34	4	35	172	96,25	28,47
Proteins (mg% tissue)	6	2000	4900	3483,3	431,6	4	2400	6000	3975	759,8
Urea (mg% tissue)	6	35	75	50,83	6,54	4	37	62	49	5,30
Creatinine (mg% tissue)	6	0,8	5,8	2,61	0,94	4	0,5	5,9	2,6	1,15

In blood**,** the most of the analysed indices (Table 3) had higher values than in the liver or muscle, and at the *Rana esculenta* L. species the values were higher than those of *Rana ridibunda* Pall. Only the GOT displayed a higher value in *Rana ridibunda* Pall., (an average of 222,66 UE/g) than in *Rana esculenta* L. (an average of 132,6 UE/g), and the urea had similar values at the two species. The analyses displayed high levels of amylases (an average of 375 UE/g at *R. ridibunda* and 415 UE/g at *R. esculenta*) and of urea (an average value of 138,57 mg/100 g tissue at *R. ridibunda* and 138,3 mg/100 g tissue at *R. esculenta*) in blood. There was an important individual variability of the analysed indices in the sanguine tissue as well. At *R. ridibunda*, the highest variability limits were evinced for GOT (between 86 and 457 UE/g) and GPT (between 20 and 93 UE/g). These variability limits are much more obvious at *R. esculenta* and they extend to almost all the investigated parameters (Table 3).

Table 3 - The values of some biochemical parameters in the blood of *Rana ridibunda* Pall. and *Rana esculenta* L.

Parameter	*R. ridibunda* (Pall.)					*R. esculenta* (L.)				
	obs. no.	min	max	average	standard error	obs. no.	min	max	average	standard error
GOT (UE/g tissue)	3	86	457	222,67	117,70	3	100	177	132,66	22,98
GPT (UE/g tissue)	3	20	93	56,67	21,07	3	34	121	64	28,51
Amylase (UE/g tissue)	3	375	375	375	0	3	213	807	415,33	195,87
Carbohydrates (mg% tissue)	3	38	48	43,33	2,90	3	23	161	74,66	43,44
Cholesterol (mg% tissue)	3	35,9	51	41,97	4,60	3	54,5	99,3	82,23	13,99
Triglycerides (mg% tissue)	3	9,4	13,1	11,17	1,07	3	4	19,5	13,6	4,84
Proteins (mg% tissue)	3	14,4	20	16,70	1,69	3	12,3	29	18,7	5,19
Urea (mg% tissue)	3	128	154,7	138,57	8,19	3	104	160,2	138,36	17,30
Creatinine (mg% tissue)	3	0,11	0,21	0,16	0,03	3	0,25	0,38	0,31	0,04

Comparing the values of the analysed indices in the investigated tissues**,** we noticed that the highest levels of GOT, GPT, amylases and urea are in blood, and the highest amounts of carbohydrates, triglycerides, cholesterol and proteins are found in the liver – for the species *Rana ridibunda* Pall. Excepting the carbohydrates, whose amount is higher in the hepatic tissue of *Rana esculenta* L., all the other parameters have maximum values in blood, (Table 1–3).

Following the information within the Tables 4–6 (which resulted after the Student t-test was applied to establish the main differences between the averages of the biochemical parameters), we may state that, for the hepatic tissue, urea has statistic significant value. For the

muscular tissue, any biochemical parameter is not statistically significant. In the case of blood, only creatinine is statistically significant.

Table 4 -Statistical values of the analyzed parameters in the hepatic tissue of *R. ridibunda* and *R. esculenta*

Biochemical parameters	Statistical parameters			
	Averages		t (calculated)	p (two tail)
	R.ridibunda	*R.esculenta*		
GOT	0	0,015	-	-
GPT	0,23	0,21	2,364	0,157
Amylase	0,38	0,24	2,306	0,083
Carbohydrates	1043,33	1332,50	2,306	0,272
Cholesterol	117,50	0	-	-
Triglycerides	979,50	177,66	2,364	0,093
Proteins	4783,33	5075	2,306	0,849
Urea	99,17	74	2,306	0,034
Creatinine	2,10	0,63	2,446	0,081

Table 5 - Statistical values of the analyzed parameters in the muscular tissue of *R. ridibunda* and *R. esculenta*

Biochemical parameters	Statistical parameters			
	Averages		t (calculated)	p (two tail)
	R.ridibunda	*R.esculenta*		
GOT	0	0	-	-
GPT	0,055	0,084	2,306	0,207
Amylase	0,111	0,08	2,306	0,108
Carbohydrates	106,66	142,5	2,306	0,494
Cholesterol	15,33	0	-	-
Triglycerides	128,5	96,25	2,306	0,533
Proteins	3483,33	3975	2,306	0,559
Urea	50,83	49	2,306	0,847
Creatinine	2,61	2,6	2,306	0,991

Table 6 - Statistical values of the analyzed parameters in the blood of *R. ridibunda* and *R. esculenta*

Biochemical parameters	Statistical parameters			
	Averages		t (calculated)	p (two tail)
	R.ridibunda	*R.esculenta*		
GOT	222,67	132,66	2,776	0,494
GPT	56,67	64	2,776	0,846
Amylase	375	415,33	2,776	0,846
Carbohydrates	43,33	74,66	2,776	0,511
Cholesterol	41,97	82,23	2,776	0,052

Triglycerides	11,17	13,6	2,776	0,649
Proteins	16,70	18,7	2,776	0,728
Urea	138,57	138,36	2,776	0,992
Creatinine	0,16	0,31	2,776	0,034

The specialist's references on the hybrid nature of the *Rana esculenta* L. species are based on the study of serical albumins, that display varied migration speeds during the electrophoretic analysis of the proteins from the blood plasma at the two species of green frogs. Thus, Uzzell and Berger (1975), Wijnands and Van Gelder (1976), Günther and Lübcke 91979) obtained electrophoregrams in which three types of migration bands were present (figure 1): the rapid migration (A) corresponds to *Rana lessonae* Cam., the slow one (B) to *Rana ridibunda* Pall., and the one in between the two other (AB), corresponds to the hybrid species *Rana esculenta* L. These results were confirmed by Csata (1998) in Covasna county investigations.

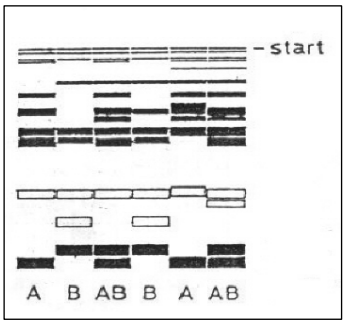

Fig. 1 - Serical albumins at green frogs (Wijnands and Van Gelder, 1976)

Reading the graphs from the electrophoretical analysis of serical proteins for the two species (figures 2**,** 3), one may notice 3 or 4 obvious peaks (each representing a proteic fraction). It may be noticed as well that, while the graph for *Rana ridibunda* Pall. has two distinct peaks in the area of albumins, the graph for *Rana esculenta* L. shows three peaks, which suggests the electrophoretic separation of a supplementary protein fraction at this species. This result sustains the specialists' opinion on the hybrid nature of *Rana esculenta* L. species.

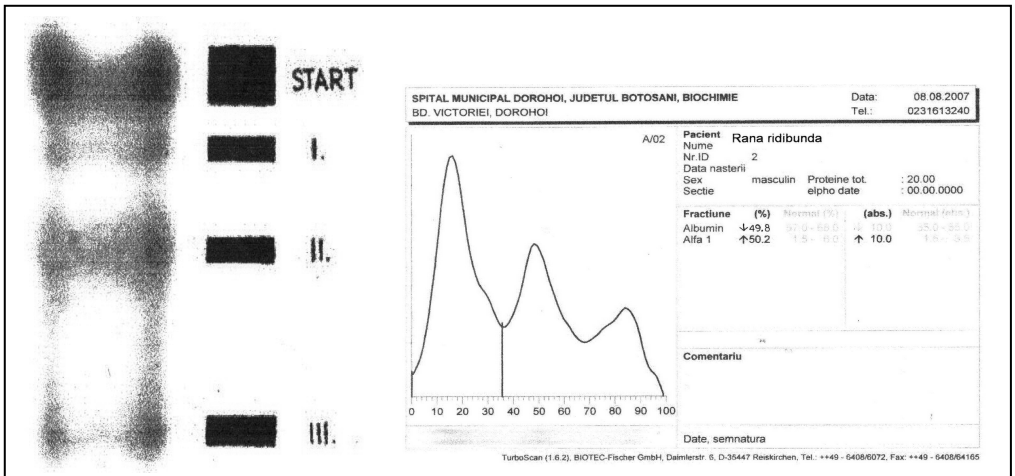

Figure 2 - The electrophoresis of serical proteins at *Rana ridibunda* (Pall.)

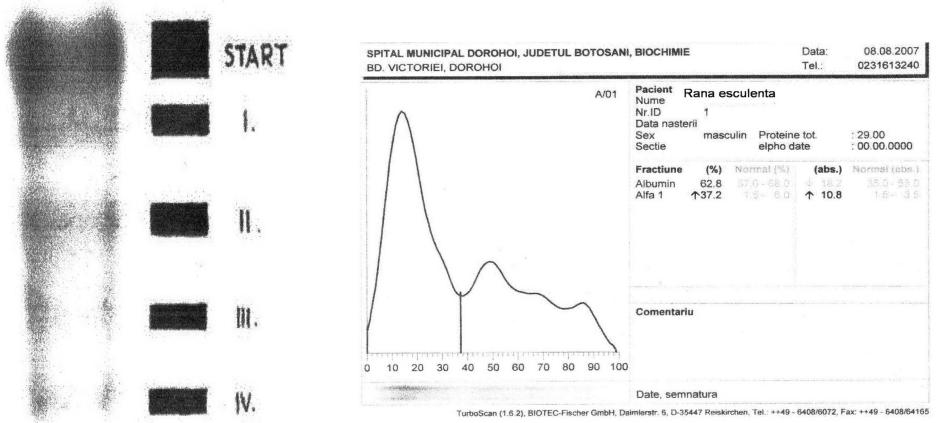

Figure 3 - The electrophoresis of serical proteins at *Rana esculenta* (L.)

CONCLUSIONS

The study of some biochemical indices in the hepatic, muscular and blood tissue at *Rana ridibunda* Pall. and *Rana esculenta* L. pointed out the existence of a high variability, depending on the species, tissue, and even on the investigated individual;

In the liver of *Rana ridibunda* Pall. is found more urea than in the *Rana esculenta* L. liver while the level of creatinine in blood of *Rana ridibunda* Pall. species is lower than in blood of *Rana esculenta* L. species;

The electrophoretical analysis of the blood plasma proteins of the two species of green frogs showed the presence of 3 to 4 proteic fractions. The albuminic fraction of *Rana esculenta* L. contains two sub-fractions, a fact that sustains the idea of a hybrid nature of this species.

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[1] National College "Grigore Ghica" Dorohoi, Botoşani county
[2] University of Bacău, Academy of Romanian Scientists
[3] University "Al. I. Cuza" Iaşi, Romania

NEW PHOTOSENSITIZERS VERSUS AMINOLEVULINIC ACID (ALA) IN EXPERIMENTAL PHOTODYNAMIC THERAPY OF ACTINIC KERATOSIS – A CASE REPORT

DANIEL BODA[1*], MONICA NEAGU[2], CAROLINA CONSTANTIN[2], ADRIANA DIACONEASA[3], SIMONA IANOSI[4], CORNELIA AMALINEI[5], BOGDAN STANOIU[6], EDUARD CRAUCIUC[5], OVIDIU TOMA[9], RODICA-MARIANA ION[7,8]

Keywords: photodynamic therapy (PDT), porphyrins, actinic keratosis, aminolevulinic acid, TSPP

Abstract: Photodynamic therapy (PDT) is increasingly being recognized as an attractive, alternative treatment modality for superficial cancer, being an emerging method for local destruction of tissue by generating toxic oxygen species using light absorbed by an administered or an endogenously generated photosensitizer (porphyrins, phthalocyanines, khellin, hypericin, riboflavin). A considerable number of PDT research over the past ten years has been devoted to the development of new sensitizers. The development of new photosensitizers for localization and treatment of tumors is a research area or current interest. The data show that, when properly used, PDT is an effective alternative treatment option in oncology. This paper will present a case study of actinic keratosis treated with aminolevulinic acid (ALA) and 5, 10, 15, 20 - tetra (4-sulfophenyl) porphyrin (TSPP) in a photodynamic therapy approach. The clinical data proved the efficacy and safety of the method and the new drug. From skin biopsies we have obtained lower absolute number of keratinocytes compared to control skin. The in vitro tests showed cells having lower viability, lower proliferation capacity, and high apoptosis/necrosis percentages; therefore after applying PDT we have obtained an active destruction of the cells.

INTRODUCTION

General Principles

Photodynamic therapy (PDT) can be considered a very promising approach for anticancer research. It consists of the selectively uptake of a photosensitizing dye, often a porphyrin, by a tumor tissue and subsequent irradiation of the tumor with a light flux of an appropriate wavelength matched to the absorption spectrum of the photosensitizing dye. Topical PDT is currently mainly used to treat **actinic keratosis (AK)** and **superficial non-melanoma skin cancers** in an increasing number of countries, with many recent publications providing new evidence in this field (Dougherty et al., 1998).

In PDT a chemical reaction activated by light is used to selectively destroy tissue. The reaction requires three basic elements: *photosensitizing compound* (porphyrins, porphyrin precursors, phthalocyanines, chlorines and others); *light*; *oxygen* ($^1O_2$) and other *free radicals* (Goldman et al., 2008)**.** Depending on the power density, light and laser light in particular causes different effects in biological tissues like photochemical reactions, coagulation, photo- and thermal ablation, plasma formation and photodisruption. The photochemical interaction occurs at very small power densities (0.01 - 50 W/cm^2) and plays significant role during the PDT. To perform this therapy, spectrally adapted chromophores are injected into the body, the most used being porphyrins and related compounds. Irradiation with a suitable wavelength may trigger selective photochemical reactions, resulting in desirable biological transformations (Ion et al., 2006).

Mechanism

PDT produces cytotoxic effects through photodamage of cellular organelles and biomolecules. It is known that PDT mediates tumor destruction by three mechanisms: direct cell killing, tumor vasculature damage and immune response activation. The combination of the three mechanisms is required to obtain long-term tumor control (Huang, 2005).

Direct cell killing. Experimental data show that in optimal conditions (optimum concentration of photosensitizer and light), PDT destroys tumor cells by necrosis (Neagu et al., 2007). In time, photosensitizer’s concentration decreases due to „photobleaching”, vascular stasis, and oedema. In these conditions, as well as in the profound regions of the tissue efficiency of photodynamic therapy lowers, some cells will enter apoptosis and some will survive by defense mechanisms or lesion repair, situation which will lead to relapse (if not destroyed by ischemia or immune response).

The subcellular localization of the photosensitizer (PS) is extremely important. Several studies showed that PS localized in mitochondria produce cell death via apoptosis, while those localized in lysosomes and plasma membranes induce cell death predominantly by necrosis (Manda et al., 2009). Apoptosis is known in clinical practice to cause less tissue reaction compared to cell death via necrosis (after release of lysosomal enzymes and other cytotoxic agents) (Triesscheijn et al., 2006). High light doses cause rapid necrosis within a few minutes, due to massive cellular damage. The potential intracellular targets of PDT are: mitochondria, lysosomes, plasmatic membrane and nuclei of the tumor cells, as well as

tumor vasculature. PS that is not uptaken by cells is inefficient, even though it can generate high amounts of singlet oxygen. PS do not accumulate in the nucleus so they have a low potential to generate DNA lesions, mutations or carcinogenesis which is another advantage of this treatment method.

Vascular damage. Targeting tumor vasculature is one promising approach to cancer treatment. Vascular shutdown (vasoconstriction, thrombus formation) after PDT leads to the limitation of oxygen supply to the tumor and consecutive inhibition of tumor growth. Some studies showed on the other hand that vascular endothelial growth factor (VEGF) and cyclooxygenase (COX-2)- both potent angiogenic factors were upregulated during PDT, presumably due to reactive oxygen species (ROS) formation and hypoxia induced by photodynamic therapy (Taub, 1995; Hasan et al., 2003).

Immune response. The curative effect of PDT arises from the death of cancer cells that initially escape from the direct cytotoxic effect, by the intervention of the antitumor activity of inflammatory cells and tumor –sensitized immune reaction. Differences between the nature and intensity of the inflammatory reaction in normal versus cancer tissues could enhance the selectivity of PDT-induced tissue damage. The inflammatory cytokines interleukin IL-6 and IL-1 (Kelty et al., 2002) but not tumor necrosis factor-α (TNF-α) have been found upregulated after PDT. Also neutrophils were found increased in number after photodynamic therapy, slowing the tumor growth. By combining PDT with immunoadjuvants, an enhancement of the host immune reaction could be obtained, and many studies are conducted recently regarding this aspect (Oleinick et al., 2002).

Light sources

Light source in PDT (typically visible or infrared) emits light at wavelengths within the absorption spectrum of the photosensitizer. Three basic events occur when light is exposed to the skin*: reflection* from the surface of the skin (used for diagnostic purposes); *scattering* by the skin after penetrating it; *absorption* by structures within the skin which conduces to the clinical effect (Goldman et al., 2008). The choice of light source for PDT can be dictated by the location of the tumor, by the light dose delivered and by the choice of PSs. Lasers and lamps have both been employed to perform PDT and activation is obtained by illuminating the lesion site with a proper wavelength light (usually between 600-900 nm) which is absorbed by the PS. This initiates a sequence of photochemical and photobiologicall processes which lead to irreversible damages in target tissues (Nowis et al., 20050. Light penetration is a very important issue in photodynamic therapy, depending on the characteristics of the tissue (pigment-rich tissues are resistant to PDT) as well as on the wavelength of the light: longer wavelength penetrate tissues better than shorter ones. Typically, the depth of penetration is from 3 to 8 mm for wavelengths of 630-800 nm. Despite this fact, PDT can eradicate tumors with up to 1 cm depth, which can be explained by the concomitant activation of local immune response.

Actinic keratosis

Actinic keratosis (AK) is the most common skin lesion with malignant potential, with a prevalence ranging from 11% to 25% in the Northern Hemisphere and from 40% to 50% in Australia (Brand et al., 2000; Frost et al., 1994). The main factors responsible for these lesions are UV light, ionizing radiations, radiant warm and exposure to carbon processing products. The most affected persons are those with Fitzpatrick I and II phototype, men being more susceptible than women; this are considered *in situ* squamous cell carcinoma (SCC) by the most clinicians (Lebwohl, 2003). In time, these lesions could remain unchanged, could spontaneously regress or could progress to SCC and further developing on the support of pre-existing actinic keratosis. 5 to 20% of AK will transform into SCC within 10 to 25 years, with reported annual transformation rates ranging widely, from 0.25% to 16%. (Salasche 2000; Glogau, 2000). This statistic data suggest the powerful impact of actinic keratosis in health population even this lesions are in most cases ignored or wrong diagnosed. The common therapy could be surgical (cryotherapy, removing with or without cauterization or laser therapy) or medication based on topic drugs like 5-flourouracyl, imiquimod, diclofenac gel or PDT. PDT works as a selective targeted treatment with high cure rates. The advantage of porphyrins-PDT is no general cutaneous photosensibility. The application time and dose could differ depending of cell type characteristics.

Photosensitizers

Different photosensitizers have been tested in human tumor tissues. Porphyrin has been the preferred drug used as a photosensitizing substance due to its high affinity for the tumor cell and its strong cellular effect after light irradiation. Ion et al. [Ion 2006] showed that porphyrins can form a variety of structures from linear head-to-tail or J-aggregates to fractal aggregates grown under different regimes of aggregation, and can exhibit rich photophysical properties. Photophysical properties of TSPP porphyrin, as a synthetic compound have been extensively studied both in vivo and in vitro with application for the photodynamic therapy of cancer.

The majority of molecules that result in photosensitization contain a heterocyclic ring structure similar to that of chlorophyll or haem. The most extensively studied PSs so far are the porphyrins. In the last years, more selective and potent sensitizers have been developed, being under investigation in clinical trials. The ideal PS should be a single compound, biologically stable and photochemically efficient, should be retained selectively in the target tissues, displays no dark cytotoxicity, has good cytotoxic oxygen species generation, generates a high quantum yield of triplets, and has increased absorbance in the red region of visible light, for a better light penetration. The localization of PS in the target tissues is of great importance to understand the mechanisms involved in the cytotoxicity of PDT. The cytotoxic product of the photochemical process- singlet oxygen- $^1O_2$ can migrate less than 0.02 μm from its production site; therefore the sites

of photodamage will reflect the localization of the sensitizer at the time of irradiation. Since most PSs are fluorescent, drug localization can be determined by fluorescence microscopy using a sensitive system.
This class of molecules is able of using that energy to produce reactive oxygen species (singlet oxygen, superoxide anion, hydroxyl radicals, so on). The mechanism of cytotoxicity involves generation of singlet oxygen and other free radicals when the light-excited sensitizer loses or accepts an electron.
The porphyrins have been the preferred drugs as photosensitizing substances due to their high affinity for the tumor cell and its strong effect under light irradiation. Different others photosensitizers have been tested for localization in human tumor tissues: fluorescein, cyanine dyes, phthalocyanines, eosin, tetracycline, acridine orange, Rhodamine 123, etc) (Braathen et al., 2007; Frackowiak et al., 2001, 2008; Alexandrova et al., 2004). In addition, several second-generation photosensitizers are undergoing clinical testing. These second-generation compounds are generally pure, can be activated by light in the range of 630-800 nm, and share in common a lower incidence of prolonged cutaneous photosensitivity than Photofrin. One of these is d-aminolevulic acid (ALA), a precursor of the photosensitive protoporphyrin IX in the haeme biosynthetic pathway. A limitation of Photofrin and ALA is their low extinction at their absorption peak furthest into the red region (630 nm).
An ideal PDT photosensitizer should meet several criteria:

- chemical purity and reproducible synthesis;
- no toxicity;
- triplet excitation state of the photosensitizer must have a sufficient life time to be able to generate singlet oxygen;
- preferential accumulation in tumor tissue;
- physical and chemical stability;
- short time interval between photosensitizer administration and its maximum accumulation in tumor tissue;
- optimum absorption properties in the therapeutical window;
- solubility in biological fluids of the body for a rapid reaching at tumor site;
- rapid removal from the body.

Two ***photosensitizers*** are currently approved for topical PDT in dermatology. Methyl aminolevulinate (MAL) marketed as Metvix® is used in Europe, Australia and New Zealand. 5-aminolevulinic acid (ALA) marketed as Levulan® is approved by the FDA and is used in the United States and Canada. The techniques and dosage regimen vary considerably from author to author and their discussion is beyond the scope of this article (Goldman et al., 2008). Promising clinical results have been obtained using them in a variety of superficial malignant and non-malignant lesions such as basal cell carcinoma of the skin, Bowen's disease, mycosis fungoides, psoriasis and actinic keratosis.
Our current study focuses on actinic keratosis treated by ALA-PDT versus a new photosensitizer, 5,10,15,20-tetra(4-sulfonatophenyl) porphyrin (TSPP). The novelty of the research is represented by innovative formulation of porphyrinic materials depending on biological system tested. Previous studies (Ion et al., 1998; Neagu et al., 2007; Ion et al., 2008), from synthesis to clinical application confirmed its safety profile and good clinical response. The preliminary results of the team, suggest that TSPP has a similar efficacy like ALA but with lower secondary effects. Also, every new data will confer a direct impact to clinicians by implementation of a new treatment procedure – photodynamic therapy with a novel porpyrinic photosensitizer in actinic keratosis.

MATERIAL AND METHODS

We present the case of a patient, 61 years old, male presenting with multiple actinic keratosis (AK) on the scalp and face, with no previous therapy. AKs were classified into 3 grades (according to Olsen et al., 1991): 1=mild, 2=moderate, 3=severe. Only isolated 1 and 2 grade AKs were chosen for the study in order to warrant accurate comparison. Grade 3 AKs were excluded from the study comparison because they are known to respond less to PDT treatment. Control photographs were taken immediately after the treatment, 5 days and 10 days after. The patient gave written consent to participate in the study.
All grade 1 and 2 lesions were evaluated by a blinded observer immediately after the treatment, 5 days and 10 days after. A clinical score was used for assessing erythema, hyperkeratosis and thickness of the lesion on a scale between 0 and 3 (0=absent, 1=slight, 2=moderate and 3 =severe). Complete response was defined as a sum score of 0.
On the left side of the scalp and face was applied 20% ALA and on the right side TSPP dissolved in an oil-in-water cream, both for 2 hours incubation time under occlusion. Identical irradiation protocol was used: one session of irradiation 130 J/cm2 fluence at 635 nm wavelength. Pain during treatment was quantified by the patient on a 0 to 10 analog scale.

5,10,15,20-tetra-(4-sulfonatophenyl)porphyrin (TSPP) was synthesized and purified in the laboratory after the literature methods (Ion, 2006). It was solubilized in water at 10^{-4} M concentration. All the stock solutions were stored at 4°C in the dark and used in the 14 days interval.

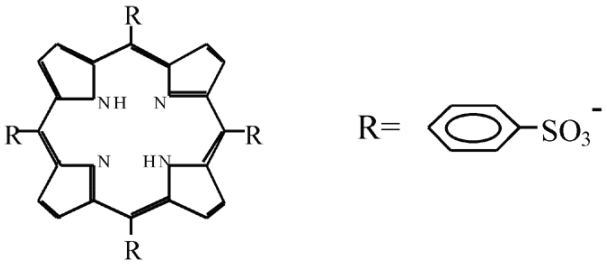


Figure 1. The chemical structure of TSPP

Keratinocyte isolation

Skin biopsies were taken before and after PDT therapy and human keratinocyte cultures were obtained after a protocol comprising several combined methods [Green et al, 1979; Gragnani et al, 2003; Boyce, 1999; E. Radu et al, 2002] in order to obtain the best yield of primary keratinocytes. Briefly, 3 mm^2 punch biopsies before and after PDT were incubated for 24h in antibiotic cocktail medium, after thorough removal of antibiotics, biopsies were incubated for another 24h in PBS without Ca++ and Mg++ containing 2 U/ml dispase (Sigma Chemical Co.), at 4^0C. The epidermis was mechanically removed with twisters. The epidermis was sliced in very small fragments with scissors and incubated for 15-30 min. in trypsin-EDTA (Sigma) at 4^0C. The suspension that contains keratinocytes was washed in PBS without Ca++ and Mg++ by centrifugation at 1000rpm for 10 min at 4^0C. After repeating the washing, cells were resuspended in KMK-2 medium (Sigma). For cultivating keratinocytes, KMK-2 medium was supplemented with bovine pituitary extract, insulin, hydrocortisone, transferine and EGF. Cell suspensions were counted in Trypan blue and further tested.

Cell viability

Besides Trypan blue exclusion test, cell viability was evaluated using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit [Technical Bulletin #TB163, Promega], that measures the lactat dehidrogenase (LDH) released in the culture medium by cultivated cells, being a suitable test for cell membrane integrity.

Proliferation capacity

Isolated keratinocytes were tested for the proliferation capacity using an indirect test that quantifies the intracellular oxidases, namely CellTiter 96 Aqueous One Solution Cell Proliferation kit (Promega) [Technical Bulletin #TB112], as a measure of metabolically active cells in culture.

Apoptosis

Annexin V-FITC (Ann-FITC-green fluorescence) propidium iodide (PI-red fluorescence) kit (BD Biosciences) was used for assessing the apoptosis by flow cytometry. The early feature of apoptosis characterized by morphological change in plasma membrane which involves the translocation of the membrane phospholipid phosphatidilserine from internal layer to external layer of cell membrane is identified by high affinity binding of Ann-FITC. Late apoptotic/necrotic cells in which phospholipid phosphatidilserine translocation has occurred and the cell membrane is damaged, cells are double stained with Ann-FITC and PI. Samples were immediately analyzed in a FACScalibur cytometer (Becton Dickinson) within one hour, using Cell Quest software. Results are presented as follows: percentage cells stained double negative – live cells, double positive – dead/necrotic cells and positive for Annexin, negative for propidium iodide – apoptotic cells (Nowis et al., 2005).

RESULTS AND DISCUSSIONS

TSPP is an anionic porphyrin, a very large disk-shape molecule which posses four negative charges sustained by the sulphonate groups from the four corners. In aqueous solutions, at neutral pH, the electronic absorption spectrum of TSPP is typical of free base porphyrins (D_{2h}

symmetry) and is characterized by an intense Soret band at around 420 nm and four Q bands in the 500-700 nm range (the aetio-type spectrum).
Aggregation of TSPP can be activated by decreasing pH or increasing ionic strength of the solution, and it can be monitored using different positions of absorption peaks corresponding to different species (Figure 2). Because of static Coulombic repulsion, the two central NH fragments are probably distorted out of the aromatic plane, as reported elsewhere (Salasche, 2000). The planar TSPP zwitterionic monomer subunit leads to the suggestion of a straightforward structure for both J- and H-aggregates. In acidic medium, new absorption bands (from 490, 707 nm) become dominant when the concentration of TSPP exceeds 10^{-5} M and they are attributed to the aggregated forms of TSPP, Table I. H and J-aggregates of some porphyrins are based on the intermolecular interactions of 3-5 Kcal/mol per porphyrin face. Such aggregates have the size of 5-6 nm in solution. The columnar structure formed by porphyrins has a length of 5 to 27 porphyrin unities (Ion et al., 2008).

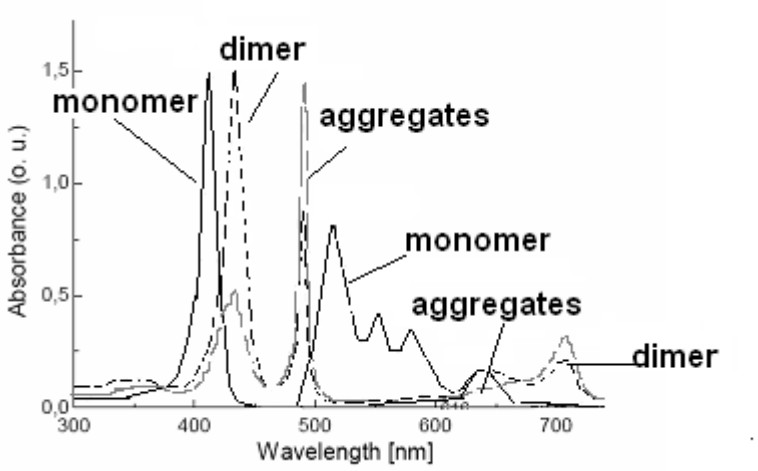


Figure 2. The absorption spectra of TSPP monomer, dimer and aggregates forms

Table I. The specific absorption bands of different TSPP forms

Porphyrinic forms	B band λ/ε x10^3(nm/M^{-1}.cm^{-1})	Q bands λ/ε x10^3(nm/M^{-1}.cm^{-1})
H_2TSPP^{-4}	412/355	515/130 551/4.5 579/1.9 33/1.01
$H_4^{+2}TSPP$ Dicationic form	433/357	550/140 594/3 644/14

H_4^{2+}TSPP J-aggregate	422	490	707
H_4^{2+}TSPP H aggregate	401		517 552 593

Ion, in 2006 and Frackowiak and co-workers, in 2001 showed that the efficiency of the incorporation of these dyes into cellular membrane changes in the same manner as the singlet oxygen generation (Ion, 2006; Frackowiak et al., 2001). The sulphonated porphyrins were better incorporated (unlike to the non-sulphonated ones). The finding can suggest that aggregated forms (J-aggregates) generated for the porphyrins in water are better penetrating the membranes and once in the cellular membrane, dyes are disaggregated by the interaction with lipids and exhibit photodynamic efficiency as the monomeric form.
In our experimental approach, we have obtained from untreated skin biopsies a mean of $2.8x10^4$ keratinocytes/cm^2 skin (Figure 3) with a mean of 65% viability. After therapy from the same skin region and from the same surface isolated keratinocytes were less than half compared to the control skin, displaying as well a lower viability (Figure 3).

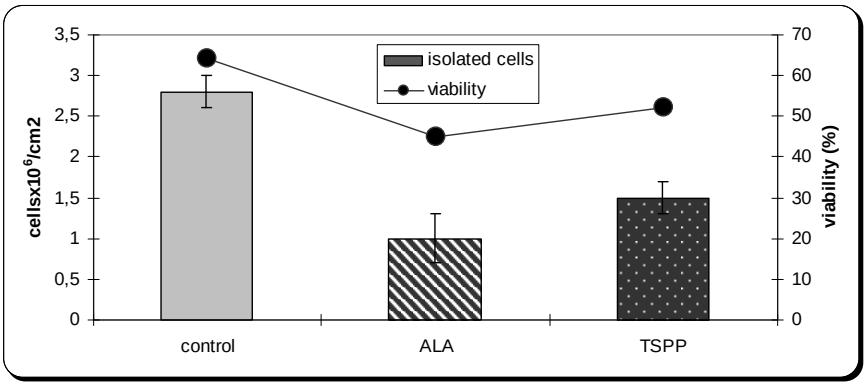


Figure 3. Primary keratinocytes isolated from normal human skin before and after PDT (isolated cells/cm^2 skin and viability) (mean±SD of triplicates)

Primary keratinocytes were further cultivated until the culture could not be maintained. The proliferation capacity of primary keratinocytes extracted from PDT skin biopsies was significantly lower compared to control skin (Figure 4). After 7-8 days from the isolation primary keratinocytes could not be maintained in culture. The functional testing of isolated keratinocytes was done at 72-96h from isolation.

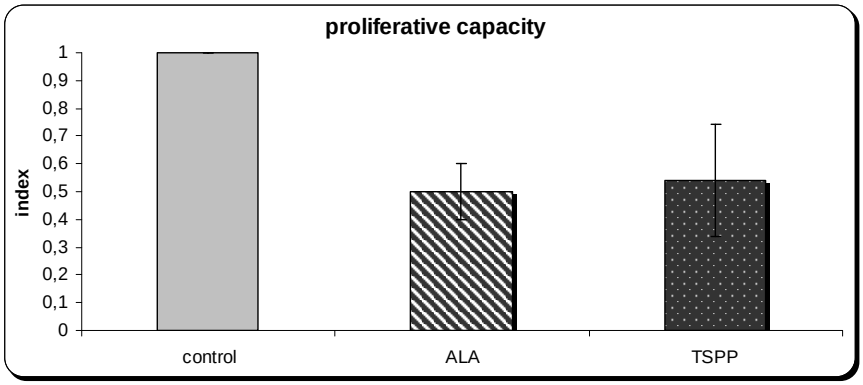


Figure 4. Proliferative capacity of isolated keratinocytes from skin subjected to ALA-PDT (mean±SD of triplicates)

The proliferation capacity matches the high apoptotic pattern of isolated cell post-PDT (Figure 5) in both compounds used in vivo.

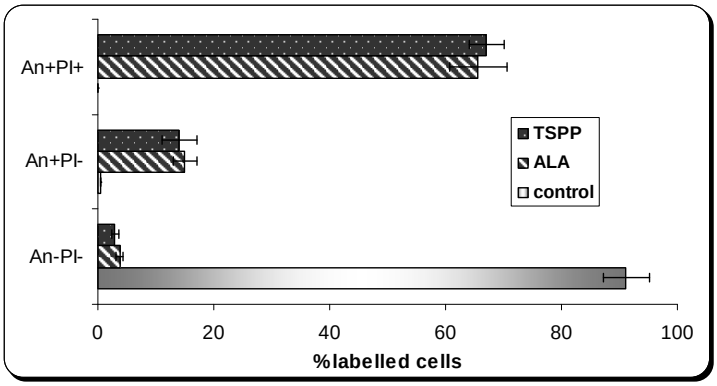
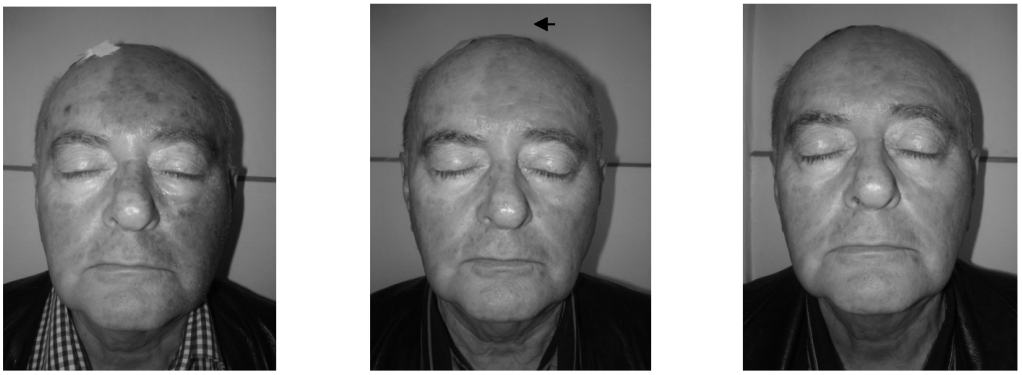


Figure. 5. Annexin-V and propidium iodide labelling of isolated keratinocytes after in vivo PDT compared to control keratinocytes (mean±SD of triplicates) – Necrosis/late apoptosis: An+PI+; Early apoptosis: An+PI-; Live cells: An-PI-.

The tested TSPP showed an effective *in vivo* destructive effect on keratinocytes in the patient with actinic keratosis doubled by a good clinical response. The adverse events to PDT (pain, erythema) were slightly lower in the TSPP treated area (Figure 6).



Day 1 – immediately after phototherapy	Day 2 – 5 days after phototherapy	Day 3 – 10 days after phototherapy

Figure 6. The volunteer patient at different stages at PDT treatment

CONCLUSIONS

We have obtained from the treated skin lower absolute number of keratinocytes compared to control skin. The cells displayed a lower viability proving a lower proliferation capacity in vitro and high apoptosis/necrosis percentages, therefore an active destruction of the cells.
Photodynamic therapy is a potentially effective treatment approach for superficial human cancers and selected benign conditions. The technique can be used as an adjuvant therapy with surgery, radiation or chemotherapy. Newer generation photosensitizers are being tested which may produce less photosensitivity.
TSPP can be a good competitor for ALA in PDT therapy considering the fact that it is cheaper and being a porphyrin by itself it doesn't induce intracellular local protoporphyrin synthesis. Further clinical studies will try to improve TSPP irradiation protocols.

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Acknowledgements: The presented research was funded by National Program CEEX 18/2005, CEEX 107/2006, PN II 41-083/2007, 11-035/2007, Capacitate 334/2007.

[1] „Carol Davila”University of Medicine and Pharmacy Bucharest, Dermatology
[2] “Victor Babes” National Institute of Pathology, Bucharest, Immunology Department
[3] „Grigore Alexandrescu” Hospital Bucharest, Dermatological Department
[4] University of Medicine and Pharmacy Craiova, Dermatology
[5] „Gr.T. Popa” University of Medicine and Pharmacy Iasi, Histology
[6] University of Medicine and Pharmacy Craiova, Cell and Molecular Biology
[7] National Institute of Research and Development for Chemistry and Petrochemistry – ICECHIM, Bucharest
[8] Valahia University, Targoviste, Romania
[9] “Alexandru Ioan Cuza” University of Iasi

*dan.boda@gmail.com

THE IMPORTANCE OF THE INDIVIDUAL CHARACTERISTICS IN THE ESTABLISHMENT OF THE DIAGNOSIS FOR THE PSYCHO-SOMATIC AFFECTIONS

ANGELA DUMITRESCU[1]*, EDUARD CRAUCIUC[1], DAN MARIAN DUMITRESCU[1], DANIELA LEPĂDATU[1], VLAD ALEXANDRU MOLFEA[2], OVIDIU TOMA[3], GEORGE IOAN PANDELE[1]

Keywords: personality traits, depression, AHT

Abstract: Starting from the statement that in the onset of the psycho-somatic disorders an important role is played by psychological factors and the personality of the patient together with cardiovascular risk factors, we believe that it is important to highlight and correlate all the possible aspects. Aim: The objectives of the study are to stress the differences in the structure of personality between the patients with arterial hypertension (AHT) and depression and the ones diagnosed only with depression syndrome. Material and methods: the 200 patients studied were divided in two groups: 100 patients with depression and 100 with depression + AHT. Results: After processing the data obtained by anamnesis and questionnaires focused on the individual characteristics of the patients, we observed that the group diagnosed with depression and AHT has a significantly increased level in dynamism, domination, perseverance and meticulosity while the group with depression has a significantly increased level of cooperation, amiability and openness for culture. Conclusions: There are important differences between the two study groups regarding their personality traits.

INTRODUCTION

The personality trait is the concept highlighting those characteristics or individualities of a person or psychological process which are relatively stable. In G.W.Allport’s opinion, the personality is a structure formed by hierarchically organized features, with each individual having 2 or 3 cardinal qualities that dominate and control the others (Allport, 1991).

A consensus was reached regarding the evaluation methods for personality traits, by the elaboration of a model integrating the basic characteristics of personality, some researchers using the phrase “the five greats”. The model proposed by Costa and McCrae represents the foundation for a widely used measurement scale – NEO-Personality Inventory-Revised (De Raad, 2000). The five large dimensions of personality are called “neurosis”, “extraversion”, “openness”, “agreeability” and “consciousness” (tab.1).

Table 1. The facets of the traits associated with the five domains in the personality model

Neurosis (N)	Anxiety, furious hostility, depression, self-consciousness, impulsivity, vulnerability
Extraversion (E)	Warmth, assertion, active spirit, looking for sensation, positive emotions prevail
Openness (O)	Fantasy, esthetic sense, feelings, actions, ideas, values
Agreeability(A)	Confidence, direct behavior, altruism, flexibility, modesty, sensitivity
Consciousness (C)	Competence, order, sense of duty, makes all efforts to achieve success, self-discipline, carefulness

It has been noted that tiredness, stressful work-environment (Serrats et al., 2005), psychological and social stress etc., psychic trauma, negative emotions such as anxiety (Clemett et al., 2000), depression (Frazer et al., 2002), alexithymia (Grewen et al., 2004), rage and hostility (Kowalik, 2004, Larkin et al., 2004) lead to a decrease in the functional ability of the brain, with interferences in the function of the hypothalamus resulting in hyper excitability and important instability of the blood-flow regulating function. The literature shows that individuals with choleric temperament present a certain degree of hostility, and these aggressive hostile persons have increased levels of nor-epinephrine hormone (nor-adrenaline) in their blood. This hormone is released in correlation with various stress factors,

indicating an internal imbalance, frequently encountered in cardiovascular pathology and in the general predisposition for illness.

Our paper concentrates on two disorders with raised incidence in the general population, having direct implications in mortality and morbidity and long-term economic consequences – arterial high blood-pressure and depression syndrome (Gallo et al., 2003, Jonas et al., 1997). A very important role is played in the onset of these affections by the psychological factors and the personality of the patient together with cardio-vascular risk factors. Thus, we believe that it is important to highlight and correlate all the possible aspects.

The aims of our study are *(i)* to emphasize the differences in the structure of the personality between the patients with AHT and depressive syndrome and those diagnosed only with depression; *(ii)* to identify the modifications determined by the depression level on the personality features; *(iii)* to detect the modifications determined by the level of blood-pressure on personality traits.

MATERIAL AND METHODS

There were investigated 200 patients admitted in the Departments of Psychiatry and Internal Medicine at several Clinical Hospitals in Romania, diagnosed with raised blood pressure according to the values of ESH/ESC guides and with depression disorder according to DSM-IV TR criteria, 71 males and 129 females. The age varied between 20 and 80 years; 99 patients came from the urban environment and 101 from the rural one. They presented different degrees of education, as follows: 14 attended only elementary school, 28 – middle school, 64 – technical school, 21 graduated high-school, 11 obtained the bachelor's degree, 45 underwent college and 17 accomplished their undergraduate studies.

The study protocol recorded the following factors: name/surname, gender (male/female), environment (rural/urban), education degree (elementary, middle or technical school, superior studies). There were evaluated several dimensions of the personality features: a. five fundamental factors: energy, kindness, conscious character, emotional stability, and openness of spirit; b. 10 specific personality traits: dynamism, dominance, cooperation, friendly attitude, meticulosity, perseverance, emotion control, impulse control, openness towards culture, openness towards experiencing – all of them evaluated with the Alter Ego questionnaire.

With respect to the application order of these instruments, we pursued a progressive particularity degree, beginning with anamnesis and continuing with Alter Ego personality inventory. For the completion of the investigation we used the observation charts and the questionnaires and for the statistical processing of the data we appealed to the SPSS (Statistical Package for the Social Sciences) program version 15.0 and to the Microsoft Office XP package as well.

RESULTS AND DISCUSSIONS

In this section we will present all the dimensions included in the Alter Ego questionnaire for the entire investigated group and we will make comparisons between the two groups of patients: with depression and with depression + AHT.

The Alter Ego questionnaire is organized on three levels which at their turn present three progressive stages, thus: the inferior level comprises stages 1, 2 and 3, the medium level presents stages 4, 5 and 6 and the superior level contains stages 7, 8 and 9. We present below the personality features graded according to the score obtained on the three stages. Thus:

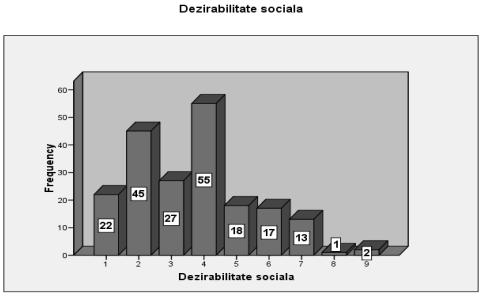

Fig. 1. Repartition of social desirability in the studied population

Social desirability (fig.1) recorded higher values in stage 4 and 2, the inferior level prevailing, followed by the medium level, while the lowest scores belonged to the superior level. In the depression+ AHT group there were recorded higher scores for stage 2 and 4 as opposed to the depression group were the scores for stage 1 and 5 predominated.

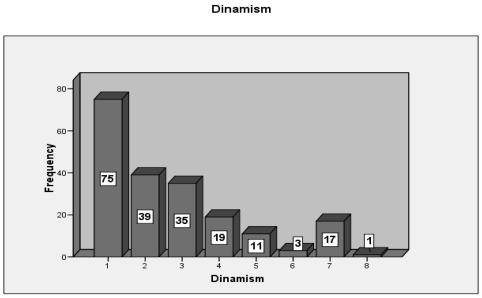

Fig. 2. Repartition of dynamism in the studied population

Dynamism (fig.2) recorded increased values in stages 1, 2 and 3, the inferior level outweighing in the entire group while the superior level was situated at the opposed pole. The depression group presented high scores for stage 1 and 2 as compared to depression+ AHT group which had higher values for stage 3, 4 and 7.

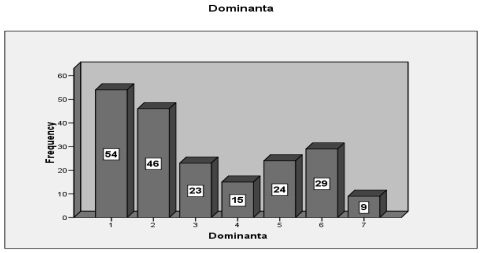

Fig. 3. Repartition of dominance in the studied population

Dominance (fig.3) had higher scores in stage 1, 2 and 5, the inferior and medium level obtaining significant values. The depression group presented increased values in stage 1 and 2, while in the depression+ AHT group the values for stage 3, 4, 5 and 6 were in the majority.

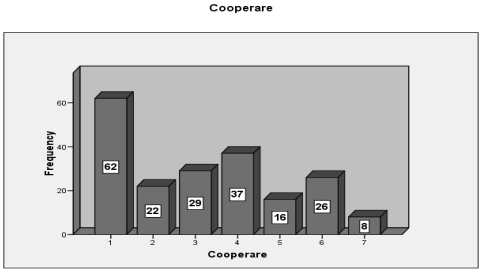

Fig. 4. Repartition of cooperation in the studied population

Cooperation (fig.4) presented raised scores for stage 1, 4 and 3, the inferior and medium level dominating. In the depression+ AHT group we noted slightly raised values for stage 1, 3 and 4, as compared to the depression group where stage 2, 5 and 7 obtained increased scores.

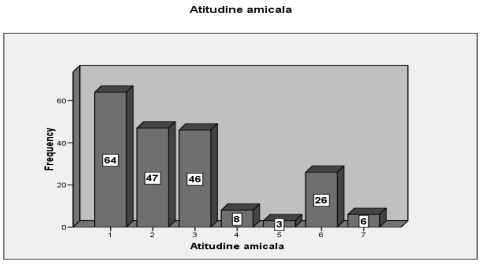

Fig. 5. Repartition of friendly attitude in the studied population

Friendly attitude (fig.5) recorded increased values in stage 1, 2 and 3, the inferior level registering maximum grades while the superior level was insignificant. In the depression+ AHT group there were higher values for the above mentioned stages (38, 24, 25) in comparison with the depression group (26, 23, 21), stage 6 being here better placed (17) than in the other group (9).

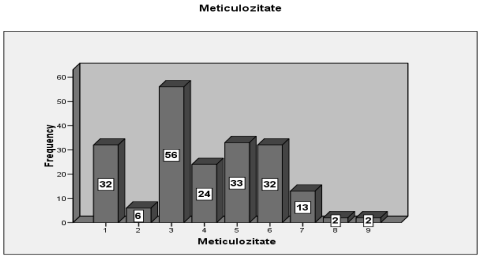

Fig. 6. Repartition of meticulosity in the studied population

Meticulosity (fig.6) presented high scores for stages 3, 5, 6, 4 and 1, which means that the inferior and medium level predominated, the superior level being very faintly represented. In the depression group stage 1, 3 and 5 recorded higher values than for the group with depression+ AHT, where stage 4 and 6 had increased marks.

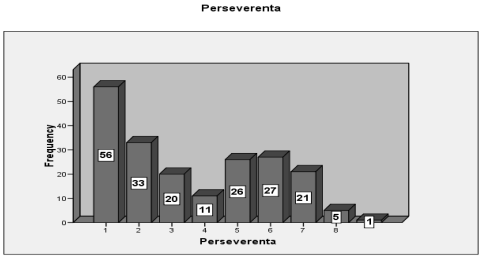

Fig. 7. Repartition of perseverance in the studied population

Perseverance (fig.7) recorded raised scores for stages 1, 2, 5, 6 and 3, meaning that the medium level was secondary to the inferior one. In the comparison of the two groups we observed that the inferior level was better represented for the depression group while for the depression+ AHT group the medium level had higher values.

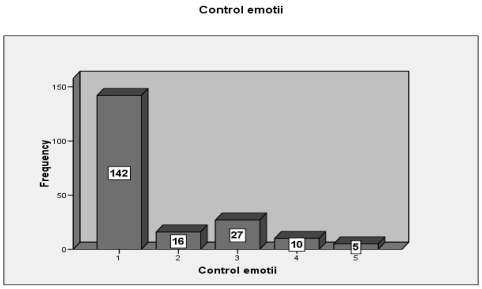

Fig. 8. Repartition of emotion control in the studied population

Emotion control (fig.8) registered maximum values for stage 1, the medium and superior level being extremely faintly represented. In the depression+ AHT group 73 points were accumulated in comparison with 69 for the depression group.

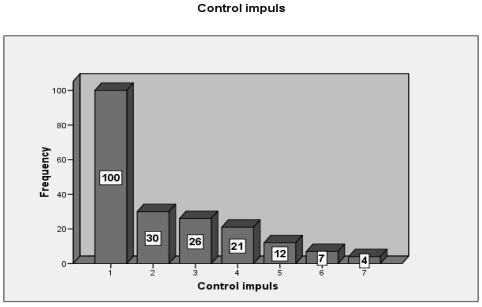

Fig. 9. Repartition of impulse control in the studied population

Impulse control (fig.9) recorded a maximum score for stage 1, followed by the scores for stage 2 and 3, meaning that the inferior level prevailed. The depression+ AHT group presented slightly raised values for stage 1 than the depression group.

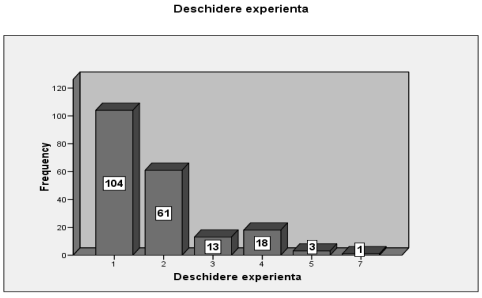

Fig.10. Repartition of openness towards experiencing in the studied population

For the openness towards experiencing item (fig.10) stages 1 and 2 present high values, which indicates that the inferior level preponderates. The depression group has slightly increased values than the depression+ AHT group for the above-mentioned stages.

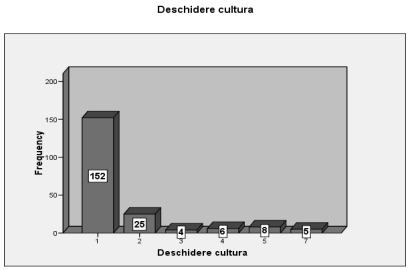

Fig. 11. Repartition of openness towards culture in the studied population

Regarding the openness towards culture (fig.11) stage 1 presented maximum values, the other being insignificant. The same thing was noticed in the comparison of the two patient groups, the depression (73) and depression+ AHT (79) study lots.

For the "social desirability" dimension we can state that the depression+ AHT study group has an increased tendency towards supplying the others with a falsely positive profile as opposed to the depression study group. Regarding "dynamism" we noted that the patients from depression+ AHT group with low depression level and first degree AHT tend to describe themselves as dynamic, active, energetic and dominant while those with third degree AHT are devoid of energy, quiet, deprived of the capacity to impose themselves and influence the others. In the depression study group, those diagnosed with mild and medium depression present acceptable values, these individuals having still some degree of energy and elocution facility.

The number of patients from the depression study group with a very low and low level of "dominance" is high, in contrast with the patients from depression+ AHT study group, these (presenting with severe and medium depression) being deprived of competitive spirit, the tendency to impose or influence other's decisions. In depression+ AHT (patients with first and second degree of AHT) the level of "dominance" characteristics is medium and low.

Following the evaluation of the "cooperation" dimension we observed that the patients in depression group are less cooperative, altruistic, generous or empathic than those in the depression+ AHT group, the latter having a better ability to understand the needs and difficulties of others, to help them and to efficiently cooperate with others (especially those with first and second degree of AHT).

By comparison, we can affirm that the patients associating depression and AHT have a better degree of "openness towards experiencing", taste for the new, openness towards values, stiles and ways of life and culture than the patients in the depression study group that describe themselves as lacking imagination and ideas, less creative, inventive or curious.

The depression+ AHT group (second and third degree) have a low level for the "friendly attitude" dimension, in other words they do not trust or feel opened towards the others and they lack benevolence as opposed to the depression study group.

Regarding "meticulosity" the patients in the depression group have lost the preoccupation for order, the care for detail in everything, they are less attentive, ordered and precise (especially those with severe and medium depression) than the ones in depression+ AHT

group who possess a better self-regulation ability (regarding the patients with mild depression and first or second degree AHT).

After the analysis of the results, we can state that the depression+ AHT study group has a medium level of ”perseverance”, which indicates a constancy and perseverance in accomplishing tasks and activities and an important care not to fail in the engagements they assume, the depression group recording low values for this dimension.

With respect to “emotion control”, subdimension of the “emotional stability” factor, we noted that the patients associating depression and AHT fail to achieve a good control of emotional tense situations, of self-control as opposed to the patients with depression. Likewise, the patients with depression+ AHT attain a lower level of “impulse control” than the patients in the depression group, these being less calm, emotional, presenting anxiety, depression, irritability, irascibility and impulsivity.

The “openness for culture”, subdimension of “openness of mind” factor that evaluates the desire to be informed, the interest towards accumulating new pieces of knowledge presents decreased values in the entire studied population, more evident for the second and third degree AHT patients associating medium and severe depression.

CONCLUSIONS

In the onset of some disorders, a very significant role is played by the psychological factors and the personality of the patient. In these cases, a careful clinical examination, with a focus on the importance of anamnesis may reveal the presence of an intra-psychological conflict.

The statistical analysis performed demonstrated significant differences between the two groups regarding the personality traits. Thus, the patients in depression+ AHT group have a significantly increased level of depression and anxiety and also a significantly raised level of dynamism, dominance, meticulosity and perseverance while the patients in depression group have a significantly higher level of cooperation, friendly attitude and openness towards culture.

Moreover, there are highly significant and negative correlations in the case of cooperation, friendly attitude and openness towards culture. Patients with first and second degree of AHT are more active, energetic, meticulous and perseverant than those with third degree AHT. We recorded decreased scores of cooperation, friendly attitude and openness towards culture for increased values of blood pressure.

According to the personality traits, the AHT patients van be divided in several groups: a. patients with first degree AHT and mild depression, who are extraverts, sociable, energetic and communicative; b. patients who are asthenic, introverts, slow, obese, with unhealthy habits and tendencies towards inactivity, irritable, irascible, somatic and impassive; c. patients with mild and medium forms of depression who are energetic, active, involved in their work, with various disease of the cardio-vascular system, having impulsive-explosive tendencies, being hostile and conflictive and d. the group comprising the greatest number of subjects, who are communicative, apparently sociable, with a hidden hostility and aggressivity, competitive, permanently tense, irritable, introverts, cooperating, meticulous and perseverant.

We noted highly significant and negative correlations between depression and personality traits, thus a raised level of depression being associated with a decreased level of social desirability, dynamism, dominance, cooperation, perseverance, emotion and impulse control, openness towards experiencing and culture.

We consider that the somatic, psychic or psycho-somatic disorder represents in the beginning only a morbid manifestation of the personality, of the individual or individuality and that personality and its newer structures are secondary influenced by the pathologic process and at their turn, these personality particularities, the temperamental ones respectively, will influence the manifestations and expressions of the disorder and conversely, in a closed morbid circuit.

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[1] University of Medicine and Pharmacy "Gr.T.Popa" Iasi, Faculty of Medicine
[2] University of Medicine and Pharmacy "Carol Davila" Bucharest, "St. Pantelimon" Hospital, Cardiology Clinic
[3] "Alexandru Ioan Cuza" University of Iasi

* dmadumitrescu@yahoo.com

Acknowledgements: We would like to acknowledge the medical team that assisted us in the achievement of this paper and also to SC. Management Design S.R.L. for the support in the accomplishment of this study and for the Alter Ego questionnaire, adjusted to the Romanian population.

PHYTOCHEMICAL STUDY OF SOME ACTIVE PRINCIPLES WITH ANTIOXIDANT ACTION FROM THE *ROSMARINUS OFFICINALIS* AND *SALVIA OFFICINALIS* SPECIES

IONELA DACIANA MIERLICI*

Keywords: hydroalcoholic extract, sage, rosemary, antioxidants
Abstract: Many natural products have been reported to contain large amounts of antioxidants other than vitamin E, C, carotenoids (Javanmardi et al., 2003). These antioxidants play a role in delaying, intercepting, or preventing oxidative reaction (Vilioglu et al., 1998) catalysed by free radicals. This antioxidant activity may be mainly do to the present of phenolic components such as flavoinoids (Pietta,1998), phenolic acids and phenolic diterpenes (Shahidi et al., 1992). It is necessary to find out if medicinal plants could provide the antioxidant substances that may help to modulate oxidative stress who are associated with many pathological disorders.

This phytochemical study emphasized the existence of some compounds with antioxidant action flavon and poliphenol types in hydroalcoholic extracts of *Salvia officinalis* and *Rosmarinus ofiicinalis*.

INTRODUCTION

The term „antioxidant” is mainly used for a chemical compound consuming molecular oxygen.

Antioxidant agents are substances capable to protect the organism against destructions caused by free radicles.

They can be divided into: primary and secodary. Most of the antioxidants used are primary antioxidants. They are phenol compunds having various substitutes attached: phenol acids, flavonoids, antocianydins, lignans, tannins, coumarins. Secondary antioxidants include metallic complex agents, singlet oxygen and others (Larson R.A., 1997).

First details regarding the antioxidating activity of the medical plants appeared in the 50’s, when Chipault and Co. did a study on 72 medical plants, testing their antioxidant capacity (Chipault and Co,1956; Chipault and Co.,1952). Rosemary and sage proved to be the most efficient resources of antioxidants. These plants have been treated very carefuly lately. Various studies proved the efficiency of these plants and had as result different commercial applications (Braco and Co, 1981).

The active compounds from the sage and the rosemary, presenting antioxidant properties are the phenol acids, flavonoids, natural pigments (capsaicin and curcumin), and terpens (rosemanol, carnosol, carnosic acid, epirosemanol, isorosemanol) (Cuvelier and Co., 1994).

Chang *et al* studied the antioxidant properties of rosemary and sage extracts in a wider variety of solvents (hexane, benzene, ethyl ether, chloroform, dichlorethylene, dioxane and methanol) applied to lard stored at 60ºC in the dark. This time, peroxide value was determined and rosemary extracts in dichlorethylene, ethyl ether and methanol showed the lowest results. Wu *et al* confirmed the antioxidant efficiency of the rosemary (0,02%) methanolic extract in lard stored in the dark for 6, 14, 21, 28 and 36 days through peroxide value determination.

In this paper we proved the presence of the polyphenols and flavones, substances with antioxidant properties, in vegetal hydroalcoholic extractsobtained from the sage (*Salvia officinalis)*) and the rosemary (*Rosmarinus officinalis*).

MATERIAL AND METHODS

As raw materials, we used 2 species of medical plants, that is sage (*Salvia officinalis)* and rosemary (*Rosmarinus officinalis).* The biological material comes from the spontaneous flora and was purchased in 2006 from SC A&C Network Ltd Brăila (rosemary) and from certified natural person in Tulcea (sage) and in that year they were also analysed.

From the two species of plants, we did more hydroalcoholic extracts obtained by processing the air part, varying the ethanolic concentration (40, 50 and 70%) as well as the report plant - solvent (1:7 and 1:10). The phytochemical analysis consisted of the assessment of the contents in polyphenols and flavones. For the polyphenols, the caffeic acid and the rosmarinic acid were dosed.

The determination of the contents in flavones was done using reactives: ethylic alcohol of 50^0; sodium acetate, sol.10%; aluminium chloride, sol. 2,5%; ruthenium (s.r.), sol. 0,1 g/l in ethylic alcohol of 50^0.

Determination completion

3-4ml for test are weighed in a 50 ml baloon and dilluted at the sign with ethylic alcohol of 50° (solution A). In a 25ml baloon we put 3ml of A solution, we add 5ml of sodium acetate solution and 3ml of aluminium chloride solution, stirring after each add. We dilute at the sign with ethylic alcohol of 50° and we stir.

Compensation solution: in a 25 ml balloon we put 3 ml of A solution, diluted up to the sign with ethylic alcohol of 50°.

After 15 minutes the absorbance of the analysed solution is measured at 430 nm, as compared to the compensation solution.

Determination of the contents in rosmarinic acid was done using the reactives: ethylic alcohol of 50^0; chloride acid of 0,5M; sodium hydroxid 1n; Arnow reactive: 10g sodium nitrite and 10g of sodium molybdate are dissolved in 100ml of distilled water.

Determination completion

1 -1-2 ml of fluid extract are weighed in a 50 ml balloon, then diluted up to the sign with ethylic alcohol of 50°(A solution).

2 -1ml of A solution is introduced in a 10 ml level test tube. We add 2 ml of chloride acid 0,5 M, 2 ml Arnow reactive, 2 ml of sodium hydroxid 1n, diluted with water up to 10ml and then we mix.

3 -Compensation solution: In another 10 ml level test tube we put: 1ml A solution, 2 ml chloride acid 0,5M, 2 ml sodium hydroxid 1n and diluted in water up to 10ml.

We immediately measure the absorbance of the analysed solution at 505 nm, as compared to the compensation solution.

RESULTS AND DISCUSSIONS

Quantitative phytochemical analysis for polyphenols and flavones at *Salvia officinalis*

From *Salvia officinalis,* air part, we did various types of hydroalcoholic extracts, using a plant: solvent report (ethylic alcohol) of 1:7 and 1:10 and three ethanolic concentrations: 40, 50 and 70%. The extracts were done both in hot and in cold (tinctures). The solutions were clear, redish brown colour, specific smell.

The ethanolic extracts obtained from the vegetal matter were analysed from a quantitative point of view for the assessment of the contents in polyphenols (expressed in caffeic and rosmarinic acids) and total flavones (expressed in rutozid). Details are found in tables 1 and 2.

Table no 1. Polyphenols and flavones content from hot extracts of *Salvia officinalis*

No.	Tests	Concentration EtOH	Polyphenols (g % s.u.)		Flavones rutozid, (g % s.u.)
			Caffeic acid	Rosmarinic acid	
1.	*Salvia officinalis* 1:7	40%	2,547	1,176	2,222
2.		50%	3,008	1,387	2,297
3.		70%	3,088	1,426	2,425
4.	*Salvia officinalis* 1:10	40%	1,902	0,878	0,488
5.		50%	1,936	0,893	0,512
6.		70%	2,087	0,963	0,681

Table no 2. Polyphenols and flavones content from cold extracts (tinctures) of *Salvia officinalis*

No.	Tests	Concentration EtOH	Polyphenols (g % s.u.)		Flavones rutozid, (g % s.u.)
			Caffeic acid	Rosmarinic acid	
1.	*Salvia officinalis* PLV (1:10)	40%	1,614	0,745	1,091
2.		50%	1,932	0,892	2,566
3.		70%	2,209	1,020	3,102
4.	*Salvia officinalis* PLV (1:7)	40%	2,001	0,924	2,047
5.		50%	2,320	1,071	2,829
6.		70%	2,777	1,283	3,111

The analysis of the results obtained in case of hot extracts (table 1), prove rather high quantities of poliphenols and flavones. There is a direct correlation between the concentration of the ethanolic extract and the quantity of the total polyphenols and flavones dosed in here.

Dosed flavones have values between 0,488 g% s.u. and 2,425 g% s.u. We can see that the lower percentage of flavones (0,488g% s.u.), belongs to the EtOH 40% and the plant: solvent report of 1:10, and the highest percentage in flavones (2,425g% s.u.), belongs to the EtOH 70% concentration and the plant: solvent report of 1:7.

Polyphenols dosed in hot extracts have values between 1,902 g% s.u. and 3,088 g% s.u., for the caffeic acid and between 0,878 g% s.u. and 1,426 g % s.u., for the rosmarinic acid. We see that the lowest percentage of polyphenols expressed in caffeic acid (1,902 g% s.u.) belongs to the 40% concentration and the plant: solvent report of 1:10, and the highest one (3,088 g% s.u.) to the 70% concentration and the plant: solvent report of 1:7. Also, the lowest percentage of polyphenols expressed in rosmarinic acid (0,878 g% s.u.) belongs to the 40% concentration and the plant: solvent report of 1:10, and the highest one (1,426 g% s.u.) to the 70% concentration and the plant: solvent report of 1:7.

Tinctures obtained from *Salvia officinalis*, have increased quantities of flavones and polyphenols, as compared to the extracts obtained in hot (table 2). This way, at the 70% concentration and the plant: solvent report of 1:7, the value for the polyphenols expressed in caffeic acid is of 2,777 g% s.u., and for the rosmarinic acid is of 1,283 g% s.u. At the same concentration (70%) for flavones we get a value of 3,111 g% s.u.

Phytochemical quantitative analysis at *Rosmarinus officinalis*

From *Rosmarinus officinalis* we did various types of vegetal extracts, using ethanolic concentrations of 40, 50 and 70% and a report between plant and solvent (ethylic alcohol) de 1:10. Extracts were done in hot and cold (tinctures – extraction time being of 8 days at room temperature). Solutions were clear, redish brown colour, specific smell.

The quantitative analyses were based on the polyphenol dosing, coffee and rosemary type and the flavones expressed in rutozid (tables 3 and 4).

Table no 3. Polyphenols and flavones content from hot extracts of *Rosmarinus officinalis*

No.	Tests	Concentration EtOH	Polyphenols (g % s.u.)		Flavones rutozid, (g % s.u.)
			Caffeic acid	Rosmarinic acid	
1.	*Rosmarinus officinalis* 1:10	40%	1,453	0,671	1,706
2.		50%	1,897	0,876	2,147
3.		70%	2,003	0,925	2,206

Table no 4. Polyphenols and flavones content from cold extracts (tinctures) of *Rosmarinus officinalis*

No.	Tests	Concentration EtOH	Polyphenols (g % s.u.)		Flavones rutozid, (g % s.u.)
			Caffeic acid	Rosmarinic acid	
1.	*Rosmarinus officinalis* 1:10	40%	2,599	1,200	1,304
2.		50%	2,859	1,320	1,429
3.		70%	3,227	1,490	1,700

For the ethanolic extracts obtained in cold, we see a quantitative variation of the polyphenols expressed in caffeic acid, from 1,453 g% s.u. (concentration EtOH 40%) to 2,003 g% s.u. (concentration EtOH 70%). To these extracts, the values of the rosmarinic acid contents are under 1 g% s.u., with the lowest value of 0,671 g% s.u. for the EtOH concentration of 40%, and of 0,925 g% s.u. for the EtOH concentration of 70%.

Flavones dosed in extracts have values between 1,706 g% s.u. (concentration EtOH 40%) up to 2,206 g% s.u. (concentration EtOH 70%). In the case of rosemary there is also a direct correlation between the concentration of the ethanolic solution and the quantity of active principles dosed in here; the highest quantities of polyphenols and flavones belong to the extract version 70%, the extraction output of this version being the highest one.

The tinctures obtained from R*osmarinus officinalis* have increased quantities of polyphenols expressed in caffeic and rosmarinic acids (Table 4), as compared to the extracts obtained in hot. For the caffeic acid, the values are between 2,599 g% s.u. and 3,277 g% s.u., and for the rosmarinic acid the values are between 1,200 g% s.u. and 1,490 g% s.u. The highest quantities of polyphenols belong to the concentration of 70%, the value for the polyphenols expressed in caffeic acid is of 3,227 g% s.u., and for the polyphenols expressed in rosmarinic acid of 1,490 g% s.u. A ligh decrease of the values for flavones at rosemary tinctures was observed, as compared to the extracts in hot, so that the minimum is of 1,304 g% s.u. and the maximum 1,700 g% s.u. At the same concentration (70%) for flavones, the highest value belongs to the extracts in hot (2,206 g% s.u.), as compared to only 1,700 g% s.u. at the tincture.

CONCLUSIONS

In case of hydroalcoholic sage extracts, the values for polyphenols and flavones range according to the ethanolic concentration and the report between solvent and vegetal matter. The minimum quantity of polyphenols espressed in caffeic acid (1,614g % s.u.) is also met in the cold extract of *Salvia officinalis* (40% ethanolic concentration and the plant: solvent report of 1:10), and the maximum (3,088 g% s.u.) is also met at the hot extract of sage (70% ethanolic concentration and the plant: solvent report of 1:7); the latter one is also characterized by the highest values for polyphenols expressed in rosmarinic acid (1,426 g% s.u.).

The maximum quantity of flavones (3,111 g% s.u.) is met in the cold sage extract (70% ethanolic concentration and the plant: solvent report of 1:7).

Tinctures obtained from *Rosmarinus officinalis* have higher quantities of polyphenols expressed in caffeic and rosmarinic acids, as compared to the hot extracts. The minimum quantity of polyphenols expressed in caffeic acid (1,453 g% s.u.) and in rosmarinic acid (0,671 g% s.u.), belong to the hot rosemary extract (ethanolic concentration of 40%), and the maximum quantity

of polyphenols expressed in caffeic acid (3,227 g% s.u.) and in rosmarinic acid (0,490 g%s.u.), belong to the cold rosemary extract (70% ethanolic concentration).

A slight decrease of the values was observed for the flavons, at the rosemary tinctures, as compared to the hot extracts, so that the minimum is of 1,304 g% s.u. in the hot extract (40% ethanolic concentration), and the maximum 2,206 g% s.u. in the cold extract (70% ethanolic concentration).

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SC CCPPM PLANTAVOREL SA, Piatra Neamt
*) ionela_daciana@yahoo.com

CELLULOSE/CHONDROITIN SULPHATE HYDROGELS AS CARRIERS FOR DRUG DELIVERY APPLICATIONS

ANA-MARIA OPREA[1*], ANDREI NEAMTU[2], BOGDAN STOICA[2], CORNELIA VASILE[1]

Keywords: drug delivery, hydrogels, biocompatibility, codeine

Abstract: The potential of the hydrogels based on natural, biodegradable and biocompatible polysaccharides, cellulose (C) and chondrotin sulphate (GAG), as sustained release vehicles, has been followed by *in vitro* swelling and drug release studies. The swelling studies were performed by mass measurements at 37 ^{0}C in twice distilled water. The release profiles and release kinetics of codeine, an opiate used for its analgesic, antitussive and antidiarrheal properties, were determined. Water-uptake data and drug release measurements are given for characterization of new solid dosage forms, the importance of the chondroitin sulphate presence being also discussed. The biocompatibility testing was made by hemolysis (plasma hemoglobin) technique. It seems that cellulose/chondroitin sulphate hydrogels are promising formulations for drug delivery.

INTRODUCTION

Hydrogels, highly hydrated polymer networks, are formed by the chemical or physical crosslinking of the hydrophilic polymer. The characteristics of hydrogels, including sensitivity to the environment, tissue-like water content and elasticity afford the potential for biomedical application. For instance, hydrogels are used for delivering drugs (Ichikawa, 2000; Matsumoto, 2003), artificially dressing burns (Choi, 1999; Yannas, 1989), cell encapsulation (Lim, 1980; Uludag, 2000), and constructing a scaffold for use in tissue engineering (Lee, 2001; Tateishi, 2002).

Among the numerous polymers that have been proposed for the preparation of hydrogels, polysaccharides have a number of advantages over the synthetic polymers which were initially employed in the field of pharmaceutics (Wichterle, 1960).

Chondroitin sulphate (CS) consists of repeating disaccharide units of D-glucuronic acid and N-acetyl galatosamine, sulfated at either 4- or 6-positions (figure 1)

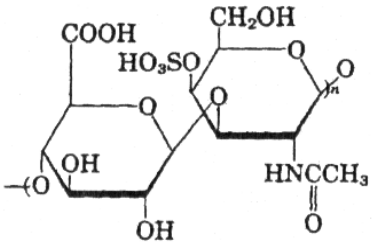

Figure 1. Chondroitin sulphate structure

The CS chains are roughly classified into types A, C, D, E, K and H (Yamada, 2000). They are named chondroitin sulfates A (C-4-S), C (C-6-S), D (C-2,6-S), E (C-4,6-S), K (C-3,4-S) and H (IdoAa1–3Gal-NAc(4S,6S)), of which C-4-S and C-6-S are the most common (Zou, 2009).

CS can bind with core protein to produce highly absorbent aggregan, which is a major structure inside cartilage and acts as a shock absorber, or it can produce sydecan, which is a cell receptor which can interact with adhesion proteins, cells and the extracellular matrix (ECM) (Bukalo, 2001). In biomedical applications, CS has shown *in vivo* anti-inflammatory effect in animal models. It also regulates metabolism *in vitro* (Bali, 2001). CS can be used for treating autoimmune and wasting joint diseases. VISCOAT™ (4% chondroitin sulphate $_{(aq)}$ and 3% sodium hyaluronate$_{(aq)}$) is used as a surgical aid in cataract extraction and lens implantation (Tomita, 2004). CS is also a component of the dermal layer of the FDA-approved skin substitute for treating burns (Phillips, 1998; Lee, 2005).

It has been demonstrated that the half-life of CS in the circulatory system is 3–15 min, based on the pharmacokinetic study of intravenously administered CS (Sakai, 2002). This indicates that orally administered CS is not systemically distributed to connective tissues such as cartilage and skin, and that exogenously administered CS may indirectly stimulate chondrocytes to synthesize ECM components. The mechanism of action of orally administered CS might be mediated by other systems (Yamada, 2008).

CS-coated poly(2-hydroxyethyl methacrylate) membranes were found to prevent adhesion in fullthickness tendon tears of rabbits (Gudemez, 2002). GAG hydrogels composed of poly(ethylene glycol) dialdehyde cross-linked with adipic dihydrazide derivatives of CS and hyaluronic acid have been evaluated as bio-interactive dressings for wound healing by Kirker et al (Kirker, 2002).

Lee et al. (Lee, 2005) prepared hydrogels based on poly(vinyl alcohol) (PVA)-chondroitin sulphate (CS) using glutaraldehyde as the crosslinking agent. These hydrogels, which have the advantages of both PVA and CS, can be used as a material for scaffolds in tissue engineering, promoting not only cell adsorption, but also cell growth. The mechanical properties of composite hydrogels facilitate the culturing of cells and make them bioactive toward the cells.

The hydrogels based on CS formed directly by crosslinking CS with poly(ethylene glycol) diglycidyl ether (EX-810) abbreviated as CS-EX or an interpenetrating polymer network named CS-EX-IPN were studied by Wang et al. (Wang, 2007) characterizing, also, the release of a model drug, diclofenac sodium (DS) and a model protein, bovine serum albumin (BSA). Diclofenac sodium released from the CS-EX and CS-EX-IPN hydrogels was rapid but BSA could be moderately controlled. The release profiles of both drugs fit in the diffusion-controlled mechanism, these preliminary results indicating that the CS-based hydrogels were more effective to sustain the release of large molecules like BSA.

A fast thermoresponsive hydrogel composed of poly(N-isopropylacrylamide) (PNIPAm) and chondroitin sulphate (CS) was synthesized using precipitation polymerization. CS was introduced to increase the water absorption of the PNIPAm hydrogel, and the precipitation polymerization method was used to induce a porous network morphology to enhance the thermal response of the hydrogel.

This hydrogel could be suitable for sensors, actuators or artificial muscle applications. In addition, the high porosity and negatively charged internal structure of PNIPAm/ChS hydrogels have the potential to load cationic drugs for controlled delivery applications (Varghesea, 2008).

Bacterial cellulose has long been used in a variety of applications in the paper, food, and electronic industries (Jonas, 1998; Miranda, 1965; Nishi, 1990; Shah, 2005; Yano, 2005).

Owing to its high porosity, water absorbance, mechanical properties, formability, and biocompatibility, bacterial cellulose has also recently attracted a great deal of attention for biomedical applications (Czaja, 2007). The structure of cellulose is showed in figure 2.

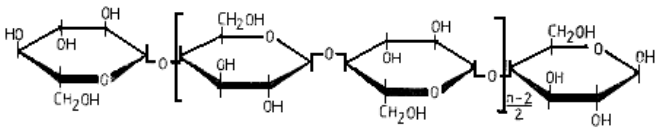

Figure 2. Cellulose structure

For instance, bacterial cellulose has been successfully used for wound dressings (Ciechanska, 1998; Czaja, 2006; Legeza, 2004) and for vascular implants (Klemm, 1999, 2001). The potential of bacterial cellulose for *in vitro* and *in vivo* tissue regeneration also continues to be explored and shows great promise (Backdahl, 2006; Helenius, 2006; Svensson, 2005; Watanabe, 1993).

Bacterial cellulose has been soaked into hydroxyapatite to develop a composite scaffold for bone regeneration (Hong, 200; Wan, 2006). Bacterial cellulose has also been augmented by immersion in solutions of polyacrylamide and gelatin, yielding hydrogels with improved toughness (Yasuda, 2005). Similarly, the immersion of bacterial cellulose into poly(vinyl alcohol) has yielded hydrogels having a wide range of mechanical properties of interest for cardiovascular implants (Millon, 2006).

In a study by Yasuda et al. microbial cellulose was immersed in two types of polymer solutions (2-acrylamide-2-methyl-propane sulfonic acid and gelatin) in order to create a hydrogel with enhanced mechanical toughness (Yasuda, 2005). The resulting double-network hydrogels (DN), consisting of two independently cross-linked networks of different polymers, can withstand high frictional forces, showing that they are resistant to wear. Thus, these microbial cellulose composites could function as replacement cartilage tissue in damaged joints (Czaja, 2007).

Sannino et al. (Sannino, 2000) tested cellulose-based hydrogels for their potential use in biomedical applications. This hydrogels main clinical application may be the treatment of oedemas of cardiac, hepatic and renal origin, which are resistant to diuretic therapy, evaluating, also, the long-term gel compatibility, in terms of morphological variations, toxicity and carcinogenic potential.

Controlled drug release systems offer numerous advantages compared to conventional dosage forms, including improved efficiency, reduced toxicity and improved patient compliance and convenience (Uhrich, 1999).

Controlled drug delivery occurs when a polymer, whether natural or synthetic, is judiciously combined with a drug or other active agent in such a way that the active agent is released from the material in a pre-designed manner. The release of the active agent may be constant over a long period, it may be cyclic over a long period, or it may be triggered

by the environment or other external events. Providing control over the drug delivery can be the most important factor at times when traditional oral or injectable drug formulations cannot be used. These include situations requiring the slow release of water-soluble drugs, the fast release of low-solubility drugs, drug delivery to specific sites, drug delivery using nanoparticulate systems, delivery of two or more agents with the same formulation, and systems based on carriers that can dissolve or degrade and be readily eliminated. The ideal drug delivery system should be inert, biocompatible, mechanically strong, comfortable for the patient, capable of achieving high drug loading, safe from accidental release, simple to administer and remove, and easy to fabricate and sterilize.

In this study it was aimed to characterize the C/GAG hydrogels containing codeine to achieve a controlled release profile suitable for subcutaneous administration so the investigation were made in pure water.

It may be mentioned that these types of formulations based on cellulose and condroitin sulphate have not been studied or proposed as hydrogels for biomedical applications.

MATERIALS AND METHODS

Microcrystalline cellulose (C) - Avicel HP-101 (Fluka) and chondroitin sulphat (GAG) powder were both separated in National Institute of Searching and Development for Biological Science laboratories, Bucharest.

The hydrogel samples were prepared in various mixing ratios: 90/10, 80/20, 70/30, 60/40, 50/50 C/GAG purified by washing with warm water and dried in air, at room temperature.

Cellulose/chondroitin sulphate hydrogels were produced by a crosslinking technique. In other study of ours the cellulose/chondroitin sulphate hydrogels have been tested for the controlled release of paracetamol and theophylline in a phosphate buffer solution (Oprea, 2008) with promising results.

In this study were used 80/20, 60/40 and 50/50 C/GAG formulations.

Codeine or methylmorphine is an opiate used for its analgesic, antitussive and antidiarrheal properties. It is one of the most effective orally-administered opioid analgesics and has a wide safety margin. It is from 8 to 12 percent of the strength of morphine in most people; differences in metabolism can change this figure as can other medications.

The *kinetics of the swelling* was carried out by weight measurements performed at 37 ^{0}C, in bidistilated water, the hydrogels samples being periodically removed and changes in weight were measured before and during swelling. The swelling degree was calculated according to the equation (1).

$$Q_{\max} = (W_t - W_d) / W_d \times 100(\%) \quad (1)$$

where W_t is the weight of the samples after swelling in water at time t and W_d is the dry weight of the sample.

The *drug loading* of the hydrogel matrices was carried out by mixing the drug used (codeine) with dried hydrogel in powder form using as a release medium physiological serum and then a certain quantity of was added and left to swell at room temperature, while the drug penetrate and/or attached into matrices. The drug-loaded samples were freeze-dried using a Labconco FreeZone device. During the *drug release* study, at predetermined time intervals, 1 ml sample was withdrawn from the release medium and concentration of codeine at 284 nm in the release medium were determined using a UV-VIS spectrophotometer HP 8450A.

To determine the kinetics of solvent diffusion into the hydrogels the following equation was used:

$$F_t = \frac{W_t}{W_{eq}} = k_{sw} t^{n_{sw}} \quad (2)$$

where W_t and W_{eq} represent the amount of water absorbed by the hydrogel at time t and at equilibrium respectively, k_{sw} is the swelling constant characteristic of the system and n_{sw} is the power law diffusion exponent which takes into account the type of solvent transport. Eq. 2 applies to initial states of swelling and linearity is observed when log F_t as a function of log t is represented.

In order to elucidate the kinetics of drug release, the data were further analyzed using the equation proposed by Korsmeyer and Peppas (Eq.3):

$$M_t / M_\infty = k_r t^{n_r} \quad (3)$$

where Mt/Mα represents the fraction of the drug released at time t, k_r is a constant incorporating characteristics of the macromolecular network system and n_r is the diffusion exponent, which is indicative of the release mechanism.

In the equations above a value of $n_{sw}/n_r = 0.5$ indicates a Fickian diffusion mechanism of solvent/drug in hydrogels, while a value $0.5 < n_{sw}/n_r < 1$ indicates an anomalous or non-Fickian behavior. When $n_r = 1$ a case II transport mechanism is involved while $n_r > 1$ indicates a special case II transport mechanism (Katime, 2001; Korsmeyer, 1984).

Percent Hemolysis Test

Blood was obtained from healthy patients drawn by routine venipuncture from the antecubital vein in tubes containing EDTA. The blood was stored refrigerated for no more than 2 days until its use. Prior to hemolysis testing all the hydrogel samples were sterilized by ultraviolet light trans-illumination for 2 min. Each hydrogel preparation was tested with blood from a single patient. Distilled water was used as positive control and plasma separated from the same blood as negative control. From each tube, 1.5 mL of blood were drawn and put into contact with hydrogels in Eppendorf centrifuge tubes (2 mL). The blood samples in contact with the biomaterials were incubated at 37 °C for 2 h. After the incubation time the samples were centrifuged at 5000 rpm for 6 min. The separated plasmas were diluted 11 fold with Tris (62.5 mmol/L, pH 8.0 adjusted with HCl) prior to spectrophotometrical measurements. The remaining 0.5 ml of blood in each tube were centrifuged at 5000 rpm and separated plasmas were diluted 11 fold with Tris, the resulting solutions being used as negative controls. The positive control was prepared by hemolysing blood with distilled water (1:11 dilution). The hemolysed solution was also incubated at 37 °C for 2 h. Finally, the positive control solution was diluted 100 fold for spectrophotometric analysis. The method used for measuring plasma hemoglobin concentration in all the specimens was the polychromatic method of Noe et al. (Noe, 1984). Absorbance was measured at 380nm, 415nm and 470nm and the formula used for evaluation was:

$$C(mg/L) = 1.65\ mA_{415} - 0.93\ mA_{380} - 0.73\ mA_{470}$$

where C is the hemoglobin concentration in mg/L, mA_{380}, mA_{415} and mA_{470} are the absorbances at 380nm, 415nm and 470nm expressed in miliabsorbance units. The results were expressed as:

$$\text{hemolysis percent (\%)} = (C - C_n)/(C_p - C_n) \times 100$$

where C is the concentration of hemoglobin in the sample, C_n the concentration of hemoglobin in the negative control and C_p the concentration of hemoglobin in the positive control.

RESULTS AND DISCUSSION

Swelling kinetic studies

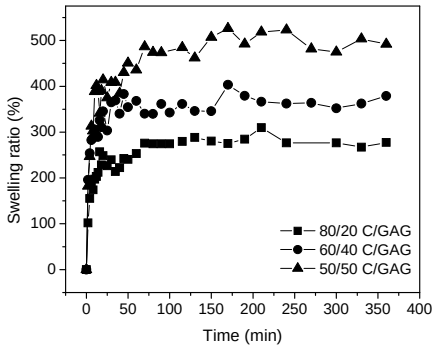

Figure 3. Swelling profiles of C/GAG hydrogels in physiological serum, at 37 ^{0}C

Table 1 presents the kinetic parameters of swelling, performed in bidistillated water, for C/GAG hydrogel samples with various compositions.

Table 1. Kinetic parameters of swelling for C/GAG hydrogels

Hydrogels	k_{sw} (min^{-1})	n_{sw}
80/20 C/GAG	0.41	0.21
60/40 C/GAG	0.58	0.14
50/50 C/GAG	0.41	0.22

The hydrogels swelling ratio (figure 3) increases with increasing GAG content and the values obtained for swelling parameter (n_{sw}) (table 1) in bidistillated water varies in range between 0.14-0.22 indicating an anomalous mechanism of swelling.

Kinetic of drug released

Codeine release

The release kinetic profiles and release rate profiles of codeine from C/GAG-based hydrogels, with different compositions, are showed in figure 4 and 5.

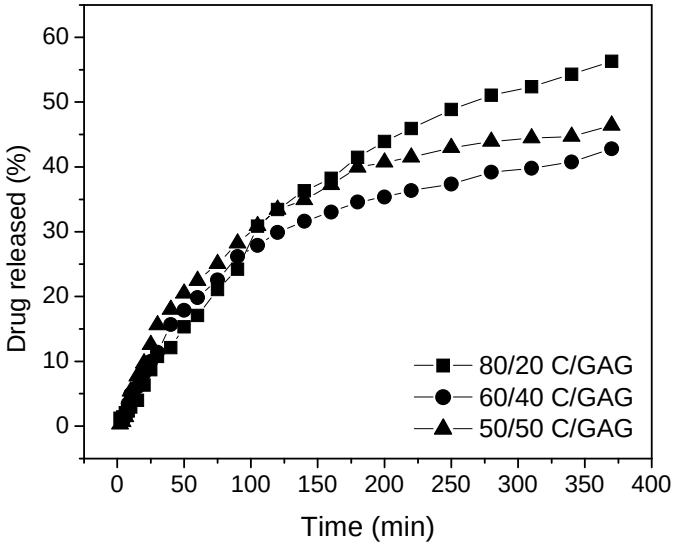

Figure 4. Release profiles of codeine from C/GAG-based hydrogels with different compositions, in physiological serum at 37 ^{0}C

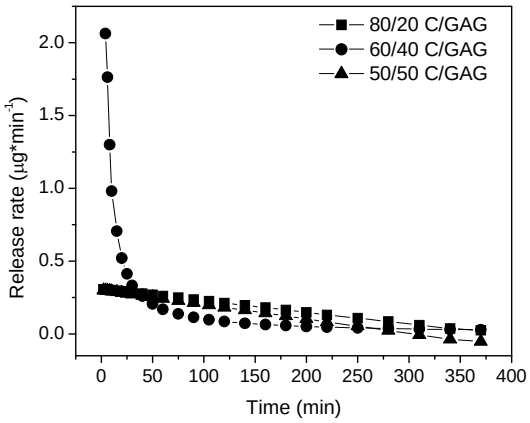

Figure 5. Release rate profiles of codeine from C/GAG-based hydrogels in, physiological serum, at 37 ^{0}C

The results showed that the release of codeine from C/GAG-based hydrogels depends on GAG content, so an increase of GAG content leads to a decrease of codeine percent released (case of 60/40 and 50/50 C/GAG compositions) with more than 15 % and a slower release rate.

The kinetic parameters for codeine released in physiological serum from C/GAG-based hydrogels with various compositions are presented in table 2.

Table 2. The kinetic parameters of codeine released from C/GAG hydrogels

Hydrogels	n_r	$k_r(min^{-n})$
80/20C/GAG	0.91	0.004
60/40C/GAG	0.78	0.008
50/50C/GAG	0.71	0.01

The values of n_r obtained for C/GAG hydrogels loaded with codeine indicate an anomalous transport mechanism for all formulations and rate coefficient (k_r) increases 100 times with increasing GAG content.

Hemolysis test

The hemolysis test showed that the hemolysis percentages of all the blood samples in contact with the hydrogels were negative. All percentages were less than 1% (table 3) compared to the positive control which is not significantly different than the negative control. The errors below 5% are admitted for this test.

Table 3. Hemolysis percentage of the three hydrogel formulations tested

Sample	Hemolysis percentage (%)
80/20 C/GAG	0.0512
60/40 C/GAG	-0.1136
50/50 C/GAG	0.1561

CONCLUSIONS

Cellulose/chondroitin sulphate hydrogels were produced by a crosslinking technique.

The swelling and drug release studies in pure water showed that an increase of GAG content in hydrogels composition leads to a higher swelling ratio and a decrease of codeine percent released and very small release rate.

The biocompatibility testing was made by hemolysis (plasma hemoglobin) technique, the results obtained showed a good biocompatibility between hydrogels and blood.

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1 „P. Poni" Institute of Macromolecular Chemistry, Physical Chemistry of Polymers Department, 41 A Gr. Ghica Voda Alley, 700487, Iasi, Romania, Tel: +40 232 217454, Fax: +40 232 211299
2 "Gr.T.Popa" Medicine and Pharmacy University, 16 University Street, 700115, Iasi, Romania, Tel: +40 - 0232/ 267.801, Fax: +40 - 0232/ 211.820
*aoprea@icmpp.ro

Acknowledgements: The authors thank for financial support of Romanian ANCS by the national grants IDEI 2561/2008 and IDEI 17/2007.

THE FUNCTIONAL FOODS: DEVELOPMENT AND OPPORTUNITIES

MICŞUNICA RUSU

Keywords: functional food, FOSHU, health claims, FUFOSE

Abstract: The development of food science in near future depends on advance in functional food science, the concept of which was proposed first in Japan nearly in the 1980s. The Japanese government developed a regulatory agency to oversee the approval of functional foods in 1991. The name of this agency is called Foods for Specified Health Use (FOSHU). Functional foods are increasingly popular in the United States. Furthermore, functional foods are reported as one of the fastest growing segments of the food economy in United States. Research on food and nutrition plays an important role in the Framework Program of Development and Research in the European Commission, established in the seventh Framework Program for Funding and in conducted Europeans projects: PASSCLAIM, FUFOSE, etc.

INTRODUCTION

The way of approaching food has suffered a lot of modifications lately. One has changed the subsistence economy, where food was a problem of survival, with the abundance economy, where the excess food causes a lot of so-called “diseases of civilization”: heart disease, obesity, diabetes, and some types of cancer. Our society tries to find an optimum diet which should try to promote the consumption of foods with benefic effects on health. This is the context where the concept of “functional food” appeared. (Secretin, M., 2001).

a) FUNCTIONAL FOOD IN JAPAN

The history of functional food is not very old. In the 1980s, Japanese society, being aware of the aging process, has become more and more concerned with the prevention of the lifestyle related diseases, through daily dietary. This gave a strong impetus to food science and to the politicians in the food sector. (Arai, S., 2001).

The first national project with the theme „functional food”, entitled Systematic Analysis and Development of Food Function was started in 1984 and it was sponsored by Ministry of Education, Science and Culture (MESC). This project establishes three functions of food:

- the regular function, of feeding the body;
- the sensory function, related to the effects of taste and smell on the sense organs;
- the body modulating function of non-nutrients (a newly-defined function), which is directly or indirectly connected with the disease prevention.

Practically speaking, the third function established the basis of functional food, which can be defined as food that can intervene effectively in reducing the risk of lifestyle related diseases. The first project (1984 – 1987) was followed by a second one, entitled „Analysis of Body-modulating functions of Foods”. The last project from the series MESC „functional food” was realized in 1992. This one focused on „Analysis and Molecular Design of Functional Foods”, having the following sub themes:

- body regulation factors of aliments;
- body protection factors of aliments;
- development of a specific technological basis in designing functional foods at the molecular level.

The Ministry of Health and Welfare established in 1991 the world’s first policy that allows the legally commercialization of functional foods using the expression Food for Specified Health Use (FOSHU). According to the new legislation, food may be declared FOSHU if each expected effect specified that it could contribute to maintain health is based on certain data concerning the relationship between food (the components of food) and health.

If an applicant wants that his product to be approved as FOSHU, he must provide a sample of the product and the necessary documentation. The Ministry of Health and Welfare’s criteria for approval are:

- food is expected to contribute to the improvement of diet and to maintain / improve health;
- health benefits of food or its components must have a clear basis of nutritional care;
- the amount of daily food and its components consumption must be determined on appropriate medical and nutrition knowledge;
- the food and its components must be safe for consumption and they must be established by experiments;
- food components must be clearly defined in terms of physico-chemical properties and they must be analytically determined (both quantitative and qualitative);
- there should not be significant loss of nutritional components of food in comparison with normal values that are present in similar food;

- food must be in the form of ordinary consumption into a daily diet rather than consumed only occasionally; it must be in the form of ordinary food (not in another one, such as capsules or pills). In other words, the food and its constituents should not be used solely as medicines.

After inspection, the food that is recognized in this manner gets the permission to have printed on its label the official approval and the special benefit for health. (Arai, S., 2001).

Policy is centered on the approval of health claims for each FOSHU product. According to the health benefit, foods can belong to the following categories:

- foods that promote the growth of bacteria which are beneficial to the intestinal micro flora and help to maintain intestinal health medium;
- foods suitable for people with increased cholesterol levels;
- foods for minerals supplement with high absorption capacity (calcium and iron);
- foods that are beneficial for hypertensive people;
- beneficial foods for people with a high concentration of glucose in blood;
- beneficial foods for people with a high concentration of triglycerides in blood.

The first FOSHU product was approved in 1993 and that was the hypoallergenic rise, and, by 2001, there have been approved 192 FOSHU products.

Most food industries have given attention to the concept of functional food introduced by the research projects of the MESC, started in 1984. In the first five years after the introduction of FOSHU products (1991 – 1996), not many of them have shown interest because the permitted claims were limited to a small number of indirect and unattractive experiments, as compared to the large volume of scientific data required. Some permutations have gradually been realized and in recent years more functional products with popular brands have entered the market very well.

On this background a Committee of functional foods has been organized in Japan, at the International Life Sciences Institute, in 1996. Its members are divided into four groups: criteria for establishing the scientific data, rules for health claims, sales situation, and research.

From the events that have marked the history of functional food in Japan, Soichi Arai selected some studies on:

- Antioxidant factors. The oxidative stress can cause the free radicals reactions that produce the damage of the membranes, enzymes and DNA. Diseases of aging, such as cancer, arteriosclerosis and diabetes are also related to oxidative stress. Starting from the idea that endogenous antioxidants from plants play an important role in defense against oxidative stress, Japanese researches obtained important results by researching the sesame lignans, the phenols and β-quinones.
- Cancer preventing factors, taken from fruit and vegetables, with special attention to plants on the families Rutaceae, Cruciferae, Umbelliferae, Zingiberaceae, which are also used for other than their nutritional values (e.g., odor, flavor characteristic and traditional medicinal proprieties). These plans contain anti-tumour promoters at high rates.
- Derived peptides from food proteins. A large number of bioactive peptides have been separated from milk proteins (casein and lactoferrin), but also from other protein sources (gluten, ovoalbumin, fish proteins, soy proteins), and the effects on health are well known (anticholesterol, antiinfectious, immunostimulative, antihypertensive effects etc.)
- Modulators of the immune system – (the probiotics and the prebiotics). The probiotics are viable microorganisms that have beneficial effects in the host's health by improving the indigenous microflora. They stimulate the immune defense of the host. The prebiotics are indigestible food ingredients that influence the host beneficial by stimulating a selective growth and / or the activity of certain species or a limited number of bacteria from the colon.
- Hypoallergenic products from wheat;
- Hypoallergenic products from soy;
- „XYZ" evaluation system. Noting the reactive oxygen with [X], the hydrogen donor with [Y], the presence of a certain substance [Z] with the role of mediator is necessary so that the photons should be emitted.
    - The phenomenon of photon emission in the visible domain was observed when studying the saponins from the soybeans.

  The photon emission in the „XYZ" system was imagined to appear through the translation of the electron or the reduction of the hydrogen between X Y and Z because the proprieties of Y are both to „clean" the radicals and to donate electrons, just like the phenolic compounds. The result is the photon mission that „sweeps" the free radicals. Experimental evidence based on XYZ system suggests that this phenomenon is fundamental for our life and also the environment.

- Building a database of safe doses of biologically active compounds. The incidents of various diseases related to lifestyle, such as cancer, BCV, diabetes, are strongly influenced by the eating habits. The polyphenols of tea and the phytoestrogens are good examples of biologically active compounds able to prevent some diseases. The consumption in excess of these substances may have toxic effects. To establish the safe dose (that avoids the toxic effects) one must know the absorption rate, the biological effect and mechanism, the rate of metabolism and the rate of elimination. Health effects of these food factors can be estimated by calculation, using tables with the composition of the suitable aliments.
- There are a lot of MAFF research programs on food and the most important conclusions are:
    - the consumption of citrus fruits is associated with a decreasing risk of cancer;
    - polyunsaturated fatty acids from the series ω - 3 and ω - 6 increase hepatic oxidation of fatty acids ad decrease their synthesis;
    - the sesamine, a lignan in sesame, is the potential inductor of the hepatic oxidation and fatty acids.

These events and activities have attracted the attention of the Western world, especially after the article entitled „Japan explores the boundary between food and medicine” appeared in 1993. In this article the term „functional food” appeared for the first time in English. (Arai, S., 2002).

b) FUNCTIONAL FOOD IN USA

Functional foods have entered quite quickly in the US, the problem being the concern both to the scientists and the ordinary people. There is no doubt that health and nutrition are in close connection. An appropriate diet is considered the first factor in worsening the genetic potential, in reducing the physical and cognitive performance, and increasing the risk of certain diseases. The introduction of the functional food on the American market and in the Americans' lives is based on studies about the health of the population and their availability to accept a change in their diet.
The strategies to optimize nutrition by using functional food or food supplements are very popular and considered to be suitable for increasing the quality of life. The importance of these strategies is accentuated by the recognition of the fact that approximately one third of cancers are related to eating habits. Actually, more than half of the US deaths are related to an inadequate nutrition. Many Americans believe that changing diets and using food supplements are the most important ways to decrease the health costs and improving the health. The functional foods were defined in different ways. The International Life Sciences Institute (ILSI) from the North America defined them as food with physiological capacities of the active components in food to bring health benefits besides the basic function of feeding. Approximately 60% of the resident adults in the US are confident in the health foods, no matter their age or sex. While young people choose foods to increase their physical or mental performances, older people select foods to decrease the risk o disease or to improve the qualities of their lives. (Milner, J., 2002).
The American researchers have focused on the study of the bioactive components. Preclinical and clinical epidemiological studies bring evidence on the dynamic relationships between nutrients (defined as any substance from the diet that is causing physiological effects) and health.

In the US special attention has been given to claims that accompany the products with a functional role. In the US legislation there are three categories of claims:

1. Health claims. Health claims describe a relationship between the active substances in food and the disease o the condition that affects health. There are three sets of laws by which Food and Drug Administration (FDA) is exercising the authority with respect to the use of health claims.

- Health claims authorized by the Nutrition Labeling and Education Act (NLEA) – they are included in the NLEA law from 1990, the law of the dietary supplements from 1992 and Dietary Supplement Heath and Education Act (DSHEA) from 1994. Under these laws FDA (Food and Drug Administration) may approve health claims for foods and food supplements based on evidence from scientific literature, using the standard for determining what the relationships are established between nutrient and disease.
- Health claims based on statements of the authorities – according to FDAHA issued in 1997. The health claims can be approved for a certain type of food under an authorized declaration of the scientific stuff from the US Government or the National Academy of Sciences.
- Qualified health claims – according to the law on consumer health information for the initiative for a better nutrition, in 2003 – it allows FDA to recognize the qualified health claims when there is an obvious relationship between a food / ingredient of food or the food supplement and the decrease of the risk for a disease or the maintaining of health. FDA prepared a guide on the procedures to obtain the qualified health claims.

2. Claims on nutrients content. NLEA allows the use of the claims that characterize the level of the nutrients in food in accordance with the rules of the FDA approval.

3. Structure / Function claims – by DSHEA in 1994 there are established some specific rules for claims on labels of food supplements. The structure / function claim describes the role of the nutrient or the food ingredient which positively affects the structure or function of the body.
There were 15 approved health claims in 2001. Here are some of these claims:

- calcium and osteoporosis;
- sodium and hypertension;
- saturated fat foods and cholesterol and cardiovascular disease;
- stanols and hart disease;
- fruits and vegetables and cancer.

To better clarify the claims of health problems, the ILSI North American Technical Committee on Food Components for Health Promotion developed in 1997 a „Strategy to develop the public health and to accept the secure foods which bring significant benefits for health". Some of the ways achieve these goals are: creating a foundation for functional food science; promoting people's trust; the development of the consumer's preferences for functional food; the optimization of the rules and creating marketing strategies for the development of the functional foods.
A large number of studies had as a theme the genetic and epigenetic action of nutrients that caused phenotypic changes. The studies on nutritional genome offer the opportunity to identify how the components of food influence the growth and increase the health level and to clarify the specific mechanisms of action. It is known that the homeostasis is regulated by the fine balance between the multiplication, controlled growth, differentiation and apoptosis the process of programmed cell death). The destruction of this balance can determine deep phenotypic changes starting from a reduction in growth to the transformation of the cell from a normal cell into a cancer cell (neoplastic cell). The disorders in the apoptosis process are frequently accompanied by the appearance of the pathogenesis, with a wide range of events including heart disease, neurodegenerative diseases, cancer, etc.
There have been identified some factors which influence the cellular homeostasis: vitamin A, vitamin D, the lignans, etc. A special attention was given to the sterols, the selenium, the folic acid, the polyphenols, and the inulin. (Milner, J., 2002).
A special attention was given to the development of the guides of the nutrition for the population.

c) FUNCTIONAL FOOD IN EUROPE

Europe had a wide variety of regulations on the approval of the products, the type of nutritional information indicated to appear on the packaging, and the type of functional claims and healthcare which are permitted in connection with the products. (http://www.fao.org/agn/agns/files/Functional_Foods_Report_Nov2007.pdf).
In functional food domain, a large number of European agencies are operating:

- European Commission – (http://ec.europa.eu). European Commission entirely supports and represents the interests of Europe. It is independent of the national governments. It proposes the plans of the new European laws which are presented in the European Parliament and the Council. It leads the implementation of the politics and it uses the EU funds. The Commission monitors the compliance of the European treats and laws. It can act against those who violate the law, appealing to the Court of Justice if necessary.
- EFSA (European Food Safety Authority) – it is an European agency based on UE budget, which operates separately from the European Committee, the European Parliament and the EU member states. EFSA (European Food Safety Authority) brings scientific evidence and scientific and technical support in all areas that affect food safety. It is an independent source of information in all areas of this field and it ensures that the public is well informed.
- EUFIC (The European Food Information Council) – it is a non profit organization which obtains scientific information related to food safety, quality and health, nutrition, for media, professionals from health and nutrition, educators and opinion leaders.
- ILSI (International Life Science Institute) – it is a world non profit organization which tries to improve the life of the public using the scientific discoveries. Its purpose is to promote the understanding of scientific issues related to nutrition, food safety, toxicology and risk assessment and it unites scientists from academia, government and industry.

The Framework Directive (the Directive 90/496/CEE) defined the nutritional labeling, in 1990, as „any representation or slogan that says, suggests or implies that a particular foodstuff has special nutritional properties due to high or low energetic content or nutrients in high or low proportion. (http://ccvista.taiex.be/Fulcrum/CCVista/RO/31990L0496-RO.doc).
In July 2003, the European Commission proposed a harmonization regulation (COM/2003/0424) on nutritional and health claims on aliments, including the food supplements.

The Regulation on nutrition and health food claims was adopted in December 2006 by the European Council and the European Parliament. The following definitions were proposed in this Regulation:

- *Claim* – any message or presentation that is not bound by Community or national legislation, including pictures, graphs, or symbolic representations, in any form, which express or suggest or imply that a certain food has particular characteristics.
- *Nutritional claims* – any claim that expresses, suggests or implies that a certain food has particular beneficial nutritional characteristics due to:
    a) the caloric value: (1) provided caloric value, (2) provided in a lower or higher caloric value, (3) it does not have a caloric value OR
    b) the nutrients or other substances that (1) they contain, (2) they contain in a higher or lower proportion and (3) they do not contain.
- *Health claims* – any claim that expresses, implies or suggests that there is a relationship between a certain food or a component of the food and health.
- *Claims to reduce the risk of disease* – any health claim that expresses, implies or suggests that through the consuming of a category of food, a food or a constituent of a food the risk factors in certain diseases development are reduced.

The medical claims for food (for example claims that express, imply or suggest that the product has healing, prevention or curing properties) are prohibited by the European and National labeling rules. In order to wear a medical claim, a product must be classified as medicine in accordance with the definition from the order 2001/83/EC of the European Parliament and the European Council, from 6th of November 2001, on the Community Code relating to medical products for human use.

EFSA is implied in the implementation of the new Regulation and it published a guide to help he companies which want to submit health claims to be authorized (EFSA, 2007). The evaluation of the health claims made by EFSA is the first step in the process of authorization. Only those claims that are scientifically sustained will be allowed to be used. The final approval of the health claims is the responsibility of the European Commission and of the EU member states, and it is based on a scientific evaluation, expressed by the opinion of the EFSA Jury. It is the first time when the harmonized approach for the authorization of the health claims has been established by the EU state members.

The European Agencies have conducted and are conducting a large number of projects in the field of functional food. From these one can mention:

1. FUFOSE – it is an action program on „The functional food science in Europe”, coordinated by ILSI Europe, with the following objectives:
    - assessment of current state of knowledge in the field;
    - the analysis of data from the perspective of the „functional effect”;
    - to reach a consensus on change for the food and their components.

The conclusions of the working group coordinated by ILSI Europe include:

- a definition of functional foods – „a food can be considered functional if it is demonstrated in a satisfactory way that it beneficially influences one or more target functions of the body, besides the basic nutritional effect – either it improves health or it reduces he risk of disease”.
- a strategy of development the functional food, base on:
    - identification and understanding the mechanisms of interaction between the components of the foods and he functions of the body;
    - the validation of the components effects using appropriate methods (being preferred the use of specific biomarkers);
    - studies on human beings (when appropriate) which demonstrate the beneficial effects on health.

2. PASSCLAIM – Process for the assessment of Scientific Support for Claims on Food) – It intends to provide the industry, academia, consulting groups, and legislators methods to assess the scientific basis of health claims (Verschuren, P., 2002).

The objectives of this project are to develop the rules for functional foods, to use the high standards and extensive processes to evaluate the scientific basis for the claims.

One can mention between the results of the project the following:

- consumers’ confidence in claims has increased;
- important documents for fixing the claims have been obtained (Salminen, S., 2005).

Research on food and nutrition play an important role in the Framework Program of Development and Research in the European Commission. In the seventh Framework Program for Funding, three important directions have been established (http://www.functionalfoodnet.eu/asp/deault.asp?p=75):

- the combat of obesity – through the development of tasty and healthy food (and clinical documentation) to prevent obesity, by using both classic and modern concepts promoted by science;
- improving heat stability of probiotics;
- food products and chronic inflammation.

The optimizing nutrition is a major challenge of the XXI century and the functional foods play a very important role.

The developing of new products and related health claims should remain a main concern of the science. This is the successful condition of which both human health and food industry will benefit.

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1 Grupul Şcolar Economic Administrativ Piatra Neamț
* rusu_mic@yahoo.com

WATER SUPPLY QUALITY FOR USE IN FOOD AND FOR CLEANING

CARMEN CORINA VIŞAN[1*], RODICA SEGAL[2]

Keywords: water quality, chlorinated drinking water, CFU 22ºC, CFU 37ºC.
Abstract: Drinking water from the supply network of the city of Focşani, Vrancea county is used in food preparation and also for cleaning the production areas, equipments and working tools. Data obtained from the analysis of microbiological parameters of reference of water supply quality are presented in this paper.

INTRODUCTION

Drinking water is water that is of sufficiently high quality that can be consumed or used without risk of immediate or long term harm. Such water is commonly called potable water. In most developed countries, all the water supplied to households, commerce and industry is drinking water, even though only a very small proportion is actually consumed or used in food preparation (often 5% or even less).

Over large parts of the world, humans drink water that contains disease vectors or pathogens or contain unacceptable levels of dissolved contaminants or solids in suspension. Such waters are not potable water and drinking such water or using it in cooking leads to widespread acute and chronic illness and is a major cause of death in many countries. (Jalan, 2004)

Throughout most of the world the most common contamination of raw water sources is from human sewage and in particular human faecal pathogens and parasites. In 2006, waterborne diseases were estimated to cause 1.8 million deaths, each year while about 1.1 billion people lacked proper drinking water. It is clear that people in the developing world need to have access to good quality water in sufficient quantity, water purification technology and availability and distribution systems for water. (Roy, 2007) In many parts of the world the only sources of water are from small streams often directly contaminated by sewage. Not even wells do not eliminate the risk of contamination.

MATERIALS AND METHODS

The relationship between water quality and food safety is currently regulated by different legislative acts on the environment and food.

Legal requirement	Guide to compliance	Advice on good practice
1. There must be an adequate supply of potable water. This potable water must be used whenever necessary to ensure foodstuffs are not contaminated.	Potable water must be used: • for the cleaning of food; • for inclusion in food recipes; • for cleaning of food equipment; • for cleaning surfaces that come into contact with food or the hands of food handlers; • for hand washing. Generally, it can be assumed that water will be potable if it comes direct from the water undertaker mains supply or from a storage system that meets the relevant requirements of any local water bye-laws. If the operation has a private water supply, that supply must be of potable quality. Non potable water may be used where this will not affect the safety and wholesomeness of the food.	Water softeners and water filter, should be maintained in good condition so that they do not contaminate water. Filter cartridges should be changed regularly in accordance with maker instructions. Softened water may not be suitable for infant foods or adults with certain medical conditions.
2. Where appropriate, ice must be made from potable water. This ice must be used whenever necessary to ensure foodstuffs are not contaminated. It must be made, handled and stored under conditions which protect it from all contamination.	All ice to be used in food and drink must be made from potable water. Ice used to cool open food in buffet displays must also be made from potable water. Ice machines must sited away from sources of contamination and be regularly cleaned as should containers and utensils used to store and dispense ice. Parts of the machine and utensils that come into direct contact with ice must be disinfected periodically.	Ice for drinks should not be handled with bar e hands. Glassware should not be used to ‘shovel’ ice.

Legal requirement	Guide to compliance	Advice on good practice
	Utensils must be made of durable materials that will not present a foreign body hazard from brittle fracture.	
3. Steam used directly in contact with food must not contain any substance which presents a hazard to health, or is likely to contaminate the product.	Potable water must be used if the steam may come into contact with, or become included in the food.	
4. Water unfit for drinking used for the generation of steam, refrigeration, fire control and other similar purposes not relating to food, must be conducted in separate systems, readily identifiable and having no connection with, nor any possibility of reflux into, the potable water systems.	Supplies of non-potable water to food preparation areas are not recommended. In some circumstances, hoses for fire fighting may be linked to a supply of water that is not potable. In those cases, the supply should be clearly marked for firefighting and hoses should not be used for cleaning.	

Parameters for drinking water quality typically fall under two categories: chemical/physical and microbiological. Chemical/physical parameters include heavy metals, trace organic compounds, total suspended solids (TSS), and turbidity. Microbiological parameters include Coliform bacteria, *E. coli*, and specific pathogenic species of bacteria (such as cholera-causing Vibrio cholerae), viruses, and protozoan parasites.

Chemical parameters tend to pose more of a chronic health risk through buildup of heavy metals although some components like nitrates/nitrites and arsenic may have a more immediate impact. Physical parameters affect the aesthetics and taste of the drinking water and may complicate the removal of microbial pathogens.

Originally, fecal contamination was determined by the presence of coliform bacteria, a convenient marker for a class of harmful fecal pathogens. The presence of fecal coliforms (like *E. coli*) serves as an indication of contamination by sewage. Microbial pathogenic parameters are typically of greatest concern because of their immediate health risk.

Drinking water from the supply network of Focşani is used by units for the food preparation and also for cleaning the production areas, equipments and working tools. About 12 month, samples of 500 ml chlorinated drinking water were collected from the urban supplying distribution network of the city of Focşani, and microbiological analyzed.

RESULTS AND DISCUSSIONS

The results of the exams are shows in the table 1.

The figures 1 and 2 reproduces the correlations between microbiological parameters of drinking water and time, CFU 22°C and CFU 37°C, in the studied samples.

Table 1. Microbiological analysis of drinking water

Month	BACTERIOLOGICAL EXAM				
	Microbiological parameters				
	CFU 22°C	**CFU 37°C**	**Bacterii coliforme**	**Escherichia coli**	**Enterococi**
	Reference				
	SR EN ISO 6222	SR EN ISO 6222	ISO 9308/1/ 2000	ISO 9308/1/ 2000	ISO 7899/2/ 2000
	CONDITIONS OF ADMISSIBILITY				
	100/ml	**20/ml**	**abs/100ml**	**abs/100ml**	**abs/100ml**
April 2008	28	5	abs	abs	abs
May 2008	8	7	abs	abs	abs

Month	BACTERIOLOGICAL EXAM				
	Microbiological parameters				
	CFU 22ºC	**CFU 37ºC**	**Bacterii coliforme**	**Escherichia coli**	**Enterococi**
	Reference				
	SR EN ISO 6222	SR EN ISO 6222	ISO 9308/1/ 2000	ISO 9308/1/ 2000	ISO 7899/2/ 2000
	CONDITIONS OF ADMISSIBILITY				
	100/ml	**20/ml**	**abs/100ml**	**abs/100ml**	**abs/100ml**
June 2008	26	8	abs	abs	abs
July 2008	32	7	abs	abs	abs
August 2008	26	10	abs	abs	abs
September 2008	17	6	abs	abs	abs
October 2008	10	7	abs	abs	abs
November 2008	8	5	abs	abs	abs
December 2008	13	6	abs	abs	abs
January 2009	7	4	abs	abs	abs
February 2009	8	3	abs	abs	abs
March 2009	15	7	abs	abs	abs

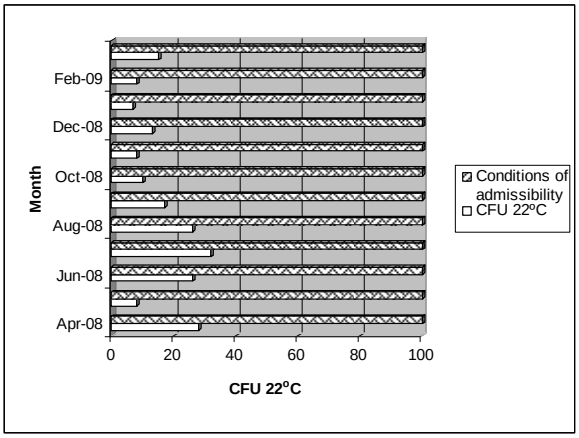

Fig. 1 CFU 22^{o}C of drinking water

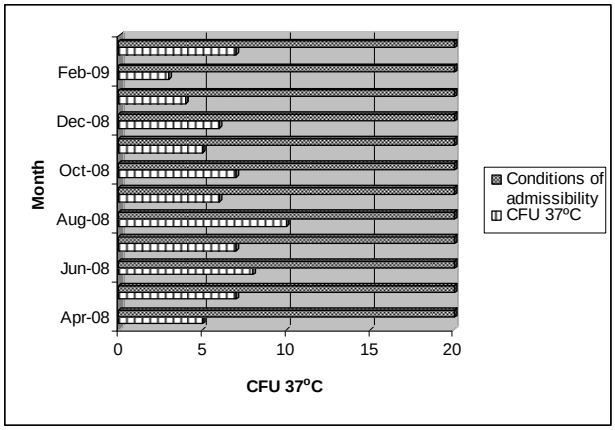

Fig. 2 CFU 37°C of drinking water

The graphic analysis obtained by processing statistical data established that a slight increases of CFU 22°C in some months has been detected: April 2008 and June-August 2008, but not exceeding the admissibility conditions.

Increased values of CFU 37°C were recorded in August 2008, but without exceeding the maximum admissible levels.

All samples that were analyzed have recorded the parameters' values lower than the maximum permissible levels, in consequence, the water is accordingly in terms of quality, and can be used in technological processes in the manufacture of food and for hygiene.

CONCLUSIONS

The food-processing industry is a large water user. Water is used as an ingredient, an initial and intermediate cleaning source, an efficient transportation conveyor of raw materials, and the principal agent used in sanitizing plant machinery and areas. Although water use will always be a part of the food-processing industry, it has become the principal target for pollution prevention, source reduction practices.

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Legea nr. 458/2002 privind calitatea apei potabile.

Legea nr. 311/2004 pentru modificarea şi completarea Legii nr. 458/2002 privind calitatea apei potabile.

Hotărâre de Guvern nr. 974/2004 pentru aprobarea Normelor de supraveghere, inspecţie sanitară şi monitorizare a calităţii apei potabile şi a Procedurii de autorizare sanitară a producţiei şi distribuţiei apei potabile.

[1] "*Mihail Kogălniceanu*" Economic College of Focşani,
[2] "*Dunărea de Jos*" University of Galaţi
* corinavisan@yahoo.com

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Hartl, D., Freifelder, D., Snyder, L., 1988. *Basic genetics*, Jones and Bartlett Publishers, Boston.

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