

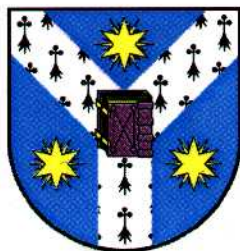
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PRELIMINARY DATA REGARDING THE KINETIC PROPERTIES OF AN ALPHA-AMYLASE FROM *ROBINIA PSEUDACACIA* L. GERMINATED SEEDS

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Key words: α -amylase, purification, alginate, kinetic properties

Abstract: We have accomplished a partial purification of a α -amylase from germinated seeds of *Robinia pseudacacia* L. by affinity precipitation. The key element is the sodium alginate, a polymer that proved affinity for this enzyme, and also has the propriety to reversibly precipitate with Ca^{2+} . The enzyme binds to the alginate and the complex is precipitated with Ca^{2+} . The amylase activity is recovered by dissolving the precipitate in 1M maltose and precipitating the alginate alone by addition of Ca^{2+} . The enzyme has a molecular weight estimated between 50 and 65 kDa, an optimum pH between 5 and 6; it is inhibited by ammonium sulfate and activated by CaCl_2 .

INTRODUCTION

α -Amylase (α -1,4-glucan 4-glucanohydrolase) hydrolyzes the α -1,4-glycosidic bonds inside the poliglucidic chains from starch, amylose, amylopectine, dextrans. This enzyme is widely spread both in animals, plants and microorganisms [1].

Inside the digestive tube of the animals, α -amylase participates in the depolymerization of the α -glucans from food, converting them to dextrans and finally to soluble oligoglucids. In plants α -amylase initiates the starch granules hydrolysis in vivo. Non-germinated cereal seeds contain very low levels of α -amylase. When the germinating process start, α -amylase biosynthesis in the aleurone layer increases dramatically. In the literature we have found many observations regarding the biosynthesis, structure and properties of α -amylase from cereals and other plants. The enzyme from legumeous species is less studied.

In this paper we report the partial purification and some properties of an α -amylase from *Robinia pseudacacia*.

MATERIAL AND METHODS

Plant material and germination. Locust tree (*Robinia pseudacacia* L.) seeds were collected from the „Anastase Fătu” Botanic Garden of Iași. After sterilization, the seeds were subdued to a mechanical treatment, in order to destroy the structural integrity of the tegument, and to a heat treatment with 80°C-90°C water. The seeds were placed on Petri plates at room temperature and dark. The germination took place in 3 days.

Amylolytic Activity Assay. Activity of α -amylase was estimated using starch as substrate, according with the Noelting-Bernfeld method [3]. One enzyme unit (U) liberates 1 μmol of reducing sugar (calculated as maltose) per minute at 40°C and pH 5.6 from soluble starch.

Estimation of protein. Protein was estimated using Bradford dye-binding method, with bovine serum albumine as standard [4].

Partial purification of α -amylase. Partial purification of α -amylase was done using the method proposed by Gupta and colab.[5], which uses sodium alginate [7] as a support for enzyme binding. 20 g of plant material was homogenized and stirred with a total of 200 ml chilled acetone in cold for 2 hours. The dry acetonetic powder obtained can be kept in the refrigerator, without any activity losses. 10 grams of this powder were used for enzyme extraction with 50 ml acetate buffer 0.5 M, pH 5.6 for 1 hour. The insoluble material was eliminated by centrifugation at 3000 rpm for 15 min.

The clear supernatant was subdued to a two step ammonium sulfate precipitation at 35% and 75%. After the last step, the precipitate is re-suspended in 10 ml acetate buffer and mixed with the 2% alginate solution (crude extract: alginate 2% 2/1). The final volume of the solution was made to 16 ml with acetate buffer. The enzyme-alginate complex is developed within 1 hour. The complex is then precipitated with CaCl_2 solution 1M to a final Ca^{2+} -concentration of 0.6M. The precipitate was centrifuged at 3000 rpm for 15 min at 25°C, and then washed with acetate buffer containing 0.6 M CaCl_2 . The complex is dissolved in acetate buffer containing 1M maltose (15 min). Without the calcium ions the polymer becomes soluble and the maltose induces the breakdown of enzyme-alginate complex. The alginate is eliminated from the solution by precipitation with calcium ions and centrifugation. The amylolytic activity of the supernatant was determined after dialysis of maltose against the buffer solution. The dialysis was done in two steps of 6 hours each; after every step the solution was renewed. The solution obtained was used as enzyme source.

PAGE-SDS Electrophoresis. For purity verification and molecular weight evaluation PAGE-SDS electrophoresis was conducted, using 10% resolving gel and 5% stacking gel. As marker we used Pharmacy Biotech-53-212 kDa; staining of the gels was done with Coomassie Brilliantblue R 250. Electrophoresis was carried out first at 15 mA-50 V, and then changed at 30 mA-150 V.

RESULTS AND DISCUSSIONS

Partial purification of α -amylase. Initially, we took samples from every purification step for protein and amylase activity assay. The ammonium sulfate proved to interfere with the determinations and we had to give up the samples with high sulfate concentrations: the supernatant after the precipitation with 35% ammonium sulfate and the supernatant with 70% sulfate. The acetone-washing step was necessary, as fractionation proved to be deficient without it (data not shown).

The evolution of the purification factor and yield along the purification steps are shown in the table:

Step	Volume (ml)	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	40	60,88	14,907	4,083	100,00	1,00
Ammonium sulfate precipitation	9,7	39,432	3,725	10,585	64,77	2,59
After dialysis	29	32,838	0,79	51,271	53,94	12,55

We carried out the PAGE-SDS electrophoresis of these three samples and the photo of the gel is presented in figure 1. The brute extract showed 10 protein fractions with different electrophoretic mobility, which proves a good extraction. The sample taken after the ammonium sulfate precipitation shows thicker bands, but the number of the fractions is the same, although the total protein decreases from 14.9 mg to 3.725 mg and the purification factor increases.

The lanes 4 and 5 show that a great number of fractions have disappeared; from the initial ten we found only three. In the literature we have found reports that highly purified α -amylase appear as multiple fractions on PAGE-SDS, because the buffer used for electrophoresis do not have Ca^{2+} . During the electrophoresis some amylase loses this ion and migrates more rapidly [2]. It is not the case of our experiment, as the purification factor that we obtained shows that the solution contains some proteins without amylolytic activity. The explanation is that alginate shows affinity and for other proteins, like pectinase, phospho-lipase [6].

The estimated molecular weight is between 50 and 65 kDa, which agrees well with the literature [8].

Substrate specificity. We have studied the substrate specificity of the enzyme from both the crude extract and the post-dialysis solution upon 3 different substrates: soluble starch, glycogen and dextrin. The results are shown in figure 2.

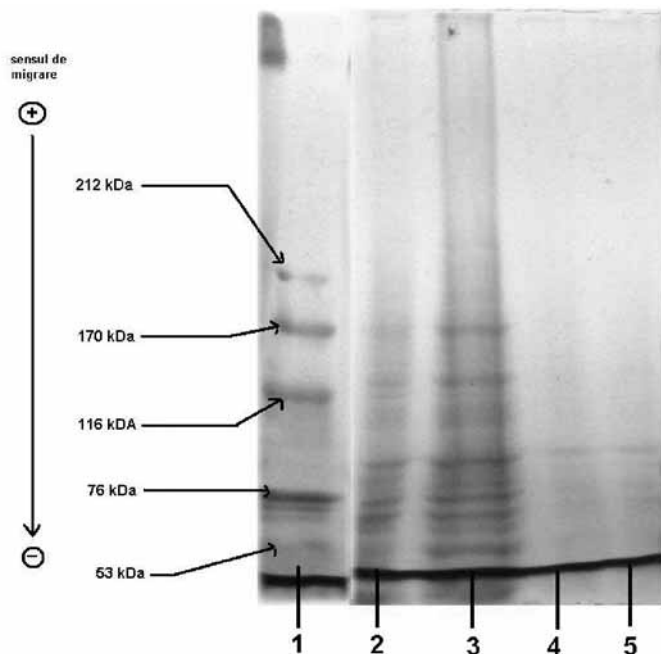


Figure 1. SDS-PAGE of samples taken in the purification steps. Lane 1-marker proteins, lane 2- crude extract, lane 3- ammonium sulfate precipitation step, lane 4 and 5- after dialysis

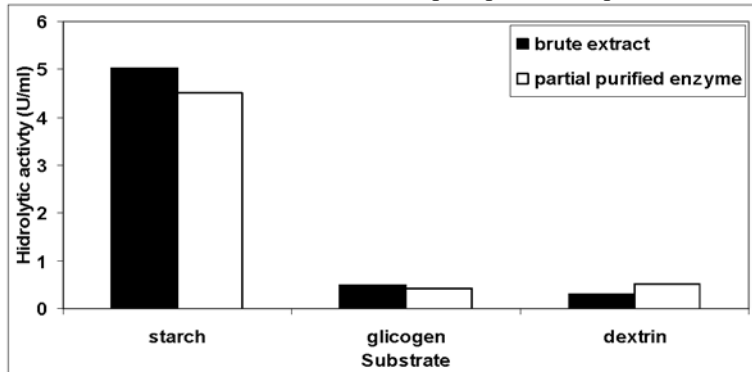


Figure 2. Hydrolytic activity of α -amylase from on different substrates

The enzyme behaves typically for a vegetal amylase. The small differences between the activity of the crude extract and the partial purified enzyme acting on starch can be explained in two ways. First, the solutions used as enzyme source are not equal regarding their amylolytic activity/ml. Second, the crude extract offers conditions much closer to those found in vivo (different types of ions, different biological molecules) that could stabilize the enzyme.

The influence of substrate concentration over the amylolytic activity. The dependence of the starch degrading rates upon starch concentration is presented in figure 3.

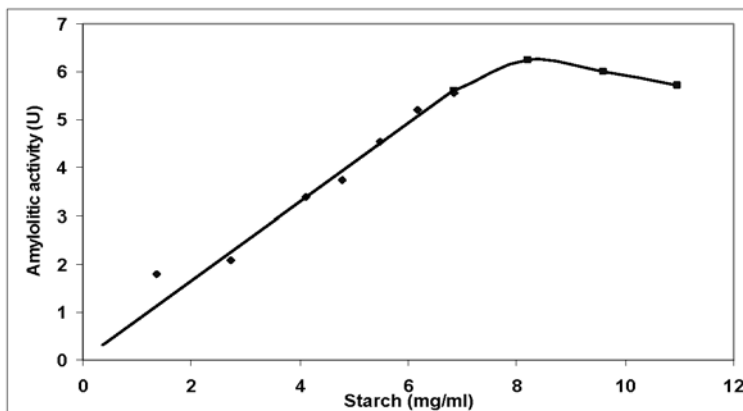


Figure 3. The relation between starch degrading rate and substrate concentration

The data shows that the enzyme does not behave typically according to a Michaelis-Menten kinetic, but more like as substrate-inhibited enzyme. This behavior has two explanations: for the hydrolases the water may be considered one of the substrates, the increasing of the starch concentration leading thus to the diminishing of water “concentration”. The second explanation is the starch degrading mechanism. According to the subsitus theory [8], the enzyme has several affinity sites for starch. By increasing its concentration a greater number of non-productive complexes are formed and the degrading rate decreases.

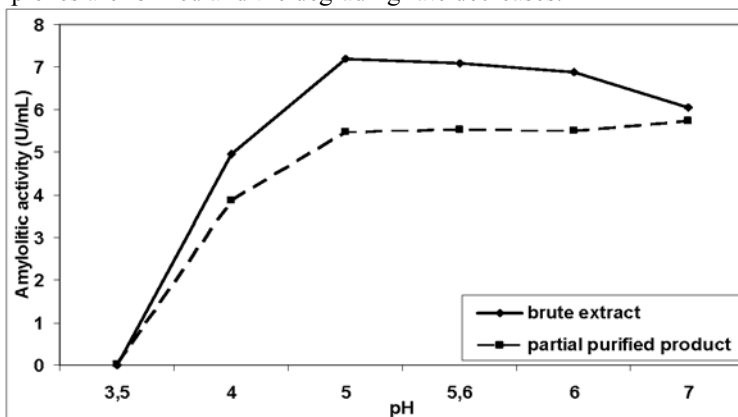


Figure 4. The influence of pH on the amylolytic activity of the α -amylase from both brute extract and partial purified product

The influence of pH and temperature on amylolytic activity. We have studied the influence ionization degree of the enzyme and substrate upon the starch degrading rate, and not the pH stability of the enzyme. The results are shown in figure 4.

For the enzyme from both sources, the optimum pH is on a rather broad plateau of 5-6 and, at low values, the starch degrading rate decreases dramatically: from 4,94 U/ml at pH 5 to 0,012 U/ml at pH 4 for the brute extract and from 3,851 U/ml to 0,036 U/ml for the partial purified enzyme. This would indicate the presence of some aminoacids with basic side chains in the catalytic site.

Concerning the temperature, we have studied its influence upon the degrading rate and not the enzyme thermostability. The results are presented in figure 5.

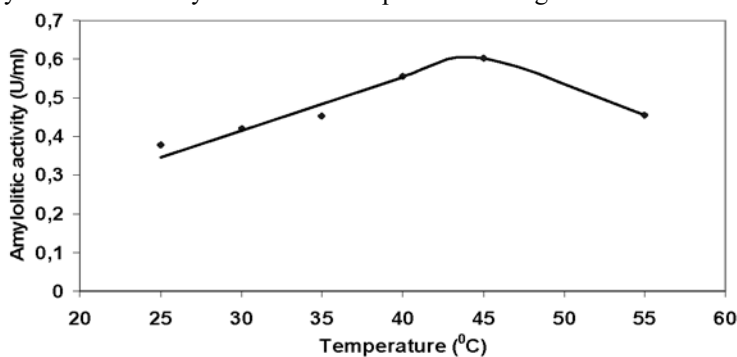


Figure 5. The influence of temperature on starch degrading rate.

The α -amylase from germinated seed of *Robinia pseudacacia* L has an optimum temperature of 45°C, similar to that from rice (45°C-50°C).

The influence of different inorganic ions over the activity of the α -amylase from *Robinia pseudacacia* L germinated seeds. During the purification process we observed that in the presence of ammonium sulfate at high concentration the enzyme activity decreases dramatically. We have studied the influence of different concentrations of the salt on the amylolytic activity of the enzyme. The results are shown in figure 6.

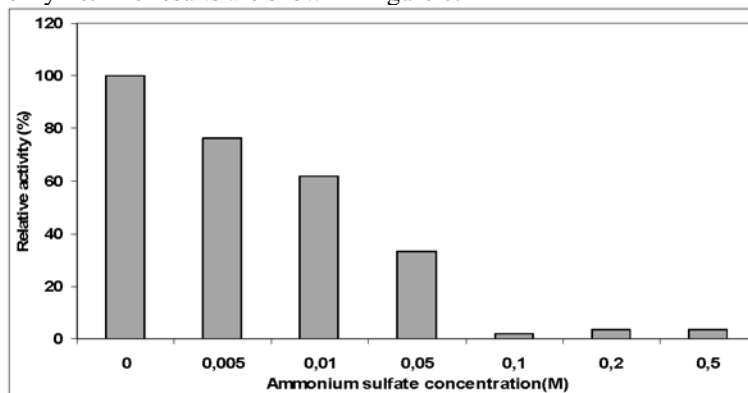


Figure 6. The inhibitory effect of ammonium sulfate on amylase activity

Concentration of only 0.005 M ammonium sulfate leads to a decrease of the amylolytic activity, from 3,165 U/ml for the control to 2,412 U/ml. By increasing the salt concentration to 0.05 M the enzyme activity decreases to about 33.33% of control. In the samples with high salt concentrations the activity decreases dramatically and we could observe a deficient development of the maltose specific color.

The calcium ion has a specific importance for the vegetal α -amylases because it has a thermostabilizing role. Without it the amylolytic activity disappears at 70°C because of thermodenaturation of the enzyme. We have investigated whether this ion has an activating effect on this enzyme. The results are presented in the following diagram (figure 7).

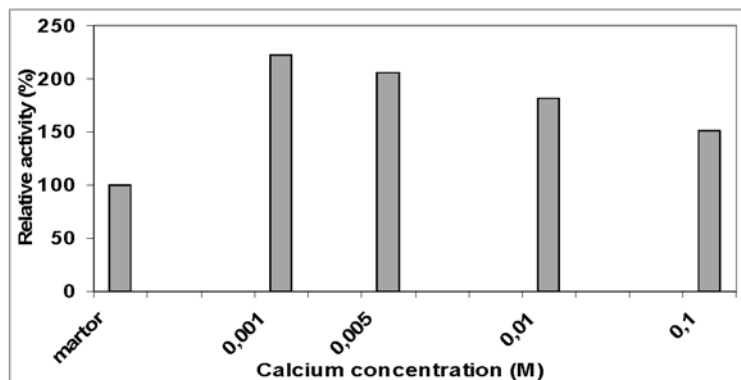


Figure 7. Activatory effect of the CaCl_2 on amylase activity

In the presence of the CaCl_2 0.001 M the amylase activity increases to about 122% of the control value. Further increases of the salt concentration lead to a lost of enzyme activity. At 0.005 M CaCl_2 the activity is 4.359 U/ml and at 0.1 M becomes 3.213 U/ml. Although the activation effect decreases, it maintains above the control level values. In order to explain this effect, we have to consider both the Ca^{2+} and Cl^- ions.

CONCLUSIONS

The relative molecular weight of the α -amylase is between 50-65 kDa.

The enzyme is inhibited by its substrate at high concentrations

The optimum pH is a broad interval between 5-6.

The optimum temperature is found between the values of 40°C and 50°C.

CaCl_2 has an activating effect, especially at low concentrations.

Ammonium sulfate has an inhibiting effect, proportional with its concentration.

REFERENCES

1. Artenie Vlad, 1991, *Biochimie*. Editura Universității „Al. I. Cuza ” Iași, p. 129
2. Beers E.P., Duke S.H., 1990, *Plant Physiology*, 92, 1154-1163
3. Bernfeld, P., *Methods in Enzymology*, vol. I, (Colowick, S. and Kaplan, N., eds.), Academic Press, New York, 149-154,
4. Bradford M.M., 1976, *Anal. Biochem.*, 72, 248-254
5. Gupta M.N., Aparna Sharma, Shweta Sharma, 2000, *Protein Expression and Purification*, 18, 111-114
6. Gupta M.N., Sacarikova M., Roy I., Safarik I, 2003, *J. Biotechnol.*, 105, 255-260.
7. Gupta M.N., Sardar M., 1998, *Bioseparation*, 7, 159-165
8. *Handbook of Amylases and related Enzymes-Their Sources*, Isolation Methods, Properties and Applications, Pergamon Press 1988

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L'ETUDE DE L'ACTIVITE DES QUELQUES ENZYMES DU STRESS OXYDATIF DANS LE TISSU MUSCULAIRE A DES DIFFERENTS CYPRINIDES DE CULTURE

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Mots clef: catalase, peroxydase, cyprinidé

Résumé. Dans le travail présent sont systématisées les dates expérimentales obtenues dans l'étude de l'activité de la catalase et de la peroxydase du tissu musculaire des quelques espèces de cyprinidé de culture. Les résultats obtenus attestent le fait que l'activité de ces enzymes est dans une corrélation serrée avec la masse corporelle et cela ne dépend pas du sexe d'individus qui sont étudiés.

INTRODUCTION

La pisciculture représente l'un des domaines les plus importants de l'économie, le poisson représentant l'un des aliments les plus riches en des éléments nourrissants nécessaires pour l'homme. Dans les dernières décennies, l'aquaculture intensive a enregistré un développement différent à cause de la croissance continue de la consommation du poisson. Cela se base sur la nutrition contrôlée des différentes espèces de poisson le but étant la croissance de la productivité.

La modification de la nutrition, comparatif à des conditions normales de nourriture d'accumulations naturelles d'eau, peut-être cependant quelques-unes modifications plus ou moins prononcées pour le métabolisme. La profondeur de ces modifications peut être mise en évidence par l'évaluation des différents paramètres biochimiques dans le sang, les muscles, le tube digestif etc.

Dans notre recherches nous avons-nous proposé d'évaluer les valeurs de ces paramètres à différentes espèces de cyprinidé par les accumulations hydrotechniques où s'exerce l'aquaculture intense pour établir les particularités possibles d'espèce, âge, sexe, habitation etc.

Parce que les recherches sont à peine au début, dans le travail présenté on systématise seulement les dates expérimentales qui se réfère à l'activité de la catalase et de la peroxydase à les espèces de cyprinidé prises pour étudier, ces enzymes étant considérées comme des indicateurs du stress oxydatif. Pour les mêmes raisons on a corrélié les valeurs trouvées avec le poids corporelle, les dates qu'on a à présent ne pas nous permettant qu'on fasse un étude complet sur l'aspect ontogénétique, à suivre que notre recherches prochaines complètent ces dates.

LE MATÉRIEL ET LES MÉTHODES

Pour les recherches proposées on a pris dans l'étude trois espèces de cyprinidé *Hypophthalmichthys molitrix* (sânger) et *Aristichthys nobilis* (novac) d'accumulation Ezăreni et respectif *Carassius auratus gibelio* Bloch (caras) du lac Tansa-Belcești, département Iași. Après la pêche et le sacrifice s'ont recueilli preuves du tissu musculaire par la zone de la nageuse de poitrine. Des tissus récoltés s'ont pesés des preuves avec la masse bien déterminée, s'ont homogénéisés sur bouteille pilée et on a obtenu l'extrait acellulaire par centrifugation. Les extraits obtenues ont servi pour sources d'enzymes pour les déterminations ultérieures.

L'activité de la catalase s'est déterminée par titrimétrie avec permanganate de potassium par le dosage de l'eau oxygénée restée non décomposée après l'interruption d'action d'enzyme sue elle, et l'activité de la peroxydase s'est déterminé d'après la méthode colorimétrique avec orto-dianisidine qui se base sur le mesurage du densité optique du produit d'oxydation de l'orto-dianisidine avec l'aide de l'eau oxygénée sur l'action d'enzyme.

LES RÉSULTATS EXPÉRIMENTALES ET DISCUSSIONS

L'étude de littérature de spécialité relève le fait que l'étude de l'analyse du stress oxydatif aux poissons d'aquaculture intense, par l'étude de l'activité de la catalase et peroxydase fait l'objet de beaucoup de recherches. Les plus articles se réfèrent à la détermination de ces paramètres biochimiques sur la truite arc-en-ciel [1, 4]. Dans nos recherches nous avons proposé à analyser

ces paramètres à trois espèces de cyprinidé (*Hypophthalmichthys molitrix*, *Aristichthys nobilis* et *Carassius auratus gibelio* Bloch) du deux accumulations hydrotechniques du département Iași où se pratique l'aquaculture intense.

Les dates expérimentales obtenues dans notre détermination on les a systématisés dans les listes I et II et nous les avons représentés graphique dans la figure 1 et 2.

Dans une première série d'expériences on a déterminé l'activité de la catalase et peroxydase au individus appartenant aux espèces *Hypophthalmichthys molitrix* et *Aristichthys nobilis* par l'accumulation Ezăreni, le département Iași. Donnée étant le numéro limité d'individus de différentes âges qu'on a eu jusqu'à cette étape des recherches, on a essayé à réaliser une corrélation d'activités enzymatiques avec la masse corporelle d'individus étudiés.

Après comme on voit les dates présentes (la liste I), aux masses corporelles entre 1650 et 3000 grammes on observe une lente diminution de l'activité de la catalase musculaire (du 2,91 U/mg pour l'individus de 1,65 kg à 1,94 U/mg pour ceux la masse corporelle 3 kg). Au individus avec la masse corporelle plus grande on observe une croissance relatif brusque de l'activité enzymatique, les valeurs trouvés approximatif 2,30 U/mg au individus de 5,9 kg et respectif 2,50 U/mg à ceux qui ont 6,2 kg.

À la fin de ces déterminations on ne peut pas faire pour le moment des corrélations entre l'activité de la catalase musculaire et le sexe d'individus pris en étude.

En ce qui concerne l'activité de la peroxydase les valeurs obtenues ne permettent pas la conclusion d'une corrélation entre l'activité enzymatique et la masse corporelle d'individus, ou leur sexe. Les valeurs obtenues sont obtenues sont situées, approximatif, entre 10,34 et 11,85 U/gramme tissu musculaire.

Pour avoir une image plus claire en ce qui concerne les différences enregistrées, on a représenté graphique l'activité des deux enzymes dans des pourcentages de la valeur maximale (figure 1).

Une dynamique en quelque sorte ressemblante à l'activité de la catalase et peroxydase a été obtenue aussi dans le cas d'individus appartenant à l'espèce *Carassius auratus gibelio* Bloch du lac Tansa- Belcești, le département Iași (la liste II). Mais à la différence de deux espèces de cyprinidé, dans le cas du *Carassius auratus gibelio* Bloch l'activité de la catalase est moins approximatif demi de celle enregistrée au *Hypophthalmichthys molitrix* et au *Aristichthys nobilis*, et l'activité de la peroxydase est de 8-9 petite.

Les valeurs qu'on obtenus oscillent entre 0,82 et 1,28 U/mg dans le cas de l'activité de la catalase et respectif 1,99 et 1,38 U/mg pour peroxydase.

Et aussi à celle espèce, la corrélation de l'activité des deux enzymes avec le sexe d'individus ne met pas en évidence une dépendance palpable.

CONCLUSIONS

De l'analyse des dates expérimentales obtenues jusqu'à ce moment de notre recherches on peut formuler les suivantes conclusions générales :

L'activité de la catalase musculaire aux espèces *Hypophthalmichthys molitrix* et *Aristichthys nobilis* est inverse proportionnelle avec la masse corporelle (au individus avec la masse entre 1,65 et 3 kg) et puis cela augmente progressivement avec la croissance de la masse jusqu'à 6,2 kg.

Dans le cas d'individus appartenant à l'espèce *Carassius auratus gibelio* Bloch l'activité des deux enzymes présente une dynamique ressemblante, les valeurs étant cependant plus petites, spécialement dans le cas de la peroxydase.

Pour toutes les trois espèces de cyprinidé prises en étude, on ne peut pas réaliser une corrélation entre l'activité des deux enzymes et le sexe d'individus étudiés, au moins à cet niveau des recherches.

BIBLIOGRAPHIE

1. Artenie Vl.- 1980, *Les travaux S.C.P. Piscicole Iași*, volume I, p. 357.
2. Battes, K., Artenie, Vl. - 1975, Les travaux du Station „Le chême”. *Limnologie*, p. 277.
3. Cojocaru, D. C. – 1976, *Enzimologie*, Ed. Gama, Iași
4. Dumitru, I. F. – 1980, *Biochimie*, Ed. Did. și Ped, Bucharest.
5. Frecht, H – 1961, *I.Z.Vergl.Physiol.*, vol. 44, p. 451.
6. Rudnick von, N.H. – 1967, *Zool. Jb. Physiol.*, vol. 73, p. 227.

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La liste I

L'activité de la catalase et peroxydase dans le tissu musculaire aux individus appartenant aux espèces *Hypophthalmichthys molitrix* (sânger) et *Aristichthys nobilis* (novac) du accumulation Ezăreni, département Iași (*)

Nr. crt.	Espèce	Sexe	Masse corporelle (grammes)	Activité de la catalase (U./mg)	Activité de la peroxydase (U/g)
1	<i>Aristichthys nobilis</i>	♂	1650	2,91 ± 0,09	10,34 ± 0,23
2	<i>Hypophthalmichthys molitrix</i>	♂	2300	2,14 ± 0,07	11,59 ± 0,18
3	<i>Hypophthalmichthys molitrix</i>	♀	3000	1,94 ± 0,07	11,25 ± 0,11
4	<i>Aristichthys nobilis</i>	♂	5900	2,30 ± 0,12	10,97 ± 0,16
5	<i>Aristichthys nobilis</i>	♂	6200	2,50 ± 0,10	11,85 ± 0,21

*) Les résultats représentent la moyenne des trois déterminations

La liste II

Activité de la catalase et peroxydase dans le tissu musculaire aux individus appartenant aux espèces *Carassius auratus gibelio* Bloch du lac Tansa-Belcești, département Iași (*)

Nr. crt.	Espèce	Sexe	Masse corporelle (grammes)	Activité de la catalase (U./mg)	Activité de la peroxydase (U/g)
1	<i>Carassius auratus</i>	♂	100	0,97 ± 0,03	1,31 ± 0,13
2	<i>Carassius auratus</i>	♂	107	1,17 ± 0,07	1,13 ± 0,09
3	<i>Carassius auratus</i>	♀	138	1,07 ± 0,02	1,22 ± 0,05
4	<i>Carassius auratus</i>	♀	138	0,92 ± 0,08	1,00 ± 0,07
5	<i>Carassius auratus</i>	♀	143	0,82 ± 0,04	1,16 ± 0,11
6	<i>Carassius auratus</i>	♀	145	1,12 ± 0,09	1,03 ± 0,08
7	<i>Carassius auratus</i>	♀	150	1,10 ± 0,07	1,06 ± 0,14
8	<i>Carassius auratus</i>	♀	189	1,22 ± 0,09	1,19 ± 0,07
9	<i>Carassius auratus</i>	♀	196	1,02 ± 0,05	1,38 ± 0,12
10	<i>Carassius auratus</i>	♀	203	1,28 ± 0,11	1,34 ± 0,09

*) Les résultats représentent la moyenne des moyenne des trois déterminations

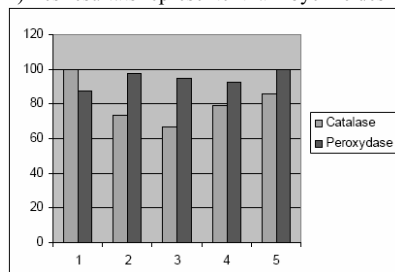


Fig.1 Activité de la catalase et peroxydase dans le tissu musculaire aux individus appartenant aux espèces *Hypophthalmichthys molitrix* et *Aristichthys nobilis* d'accumulation Ezăreni, département Iași

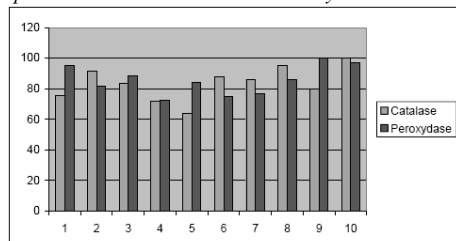


Fig. 2 Activité de la catalase et peroxydase dans le tissu musculaire aux individus appartenant aux espèces *Carassius auratus gibelio* Bloch du lac Tansa- Belcești, département Iași

STUDY OF A MICROBIAL INOCULUM ON SEVERAL BIOCHEMICAL INDICES IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

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Keywords: rhizosphere, assimilating pigments, catalase, *Helianthus annuus* L.

Abstract: The study of the complex interaction between rhizospheric bacteria and plant roots represents a very important and actual problem in microbiology. The use of bacteria that stimulate plant growth – PGPR (plant growth promoting rhizobacteria) – as biofertilizers is one of the most promising biotechnologies used for the increase of the primary production with reduced amounts of chemical fertilizers (Lemanceau, Alabouvette, 1993).

INTRODUCTION

It is well known that by their roots, plants secrete in the environment an important diversity of organic substances. These products are used by microorganisms as important sources of nutrients. On their part, the microbial populations adapted to rhizospheric environment influence plant development and production quality in a significant manner. (Whipps and Lynch, 1980). Lately it was shown that several rhizospheric bacteria could stimulate the plant metabolic process and implicitly the plant growth (Merbach and Ruppel, 1992). The microorganisms' activity can produce these effects by:

- Secretion of vitamins and phytohormones (Polonskaya, 1995);
- Production of antibiotics which inhibit the growth of the pathogen fungi
- Conversion of some minerals to more accessible forms for the plants.

In this context, the goal of our paper is the study of the influence of several rhizospheric bacteria on both the catalase activity and the assimilating pigment content in the sunflower plants.

MATERIALS AND METHODS

Six bacterial strains (conventionally marked as F1, F2, F3, F4, F5, and F6) isolated from *Helianthus annuus* rhizosphere were used to prepare a suspension in distilled water = the inoculum. Biopreparate concentration was calculated by counting the colonies in plates and estimated at 64×10^6 UFC/ml.

After the initial sterilization of all sunflower caryopses, the inoculation by immersion in biopreparate was performed only on the treated group of caryopses. Both groups (control and treated) were sown by using a mini experimental sower. The experiment was done in 2004 in the Didactic Farm Ezăreni, Didactic Station of USAMV „Ion Ionescu de la Brad” Iași on a cambic chernozem with argilo-sandy texture and middle to good fertility, with a moderate content in humus and a relatively high amount of nitrogen, well enriched in potassium and a slightly acid to neutral pH.

The testing of catalase activity and assimilating pigment content in plant leaves was performed on the foliar tissue of both control and treated plants in two stages: 29.06.2004 (prior to the bloom) and 19.07.2004 (when the heads were formed completely).

Determination of the catalase activity was performed by using the iodometric titration (V. Artenie, Elvira Tănase, 1981), the results being displayed in catalase units (CU)/ g vegetal material.

Chlorophyll and carotene concentration determination in leaves was performed by using photocolometric technique (V. Artenie, Elvira Tănase, 1981) and the results were shown in mg/100 g vegetal material. 90% acetone was used for extraction, while the readings were performed on a Metertek SP-830 spectrophotometer using the following wavelengths: chlorophyll a – 662 nm, chlorophyll b 644 nm, and carotene – 440.5 nm.

Statistical analysis

The results expressed as mean \pm standard error were statistically analyzed by using T-test (Student). Theoretical probability of the test was set at $p < 0.05$.

RESULTS AND DISCUSSIONS

Catalase activity

The results obtained showed that the studied biopreparate influenced the catalase activity in the foliar tissues of *Helianthus annuus* plants. Thus, Fig. 1 shows that catalase activity prior to the bloom is bigger in the treated group (363.41 CU/100 g vegetal matter) than that in the control (259.41 CU/100 g vegetal matter), with a significant t-test ($p < 0.03$).

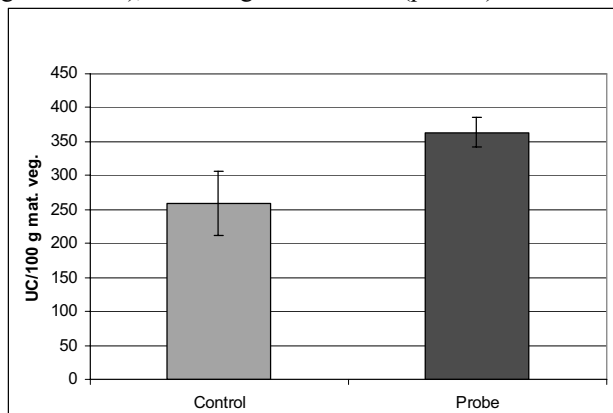


Fig. 1 - Catalase activity of control and treated groups of *Helianthus annuus* prior to the bloom period (29.06.2004)

This difference observed is the result of the rhizospheric microorganism activity (the initial inoculum) because the other factors that could take place (neighbor influence, non-uniformity of the soil, climatic factors, etc) have no influence in this case. Having in consideration the fact that the probes were taken from physiologically normal plants we suggest that an increased catalase activity in the treated group can eventually be correlated with an intensification of respiratory processes that metabolically underlie the growth process.

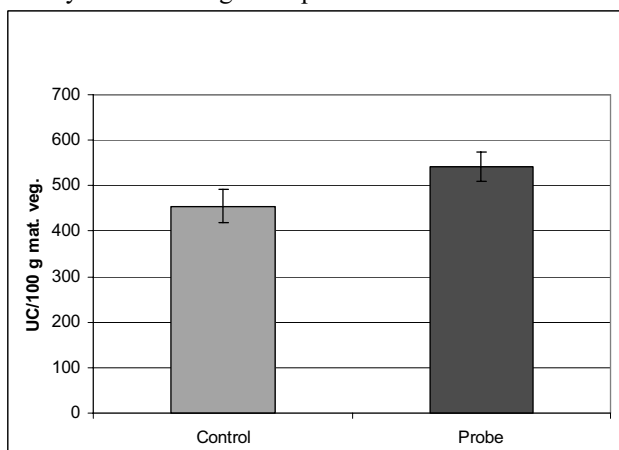


Fig. 2 - Catalase activity of control and treated group of *Helianthus annuus* after the bloom. (19.07.2004)

Fig. 2 shows that the differences between catalase activity of the control and treated group after the bloom (19.07.2004) become statistically non-significant ($0.005 < p < 0.05$). This result can be explained by the fact that, after the bloom, the decreased intensity of the majority of the plant metabolic processes is followed by a decreased exchange of substances mediated at the radicular

level by the rhizobacteria. This reduction of the positive influence of the rhizospheric microorganisms can be considered as a normal one in this situation.

Concentration of assimilating pigments

The concentration of assimilating pigments (chlorophyll a and b and carotenes) in the foliar tissue of control and treated group was determined in two stages, before and after the bloom. The results are presented in Fig. 3 and Fig. 4.

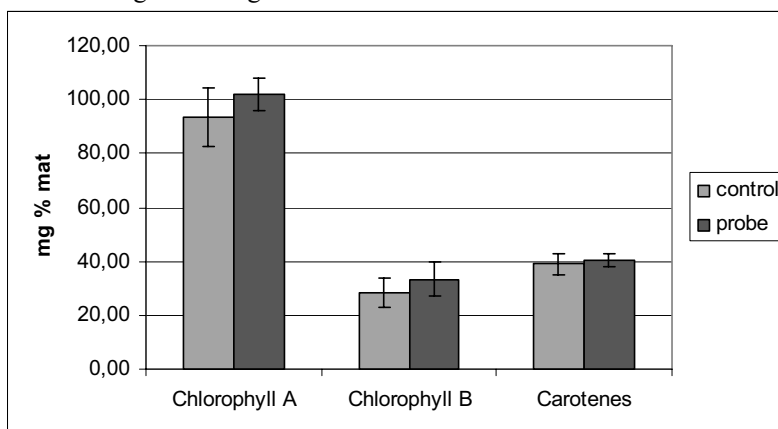


Fig. 3 Concentration of assimilating pigments in the foliar tissue of *Helianthus annuus* prior to the bloom (29.06.2004)

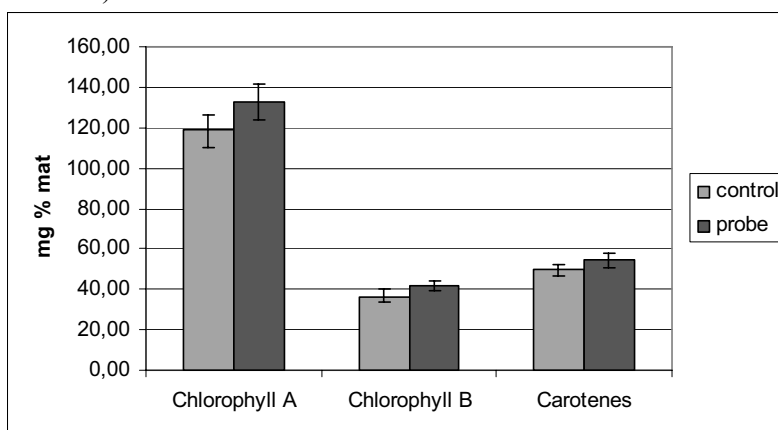


Fig. 4 Concentration of assimilating pigments in the foliar tissue of *Helianthus annuus* after the bloom (19.07.2004)

The data analysis has pointed out that although in the maximum activity period (prior to the bloom) the amount of the assimilating pigments is bigger in the treated group (101.98 mg chlorophyll a /100 g vegetal matter, 33.34 mg chlorophyll b /100 g vegetal matter, 40.43mg carotenes /100 g vegetal matter), the difference is not statistically significant (T test is non-significant $p=0.24>0.05$, $p=0.28>0.05$ respectively $p=0.39>0.05$).

The same situation was also observed after the bloom when as a result of the decreased influence exerted by the rhizospheric microorganisms, the difference regarding the content of assimilating pigments between the control and treated group become non-significant.

The lack of statistical significance of the values obtained as a result of quantitative determination of assimilating pigments should not be exclusively interpreted as a consequence of the positive non-influence of rhizospheric microorganism on plant metabolism. A possible explanation of this result could relay on the fact that the positive influence of rhizospheric microorganisms weakly manifests at the level of photosynthetic pigment metabolism maybe because of the complexity of the metabolic chains involved in this process. The more obvious manifestation of the positive action exerted by the rhizospheric microorganisms on the catalase activity level comparatively with the action on photosynthetic pigment biosynthesis can also be associated with a larger involvement of catalase in energetic processes associated with the plant growth and development, the photosynthetic pigments representing end products of several metabolic chains compared to catalase which is directly involved in more metabolic chains.

CONCLUSIONS

Catalase activity of treated group is higher (statistically significant) than that observed in the control group both before and after the bloom period, with a smaller difference between the groups after the bloom.

The amount of photosynthetic pigments in the foliar tissue of treated group is smaller but statistically non-significant compared to that in the foliar tissue of control group.

Taking in consideration the intensification of catalase activity and the increase of assimilating pigments in treated group as a result of biopreparate administration, we suggest that the metabolic modifications induced by the rhizobacteria prior to the bloom could lead to a stimulation of plant growth and development.

After the bloom, this beneficial influence becomes non-significant, maybe because of the normal physiologic decrease in the intensity of metabolic processes.

REFERENCES

1. Artenie, V., Elvira Tănase, 1981 – *Practicum de biochimie generală*, Ed. Univ. „Al. I. Cuza” Iași, 110-111, 135-138.
2. Burzo, I. Toma, S., Dobrescu, A., Ungurean, L., Ștefan, V., 1999 - *Fiziologia plantelor de cultură*, vol. 2, Fiziologia culturilor de câmp, Întreprinderea Editorial-Poligrafică Știința, Chișinău.
3. Merbach, W., Ruppel, S., 1992 – Influence of microbial colonization on $^{14}\text{CO}_2$ assimilation and amopunts of rootborne ^{14}C compounds in soil, *Photosynthetica*, vol. 26, no. 4, 551-554.
4. Orazova, M.K., Polyanskaya, L.M., Zviagintsev, D.G., 1999 – The structure of the microbial community in the barley root zone, *Microbiology*, vol. 68, No. 1, 109-115.
5. Polonskaya, J.E., 1995 – The influence of epiphytic bacteria on growth and nitrogen absorbtion of pine seedling roots (sterile conditions and field test), *Abstracts of papers of Inst. Long Ashton Int. Symp.* Bristol, England, 13-15 Sept., 100.
6. Thuar, A.M., Olmedo, C.A., Bellone, C., 2000 – Greenhouse studies on growth promotion of maize inoculated with plant growth-promoting rhizobacteria (PGPR), *Proceedings of the Fifth International PRPR Workshop*, Cordoba, Argentina.
7. Whipps, J.m., Lynch, J.M., 1986 – The influence of the rhizosphere on crop productivity, *Adv. Microb. Ecol*, vol. 9, 187-244.

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ROLE OF THE SEROTONIN IN MEMORY PROCESSES IN THE RAT

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Key words: Serotonin; learning; memory, 5,7-DHT; parachlorophenylalanine (PCPA).

Abstract: Chronic 5, 7-dihydroxytryptamine (5, 7-DHT, 150 μ g.i.c.v.) disruption of the central serotonergic function, is able to interfere with learning and memory processes in the rat. Serotonin depletion significantly diminished spontaneous alternation % in Y-maze task, which suggest the impairment of short-term memory. Long-term memory does not undergo significant changes. Parachlorophenylalanine (200 μ g i.c.v. x 3 days) a semichronic serotonin neurotoxin, do not impaired long-term memory. This effect of serotonin depletion was not produced at the level of organism motricity that, in turn, would allow an enhancing efficiency of another neurotransmitters contribution to memory processes, as number of arm entries was not affected by serotonin depletion. It is concluded that learning and memory processes is a multitransmitter system function, in which serotonin play an important role.

INTRODUCTION

There are several reason for the suggestion that cholinergic neurotransmission mediate cognitive processes: cholinergic drugs alter cognitive performance in humans (Drachman and Leavitt, 1974) and animals (Hefco et. 2003); cholinergic neurons undergo degeneration in dementing disorders (Davies and Maloney, 1976), and cognitive deficits in demented patients have been correlated with cholinergic dysphunction (Perry et al. 1978), suggesting that at least some of the cognitive impairments in dementia are due to cholinergic damage; lesions of cholinergic systems induce cognitive deficits in animals (Flicker et al. '1983).

Other neurotransmitter systems have also been implicated in these processes and serotonin (5-HT) is though to be involved. Drugs acting at serotonergic system influence human (Crook 1991) as well as animal (Steckler, Sahgal, 1995) cognition; serotonergic neurons degenerate in disorders associated with dementia (Steckler and Sahgal, 1995) although the literature concerning the role of 5-HT in cognition is more controversial than the literature covering the function of cholinergic systems in mediating these behavioral processes.

The majority of serotonergic neurons are restricted to clusters of cell lying within or near the raphe regions of the pons and medulla. The dorsal raphe nucleus (DR) containing approximately half of all serotonergic neurons in the brain (Steckler and Sahgal, 1995) and the medial raphe nucleus (MR) have received most attention in the literature. Ascending projections of these two nuclei travel through the medial forebrain bundle and projections from the DR reach hypothalamic nuclei, thalamic nuclei, basal ganglia, septal area, cerebral cortex and hippocampus. The MR projects predominantly to hippocampus and septum. Further, there are serotonergic neurons located outside these areas, for example, within the hypothalamus, the interpeduncular complex and reticular formation of the brain.

The aim of the present work was to study the effects of lesions of all these different serotonergic neurons by means of i.c.v. administration of 5, 7 -DHT, a chronic serotonergic neurotoxin, acting on the neuronal cell bodies, and parachlorophenilalanine (PCPA), a semichronic serotonergic neurotoxin, which blocks the synthesis of serotonin, on learning and memory processes evidenced by means of Y-maze task and multi-trial passive avoidance task. Our data suggest that serotonin play one important role on learning and memory processes.

MATERIAL AND METHODS

Animals

The experiments were carried out on male Wistar rats weighing 225-250g at the start of the experiment. The rats were treated in accordance with the Guidelines for Animal Experiments of the "A.I.I.Cuza" University and of the U.S. National Institute of the Health Guide for the Care and Use of Laboratory Animals. The rats were housed three per cage with free access to food and water under controlled laboratory conditions (a 12-h light/dark cycle with light on at 8:00 a.m., 22 \pm 0.5 O C).

Surgery

All surgical procedures were conducted under aseptic conditions, under sodium pentobarbital (45 mg /kg b.w. i.p.) anesthesia. Injections into the lateral ventricle were performed stereotaxically through a 10 μ l Hamilton syringe at the following coordinates (in mm): A-0.5mm (from bregma), L \pm 1.3 (from midline), V- 4.3 (from bregma), with the incisor bar set at 3.3 mm beneath the level of the interaural line (Paxinos and Watson 1986). After each injection, the needle was left in situ for 5 min, retracted 2 mm, and a second delay of 4 min was allowed before complete retraction.

All rats sustaining a 5, 7-DHT lesion were pretreated with desipramine (25 mg/kg, i.p. in saline, Sigma) 20 min before anesthesia in order to protect noradrenergic system. Rats were injected (4.5 μ l/ ventricle) with 150 μ g/free base) of 5,7-DHT (creatine sulfat salt; 338 μ g dissolved in 20 μ l of physiological saline containing 0.2 mg/ml ascorbic acid, Sigma). Control rats were treated exactly as the rats subjected to the 5,7-DHT lesions except that no 5,7-DHT was present in the saline solution

Parachlophenylalanine (PCPA), which blocks the synthesis of serotonin, was administered i.c.v. through a plastic (silastic) cannula (0.9 mm O.D), implanted stereotaxically in the left cerebral ventricle at the following coordinates (in mm): A-0.5 (from bregma), L 1.3, V-4.3 (from bregma) (Paxinos and Watson, 1986). The cannula was positioned with acrylic dental cement and secured by one stainless steel screw. After surgery the rats were isolated in separate cage and protected with large spectrum antibiotic (Manolidis et al. 2004). Five to seven days after surgery, the cannula was connected to a Hamilton micro syringe and 200 μ g PCPA/ 5 μ l for three consecutive days, was administered at a rate of 1 μ l/min in desipramine pretreated rats. Before withdrawal the syringe was left in place for an additional 3 min to minimize dragging the injected solution. Learning and memory test was started 48 h after the last PCPA administration.

The correct placement of the cannula was controlled at the end of experiment using a methylene blue dye injected through the implanted cannula.

Learning and memory tasks

Step-through passive avoidance task

In brief, a step-through type passive avoidance apparatus consisting of two compartments (25 X 15 X 15 high), one illuminated and one dark, both equipped with a grid floor, was used. The two compartments were separated by a guillotine door. In the acquisition trials, each rat was placed in the illuminated compartment; when the animal entered the dark compartment, the door was closed and an inescapable foot shock (0.3 mA, 5 s) was delivered through the grid floor. The rat was removed after receiving the foot shock and was placed back into the light compartment. The door was again opened 30 s later to start the next trial. The training continued until the rat stayed in the light compartment for a 120-s period on a single trial. After 24 h, each rat was placed in the compartment and the step-through latency was recorded until 300 s had elapsed (retention trial). The step-through latency in the retention trial was used as the index of retention of the training experience (Yamada et al., 1996). Longer retention latencies were interpreted as indicating better retention of the training experience.

Y-maze task

Short-term memory was assessed by spontaneous alternation behavior in the Y-maze task. The Y-maze used in the present study consisted of three arms (35 cm long, 25 cm high and 10 cm wide) and an equilateral triangular central area. The rat was placed at the end of one arm and allowed to move freely through the maze for 8 min. An arm entry was counted when the hind paws of the rat were completely within the arm. Spontaneous alternation behavior was defined as entry into all three arms on consecutive choices. The number of maximum spontaneous alternation behaviors was then the total number of arms entered minus 2 and percent spontaneous alternation was calculated as (actual alternations/maximum alternations) X 100 (Yamada et al., 1996). Spontaneous alternation behavior is considered to reflect spatial working memory, which is a form of short-term memory.

Statistical analysis

The results are expressed as means \pm S.E.M. The results were analyzed statistically using Student's t-test. Values of P < 0.05 were regarded as significant.

RESULTS AND DISCUSSIONS

1. Effects of the 5, 7 –DHT serotonergic lesion on memory.

1.5 months after 5, 7-DHT serotonergic lesion, short-term memory, explored by means of Y-maze task, undergo a significant impairment indicated by a decrease of spontaneous alternation %. This decrease can not be attributed to a change of locomotors activity, because the number of arms entries is not significantly changed (Fig.1).

Chronic serotonin depletion with 5, 7-DHT does not produce a significant change in long term memory as measure with multi-trial passive avoidance test (Fig.2).

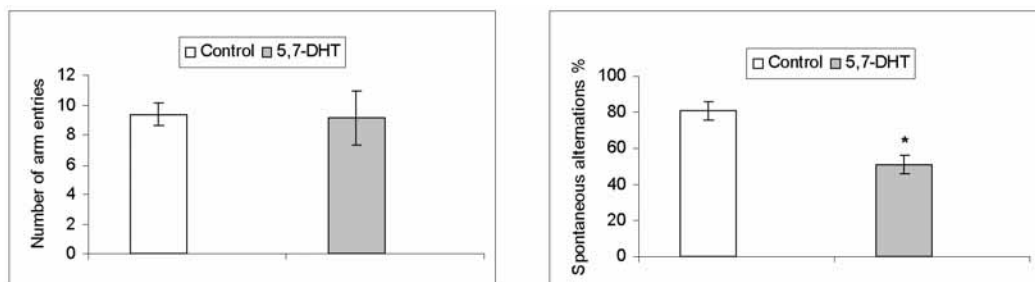


Fig.1. Alterations of number of arm entries and spontaneous alternation % in Y-maze task induced by serotonergic depletion with 5, 7 –DHT. Values are means \pm S.E.M. * $P < 0.05$ vs. control group.

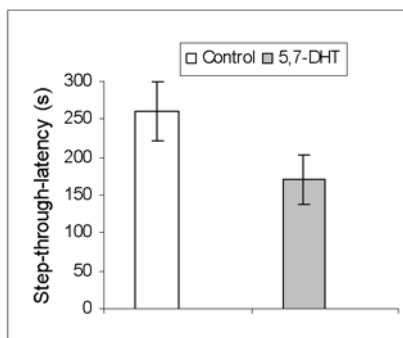


Fig.2.Alterations of step-through latency induced by serotonergic system lesion with 5, 7 – DHT at 24 h after acquisition training in multi-trial passive avoidance test. Values are means \pm S.E.M.

2. Effect of parachlorophenylalanine serotonergic lesion on memory processes.

48 hours after last PCPA lesion of the serotonergic system, long-term memory, explored by means of multi-trial passive avoidance test, do not suffer significant impairment (Fig.3).

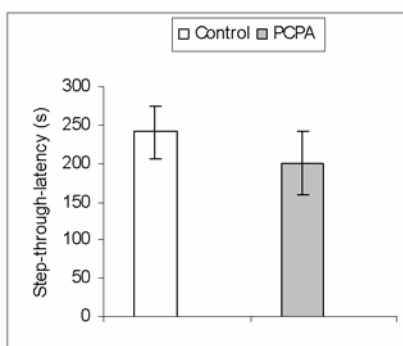


Fig.3. Effects of parachlorophenylalanine lesion of serotonergic system on step-through-latency at 24 h after acquisition training in multi-trial passive avoidance test. Values are means \pm S.E.M.

Chronic serotonin depletion by means of 5, 7 -DHT, impaired significantly short-term memory, in Y- maze test, without affecting significantly long-term memory and locomotion.

PCPA, semichronic serotonin synthesis inhibition (Routhsalainen et al. 1998), do not induce a significant impairment of long-term memory performance in the rats.

As seen from our present data as well as data obtained in serotonin-depleted rats (5, 7 -DHT) in radial arm-maze performance (Hefco et al., 2005), the normal concentration of serotonin is more important for short-term memory than for long-term memory storage, the last form of memory being not affected in chronic (5, 7,-DHT) or semichronic (PCPA) serotonin-depleted rats.

Long term-memory ensures the consolidation of information for long-term retrieval or recognition, and short-term memory allows the maintenance of information during short periods of time to execute a particular action or sequential actions (Baddeley, 1995). The different effect of serotonin on short-term and long-term memory can be attributed to the fact that different brain regions are involved in storage and retrieval in these two categories of memory. In addition, the molecular mechanisms that underlie short- and long-term memory are different (DeZazzo and Tully, 1995).

Knowledge of the mechanism by which serotonin contributes to learning and memory requires an in-depth investigation of the role played by specific types or subtypes of 5-HT receptors, especially those localized in particular cerebral structures underlying defined cognitive functions. The hippocampus is conceived as a key structure involved in long-term memory (O'Keefe and Nadel, 1978), but also in working (short-term) memory (Olton et al. 1979), the prefrontal cortex is also considered as a key structure to complete working memory tasks (Fuster, 1989; Tulving, 1991). To mediate the actions of 5-HT, at least 15 distinct 5-HT receptors have been identified which are divided into seven main families (For ref. Roth et al. 2004)

5-HT may exert neurotrophic effect on cholinergic neurons mediated by at least 5-HT_{1A} receptors (Riad et al. 1994; Whitaker-Azmitia et al, 1990, Buhot et al. 2003).

5-HT_{2A} receptors are also found on the cell bodies of dopaminergic neurons in the ventral tegmental area, where they may modulate dopamine neuronal activity (Nocjar et al. 2002, Roth et al. 2004).

Also there are studies which suggest an intimate association between NMDA and 5-HT_{2A} and imply that drugs with potent 5-HT_{2A} antagonistic actions may prove beneficial at improving cognition in schizophrenia, perhaps by normalizing NMDA receptor functioning (Varty et al. 1999).

5-HT₄ receptors modulate the release of acetylcholine, dopamine, GABA and serotonin (Barnes and Sharp, 1999).

5-HT₆ receptors exert a tonic inhibitory control over acetylcholine release in cortex and hippocampus.

Parachlorophenilalanine (PCPA) may induce catecholaminergic alterations in most regions of the brain. Also, it does not affect all functional markers of the serotonergic innervation (i.e. 5-HT uptake sites are preserved, (Cassel and Jeltsch, 1995; Dewar et al. 1992).

The correlation between the decrease in cholinergic activity and cognitive impairment has lead to attempts to develop cholinergic replacement therapy (acetylcholine precursors, acetylcholinesterase inhibitors, muscarinic receptor agonist) (Davis et al. 1993, Iversen, 1993,; Patel, 1995). However, this replacement therapy has produced only a slight improvement in cognitive decline of Alzheimer's disease (AD) patients (Patel, 1995). Thus, it could be hypothesized that cognitive impairment of AD patients is not entirely owing to the cholinergic

degeneration, but it could be a result of combined degeneration of cholinergic and other ascending pathways, e.g. serotonergic projections. The clinical effectiveness of cholinergic replacement therapy in AD could perhaps be augmented by an amelioration of the serotonergic neurotransmissions. This combined therapy could lead to an improvement in learning and memory.

CONCLUSIONS

Chronic (1.5 months postoperatively) serotonin depletion (5, 7 –DHT) significantly impaired short-term memory as evidenced in Y-maze task by significantly diminished spontaneous alternation %. Locomotion was not affected.

In the same experimental condition, long-term memory is not significantly impaired.

Parachloophenylalanine, a semichronic serotonin neurotoxin, do not impaired long-term memory.

The effects of serotonin on memory processes can be attributed to its own cognitive effects and its interactions with other neurotransmitter systems.

REFERENCES

- Baddeley, A. 1995, In: *The cognitive neuroscience* (Ed. Gazzaniga, M.S.) pp 755-764, MIT Press, Cambridge, MA
- Barnes, N.M., Sharp, T. 1999, *Neuropsychopharmacol.* 38, 1083-1152
- Buhot, M.-C., Wolf, M., Benhassine, N., Costet, P., Hen, R., Segu, L. 2003, *Learn. Mem.* 10, 466-477
- Cassel, J.-C., Jeltsch H., 1995, *Neuroscience*, 69, 1, 1-41.
- Crook, T.H., 1991, 5th World Congress of Biological Psychiatry, *Satellite symposium*. Pp 21-24
- Davies, P., Maloney, A.J.F., 1976, *Lancet*, 25, 1403
- Davis, R., Raby, C., Callahan, M.J., Lipinski, W., Schwarz, R., Dudley, D.T., Lauffer, D., Reece, P., Jaen, J., Tecle, H., 1993, *Prog. Brain Res.* 98, 439-445
- DeZazzo, J., Tully, T., 1995, *Trend. Neurosci.* 18, 212-218
- Dewar, K.M., Grondin, L., Carli, M., Lima, L., Reader, T., 1992, *J. Neurochem.* 58, 250-257
- Drachman, D.A., Leavitt, T. 1974, *Arch. Neurol.* 30, 113-121
- Flicker, C., Dean, R.L., Watkins, D., Fisher, S., Bartus, R., 1983, *Pharmacol. Biochem. Behav.* 18, 973-981.
- Fuster, J.M., 1989, *The prefrontal cortex: anatomy, physiology and neuropsychology of the frontal lobe*. Raven Press, N.Y
- Hefco, V., Hefco, A., Stratulat, S., Hritcu, L., 2005, *Ann. Univ. Iassi. Genet. Mol. Biol.* (in press)
- Hefco V., Yamada, K., A. Hefco, Hritcu, L., Nabeshima T. 2003 *Eur. J. Pharmacol.*, 474, 227-232
- Iversen, L.L., 1993, *Prog. Brain Res.*, 98, 423-426
- Manolidis, G., Neamtu, C., Vasincu, D., Jaba, I., Radasanu, O., Mungiu, O. 2004, *Rom. J. Physiol.* 41, 1-2, 83-89
- Nocjar, C., Roth, B.L., Pehek, E.A., 2002 *Neurosci.* 111, 163-176
- O'Keefe, J., Nadel, L. 1978, *The hippocampus as a cognitive map*, Clarendon Press, Oxford, UK
- Olton D., Becker, J., Handelmann, G., 1979, *Behav. Brain Sci.* 2, 313-365
- Patel, S.V. 1995, *J. Geriatr. Psychiatry. Neurol.* 8, 81-95
- Paxinos, G., Watson, C., 1986. *The rat brain in stereotaxic coordinate's*. Acad. Press

- Perry, E.K. Tomlinson, B.E., Blessed, G., Bergman, K., Gibson, P.H., Perry, R.H., 1978, *Br. Med. J.*, 2, 1457-1459
- Riad, M., Emeritt M.B., Hamon, M., 1994, *Devl. Brain Res.* 82, 245-258
- Roth, B.L., Hanizavareh, S.M., Blum, A.E. 2004, *Psychopharmacol.* 174, 17-24
- Ruotsalainen, S., Miettinen, R., MacDonald, E., Riekkinen, M., Sirvio, A.J., 1998, *Neurosci. Behav. Rev.*, 22, 1, 21-31
- Sirvio, J., Riekkinen P.Jr., Jakala, P., Riekkinen, P., 1994 *Progr. Neurobiol.* 43, 363-379
- Steckler, T., Sahgal, A., 1995, *Behav. Brain Res.* 165-199
- Tulving, E. 1991, *In: Memory: organization and locus of change* (Eds. L.R. Squire et al.) pp 3-32, Oxford Univ. Press, Oxford, UK
- Varty, G.B., Bakshi, V.P., Geyer, M.A., 1999, *Neuropsychopharmacol.* 20, 311-321
- Whitaker-Azmitia, P.M., Shemer, A.V., Caruso, J., Molino, L., Azmitia, E.C., 1990, in: *The neuropharmacology of Serotonin*, *Ann. N.Y. Acad. Sci.* vol. 600 (Eds. Whitaker-Azmitia P. Nad Peroutka J) pp 315-330, New York
- Yamada, K., Noda, Y., Hasegawa, T., Komori, Y., Nikai, T., Sugihara, H., Nabeshima, T., 1996, *J. Pharmacol. Exp. Ther.* 276, 460-466

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EFFECTS OF 5, 7-DIHYDROXYTRYPTAMINE-INDUCED DEPLETION OF BRAIN SEROTONIN ON RADIAL ARM-MAZE TASK IN RATS

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Key words: 5, 7-dihydroxytryptamine, radial arm-maze, working and reference memory

Abstract: Adult rats pretreated with desipramine (25 mg/kg i.p. 30 min before anesthesia) in order to protect noradrenergic system, were subjected to intracerebroventricular injection of 5, 7-dihydroxytryptamine (5, 7-DHT, 150 µg, 4.5 µl/ventricle), a chronic neurotoxin of the central serotonergic function. After 1.5 months later, we assessed the working memory and reference memory in radial 8 arm-mazes. Serotonergic depletion impaired more significantly short-term memory tested by means of the average working memory errors, entries to repeat and average time taken to consume all five baits during 12 days training. Long-term memory, explored by means of reference memory errors, was less impaired. It is concluded that serotonin, among other neurotransmitters, play one important role in cognitive functions, including learning and memory.

INTRODUCTION

Studies directed at understanding the mechanism for action of serotonin (5-HT) are based on evidence that 5-HT may be involved in the modulation of brain function such as appetite, thermoregulation, aggression and sexual behavior (Carlsson 1987; Sandyk 1992). 5-HT is also known to be associated with various pathophysiological processes, including depressive disorders, schizophrenia and dementia (Sandyk, 1992). 5HT also appears to facilitate synapse formation and maintenance, and thereby to modulate the net number of synapse in the developing and mature brain (Okado et al. 1993; Chen et al. 1994; Matsukawa et al. 1997).

The serotonergic neural system derives mainly from neurons in the dorsal and ventral raphe nuclei with projection to virtually every brain region that subserves cognition. There is neurochemical and pathological evidence of a decline in the function of the serotonergic system of Alzheimer's disease (AD) patients (Cross, 1990; Greenamyre and Maragos, 1993; Reinikainen et al. 1990; 1988; Young and Penney, 1994). It has been proposed that the abnormalities in the serotonergic system in AD may be related to behavioral disturbance such as depression, aggressive behavior and anxiety rather to cognitive dysfunction (Palmer et al. 1988). However, studies with experimental animals, have provided evidence for the serotonergic system being involved in cognitive processes (see for review Cassel and Jeltsch, 1995; Steckler and Sahgal, 1995). For example, lesions of raphe nuclei, induced by 5, 7, DHT, impaired delayed spatial alternation in a T-maze at a delay (60 sec) but not at shorter delays (5 and 30 sec) (Wenk et al. 1987), such indicating an impairment in working memory. However, there are also studies which have shown that manipulations at the serotonergic system do not alone cause changes in cognitive function per se (Jakala et al. 1993; Richter-Lewin and Segal, 1989; Riekkinen et al. 1991; Sahgal and Keith 1993; Sakurai and Wenk, 1990,). Thus, the role of serotonergic system in cognition is rather controversial.

The majority of behavioral paradigms used in order to elucidate the role of serotonergic function were based on punishment or aversive situations (passive/active avoidance, water maze). Although these experiments are valid in their own right, it would also be of importance to examine serotonergic function in other learning tasks which should hopefully allow a clearer statement. The aim of the present study was to investigate whether the serotonergic system is involved in working and reference memory as tested in radial arm-maze where the behavior of rats is food motivated. Working memory is an essential part of cognition and its deficit is one of the earliest cognitive problems seen in the AD (Bartus and Dean, 1988). Working memory consists of information which is useful in the immediate future and working memory also permits the manipulation of this information. Working memory is also linked to retrieval of stored knowledge (Carlson, 1994; Goldman-Rakic, 1995). The prefrontal cortex is an important area for working memory processing. Reference memory is a long-term memory. In the present study we used the radial 8 arm-maze task in order to measure the working and reference memory. Depletion of serotonin in rat brain was attained by 5, 7-DHT, a chronic neurotoxin of the central serotonergic function, which induced neural degeneration acting on the neuronal cell bodies.

MATERIALS AND METHODS

Animals

The experiments were carried out on male Wistar rats weighing 225-250g at the start of the experiment. They were fed and allowed to drink water at libitum. Rats were treated in accordance with institutional guidelines.

Surgery

All surgical procedures were conducted under aseptic conditions, under sodium pentobarbital (45 mg /kg b.w. i.p.) anesthesia. Injections into the lateral ventricle were performed stereotaxically through a 10 μ l Hamilton syringe at the following coordinates (in mm): A-0.5mm (from bregma), L \pm 1.3 (from midline), V- 4.3 (from bregma), with the incisor bar set at 3.3 mm beneath the level of the interaural line (Paxinos and Watson 1986). After each injection, the needle was left in situ for 5 min, retracted 2 mm, and a second delay of 4 min was allowed before complete retraction.

All rats sustaining a 5, 7-DHT lesion were pretreated with desipramine (25 mg/kg.i.p. in saline, Sigma) 20 min before anesthesia in order to protect noradrenergic system. Rats were injected (45 μ l/ ventricle) with 150 μ g/free base) of 5,7-DHT (creatine sulfat salt; 338 μ g dissolved in 20 μ l of physiological saline containing 0.2 mg/ml ascorbic acid, Sigma). Control rats were treated exactly as the rats subjected to the 5,7-DHT lesions except that no 5,7-DHT was present in the saline solution.

Radial arm-maze task

The radial arm-maze used in the present study consisted of 8 arms, numbered from 1 to 8 (48 x 12 cm), extending radially from a central area (32 cm in diameter). The apparatus was placed 40 cm above the floor, and surrounded by various extra maze cues placed at the same position during the study. At the end of each arm there was a food cup that had a single 50 mg food pellet. Prior to the performance of the maze task, the animals were kept on restricted diet and body weight was maintained of 85% of their free-feeding weight over a week period, with water being available ad libitum.

Before the actual training began, the animals were shaped for 4 days to run to the end of the arms and consume the bait. The bait was initially available throughout the maze, but gradually was restricted to the food cup. Briefly, each animal was placed individually in the center of the maze and subjected to working and reference memory tasks, in which same 5 arms (no. 1, 2, 4, 5, and 7), were baited for each daily training trial. The other 3 arms (no. 3, 6, 8) were never baited. The training trial continued until all 5 baits had been consumed or until 5 minutes had elapsed. An arm entry was counted when all four limbs of the rat where within an arm. Measures was made of the number of working memory errors (entering an arm containing food, but previously entered), and reference memory errors (entering an arm that was not baited). The time taken to consume all five baits was also recorded. Reference memory is regarded as a long-term memory for information that remains constant over repeated trials (memory for the positions of baited arms), whereas working memory is considered a short-time memory in which the information to be remembered changes in every trial (memory for the positions of arms that had already been visited in each trial) (Durkin, 1994; Olton et al., 1979). Each animal was subjected to one trial each day.

Entries to repeat, choice accuracy was measured by entries to repeat, which was the number of arms entered until a repeat entry was made in the same arm in working or reference type memory, respectively.

Statistical analysis

Results were expressed as mean \pm S.E.M. The results were analyzed statistically by means of the Student's "t" test. $p < 0.05$ was taken as the criterion for significance.

RESULTS AND DISCUSSIONS

1. Effects of 5, 7-DHT lesion on memory performance in rats.

Experimental data were registered 1.5 months after 5, 7-DHT lesions. Serotonin depletion impaired more significantly short-term memory, tested by means of the numbers of working memory errors and average working memory errors (Fig 1), average time taken to consume all five baits (Fig.2) and entries to repeat (Fig. 3) during 12 days training.

Long-term memory, explored by means of number of reference memory errors (Fig 4), average reference memory errors (Fig. 4) and entries to repeat (Fig 3), was less impaired during 12 days training of rats with 5, 7-DHT lesions.

Chronic (5, 7-DHT) disruption of the central serotonergic function is not sufficient to produce reliable effects on learning and memory processes. In rats with chronic serotonergic dysfunctions, a significant impairment, but of less amplitude, undergoes only working memory.

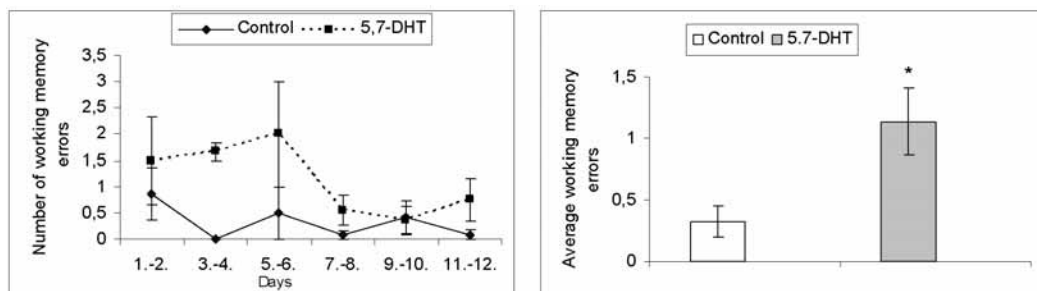


Fig. 1 Effects of chronic serotonin depletion on numbers of working memory errors and average working memory errors during 12 days training. Values are means \pm SEM. * $p < 0.05$ vs. control group

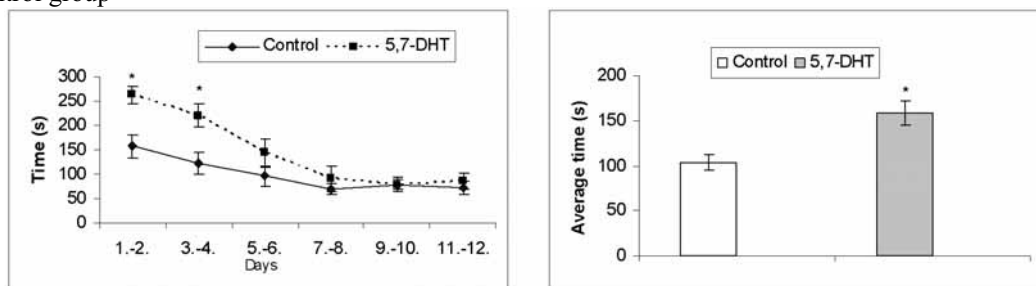


Fig.2. Effects of chronic serotonin depletion on time taken to consume all five baits during 12 days training. and average time during this period. Values are means \pm SEM. * $p < 0.05$ v.s. control groups

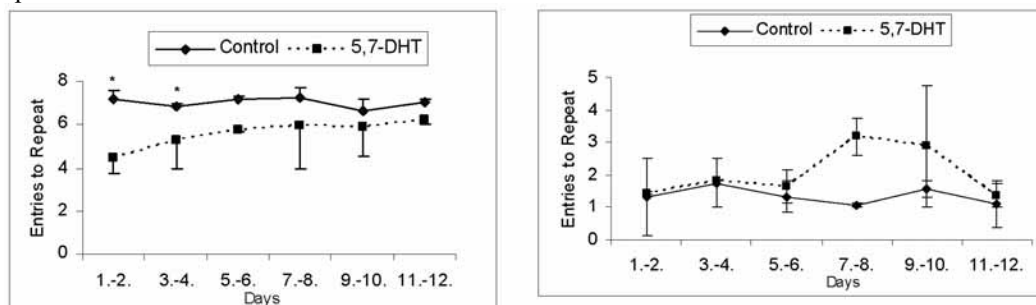


Fig.3. Effects of 5,7-DHT lesion on entries to repeat in working memory (left) and reference memory (right). Values are means \pm E.S.M for two successive days. * $p < 0.05$ v.s. control groups

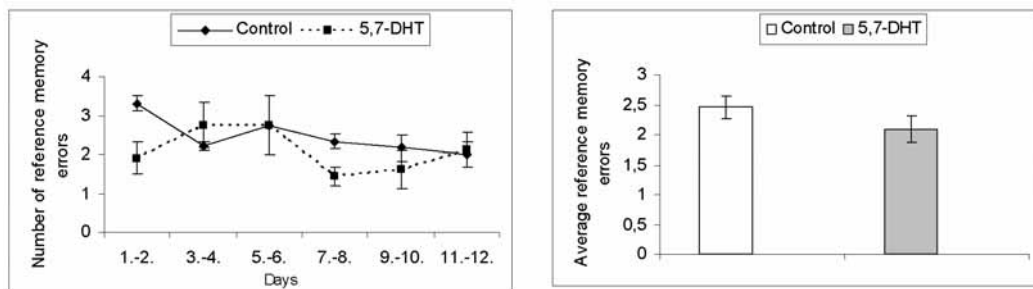


Fig.4. Effects of chronic serotonin depletion on numbers of reference memory errors and average reference memory errors during 12 days training. Values are means \pm SEM.

I.c.v. injections of 5, 7 -DHT, depleted cortical 5-HT by about 50% and produced a nearly complete lesion of the serotonergic projections to the hippocampus (95% depletion of 5-HT) (Steckler and Sahgal, 1995). Moreover i.c.v. 5, 7-DHT do not affect norepinephrine and dopamine release at the prefrontal cortex (Temel et al. 2003) or hippocampal acetylcholine release (Nilsson et al. 1992). In rat hippocampal slices, 5, 7-DHT lesions enhance the evoked overflow of acetylcholine (Birther et al., 2002).

Cholinergic systems have been linked to cognitive processes such as attention, learning and mnemonic function. However, other neurotransmitters system, such as serotonergic one, which may have only minor effects on cognitive functions on their own, interact with cholinergic function and their combined effects may have marked behavioral actions. Serotonergic-cholinergic interactions could be of importance in the mediation of learning processes and working memory. It was hypothesized that it is primarily the hippocampus where serotonergic and cholinergic systems interact in the mediation of working memory (Steckler and Sahgal 1995). As concerned long-term memory, our data do not allow an unambiguous conclusion about the role of these interactive processes in the mediation of long-term reference memory.

To mediate the actions of 5-HT, at least 15 distinct 5-HT receptors have been identified which are divided into seven receptor main families (Roth et al. 2004; Barnes and Sharp, 1999) on the basis of their structural, functional and to some extent pharmacological characteristics (Barnes and Sharp 1999). In rats, the activation of 5-HT_{1A} or 5-HT_{1B} receptors as well as the inhibition of 5-HT₂ receptors was found to exacerbate the deficit due to central muscarinic blockade or to cholinergic lesions (Cassel and Jeltsch 1995). Conversely, the blockade of 5-HT₃ receptors was found to attenuate the cognitive deficits due to central cholinergic disruption.

In vivo and in vitro, 5-HT_{1B} agonists attenuate the release of acetylcholine in at least the hippocampus. Thus, the cognitive perturbations induced by such agonist might, in some respects, be explained by their inhibitory effects on central cholinergic function. The cognitive alteration induced by serotonergic depletion cannot be reduced to a simple and direct modulatory influence that the drug might exert on central cholinergic function.

5-HT appears closely involved in the modulation of neuronal functions in a diverse region of the brain by changing activities of the glutamatergic system and long term depletion of serotonin leads to selective changes in glutamate receptor subunits (Shutoh et al. 2000). In addition to the effects of serotonin on cholinergic and glutamatergic function, one has to consider the possibility that serotonin has its own cognitive actions, for instance in modulating functions that may be essential for mnemonic processes to occur efficiently (e.g., anxiety, arousal, attention etc). In view to

decipher the mechanisms involved in serotonin action on learning and memory in rats, future research is required.

CONCLUSIONS

On the basis of our results obtained by chronic (5, 7 –DHT) disruption of the central serotonergic function, we can conclude that in the rats, chronic serotonin depletion affect more obvious short-term memory. Long-term memory, explored by means of reference memory in radial-arm maze, was less affected by chronic (1.5 month postoperatively) serotonin depletion. The effects of serotonin depletion can be attributed to the interaction between serotonergic system with other neurotransmitters systems or to its own cognitive actions.

REFERENCES

- Barnes, N.M., Sharp, T. 1999, *Neuropharmacol.* 38, 1083-1152
- Bartus R.T., Dean R.L., 1988. In: Giacobini, E., Becker, R. (Eds). Current research in Alzheimer's disease. N.Y.: Taylor and Francis Birtjelmer A., Schweizer T., Jeltsch H., Jackisch R., Cassel J-C., 2002, *Eur.J.Neurosci.* 16, 1839-1849
- Carlsson, A., 1987, *Annu.Rev.Neurosci.* 10, 19-40
- Carlsson, N.R., 1994, In: *Physiology of behavior*. Boston: Allyn and Bacon, 481-509
- Cassel, J-C., Jeltsch, H., 1995, *Neuroscience*, 69, 1-41
- Chen, L., Hamaguchi, K., Ogawa, M., Hamada, S., Okado, N., 1994, *Neurosci.Res.* 19, 11-115
- Durkin, T.P., 1994, *Neurosci.*, 62, 681-693
- Goldman-Rakic, P.S., 1995, *Neuron*, 14, 477-485
- Greenamyre, J. T., Maragos, W.F., 1993, *Cerebrovasc. Brain Metab. Rev.*, 5, 61-94
- Jagala, P., Sirvio, J., Riekkinen P., Ascady, L., Riekkinen, P., 1993, *Pharmacol. Biochem.Behav.*, 44, 411-418
- Matsukawa, M., Ogawa, M., Nakadate, K., Maeshima, T., Ichitani, Y., Kawai, N., Okado, N., 1997, *Neurosci.Lett.* 230, 13-16
- Nilsson, O.G., Leanza, G., Bjorklund, A., 1992, *Brain Res.* 584, 132-140
- Okado, N., Cheng, L., Tanatsuku, Y., Hamada, S., Hamaguchi, K., 1993, *J.Neurobiol.* 24, 687-698.
- Olton, D.S., Becker, J.T., Hanndelman, G.E., 1979, *Behav.Brain Sci.* 2, 313-365
- Palmer, A.M., Stratmann, M.A., Procter, A.W., Bowen, D.M., 1988, *Ann.Neurol.*, 23, 616-620
- Paxinos, G., Watson C., 1986, *The rat brain in stereotaxic coordinates*. Acad. Press, New York
- Reinikainen, K.J., Soininen, H., Riekkinen, P.J., 1990, *J.Neurosci.Res.* 27, 576-586
- Reinikainen, K.J., Paljarvi, L., Houskonen, M., Soininen, H., Laakso, M., Riekkinen, P.J., 1988, *J.Neurol. Sci.* 84, 101-116
- Richter-Levin, G., Segal, M., 1989, *Brain Res.*, 477, 404-407
- Riekkinen, P.Jr., Sirvio, J., Valjakka, A., Miettinen, R., Riekkinen, P., 1991, *Brain Res.*, 552, 23-26
- Roth, B.L., Hanizavarech S.M., Blum, A.E., 2004, *Psychopharmacol.* 174, 17-24
- Ruotsalainen, S., Miettinen, R., MacDonald, E., Riekkinen M., Sirvio J., 1998, *Neurosci. Biobehav. rev.* 22, 21-31
- Sahgal, A., Keith, A.B., 1993, *Pharmacol. Biochem.Behav.*, 45, 995-1001
- Steckler, T., Sahgal, A., 1995, *Behav.Brain Res.*, 67, 165-199
- Sandyk, R., 1992, *I.J.Neurosci.* 67, 127-144

- Shutoh,F., Hamada, S., Shibata,M., Narita,M., Shiga,T., Azmitia, E., Okado,N., 2000,*Neurosci.Res.* 365-371.
- Temel, Y., Helmy,A., Pinnock,S., Herbert,J.,2003, *Neurosci.Lett.* 139-142
- Wenk, G., Hughey, D., Boundy, V., Kim,A., Walker,L., Olton,D., 1987, *Behav.neurosci.*, 101, 325-332
- Young, A.B., Penney,J.B.Jr. 1994, In: Terry,R.D., Katzman,R., Bick, K.L. (eds), Alzheimer Disease. New York, *Plenum Press*, Chap. 16, 293-303

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CARACTÉRISATION BIOCHIMIQUE DE CERTAINS HYBRIDES DE MAÏS UTILISÉS COMME SUBSTRAT DANS L'OBTENTION DES SPORES DE *PENICILLIUM CHRISOGENUM* : MISE EN ÉVIDENCE DE LA COMPOSITION BIOCHIMIQUE OPTIMALE PRO *PENICILLIUM*

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Mots clef : pénicilline, spore, mycélium, culture submergée, substrat pro *Penicillium*

Résumé : La présente étude a pour but l'identification des différents caractères biochimiques de quelques 23 hybrides de maïs cultivés dans notre pays, ainsi que l'analyse de leur influence sur le comportement du mycélium de *Penicillium chrysogenum* développé en culture submergée à partir de spores poussées sur des caryopses de maïs. Les données accumulées nous ont permis l'établissement d'un tableau biochimique contenant des intervalles de valeurs définissant un substrat optimal pour l'obtention de spores donnant des mycéliums productifs de *Penicillium*.

INTRODUCTION

La biosynthèse de la pénicilline par *Penicillium chrysogenum* [8] représente un processus complexe qui, quoique apparemment très bien connu, nous surprend avec de nouveaux aspects concernant les variables le caractérisant. Ainsi, alors que dans le passé il était considéré comme certain le fait que la biosynthèse de la pénicilline débute à la fin de la période de croissance logarithmique, de nos jours il a été constaté que la vitesse de biosynthèse atteindrait sa valeur maximale pendant la période de croissance maximale du mycélium. Après cette période, la valeur de la vitesse de biosynthèse serait comprise entre 30-50% par rapport aux valeurs enregistrées en phase logarithmique. Ce comportement étudié par nous est également soutenu par les recherches de Brakhage [2,3] concernant les mécanismes intimes de régulation moléculaire de la biosynthèse de la pénicilline. En suivant, tout au long de la végétation, l'évolution de la concentration des ARNm correspondants aux enzymes impliquées dans la biosynthèse de la pénicilline, il a été constaté que la biosynthèse de la aminoadipyl-cystéinyl-valine synthétase (ACVS), enzyme catalysant la première étape de biosynthèse, c'est-à-dire la formation du tripeptide δ (L- α -aminoadipyl)-L-cystéinyl-D-valine, se réalise jusqu'à l'âge de 60 heures. Après ce moment, la biosynthèse du tripeptide reposera uniquement sur les réserves enzymatiques accumulées dans la cellule pendant la période de croissance. Ce comportement démontre le fait que dans la formation des enzymes impliquées dans la biosynthèse de l'antibiotique, un rôle déterminant est détenu par la composition chimique du très jeune mycélium, et implicitement, de la spore à partir de laquelle ce mycélium se développera. À son tour, la composition chimique de la spore est déterminée par l'information génétique du mycélium ayant généré la spore et par la composition chimique du substrat sur lequel ce mycélium s'est développé.

MATÉRIEL ET MÉTHODES

Nous avons utilisé comme matériel de recherche les caryopses de maïs provenues de 23 hybrides de maïs obtenus au Centre de Recherches Agricoles « Podu Iloaiei », ainsi qu'une souche hautement productive de *Penicillium chrysogenum* issue de la collection de microorganismes de la S.C. « Antibiotice » Iași.

Pour l'obtention des spores de *Penicillium chrysogenum* nous avons utilisé comme milieu de culture des caryopses de maïs stériles, humectés.

La vérification de la capacité des spores à produire la biomasse mycélienne en culture submergée s'est réalisée sur un milieu végétatif à base d'extrait de maïs (extrait de maïs 20g/l, sucrose 20g/l, Carbonate de calcium 5g/l, à pH 6,5) [6,7].

Le potentiel biosynthétique du mycélium de *Penicillium chrysogenum* a été vérifié en utilisant un milieu de fermentation, dont la composition a été indiquée par Hersbach [4] (glucose 5g/l, lactose 70g/l, extrait de maïs 50g/l, nitrate d'ammonium 5g/l, sulfate de sodium 1g/l, carbonate de calcium 8g/l, phosphate mono potassique 4g/l, sulfate d'ammonium 5g/l, sulfate de magnésium 0,25g/l, sulfate de zinc 0,04g/l, sulfate de manganèse 0,02 g/l, acide phénylacétique en addition continue et en maintenant la concentration entre 0,2 et 0,6g/l, antimoussant : huile de soja, à pH 7-7,4).

Le dosage de la pénicilline dans les milieux s'est effectué par la méthode HPLC (High Performance Liquid Chromatography) [5].

Les variantes de substrat (hybrides de maïs) testées sont présentées dans le Tableau 1. Comme témoin, il a été utilisé un hybride d'importation.

Tableau 1. Les différents hybrides de maïs utilisés comme substrat

Variante	Hybride de maïs	Variante	Hybride de maïs
V1	Helga	V12	P 226
V2	Merlin	V13	SV 95
V3	Turda	V14	Carpatin
V4	Seorumpic	V15	Presto
V5	Orange de Tg. Frumos	V16	Moldova 425
V6	Orange de Ezăreni	V17	Perlis
V7	Podu Iloaiei	V18	Podu Iloaiei 110
V8	LC 255	V19	Montana
V9	Simona	V20	P 348
V10	Oana	V21	Eva
V11	Cicantîn	V22	Élan
TÉMOIN	-----	V23	P 223

L'établissement de la composition biochimique des caryopses de maïs a comporté la détermination des indicateurs suivants [1] :

- Le contenu en matière sèche par thermostatisation à 105°C ;
- Le contenu en protéines, selon la méthode Kjeldha [A+T] ;
- La quantité de sucres solubles, selon la méthode Fehling ;
- La quantité d'azote aminique, selon la méthode Sorensen ;
- Le niveau du phosphore, par la méthode spectrophotométrique ;
- Le contenu en calcium et en magnésium, par la méthode complexométrique ;
- Le contenu en acides aminés, à l'aide de l'appareil « Aminoacid Analyser T339 Microtechna Praha ».

L'expérience s'est déroulée en deux étapes :

➤ Durant la première étape, les caryopses des hybrides de maïs ont été inoculés avec des spores de *Penicillium chrysogenum* et incubées à 25°C jusqu'à la sporulation du mycélium développé sur ces caryopses. Les spores obtenues ont été inoculées en cultures submergées afin de vérifier le potentiel de biosynthèse du mycélium issu des spores respectives ;

➤ Dans la deuxième étape, il a été établi la composition biochimique des hybrides de maïs.

➤ Des limites de variation ont été préfigurées pour les caractères biochimiques analysés correspondant aux hybrides de maïs possédant des qualités pro *Penicillium*.

RÉSULTATS ET DISCUSSIONS

Les valeurs des variables biochimiques déterminées ont été centralisées dans les tableaux 2 et 3. Les analyses biochimiques ont été effectuées uniquement pour les hybrides ayant favorisé l'obtention de résultats en biosynthèse équivalents ou supérieurs à ceux enregistrés sur le maïs témoin. Les hybrides associés à des résultats non appropriés en fermentation (V5: Orange de Tg. Frumos et V6: Orange de Ezăreni) ont été éliminés.

Dans le tableau 2 on peut observer que les hybrides testés présentent des valeurs proches en ce qui concerne leur contenu en matière sèche, les petites différences enregistrées n'influençant pas la qualité des spores de *Penicillium*.

Le contenu en protéines des hybrides de maïs est différent et, généralement, les meilleurs résultats en biosynthèse ont été associés avec un contenu protéique compris entre 9,5 et 11%. Le contenu en sucres solubles est différent d'un hybride à l'autre et on peut observer que les meilleurs résultats en biosynthèse ont été réalisés avec les spores obtenues sur des caryopses de maïs avec un contenu glucidique situé entre 63 et 75%. En ce qui concerne l'azote, les meilleurs résultats ont été enregistrés avec les hybrides dont les valeurs de l'azote aminique comprises entre 0,042-0,065%. Les macroéléments dosés (phosphore, calcium, magnésium), quoique présentant des valeurs très diverses et différenciant d'une manière intéressante les hybrides étudiés, ne semblent pas influencer directement la qualité des spores de *Penicillium*.

Tableau 2. Les valeurs des variables biochimiques déterminés pour les hybrides de maïs étudiés

Variante	Matière sèche %	Protéines %	Sucres solubles %	Azote aminique %	Phosphore mg/100g	Calcium, Magnésium mg/100g	Pénicilline % /témoin
M	89	13.7	60.8	0.015	25	0.034	100
V1	87	11.66	70	0.034	176	0.070	105
V2	88	9.57	71	0.050	190	0.065	105
V3	87	7.48	63	0.030	198	0.072	103
V4	88	7.61	71	0.054	177.4	0.080	105
V8	89	7.67	72.61	0.034	200.5	0.060	105
V9	89	11.12	60	0.050	222.4	0.069	103
V10	89	10.85	72.04	0.056	197.8	0.069	106
V12	87	9.11	66.82	0.030	174.6	0.065	107
V13	87	14.0	61	0.042	220	0.050	115
V14	87	12.6	60	0.040	205	0.050	116
V15	88	11.66	63.55	0.040	172.1	0.052	120
V16	87	9.8	66.82	0.040	217.8	0.133	132
V17	87	9.76	74.4	0.056	215.1	0.075	128
V18	87	9.56	69.78	0.056	65.6	0	122
V19	91	9.7	72.56	0.056	81.3	0.028	124
V20	88	7.9	74.9	0.042	74.8	0.055	124
V21	88	9.7	65.37	0.042	90.8	0.086	121
V23	88	11.3	69.18	0.065	131.1	0.07	126

Tableau 3. Composition en acides aminés des différentes variantes de maïs (g% * 10⁻³)

V	Asparagine	Thréonine	Sérine	Acide glutamique	Proline	Glycine	Alanine	Valine	Méthionine	Isoleucine	Leucine	Tyrosine	Phénylalanine	Histidine	Lysine	Arginine
M	897	476	696	2738	1122	469	1697	626	264	475	1911	627	1702	714	254	510
V1	680	391	499	1210	1006	234	659	465	321	428	1020	530	1269	645	210	302
V2	560	297	470	1345	1084	198	720	432	308	379	1243	378	1196	587	198	295
V3	699	397	531	1997	1078	210	910	488	337	430	1486	507	1233	600	245	390
V4	550	281	349	1184	269	126	459	333	259	247	774	389	1353	657	177	0
V7	743	389	558	2249	1095	185	1077	527	286	471	1716	564	1359	638	192	250
V8	504	275	394	1493	550	151	623	365	238	297	1050	380	766	570	158	280
V9	602	303	410	1594	688	133	710	455	133	139	1035	578	478	744	117	250
V10	581	299	397	1684	576	135	660	405	169	329	1186	404	958	592	152	200
V11	668	360	518	2005	604	210	793	611	369	490	1525	665	1212	1070	250	320
V12	470	249	363	1250	910	220	530	390	350	316	910	420	710	520	250	260
V13	616	323	485	1885	990	266	825	543	380	390	1420	410	1150	493	250	170
V14	710	365	586	2382	1290	276	1069	550	285	432	1720	626	1290	563	230	380
V15	710	373	548	2020	933	232	874	650	260	395	1514	517	1180	575	244	400
V16	615	358	533	2150	1317	250	934	623	337	433	1636	717	1550	542	226	270
V17	613	334	514	1757	1350	236	742	436	240	210	1135	536	798	500	191	350
V18	528	276	453	1690	988	240	750	490	220	338	1217	528	1040	533	260	360
V19	614	350	540	2017	1130	280	865	488	233	358	1390	545	1060	740	224	320
V20	487	264	383	1364	880	245	621	430	310	310	988	556	880	490	260	280
V21	596	273	453	1577	630	220	710	411	188	350	1159	414	430	460	270	300
V22	429	236	375	1390	610	200	610	353	257	295	1040	490	860	450	240	310
V23	699	366	630	2550	1320	268	1112	620	357	510	1960	630	1580	660	250	310

Dans le tableau 3 est présentée la composition en acides aminés des caryopses de maïs. La variante témoin se caractérise par les valeurs les plus élevées pour la plupart des acides aminés dosés, la méthionine et l'histidine étant les seules exceptions. La variante V23 (l'hybride P223) présente une composition semblable à celle du témoin, mais aussi un contenu élevé en méthionine. Cette différence est accompagnée par un comportement différent du mycélium issu des spores élevées sur les deux variantes de maïs. Ainsi, les spores formées sur les caryopses de l'hybride P223 ont développé en culture submergée un mycélium dont le potentiel biosynthétique est supérieur avec 26% à celui du mycélium développé à partir des spores obtenues sur le témoin. Les variantes qui ont favorisé le potentiel biosynthétique le plus élevé (V16, V17 et V23) se caractérisent par un contenu important en proline, phénylalanine, leucine (V16 et V23), valine et alanine. De façon générale, le meilleur hybride de maïs utilisé comme substrat pour l'obtention des spores de *Penicillium*, c'est-à-dire le V16 (Moldova 425), se caractérise par un contenu élevé ès aminoacides présentés dans le tableau 3.

Parmi toutes les variantes du lot étudié, 8 hybrides de maïs (présentés dans le tableau 4) se remarquent par l'induction de qualités supérieures aux spores de *Penicillium*, qualités concrétisées par des résultats en biosynthèse supérieurs de 20 à 32% à ceux obtenus en utilisant les spores élevées sur le témoin.

Tableau 4. Les 8 hybrides représentant de meilleurs substrats que le témoin

Variante	Nom de l'hybride	Résultat en biosynthèse, en % par rapport au témoin
Témoin	-----	100
V16	Moldova 425	131.54
V17	Perlis	128.46
V23	P 223	125.96
V19	Montana	122.34
V20	P 348	124
V18	Podu Iloaiei 110	122.34
V21	Eva	120.59
V15	Presto	119.96

À cause du fait que parmi les 23 hybrides étudiés 21 ont présenté des qualités supérieures au témoin et que chaque caractère biochimique a présenté une large intervalle de variabilité à l'intérieur de lot testé, il a été difficile de réaliser un tableau biochimique aux intervalles strictes qui puisse définir un substrat optimum pour l'obtention des spores de *Penicillium*. Pour cette raison, nous avons considéré que l'établissement de deux intervalles de valeurs pour chaque caractère biochimique serait plus efficace.

Tableau 5. Intervalles de données biochimiques pour les meilleurs hybrides étudiés

Composé analysé	Intervalle de valeurs	
	Résultats très bons	Résultats bons
Matière sèche %	87.4 – 87.9	86.5 – 90.7
Contenu en protéines %	9.7 – 9.8	7.9 – 11.3
Contenu en sucres %	66.8 – 74.7	63.55 – 74.9
Azote aminique %	0.04 – 0.065	0.040 – 0.065
Soufre	1.19 – 1.22	0.2 – 2.34
Phosphore mg/100g	131.1 – 216.8	65.6 – 216.8
Ca ²⁺ et Mg ²⁺ %	0 – 0.075	0 – 0.133
Asparagine %	0.613 – 0.699	0.429 – 0.71
Thréonine %	0.334 – 0.336	0.236 – 0.366
Sérine %	0.514 – 0.63	0.375 – 0.630
Acide glutamique %	1.757 – 2.55	1.364 – 2.550
Proline %	1.317 – 1.35	0.61 – 1.35
Glycine %	0.236 – 0.268	0.20 – 0.268
Alanine %	0.742 – 1.112	0.610 – 0.934
Valine %	0.436 – 0.62	0.353 – 0.623
Méthionine %	0.240 – 0.357	0.188 – 0.357
Isoleucine %	0.21 – 0.51	0.21 – 0.51
Leucine %	1.135 – 1.96	1.04 – 1.96
Tyrosine %	0.536 – 0.717	0.49 – 0.717
Phénylalanine %	0.798 – 1.58	0.43 – 1.58
Histidine %	0.5 – 0.66	0.45 – 0.74
Lysine %	0.191 – 0.25	0.191 – 0.27
Arginine %	0.27 – 0.35	0.27 – 0.4

Le tableau 5 présente ces deux intervalles de valeurs. Dans la première sont notées les valeurs correspondant aux hybrides sur lesquels les meilleurs résultats ont été obtenus (V16, V17 et V23 avec des résultats de 126-132% par rapport au témoin), alors que dans la deuxième sont présentées les valeurs pour les hybrides ayant donné de bons résultats (V15, V16, V19, V20, V21 avec des résultats entre 120-125% par rapport au témoin).

CONCLUSIONS

Les résultats enregistrés démontrent que de petites variations dans la composition biochimique du substrat sur lequel sont obtenues les spores de *Penicillium* peuvent induire des variations dans la composition biochimique de la spore même et, implicitement, du jeune mycélium développé à partir de cette spore. Ces modifications affectent la capacité de biosynthèse du mycélium, probablement en influençant la synthèse des enzymes impliquées dans la formation de la pénicilline.

Par la connaissance du tableau biochimique optimal pour le substrat utilisé dans l'obtention des spores de *Penicillium*, ainsi que des hybrides de maïs correspondant à ce tableau, d'autres hybrides possédant des qualités pro- *Penicillium* peuvent être créés.

BIBLIOGRAPHIE

1. Artenie, V. și Tănase, E., 1981, *Practicum de biochimie generală*. Centrul de Multiplicare al Universității „Alexandru Ioan Cuza” din Iași.
2. Brakhage, A.A., Sept.1998, Molecular Regulation of β -Lactam Biosynthesis in Filamentous Fungi. *Microbiology and Molecular Biology Reviews*, 62(3), 547-585.

3. Brakhage, A.A., Sprote, P., Al-Abdallah, Q., Gehrke, A., Plattner, H. and Tuncher, A., 2004, Regulation of penicillin biosynthesis in filamentous fungi. *Adv. Biochem. Eng. Biotechnol.*, 88, 45-90.

4. Hersbach, G.J.M., van der Beek, C.P. and van Dijck, P.W.M., 1984, The penicillins : properties, biosynthesis and fermentation. *Biotechnology of industrial antibiotics*. Vandamme EJ editor, New York, Marcel Dekker Inc., p. 45–140.

5. Knox, J.H. and Kauer, B., 1989, *High Performance Liquid Chromatography*. Brown. P.R. and Hartwick, R.A. Eds., Wiley Interscience: New York, Chapter 4.

6. Luengo, J.M., Iriso, J.L., Lopez-Nieto, M.J., 1986, Direct enzymatic synthesis of natural penicillins using phenyl-acetyl-CoA : 6aAPA phenylacetyl transferase of *Penicillium chrysogenum*, minimal and maximal side chain length requirements. *J. Antibiotics*, 39(12), 1754-1759.

7. Revilla, G., Lopez-Nieto, M.J., Luengo, J.M., Martin, J.F., 1984, Carbon catabolite repression of penicillin biosynthesis by *Penicillium chrysogenum*. *J. Antibiotics*, 37(7), 781-789.

8. Tudose, G., 1982, *Genetica microorganismelor*. Editura Didactică si Pedagogică, Bucuresti.

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L'INFLUENCE DES PARTICULARITÉS BIOCHIMIQUES DE CERTAINS HYBRIDES DE MAÏS UTILISÉS COMME SUBSTRAT SUR LA QUALITÉ DES SPORES DE *PENICILLIUM CHRISOGENUM*

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Mots clef : spore, mycélium, caryopses de maïs, biosynthèse de la pénicilline

Résumé : Dans ce travail, nous avons essayé de mettre en évidence la modalité dont le substrat sur lequel sont obtenues les spores utilisées comme inocule peut influencer le taux de biosynthèse du mycélium issu de ces spores en culture submergée (en flacons agités). Nous avons également cherché à déceler la variante optimale de substrat et la mise en évidence des éventuels aspects biochimiques qui pourraient influencer négativement la qualité des spores. Pour des raisons pratiques, on a opté pour des variantes de substrat naturel appartenant à une même espèce végétale (caryopses de 23 hybrides de maïs autochtones).

INTRODUCTION

L'optimisation des processus industriels de biosynthèse de la pénicilline a été orientée spécialement vers la modélisation de la composition du milieu de fermentation et vers l'obtention de mutants au potentiel biosynthétique élevé. Au contraire, la qualité des spores utilisées comme inocule, ainsi que l'espacement temporel de la synthèse des systèmes enzymatiques impliqués dans ce processus ont été insuffisamment traités. Ces deux aspects peuvent influencer décisivement l'évolution d'un système industriel, même avec un fort potentiel de la souche utilisée et un milieu de fermentation optimal.

Généralement, pour l'obtention des spores de *Penicillium* sont utilisés des substrats naturels ou synthétiques, capables d'induire une sporulation abondante. Cependant, la façon dont la composition biochimique de la spore influence les mécanismes intimes de synthèse des enzymes impliquées dans la biosynthèse de la pénicilline au sein du jeune mycélium développé à partir de la spore est très peu connue et dépendante d'une série de variables intrinsèques.

MATÉRIEL ET MÉTHODES

Nous avons utilisé comme matériel de recherche les caryopses de maïs provenues de 23 hybrides de maïs obtenus au Centre de Recherches Agricoles « Podu Iloaiei », ainsi qu'une souche hautement productive de *Penicillium chrysogenum* issue de la collection de microorganismes de la S.C. « Antibiotice » Iași.

Pour l'obtention des spores de *Penicillium chrysogenum* nous avons utilisé comme milieu de culture des caryopses de maïs stériles, humectées.

La vérification de la capacité des spores à produire la biomasse mycélienne en culture submergée s'est réalisée sur un milieu végétatif à base d'extrait de maïs (extrait de maïs 20g/l, sucrose 20g/l, Carbonate de calcium 5g/l, à pH 6,5) [6,7].

Le potentiel biosynthétique du mycélium de *Penicillium chrysogenum* a été vérifié en utilisant un milieu de fermentation, dont la composition a été indiquée par Hersbach [4] (glucose 5g/l, lactose 70g/l, extrait de maïs 50g/l, nitrate d'ammonium 5g/l, sulfate de sodium 1g/l, carbonate de calcium 8g/l, phosphate mono potassique 4g/l, sulfate d'ammonium 5g/l, sulfate de magnésium 0,25g/l, sulfate de zinc 0,04g/l, sulfate de manganèse 0,02 g/l, acide phénylacétique en addition continue et en maintenant la concentration entre 0,2 et 0,6g/l, antimoissant : huile de soja, à pH 7-7,4).

Les variantes de substrat (hybrides de maïs) testées sont présentées dans le Tableau 1. Comme témoin, nous avons utilisé un hybride d'importation.

Tableau 1. Les différents hybrides de maïs utilisés comme substrat

Variante	Hybride de maïs	Variante	Hybride de maïs
V1	Helga	V12	P 226
V2	Merlin	V13	SV 95
V3	Turda	V14	Carpatin
V4	Seorumpic	V15	Presto
V5	Orange de Tg. Frumos	V16	Moldova 425
V6	Orange de Ezăreni	V17	Perlis
V7	Podu Iloaiei	V18	Podu Iloaiei 110
V8	LC 255	V19	Montana
V9	Simona	V20	P 348
V10	Oana	V21	Eva
V11	Cicantini	V22	Élan
TÉMOIN	-----	V23	P 223

Les variables suivies ont été les suivantes :

- L'aspect du mycélium en fin de thermostatisation des flacons contenant les caryopses inoculés avec des spores de *Penicillium chrysogenum* ;

- La biomasse microbienne obtenue en culture végétative, calculée en pourcentage de mycélium humide compacté obtenu par la centrifugation du milieu pendant 15 minutes à 1500 rpm ;

- La vitesse spécifique moyenne de biosynthèse de pénicilline en milieu de fermentation, calculée en fonction de la concentration en pénicilline (Déterminée, quant à elle, par HPLC (High Performance Liquid Chromatography) [5].), par rapport à la quantité de matière sèche et par unité de temps.

Les flacons contenant des caryopses stériles ont été inoculés avec une suspension de spores de *Penicillium chrysogenum* et incubés à 25°C jusqu'à l'obtention des spores pigmentées. En fin de thermostatisation, des observations ont été effectuées sur l'épaisseur de la couche de mycélium développée sur les caryopses, sur le degré de sporulation et celui de pigmentation des spores. Les caryopses enveloppés de mycélium sporulé ont été lavés et les suspensions obtenues ont été diluées à l'eau distillée stérile jusqu'à une densité d'environ 1010 spores/ml. La suspension résultée a servi pour inoculer les flacons contenant du milieu végétatif. Après cette inoculation, les flacons ont été incubés à 25°C sur un agitateur rotatif, pendant 20 heures à 240 rpm. En fin de thermostatisation on a déterminé le pourcentage de mycélium humide compacté. 10 ml de milieu végétatif développé a été centrifugé dans des éprouvettes stériles pendant 15 minutes à 1500 rpm, le surnageant a été enlevé et le mycélium a été redissout dans de l'eau distillée stérile en complétant le volume à 10 ml. 2 ml de ce volume ont servi comme inocule pour le milieu de fermentation. Les flacons ainsi inoculés ont été incubés 25°C sur un agitateur rotatif, pendant 120 heures à 240 rpm. À chaque 24 heures d'intervalle on a déterminé la vitesse spécifique de biosynthèse, en fin du processus la vitesse spécifique moyenne étant calculée pour chaque variante. Afin d'obtenir les résultats les plus précis possibles, chaque hybride de maïs a été vérifié sur 4 cultures pures différentes appartenant à la même souche de *Penicillium chrysogenum*.

RÉSULTATS ET DISCUSSIONS

Dans le Tableau 2 sont centralisées les valeurs des variables suivies, correspondant à chaque hybride de maïs. L'épaisseur du mycélium, le degré de sporulation et le degré de pigmentation des spores ont été appréciés en comparaison avec l'aspect du mycélium développé sur les caryopses témoin. Ainsi, pour le symbole 0 la variable caractérisée a été approximativement identique au témoin, le symbole + signifie que la variante a été plus intense que dans le cas du témoin (mycélium plus épais, degré de sporulation plus intense ou degré de pigmentation plus accentué), alors que – signifie que la variable a été représentée plus faiblement que sur le témoin. La vitesse spécifique moyenne de biosynthèse de pénicilline a été donnée en pourcentages par rapport à la valeur correspondant au témoin.

Tableau 2. Variables suivies pour les différentes variantes par rapport aux valeurs du témoin

Variante	Épaisseur du mycélium	Degré de sporulation	Degré de pigmentation	Mycélium humide compacté (%)	Vitesse spécifique moyenne de biosynthèse (%)
M	0	0	0	100	100
V1	0	0	+	118	107
V2	0	0	0	107	101
V3	+	+	+	118	105
V4	0	0	0	107	105
V5	+	+	+	121	88
V6	+	+	+	121	94
V7	0	0	0	107	109
V8	0	0	-	111	105
V9	0	0	0	111	103
V10	0	0	0	111	106
V11	0	0	0	111	113
V12	0	0	-	111	107
V13	0	0	0	104	115
V14	+	+	+	114	116
V15	0	0	+	125	120
V16	-	-	-	111	132
V17	+	+	0	111	128
V18	-	-	-	118	122
V19	+	+	+	114	124
V20	0	0	-	118	125
V21	-	-	-	118	121
V22	0	0	0	109	100
V23	+	+	-	107	126

On peut observer dans ce tableau que les différentes variantes de substrat végétal appartenant à la même espèce ont induit des différences significatives d'ordre morphologique et physiologique, aussi bien dans la première génération (mycélium développé sur les caryopses) que dans la génération suivante (mycélium développé à partir des spores obtenues sur les caryopses, en culture submergée). Nous allons présenter ces différences plus en détail :

- L'aspect macroscopique du mycélium développé sur les caryopses de maïs

En analysant l'influence du substrat, d'un côté sur la vitesse de croissance de la biomasse en culture de surface, appréciée par l'épaisseur de la couche de mycélium sur la surface des caryopses, et de l'autre côté sur le degré de sporulation, apprécié en fonction de l'aspect plus ou moins velouté, ainsi que par le calcul du nombre de spores dans la suspension obtenue lors du lavage des caryopses, on constate les différences suivantes :

- Les variantes V3, V5, V6, V14, V17, V19 et V23 ont représenté des substrats facilement métabolisables, par conséquent la couche de mycélium développée sur ces caryopses a été plus épaisse et plus intensément sporulée que celle développée sur le témoin ;
- Les variantes V1, V2, V4, V7, V8, V12, V13, V15 et V20 ont favorisé le développement d'une couche de mycélium comparable à celle développée sur le témoin ;
- Sur V16, V18 et V21 la couche de mycélium a été moins épaisse et moins intensément sporulée que sur le témoin.

En ce qui concerne le degré de pigmentation des spores :

- Sur V1, V3, V5, V6, V14, V15 et V19 – pigmentation plus intense que celle des spores obtenues sur le témoin ;
- Sur V2, V4, V7, V8, V10, V11, V13 et V17 – pigmentation approximativement identique avec le témoin ;

- Sur V12, V16, V18, V20, V21, V22 et V23 – pigmentation moins intense que sur le témoin.

- La capacité des spores à produire de la biomasse en culture submergée, appréciée par le pourcentage de mycélium humide compacté, a démontré que :

- Les spores obtenues sur les variantes V3, V5, V6, V14, V15 et V19 ont développé en cultures submergées un pourcentage de mycélium humide compacté supérieur à celui obtenu avec les spores développées sur le témoin ;

- Les spores obtenues avec les autres variantes ont développé un pourcentage de mycélium comparable au témoin.

- En analysant le potentiel de biosynthèse du mycélium développé en culture submergée à partir des spores obtenues sur les caryopses de maïs, apprécié par la vitesse spécifique moyenne de biosynthèse de benzylpénicilline, on constate que :

- Le mycélium développé à partir des spores obtenues sur les variantes V5 et V6 (respectivement les hybrides Orange de Tg. Frumos et Orange de Ezăreni) se caractérise par un potentiel biosynthétique inférieur au témoin ;

- On distingue huit hybrides de maïs (présentés dans le Tableau 3) qui, utilisés comme substrat, ont induit aux spores de *Penicillium chrysogenum* et par conséquent au mycélium développé à partir des spores en culture submergée des qualités pro *Penicillium* supérieures. Dans le cas de ces huit variantes (V15, V16, V17, V18, V19, V20, V21 et V23), la vitesse spécifique moyenne de biosynthèse a été avec 20-30% supérieure à celle enregistrée sur le témoin ;

- Le mycélium issu des spores développées sur les autres variantes de maïs a présenté une vitesse spécifique moyenne de biosynthèse comparable au témoin.

Tableau 3. Les huit variantes donnant des spores aux qualités supérieures au témoin

Variante	Hybride	Résultats en biosynthèse [% par rapport au témoin]
Témoin	-----	100
V16	Moldova 425	132
V17	Perlis	128
V23	P223	126
V19	Montana	122
V20	P348	124
V18	Podu Iloaiei 110	122
V21	Eva	121
V15	Presto	120

Généralement, les hybrides de maïs sur lesquels le mycélium se développe rapidement, sporule abondamment avec des spores intensément pigmentées peuvent être utilisés comme substrat pour *Penicillium* uniquement dans les situations où on cherche l'obtention de biomasse mycélienne ou de spores capables de produire rapidement de la biomasse en cultures submergées. Par contre, ces hybrides ne doivent pas être utilisés comme substrat lorsqu'on cherche l'obtention de spores utilisées dans la biosynthèse de la pénicilline en cultures submergées, car les résultats obtenus seront assez modestes. Lors des applications industrielles qui ont pour but la biosynthèse de la pénicilline, il est conseillé d'éviter les hybrides de maïs pigmentés en rouge (Orange de Tg. Frumos et Orange de Ezăreni) qui, par leur composition biochimique, influencent négativement la composition biochimique de la spore de *Penicillium* et implicitement du mycélium développé

à partir de la spore. Les mécanismes enzymatiques mis en œuvre dans ce mycélium facilitent les processus anaboliques, au détriment de ceux biosynthétiques. Ce comportement se traduit finalement par une biomasse abondante et vigoureuse, mais d'un potentiel biosynthétique bas.

Les meilleurs résultats en biosynthèse ont été obtenus avec la variante 16 (Moldova 425), la couche de mycélium qui s'y est développée étant peu épaisse, faiblement sporulée et faiblement pigmentée. La composition biochimique du hybride Moldova 425 a influencé la composition biochimique de la spore de *Penicillium* et donc du mycélium développé à partir de la spore dans le sens d'une favorisation des systèmes enzymatiques impliqués dans la biosynthèse de la pénicilline.

La présente étude démontre que l'optimisation de la biosynthèse de la pénicilline doit être initiée à partir du substrat sur lequel sont obtenues les spores de *Penicillium*. La composition de la spore, ainsi que les systèmes enzymatiques qui se forment au sein de la spore en germination et du jeune mycélium sont décisives pour la biosynthèse de la pénicilline. Ces observations sont soutenues par les études effectuées sur les mécanismes intimes de régulation de la biosynthèse, qui ont démontré que l'enzyme ACVS (aminoadipyl-cystéinyl-valine synthétase) qui catalyse la première étape de biosynthèse est synthétisée dans la cellule uniquement pendant les 60 premières heures de végétation. Le déroulement de la biosynthèse après cet âge est assuré par les réserves enzymatiques accumulées dans la cellule auparavant.

À quelques exceptions près, les spores obtenues sur les hybrides de maïs sur lesquels le mycélium de *Penicillium* a formé une couche pas très épaisse et pas intensément pigmentée se sont caractérisées par des qualités supérieures, en conférant au mycélium développé en culture submergée un potentiel biosynthétique supérieur au mycélium développé à partir des spores élevées sur les variantes de maïs couvertes de mycélium épais et aux spores intensément pigmentées.

CONCLUSIONS

Généralement, les hybrides de maïs qui, par leur composition, ont favorisé les systèmes métaboliques impliqués dans l'anabolisme mycélien, ce qui a conduit à un mycélium abondant, fortement sporulé et bien pigmenté, ont été associés à des faibles résultats en biosynthèse. Les spores respectives cultivées en submersion ont donné naissance à une biomasse abondante, formée de hyphes vigoureuses, mais avec un faible potentiel de biosynthèse.

Les hybrides de maïs intensément pigmentés (Orange de Tg. Frumos et Orange de Ezăreni) doivent être évités dans les applications industrielles. Ces hybrides, utilisés comme substrat pour *Penicillium*, favorisent l'obtention d'un mycélium de surface épais, fortement sporulé, aux spores intensément pigmentées capables de produire rapidement un mycélium submergé mais ayant un faible potentiel de biosynthèse. Pour les applications industrielles sont recommandées les huit variantes que nous avons caractérisées comme ayant des qualités biosynthétiques supérieures.

Les résultats obtenus démontrent l'importance du substrat sur lequel sont obtenues les spores utilisées comme inocule dans la biosynthèse de la pénicilline en cultures submergées.

Avec l'identification des hybrides de maïs aux propriétés pro *Penicillium*, peuvent être établies les caractéristiques biochimiques optimales du substrat, ainsi que les aspects biochimiques non appropriés.

BIBLIOGRAPHIE

- Artenie, V. și Tănase, E., 1981, *Practicum de biochimie generală*. Centrul de Multiplicare al Universității „Alexandru Ioan Cuza” din Iași.
- Brakhage, A.A., Sept.1998, Molecular Regulation of β -Lactam Biosynthesis in Filamentous Fungi. *Microbiology and Molecular Biology Reviews*, 62(3), 547-585.
- Brakhage, A.A., Sprote, P., Al-Abdallah, Q., Gehrke, A., Plattner, H. and Tuncher, A., 2004, Regulation of penicillin biosynthesis in filamentous fungi. *Adv. Biochem. Eng. Biotechnol.*, 88, 45-90.
- Hersbach, G.J.M., van der Beek, C.P. and van Dijk, P.W.M., 1984, *The penicillins : properties, biosynthesis and fermentation. Biotechnology of industrial antibiotics*. Vandamme EJ editor, New York, Marcel Dekker Inc., p. 45–140.
- Knox, J.H. and Kauer, B., 1989, *High Performance Liquid Chromatography*. Brown. P.R. and Hartwick, R.A. Eds., Wiley Interscience: New York, Chapter 4.
- Luengo, J.M., Iriso, J.L., Lopez-Nieto, M.J., 1986, Direct enzymatic synthesis of natural penicillins using phenyl-acetyl-CoA : 6aAPA phenylacetyl transferase of *Penicillium chrysogenum*, minimal and maximal side chain length requirements. *J. Antibiotics*, 39(12), 1754-1759.
- Revilla, G., Lopez-Nieto, M.J., Luengo, J.M., Martin, J.F., 1984, Carbon catabolite repression of penicillin biosynthesis by *Penicillium chrysogenum*. *J. Antibiotics*, 37(7), 781-789.
- Tudose, G., 1982, *Genetica microorganismelor*. Editura Didactică si Pedagogică, Bucuresti.

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LE TRANSPORT MEMBRANAIRE ACTIF DE L'ACIDE PHÉNYLACÉTIQUE CHEZ *PENICILLIUM CHRISOGENUM* ET SON INFLUENCE SUR LA VITESSE SPÉCIFIQUE DE BIOSYNTHÈSE DE LA PÉNICILLINE

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Mots clef : transport actif, acide phénylacétique, vitesse spécifique de transport, mycélium, pénicilline

Résumé : Le but du présent travail consiste en la mise en évidence d'une corrélation existante entre la cinétique du transport membranaire actif de l'acide phénylacétique et la vitesse de biosynthèse de la benzylpénicilline, ainsi qu'en l'espace temporel du moment optimal d'activation du système de transport de l'acide phénylacétique. Les informations présentées peuvent être particulièrement utiles dans les programmes d'optimisation des processus industriels. Elles permettent d'éviter le gaspillage d'acide phénylacétique, par son administration dans le milieu de fermentation au moment où le système membranaire de transport actif est prêt.

INTRODUCTION

La première étape dans la biosynthèse de la benzylpénicilline consiste en la condensation des acides aminés avec formation du tripeptide δ (L- α -aminoadipyl)-L-cystéinyl-D-valine (ACV). Cette étape est catalysée par la ACV synthétase (ACVS), qui se trouve libre dans le cytosol. Ultérieurement a lieu la transformation oxydative du tripeptide en une structure bicyclique formée d'un noyau β -lactame et d'un noyau thiazolidinique. Le composé qui en résulte se nomme isopénicilline N (IPN) et représente le premier intermédiaire bioactif des voies biosynthétiques spécifiques aux pénicillines et aux céphalosporines [1,4,8]. La réaction de cyclisation est catalysée par la IPN synthétase (IPNS). La dernière étape dans la biosynthèse de la pénicilline consiste en le remplacement de la chaîne latérale hydrophile de l'isopénicilline N, représentée par l'acide L- α -aminoadipique, par le radical de l'acide phénylacétique qui est, lui, hydrophobe. Ce changement est catalysé par la acyl-coenzyme A : isopénicilline N-acyl transférase (IAT). Dans les milieux naturels, avec les pénicillines G et V, sont synthétisées les pénicillines D, F, DF, K, dont les chaînes latérales sont représentées par les radicaux des acides n-héxanoïque, Δ^3 -héxanoïque et n-octanoïque. Étant donné que ces pénicillines ne présentent pas d'importance économique, afin d'orienter la biosynthèse vers la production de pénicilline G, on ajoute dans le milieu de fermentation de l'acide phénylacétique (PA).

Dans la cellule, l'assimilation de l'acide phénylacétique se réalise par diffusion passive à des valeurs de pH comprises entre 3 et 5,5 et par un mécanisme de transport membranaire actif pour des valeurs de pH entre 5 et 8,5. Le transport passif induit un effet toxique, par l'abaissement du gradient de pH transmembranaire et, implicitement, par le blocage des systèmes de transport membranaire. Pour cette raison, lors de la biosynthèse industrielle la diffusion passive de l'acide phénylacétique est évitée par la modulation de la concentration en PA et des valeurs du pH. Le système de transport membranaire actif du PA est activé par l'administration de l'acide phénylacétique dans le milieu de culture. Les tentatives d'activer ce système de transport avec d'autres inducteurs se sont montrées insatisfaisantes.

MATÉRIEL ET MÉTHODES

Nous avons utilisé comme matériel d'étude une souche hautement productive de *Penicillium chrysogenum* issue de la collection de microorganismes de la S.C. « Antibiotice » Iași.

Le milieu végétatif a été formé de : extrait de maïs 20g/l, sucrose 20g/l, carbonate de calcium 5g/l, à pH 6,5) [7].

Le milieu de fermentation pour la vérification du potentiel biosynthétique du mycélium a été préparé d'après Hersbach [5] (glucose 5g/l, lactose 70g/l, extrait de maïs 50g/l, nitrate d'ammonium 5g/l, sulfate de sodium 1g/l, carbonate de calcium 8g/l, phosphate mono potassique 4g/l, sulfate d'ammonium 5g/l, sulfate de magnésium 0,25g/l, sulfate de zinc 0,04g/l, sulfate de manganèse 0,02 g/l, acide phénylacétique en addition continue et en maintenant la concentration entre 0,2 et 0,6g/l, antimoussant : huile de soja, à pH 7-7,4).

75 ml de milieu végétatif ont été inoculés avec 1 ml de suspension de spores de *Penicillium chrysogenum* avec une concentration de 108 spores/ml et incubé pendant 30 heures à 25°C, sur agitateur rotatif à 220 rpm. En fin d'incubation, le mycélium développé dans les flacons contenant du milieu végétatif a été lavé par centrifugation 8 minutes à 2000 rpm dans de l'eau distillée stérile, resuspendu dans de l'eau distillée stérile et utilisé pour l'inoculation des flacons de

fermentation. Chaque flacon de fermentation, contenant 75 ml de milieu, a été inoculé avec 4 ml de suspension de jeunes hyphes avec une concentration de 25% en mycélium humide compacté. Une fois inoculés, ces flacons ont été incubés dans des conditions similaires à ceux de végétatif, pendant 160 heures.

L'expérience s'est déroulée en trois étapes :

En une première étape, nous avons suivi la cinétique de consommation du PA dans un milieu riche en acide phénylacétique, 100 mM. Les études sur la biosynthèse de la pénicilline publiées auparavant [2] ont utilisé une concentration de 6,4 mM en précurseur, concentration entretenue par ajouts fractionnés sur toute la durée du processus. Dans le cas présent, toute la quantité de PA a été administrée dans le milieu avant son inoculation avec la suspension d'hyphes. À chaque 12 heures d'intervalle ont été déterminées les concentrations en benzylpénicilline et en PA par HPLC (High Performance Liquid Chromatography) [6], ainsi que la concentration du mycélium humide compacté par centrifugation 15 minutes à 3500 rpm.

Lors de la deuxième étape, la concentration en acide phénylacétique dans le milieu de fermentation a été maintenue constante, entre 7-10 mM, par ajouts fractionnés. Le système membranaire de transport du PA a été activé, comme dans l'étape précédente, à partir du moment initial de la fermentation, par la présence de l'acide phénylacétique dans le milieu de culture.

En une troisième étape, le système de transport membranaire du PA a été activé à différents âges du mycélium. Ainsi, la première dose d'acide phénylacétique a été administrée dans le système à 12, 24, 36, 48, 60, 72 et 84 heures. Après la première administration, la concentration en acide phénylacétique a été maintenue entre 7-10 mM par ajouts fractionnés.

Durant les trois étapes présentées ci-dessus, la dynamique du transport de l'acide phénylacétique a été suivie jusqu'en fin de fermentation, en déterminant la vitesse spécifique de consommation du PA à 12 heures d'intervalle.

Afin d'éviter l'influence de la composition du milieu sur le système de transport membranaire, la vitesse de transport du PA a été également déterminée en solution tampon phosphate. Dans ce cas, aux âges où a été déterminée la concentration en acide phénylacétique lors de la troisième étape (voir ci-dessus), respectivement à 12 heures d'intervalle, le mycélium a été lavé par centrifugation dans de l'eau distillée stérile et introduit dans une solution tampon phosphate 0,06 M à pH 6,5. Les flacons ainsi préparés ont été préincubés 5 minutes à 25°C, l'acide phénylacétique étant quant à lui ajouté par la suite en concentration de 6,4 mM. Les flacons ont ensuite de nouveau été incubés deux heures sur l'agitateur rotatif et finalement a été déterminée la vitesse spécifique de consommation du PA.

RÉSULTATS ET DISCUSSIONS

La vitesse spécifique de transport membranaire actif du PA représente la quantité d'acide phénylacétique consommé à partir du milieu de culture, rapportée à l'unité de volume (cm^3) de mycélium humide compacté et à l'unité de temps (heure). La formule de calcul est la suivante :

$$v_{\text{PA}} = \Delta [\text{PA}] / m \times \Delta t, \text{ avec :}$$

v_{PA} – vitesse spécifique de transport membranaire actif de l'acide phénylacétique ;

$\Delta [\text{PA}]$ – différence entre les concentrations en acide phénylacétique au début de l'intervalle de temps et à sa fin ($\text{PA}_i - \text{PA}_f$), pour les variantes pour lesquelles l'entière quantité d'acide phénylacétique a été administrée dans le milieu au moment initial

– différence entre la somme de la concentration initiale d'acide phénylacétique du milieu plus la quantité de PA administrée au cours de l'intervalle de temps d'une part, et la concentration finale d'acide phénylacétique d'autre part ($\text{PA}_i + \text{PA}_{\text{ad}} - \text{PA}_f$), pour les variantes pour lesquelles la concentration de PA du milieu a été maintenue par des ajouts ;

m – volume du mycélium humide compacté ;

Δt – intervalle de temps, qui a été de 12 heures pour toutes les déterminations.

Les valeurs obtenues ont été rapportées en pourcentage vis-à-vis de la plus grande valeur, considérée comme étalon et enregistrée dans l'intervalle 84-96 heures par l'échantillon V3a, où l'activation du système de transport a été effectuée à 60 heures.

Dans le Tableau 1 sont présentés les résultats enregistrés dans la première étape de l'expérience, quand toute la quantité de PA a été administrée dans le milieu en début de fermentation. Afin de mettre en évidence l'effet de la concentration élevée en acide phénylacétique sur la biosynthèse de la pénicilline, nous avons présenté dans le même tableau les

valeurs des vitesses spécifiques de biosynthèse (Vp). Vp est représentée en pourcentage par rapport à la valeur étalon (vitesse spécifique de biosynthèse de la pénicilline enregistrée dans l'intervalle 0-24 heures dans les échantillons où la concentration en acide phénylacétique a été maintenue constante entre 7-10 mM par ajouts au cours de la fermentation.

Tableau 1. Vitesse spécifique de transport de l'acide phénylacétique avec toute la quantité administrée au début (vPA) et vitesse spécifique de biosynthèse de la pénicilline (Vp)

Âge (heures)	12	24	36	48	60	84	96	108	120
Vp (% étalon)	70	8	23	28	49	46	40	23	28
vPA (% étalon)	2	4	8	12	18	40	60	52	41

Les données présentées dans le Tableau 1 montrent qu'une concentration élevée en PA dans le milieu de fermentation n'influence pas positivement le transport membranaire actif du précurseur, tout en influençant cependant l'évolution de ce même transport au cours du temps. Ainsi, la vitesse de transport a augmenté plus lentement que dans les échantillons où la concentration en PA n'a pas dépassé 7-10 mM, les valeurs maximales enregistrées étant inférieures à ces échantillons. La concentration élevée en acide phénylacétique a également influencé négativement la vitesse spécifique de biosynthèse de la pénicilline. Les valeurs enregistrées dans les premières 24 heures, quand la vitesse de biosynthèse est la plus élevée, n'ont pas atteint la valeur étalon, étant enregistrées à 70% de cette valeur. Dans tous les cas, on peut observer que, même si le système de transport membranaire a été activé par la présence de l'acide phénylacétique dans le milieu dès le début de la fermentation, la vitesse maximale de transport a été atteinte tardivement, à l'âge de 96 heures.

Au cours de la deuxième étape de l'expérience, nous avons suivi l'évolution de la vitesse spécifique de transport actif du PA, ainsi que l'évolution de la vitesse spécifique de biosynthèse, dans le milieu de fermentation où la concentration en acide phénylacétique a été maintenue constante, entre 7 et 10 mM, par ajouts fractionnés. Les résultats obtenus sont présentés dans le Tableau 2.

Tableau 2. Vitesse spécifique de transport de l'acide phénylacétique (vPA) et vitesse spécifique de biosynthèse (Vp), en pourcentages par rapport à l'étalon, avec la concentration en PA maintenue constante

Âge (heures)	12	24	36	48	60	84	96	108	120
Vp (% étalon)	96	100	61	6	28	39	42	40	31
vPA (% étalon)	8	12	30	54	30	81	68	50	51

Le maintien de la concentration en PA à des valeurs basses a influencé positivement le transport membranaire actif, mais également la vitesse spécifique de biosynthèse de pénicilline. La différence entre les situations apparues dans les deux étapes pourrait être expliquée par la pression qu'une concentration élevée en PA exercerait sur la membrane cellulaire, en actionnant aussi bien sur les systèmes actifs de transport que sur les systèmes entretenant le gradient de pH transmembranaire.

Dans la troisième étape de l'expérience a été suivie la vitesse spécifique de transport membranaire actif du PA par l'activation du système membranaire de transport à différents âges

de la culture (24, 36, 48, 72 et 84 heures). Afin d'éviter l'influence éventuelle de la composition du milieu de fermentation sur la vPA, la vérification s'est effectuée d'un coté dans ce milieu de fermentation (données présentées dans le Tableau 3), et d'un autre coté en milieu tampon phosphate (données présentées dans le Tableau 4).

Tableau 3. Vitesse spécifique de transport actif du PA dans le milieu de fermentation, sur les variantes où l'activation du système de transport s'est effectuée à différents âges de la culture

Variante	T induction PA (heures)	36 heures	48 heures	60 heures	72 heures	84 heures	96 heures	108 heures	120 heures
V1a	24	8	12	30	81	56	20	50	51
V2a	36	----	14	28	50	86	38	51	54
V3a	48	----	-----	30	57	68	92	60	54
V4a	60	----	-----	-----	29	60	100	85	74
V5a	72	----	-----	-----	-----	20	64	95	76
V6a	84	----	-----	-----	-----	-----	34	70	98

Tableau 4. Vitesse spécifique de transport actif du PA dans du tampon phosphate 0,06 M, à pH 6,5, sur les variantes où l'activation du système de transport s'est effectuée à différents âges de la culture

Variante	T induction PA (heures)	36 heures	48 heures	60 heures	72 heures	84 heures	96 heures	108 heures	120 heures
V1	24	10	12	38	50	80	52	63	61
V2	36	----	10	35	62	86	58	64	63
V3	48	----	----	15	31	47	89	60	63
V4	60	----	----	----	20	52	100	70	68
V5	72	----	----	----	----	21	62	98	75
V6	84	----	----	----	----	----	34	68	96

L'activité du système de transport membranaire du PA est dépendante de l'âge du mycélium. Les résultats enregistrés aussi bien en milieu de fermentation qu'en solution tampon phosphate ont démontré que l'assimilation du PA enregistre ses valeurs maximales lorsque le système de transport est activé à 60 heures de végétation. Son activation à des âges plus bas induit un transport membranaire d'une intensité réduite, alors que les valeurs maximales enregistrées dans ces situations sont placées au-delà de 60 heures. L'activation du système de transport après 60 heures d'âge est suivie par l'augmentation rapide de la consommation de PA les valeurs maximales étant atteintes dans ces cas très rapidement.

Dans tous les cas étudiés, lorsque les valeurs maximales de transport du PA sont atteintes on observe par la suite une diminution de ces valeurs, alors que le taux d'accumulation du PA se maintient à des valeurs importantes même si le taux de biosynthèse de la pénicilline G est en diminution. Ce comportement démontre que l'acide phénylacétique n'est pas utilisé dans la cellule uniquement pour la substitution du radical latéral de la molécule d'isopénicilline, mais qu'il est consommé également dans d'autres voies métaboliques. Les études antérieures sur le transport membranaire de l'acide phénylacétique par l'utilisation de PA marqué au ^{14}C , études menées par Susanne Havn Ericksen [3], avait démontré que 17-50% de la quantité de PA assimilé à partir du milieu serait métabolisé au cours de son transport membranaire, comportement spécifique pour la souche Wisconsin. En échange, en utilisant pour la même expérience une souche hautement productive, on constate que la conversion de l'acide phénylacétique assimilé en benzylpénicilline a été quasiment totale. Ces résultats confirment nos conclusions, ainsi que le fait que les données obtenues sont caractéristiques pour la souche utilisée dans notre travail ; leur adaptation à d'autres souches devrait par conséquent être effectuée avec beaucoup de précaution.

CONCLUSIONS

Les concentrations élevées en acide phénylacétique dans le milieu de fermentation n'influencent pas d'une manière significative le comportement de son système membranaire de transport actif, mais influencent négativement le métabolisme de l'organisme à long terme. Par conséquent, même lorsque la concentration en acide phénylacétique dans le milieu a baissé, le taux de biosynthèse a été moindre que dans le cas où la concentration du PA a été maintenue à des valeurs faibles sur toute la durée de l'expérience.

L'activité du système membranaire de transport du PA est dépendante de l'âge du mycélium. L'assimilation (vPA) enregistre les valeurs maximales lorsque le système de transport est activé à 60 heures d'âge, c'est-à-dire en fin de la période de croissance maximale et de biosynthèse maximale de la pénicilline N. Ce comportement pourrait être expliqué par la façon dont la cellule administre ses réserves énergétiques. Ainsi, durant les premières 60 heures ces réserves sont consommées principalement dans des processus impliqués dans la croissance cellulaire et dans la formation des systèmes enzymatiques dont fait partie celui impliqué dans la biosynthèse de la pénicilline.

La composition du milieu de fermentation n'influence pas significativement la cinétique du système de transport actif de l'acide phénylacétique.

La biosynthèse de la benzylpénicilline n'est pas strictement dépendante de la quantité de PA assimilé, mais la présence du précurseur est nécessaire pour la modification du rapport entre les pénicillines synthétisées, et ceci en faveur de la benzylpénicilline.

BIBLIOGRAPHIE

1. Brakhage, A.A., 1998, Molecular Regulation of β -Lactam Biosynthesis in Filamentous Fungi. *Microbiology and Molecular Biology Reviews*, 62(3), 547-585.
2. Demain, A.L., 1983, Biosynthesis of β -lactam antibiotics. *Handbook of Experimental Pharmacology*, 189-228.
3. Ericsen, S.H. and Soderblom, T.B., 1998, Uptake of phenylacetic acid by two strains of *Penicillium chrysogenum*. *Biotechnol.Bioeng.*, 60, 310-316.
4. Fernandez-Canon, J.M., Reglero, A., Martinez-Blanco, H. and Luengo, J.M., 1989, Uptake of phenylacetic acid by *Penicillium chrysogenum* Wis 54-1255: a critical regulatory point in benzylpenicillin biosynthesis. *J Antibiot.*, 42(9), 1398-1409.
5. Hersbach, G.J.M., van der Beek, C.P. and van Dijck, P.W.M., 1984, *The penicillins : properties, biosynthesis and fermentation. Biotechnology of industrial antibiotics*. Vandamme EJ editor, New York, Marcel Dekker Inc., p. 45-140.
6. Knox, J.H. and Kauer, B., 1989, *High Performance Liquid Chromatography*. Brown. P.R. and Hartwick, R.A. Eds., Wiley Interscience: New York, Chapter 4.
7. Luengo, J.M., Iriso, J.L. and Lopez-Nieto, M.J., 1986, Direct enzymatic synthesis of natural penicillins using phenyl-acetyl-CoA : 6aAPA phenylacetyl transferase of *Penicillium chrysogenum*, minimal and maximal side chain length requirements. *J. Antibiotics*, 39(12), 1754-1759.
8. MacCabe, A.P., van Liempt, H., Palissa, H., Unkles, S.E., Riach, M.B., Pfeifer, E., von Dohren, H. and Kinghorn, J.R., 1991, Delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-

valine synthetase from *Aspergillus nidulans*. Molecular characterization of the acvA gene encoding the first enzyme of the penicillin biosynthetic pathway. *J Biol Chem*, 266(19), 12646-12654.

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INFLUENCE DU CHAMP ÉLECTROMAGNÉTIQUE SUR L'ACTIVITÉ DE LA CATALASE ET DE LA PEROXYDASE DANS DES CULTURES MIXTES DE *CHAETOMIUM GLOBOSUM* ET *TRICHODERMA VIRIDE*

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Mots clef : Fungi cellulolytiques, champ électromagnétique, cultures mixtes, activité catalasique et peroxydasique

Résumé : Les auteurs de cette étude présentent les résultats de leurs recherches sur l'influence du champ électromagnétique (uniforme et pulsatoire) sur l'activité de la catalase et de la peroxydase en cultures mixtes de *Chaetomium globosum* et *Trichoderma viride*, à 7 et 14 jours d'ensemencement. Il a été constaté que l'évolution de ces enzymes dépend du type de champ électromagnétique et de l'âge de la culture.

INTRODUCTION

L'importance d'étudier l'influence des champs magnétique électromagnétique sur les organismes s'est concrétisée par l'apparition de nouveaux domaines de la science, le biomagnétisme et le bioélectromagnétisme [2] ; de nombreuses manifestations scientifiques consacrées à ces domaines ont été organisées, dont on peut mentionner les conférences internationales de biomagnétisme, les dernières en date ayant lieu à Santa Fe, au Mexique (1996), à Sendai, au Japon (1998), à Espoo, en Finlande (2000), à Jena, en Allemagne (2002) et à Boston, au Massachusetts (2004). Une place appart occupe le Symposium International de Biomagnétisme organisé par Korea Research Institute of Standards and Science (KRISS) à Daejeon, en Corée, en mai 2002. Un groupe de travail en biomagnétisme a également été créé, auquel participent 550 spécialistes de toute la planète.

En ce qui concerne les recherches sur l'influence du champ magnétique sur le métabolisme des microorganismes, nous pouvons citer les travaux publiés par Miro et coll. En 1967 [8], Grosman et coll. en 1992 [3] ou Kikuo Shimizu et coll. en 200 [4].

L'école de bioélectromagnétisme de Iași, initiée il y a plus de 50 ans par l'académicien Petre Jitariu, est reconnue pour les recherches effectuées, une synthèse récente ayant été publiée par Octăvița Ailiesei [1]. Des études concernant l'influence du champ magnétique sur le métabolisme des microorganismes ont été publiées à Iași entre 1965 et 1978 [9-19].

Les recherches ont été reprises en 2003 à l'Institut de Recherches Biologiques de Iași, dans le cadre de recherches plus complexes sur la biologie des Fungi cellulolytiques, plusieurs publications étant déjà parues [5-7].

Dans le présent travail sont présentés les résultats des recherches concernant l'influence du champ électromagnétique sur l'activité de la catalase et de la peroxydase en cultures mixtes de *Chaetomium globosum* et *Trichoderma viride*.

MATÉRIEL ET MÉTHODES

Les recherches ont été effectuées avec des cultures mixtes de *Chaetomium globosum* et *Trichoderma viride*, cultures sélectionnées dans le cadre des études menées au Département de Microbiologie et faisant partie actuellement de la collection du laboratoire, maintenues dans du milieu Haynes.

Pour effectuer ces études, nous avons utilisé le milieu Czapek-Dox liquide (modifié), qui a été ensemencé avec des disques de 0,8 cm en diamètre d'une culture mixte âgée de 7 jours de *Chaetomium globosum* et *Trichoderma viride*. Ces cultures ont été exposées à l'action d'un champ électromagnétique uniforme et pulsatoire, durant 15 minutes chaque jour. La détermination de l'activité de la catalase et de la peroxydase s'est effectuée à 7 et à 14 jours de l'ensemencement, dans le mycélium ainsi que dans le liquide de culture ; une variante témoin non exposée au champ électromagnétique a également été utilisée.

L'activité de la catalase a été déterminée par la méthode iodométrique avec du thiosulfate de sodium, méthode qui consiste en le dosage de l'eau oxygénée restée non décomposée par l'interruption de l'action de l'enzyme. L'eau oxygénée oxyde l'iodure de potassium en milieu acide avec formation d'une quantité équivalente de iode, déterminée par titrage avec du thiosulfate de sodium.

L'activité de la peroxydase a été déterminée en mesurant l'intensité de coloration du produit d'oxydation de l'ortho-dianisidine par l'eau oxygénée, sous l'action catalytique de l'enzyme.

RÉSULTATS ET DISCUSSIONS

Les données concernant l'influence du champ électromagnétique sur l'activité de la catalase dans le mycélium du champignon sont présentées dans la Figure 1, où on peut observer qu'à 7 jours de l'ensemencement l'activité de cette enzyme se situe à 201,93 UC/g pour la variante témoin, valeur qui baisse pour la variante exposée au champ électromagnétique pulsatoire à 119,34 UC/g et qui augmente pour celle exposée au champ électromagnétique uniforme jusqu'à 209,85 UC/g. À 14 jours de l'ensemencement, l'activité maximale de l'enzyme est également trouvée pour la variante exposée au champ électromagnétique uniforme (243,47 UC/g), alors que cette valeur descend à 214,2 UC/g pour la variante exposée au champ électromagnétique pulsatoire et à 191,91 UC/g pour la variante témoin.

Une analyse dynamique de l'évolution de l'activité de la catalase montre qu'à 14 jours de l'ensemencement, comparativement aux valeurs enregistrées après 7 jours, l'activité a augmenté de 209,85 UC/g à 243,47 UC/g - pour la variante exposée au champ électromagnétique uniforme, de 119,34 UC/g à 191,91 UC/g - pour la variante exposée au champ électromagnétique pulsatoire, et de 201,93 UC/g à 214,2 UC/g - pour la variante témoin.

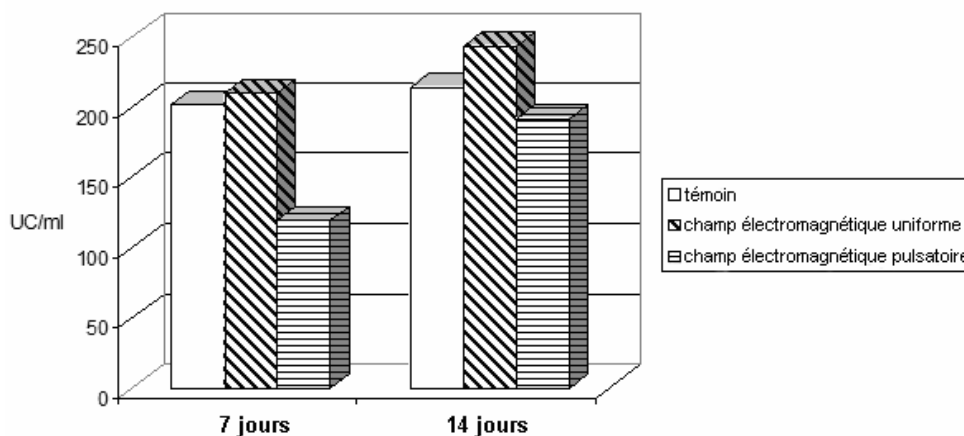


Figure 1. Influence du champ électromagnétique sur l'activité de la catalase dans des cultures mixtes de *Chaetomium globosum* et *Trichoderma viride* – mycélium (UC/g)

Les valeurs de l'activité de la catalase dans le liquide de culture sont présentées dans la Figure 2, où on peut constater qu'à 7 jours de l'ensemencement elles étaient de : V_1 – 130 UC/ml, V_2 – 240 UC/ml, et V_3 – 184 UC/ml. À 14 jours de l'ensemencement, l'activité maximale de l'enzyme a été enregistrée à V_2 – 280 UC/ml, suivie dans l'ordre décroissant par V_1 – 202 UC/ml et V_3 – 172 UC/ml.

En comparant les valeurs de l'activité en fonction de l'âge de la culture, on constate une augmentation pour V_1 – de 130 UC/ml à 202 UC/ml et pour V_2 – de 240 UC/ml à 280 UC/ml, alors que pour V_3 il y a une baisse de 184 UC/ml à 172 UC/ml.

(N.B. Nous avons noté V_1 - la variante témoin, V_2 - la variante exposée au champ électromagnétique uniforme et V_3 - la variante exposée au champ électromagnétique pulsatoire).

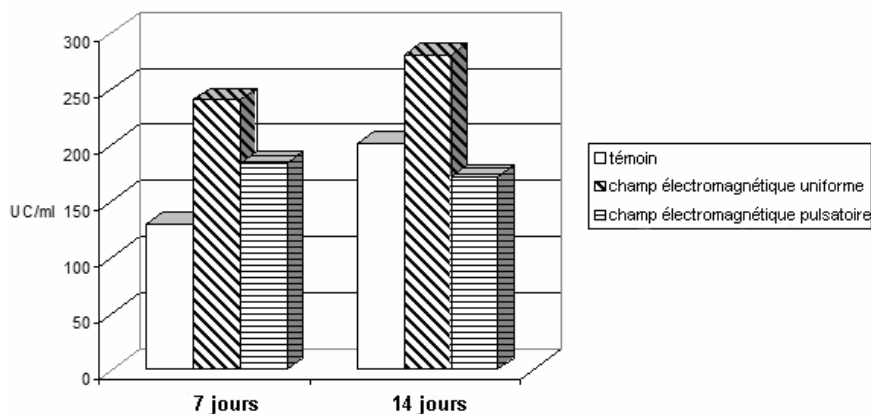


Figure 2. Influence du champ électromagnétique sur l'activité de la catalase dans des cultures mixtes de *Chaetomium globosum* et *Trichoderma viride* – liquide de culture (UC/ml)

Les données concernant l'influence du champ électromagnétique sur l'activité de la peroxydase dans le mycélium du champignon sont présentées dans la Figure 3, où on peut observer qu'à 7 jours de l'ensemencement les valeurs de l'activité ont été les suivantes : $V_1 = 4,0 \text{ UP/g} \times 10^{-3}$, $V_2 = 7,4 \text{ UP/g} \times 10^{-3}$ et $V_3 = 1,5 \text{ UP/g} \times 10^{-3}$, ce que signifie que le champ électromagnétique uniforme a eu un effet stimulateur. À 14 jours de l'ensemencement, la plus grande valeur de l'activité de cette enzyme a été déterminée pour V_3 - champ électromagnétique pulsatoire ($3,04 \text{ UP/g} \times 10^{-3}$), suivie de V_2 - champ électromagnétique uniforme ($1,08 \text{ UP/g} \times 10^{-3}$) et de V_1 – variante témoin ($0,05 \text{ UP/g} \times 10^{-3}$).

La comparaison des dynamiques de l'activité de la peroxydase déterminée dans les deux intervalles de temps montre qu'à 14 jours de l'ensemencement cette activité a augmenté de $1,5 \text{ UP/g} \times 10^{-3}$ à $3,04 \text{ UP/g} \times 10^{-3}$ pour V_3 , alors qu'elle a baissé de $4,0 \text{ UP/g} \times 10^{-3}$ à $0,05 \text{ UP/g} \times 10^{-3}$ pour V_1 et, respectivement, de $7,4 \text{ UP/g} \times 10^{-3}$ à $1,08 \text{ UP/g} \times 10^{-3}$ pour V_2 .

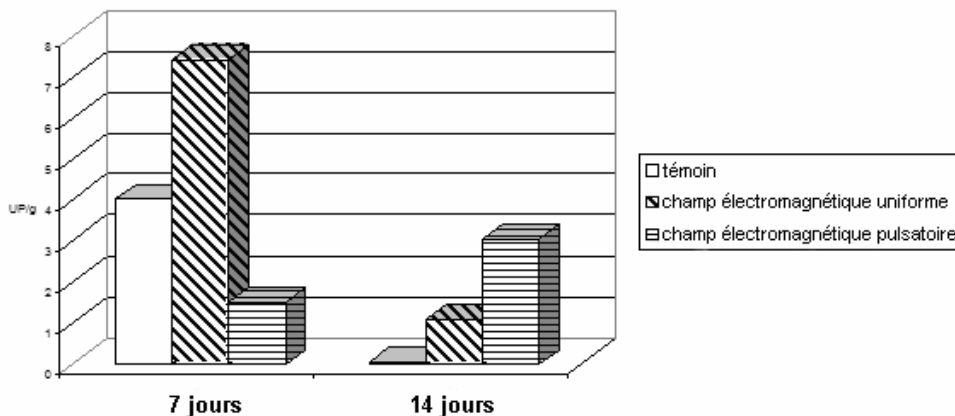


Figure 3. Influence du champ électromagnétique sur l'activité de la peroxydase dans des cultures mixtes de *Chaetomium globosum* et *Trichoderma viride* – mycélium (UP/g x 10⁻³)

Les résultats concernant l'influence du champ électromagnétique sur l'activité de la peroxydase dans le liquide de culture sont présentés dans la Figure 4. À 7 jours de l'ensemencement, la valeur de l'activité a été nulle pour V_1 et V_3 , alors que pour V_2 (champ électromagnétique uniforme) elle a été enregistrée à $0,05 \text{ UP/ml} \times 10^{-3}$. À 14 jours toutes les valeurs ont augmenté, en atteignant $0,89 \text{ UP/ml} \times 10^{-3}$ pour le témoin V_1 , $21,45 \text{ UP/ml} \times 10^{-3}$ pour V_2 et $5,61 \text{ UP/ml} \times 10^{-3}$ pour V_3 .

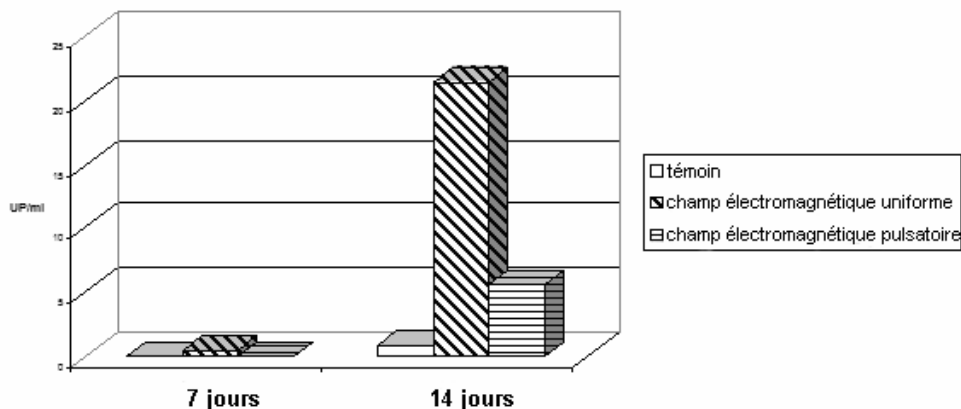


Figure 4. Influence du champ électromagnétique sur l'activité de la peroxydase dans des cultures mixtes de *Chaetomium globosum* et *Trichoderma viride* – liquide de culture (UP/ml)

CONCLUSIONS

L'activité de la catalase dans le mycélium du champignon, ainsi que dans le milieu de culture, a été stimulée par l'exposition au champ électromagnétique uniforme, aussi bien à 7 qu'à 14 jours de l'ensemencement.

L'activité de la peroxydase dans le mycélium du champignon a été stimulée par l'exposition à 7 jours au champ électromagnétique uniforme et à 14 jours par le champ électromagnétique pulsatoire. Dans le liquide de culture, à 7 jours les valeurs de l'activité de la peroxydase ont été très faibles pour les trois variantes étudiées, alors qu'à 14 jours de l'ensemencement cette activité a été stimulée d'une manière significative pour la variante exposée au champ électromagnétique uniforme.

BIBLIOGRAPHIE

1. Ailiesei, O., 1996, *Elemente de magnetobiologie*. Edit. Univ. "Alexandru Ioan Cuza" Iași.
2. Coetzee, H., 1998, *Biomagnetism and Bioelectromagnetism*. The Foundation of Life, Future History Publications, vol. 8.
3. Grosman, Z., Kolar, M. and Tesarikova, E., 1992, Effects of static magnetic field on some microorganisms. *Acta Univ. Polacki Olomuc Fac. Med.*, 134-179.
4. Shimizu, K., Nakaoka, Y. and Yamamoto, T., 2000, Biological effect of non-ionizing radiations on microorganisms. *Bioelectromagnetics Newsletters*, 21(8), 584-588.
5. Manoliu, A., Oprică, L., Olteanu, Z., Creangă, D.E. and Bodale, I., 2003, Static magnetic field influence on dehydrogenase activity in the cellulolytic fungus *Trichoderma viride*. *Analele Științifice, Seria Agronomie*, Univ. Agronomică și Medicină Veterinară, 1(46), 193 – 196.

6. Manoliu, A., Oprică, L., Olteanu, Z., Humă, A., Artenie, V. and Creangă, D.E., 2004, Magnetic field effect on some cellulolytic fungi. *3rd International Workshop on "Biological effects of Electromagnetic fields"*, October 4-8, Kos, Greece.
7. Manoliu, A., Oprică, L., Bodale, I. and Diaconeasa, S., 2004, Influența câmpului electromagnetic asupra dehidrogenazelor ciclului Krebs în culturi mixte de *Chaetomium globosum* și *Trichoderma viride*. *Lucrările celui de-al X-lea Simpozion de Microbiologie și Biotehnologie – Proceedings of the X – th Symposion of the Microbiology and Biotechnology*, Ed. Corson, Iași, sub tipar.
8. Miro, L., Deltour, G., Pfister, A., Donadieu, L. and Royet, J., 1967, Effect of magnetic field variations on the growth of various microorganisms. *J. Physiol.*, 59 (1 Suppl.), 267.
9. Nimițan, E., 1974, Reacția de dehidrogenare la bacterii și levuri sub influența câmpului magnetic. *Analele științifice ale Universității „Al. I. Cuza” Iași*, t. XX, fasc. 1, p. 56– 58.
10. Nimițan, E., 1976, *Influența câmpurilor magnetice asupra microorganismelor*. Rezumatul tezei de doctorat, Universitatea „Al. I. Cuza” Iași.
11. Nimițan, E., 1978, The influence of the magnetic field (MF) on the enzyme activity in microorganisms, *Trav. Mus. Hist. Nat. “Grigore Antipa”*, XIX, p. 107-109.
12. Nimițan, E., 1978, Acțiunea câmpurilor magnetice asupra dezvoltării bacteriilor, *Analele științifice ale Universității „Al. I. Cuza” Iași*, t. XXIV, p. 137 – 138.
13. Nimițan, E. și Topală, N.D., 1972, Influența câmpurilor magnetice asupra activității citrocromoxidazei la *Sacchromyces cerevisiae*. *Analele științifice ale Universității „Al. I. Cuza” Iași*, t. XVIII, fasc. 1, p. 19 – 21.
14. Nimițan, E. și Topală, N.D., 1972, Influența câmpurilor magnetice asupra activității dehidrogenazei la *Sacchromyces cerevisiae*. *Analele științifice ale Universității „Al. I. Cuza” Iași*, t. XVIII, fasc. 2, p. 259 – 264.
15. Nimițan, E. și Topală, N.D., 1973, Influența câmpurilor magnetice asupra activității dehidrogenazei la *Sacchromyces ellipsoideus*. *Analele științifice ale Universității „Al. I. Cuza” Iași*, t. XIX, fasc. 1, p. 62– 66.
16. Nimițan, E. și Topală, N.D., 1977, Influența câmpurilor magnetice asupra sporogenezei și germinării sporului la bacterii. *Analele științifice ale Universității „Al. I. Cuza” Iași*, t. XXIII, p. 122 – 124.
17. Topală, N.D., Ailiesei, O. și Nimițan, E., 1965, Influența câmpurilor magnetice asupra dinamicii dezvoltării la *Staphylococcus aureus* (Oxford). *Analele științifice ale Universității „Al. I. Cuza” Iași*, t. XI, fasc. 2, p. 201 – 209.
18. Topală, N.D., Nimițan, E. și Ailiesei, O., 1966, Influența câmpurilor magnetice asupra respirației la *Staphylococcus aureus*. *Analele științifice ale Universității „Al. I. Cuza” Iași*, t. XII, fasc. 2, p. 277 – 281.
19. Topală, N.D. and Nimițan, E., 1977, The influence of homogeneous magnetic fields on the dehydrogenasic activity of bacteria. *Revue Roumaine de Biologie*, 14(5), 293 – 297.

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INFLUENCE DE LA PROPOLIS SUR LA MITOSE DANS LE MERISTEME DE *SECALE CEREALE* L.

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Mots clef: propolis, influence, mitose, *Secale cereale* L. (2n=14)

Résumé: Cette recherche présente l'influence de propolis sur la mitose dans le meristeme de *Secale cereale* L. (2n=14). On confirme l'utilisation de plus en plus large de ce produit apicole - propolis - au niveau de la thérapeutique.

INTRODUCTION

Propolis, se trouve à l'intérieur de la ruche, sur les parois. Chaque cellule des rayons du miel est enveloppée par une couche élastique et uniforme de propolis.

Les composants majeurs de propolis sont les résines, la cire et les substances flavonoïdes. Le grand contenu en acides aminés libres de la résine confère à la propolis une réaction très acide, dont l'indicateur de l'acidité est plus grand que celui de la cire d'abeilles (1, 3)

La cire a une double origine étant donné la cire végétale - existante dans la sécrétion des bourgeons récoltés par les abeilles et la cire d'abeilles - ajoutée par eux au produit primaire pendant sa production, dans un rapport 1 : 3. À cause de l'origine et composition spécifique, les caractéristiques organoleptiques et physico-chimiques de la cire de propolis diffèrent de celles de la cire d'abeilles (2, 3).

Les substances flavonoïdes représentent 15-20% de la masse de la propolis. À côté des résines, elles représentent les composants les plus précieux du point de vue thérapeutique. Étant donné leurs fonctions biologiques actives très importantes, on les a nommé bioflavonoïdes ; on les retrouve dans un total de 21 (flavones, flavonoles et flavonones) au niveau de la propolis (3).

Les composants mineurs sont les huiles volatiles, enzymes, tanins etc. ; les constituants auxiliaires sont représentés par le pollen et impuretés.

Les huiles volatiles et les balsames existent en quantité modérée aussi que les substances minérales (0,5-1 %) surtout des microéléments (3).

On a identifié aussi de la glucose, fructose et mélezitose, des vitamines liposolubles (A, E) et hydrosolubles (B, PP, C) et des enzymes de la groupe des transhydrogénases.

La plus grande partie des composants chimiques ont leur origine dans le pollen.

Les impuretés cumulent des fragments de tissu végétal afférents aux bourgeons des plantes abordés par les abeilles aussi que des impuretés mécaniques incorporés dans propolis pendant le moissonnage au niveau de la ruche (3).

Ce produit biologique - propolis - réalise des fonctions multiples à l'intérieur de la ruche, peut prévenir l'humidité par l'imperméabilisation des parois de la ruche, arrête le développement des moisissures sur les parois et les cadres.

Avec propolis, les abeilles couvrent les insectes nuisibles - qui ont déjà pénétré la ruche et qui ont été tués - qui ne peuvent pas être évacués, en protégeant la famille des abeilles contre les conséquences de la décomposition par leur putréfaction.

Propolis assure une très bonne antiseptique contre plusieurs bactéries, levures et moisissures, ayant fortes caractéristiques antiseptiques et purifie l'air de l'intérieur de la ruche par les huiles étheriques et les balsames qui s'évaporent.

L'apithérapie, soutenue par un fort support scientifique, réaffirme la liaison avec la nature, avec sa source permanente de vigueur et longévité (4).

MATÉRIEL ET MÉTHODE DE RECHERCHE

Dans cette recherche on a utilisé comme matériel biologique pour le déroulement des analyses cytologiques les plantules de seigle en provenant de caryopses de *Secale cereale* L., variété "Moara domneasca" (2n = 14) germinées sur un substrat avec propolis. Propolis dissoute dans l'alcool a été distribuée sur les papiers de filtre avec surface connue, d'une manière de réaliser les dosages suivants :

- 500,0 mg/dm² ;
- 100,0 mg/dm² ;
- 20,0 mg/dm² ;
- 2,4 mg/dm².

Comme méthode de recherche on a utilisé les testes suivantes : fréquence des cellules dans la division mitotique, distribution par phases, procès aberrational, test de croissance.

Le procès aberrational est résultat de la somme des effets mitoclaiziques et cromatoclaiziques, du rapport par phases et pourcentages vis-à-vis de numéro total des cellules en division sur 5 préparâtes.

Pour le réalisation du examen cytologique on a récolté des échantillons « en action » à 3, 5, 7 jours après la germination et après-ça on a transféré les plantules sur un milieu maquant de propolis.

RÉSULTATS ET DISCUSSIONS

Propolis a été caractérisé comme un produit naturel qui possède une activité mitodépressive qui peut être calculé et considéré « facile » en fonction des dosages et des temps d'action avec lesquels on a conduit la recherche.

Dans le cas des doses modérées une tendance d'accommodation à la présence de la substance après quelques jours d'action s'est bien relevée.

Les moments du cycle cellulaire affectés par propolis sont les préparations pour la mitose mais plus accentués sont ceux afférentes à la métaphase et la despiralisation telophasique des chromosomes. Le plus facile s'adaptent à la présence de la substance les préparations pour la mitose.

Le processus aberrational déterminé par la présence de la propolis se résume à une faible dérégulation de la fonctionnalité du fuseau achromatique qui conduit aux figures modifiées (statométaphases, métaphases avec retardants etc.) sans être affecté l'intégrité des chromosomes.

CONCLUSIONS

À cause du niveau génomique sur lequel se manifeste l'action de propolis comme mutagène le processus aberrational commence à disparaître lentement à l'aide de l'effet réparatoire de l'anaphase.

Le processus de la croissance de la parte aérienne est très fort affecté, surtout pendant l'action, ce qui montre que certains composants chimiques présentant des effets sur la croissance sont solubilises et transloqués à l'intérieur du corps de la plante, arrivant au méréstème apical (aussi que les méréstèmes apicales de la tige) où l'action devienne inhibitoire.

Etant donné le faible effet mitodépressive de la propolis et en supposant une homéostasie physiologique des cellules tumorales diminuée, on considère vraisemblables les remissions de cancer et leucémies.

BIBLIOGRAPHIE

1. Derevici A., Popescu Al., Popescu N., 1965 - Recherches sur certaines propriétés biologiques de propolis. *Rev. De Pathologie Comparée*, T2, 1, 764
2. Derevici A., Popescu Al., Popescu N., 1967 – Ulteriori contributi delle proprieta biologiche del propolis. *Rev. Di Veterinaria*, XVI, 336
3. Popescu N., Meica S., 1995 – *Bazele controlului sanitar veterinar al produselor de origine animală*, Editura Diacon Coresi, București
4. Vasilescu A., Paloș E., Mateescu C., 1990 – *Din tainele stupului : Propolis*, Ed. Apimondia, București

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ADRENALINE EFFECT ON THE ACTIVITY OF CARDIAC CELLS

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Keywords: neurotransmitter, emotion stress, electrocardiogram, computational tests

Abstract. The increased content of adrenaline in emotionally stressed people is known as a major source of heart beat acceleration. Using the electrocardiographic recordings (ECG) the influence of the adrenaline on the electric activity of heart muscle cells has been indirectly investigated. Physiologically normal and emotional loaded voluntary human subjects have been studied. Computationally tests based on the Hurst exponent and the capacity dimension have been used to get numerical data able to make the difference between the two physiological situations. While Hurst's exponent led to similar values in both situations the capacity dimension has provided higher values for correspondingly higher adrenaline content indicating higher complexity in the electric activity of emotional subjects' heart

INTRODUCTION

Among the mostly often invoked neurotransmitters, the adrenaline is known especially due to its deep involvement in the daily stress situations of the human modern society. The study of adrenaline metabolism is based both on direct and indirect investigation methods: molecular, biochemical and genetic methods on a hand – electrophysiological, clinical and behavioral methods on the other hand. In the last decades the computational methods became an appreciated alternative to the experimental data interpretation and even for prediction of future behavior. Since one of the first applications of nonlinear methods to the analysis of emotional stress effect on cardiac muscle cell physiology was reported in the '90 years (Reidbord & Redington, 1992), many other computational studies have been developed since our days. Computational analysis of the ECG signal are related, for instance, to the next aspects:

- the evidence of the changes in heart rate variability during induction of general anesthesia (Sleight, 1999, Pomfrett, 1999);
- the study of the respiratory influences on non-linear dynamics of heart rate variability (Fortrat et al., 1997);
- the theoretical investigation of turbulence (Lin et al., 2001) and non-stationarity in human heart (Bernaola-Galvan et al., 2001);
- the study of heart rate variability in different generations (Yoshikawa and Yasuda, 2003);
- the study of cardiac arrhythmia (Lass, 2002);
- the application of wavelet analysis of heartbeat intervals discriminates healthy patients from cardiac ones (Thurner et al., 1998);

In this paper we present the results obtained in the analysis of two series of electrocardiographic (ECG) signals recorded for health subjects in different emotional situations: relaxed and respectively stress loaded (when the adrenaline is supposed to be delivered in higher amount in the body).

MATERIALS AND METHODS

ECG Data series. Two lots of ten ECG data series each have been recorded in digital version using a portable electrocardiograph convenient for laboratory investigations (10) (Creanga et al., 2000) (signal sampling was done with a frequency of 5000 Hz). Relaxed physiological state was assigned to young healthy students invited as voluntary for the electrocardiographic recording while high adrenaline level was supposed to be generated by emotional stress induced further in similarly subjects by submitted them to a non-announced test of evaluation of their activity. The linear and non linear computational tests have been applied on series of 10.000 data each). Student t-test, pair, two tailed was applied using Ms Excell soft to compare normal and stress loaded cases.

Computational algorithms. (i) The Hurst Exponent (11). One common type of time series arises from a random “walk” within the data series, sometimes called Brownian motion. In such a case, the value of $X(t)$ (the measured temporal parameter in the studied system) on average moves away from its initial position by an amount proportional to the square root of time (the power equal to 1/2), and we say the Hurst exponent is 0.5. The root-mean-square displacement ($DX(t)$) is plotted here versus time, using each point in the time series as an initial condition. The slope of this curve is the Hurst exponent. Exponents greater than 0.5 indicate persistence (past trends persist into the future), whereas exponents less than 0.5 indicate antipersistence (past trends tend to reverse in the future). Thus, when the analyzed data present an appearance of randomness one might integrate it and search if the exponent is close to 0.5, which would imply that it is random and uncorrelated (higher complexity or chaotic trend in the studied system). Exponents higher than 0.5 are indicating correlated data (higher linearity in the studied system).

(ii) **The capacity Dimension.** Similar to the Hausdorff dimension (11), the capacity dimension is calculated by successively dividing the phase space (a hyperspace which can be recovered from a unique measured temporal parameter) with embedding dimension D (the embedding dimension being a measure of the observation scale used to study the system) into equal hypercubes and plotting the log of the fraction of hypercubes that are occupied with data

points versus the log of the normalized linear dimension of the hypercubes. The average slope of the line for the two middle segments is taken as the capacity dimension. As the embedding dimension is increased, the capacity dimension should increase but eventually saturate at the correct value. Many data points are required to get an accurate estimate of the capacity dimension if the dimension is high. A dimension greater than about five implies essentially random data while smaller values are indicating the presence of high complexity degree (chaotic trends). In the following we present the results of the calculation of the Hurst exponent and of the capacity dimension in the two lots (of ten data series each) of ECG recordings.

RESULTS AND DISCUSSIONS

In figure 1 a, b the raw data recorded in normal and respectively nervous subjects are given. Differences at the level of the ECG amplitudes and durations can be seen in the case of emotional stressed subject, probably due, mainly, to the muscle cell increased excitability under the influence of higher level of adrenaline (the time duration between two consecutive ECG signals appeared significantly shorter for stressed subjects). In previous published article (Creanga, 2004) it was shown that the power spectrum of the ECG signal exhibits significant flat regions for medium and high frequencies for both normal and emotionally stressed subjects. This could suggest either randomic (noisy) or chaotic trend overlapped onto the quasi-period dominant dynamics (the linear behavioral trend). The Hurst exponent is able to show if the flat power spectrum corresponding to the analyzed signal is related to a randomic or to a chaotic system. In the present study the application of the Hurst exponent algorithm revealed in both situations significant high values (over 0.99) excluding the randomic fluctuation presumption (fig. 2 a, b). So, the deterministic (or liner) trend (quasi-periodic component of the ECG signal) is strongly dominating the heart dynamics in both analyzed situations. In figure 3 a, b the capacity dimension is represented for the raw data series. As mentioned above, the result of this computational test is able to provide information upon the fractal dimension of the system attractor. As reported in other paper (Neacsu et al., 2005, General Physiology and Biophysics, Bratislava, in press) the ECG data series recorded in adrenaline loaded subjects are characterized by rather similar attractors (the totality of the points representing the possible equilibrium states in the system evolution) with non-integer correlation dimension values (the correlation dimension being an alternate way of expressing the fractal dimension of the system attractor). No saturation tendency was noticed in the two groups of subjects suggesting the significant fluctuation role (either from the recording noise or from intrinsic causes).

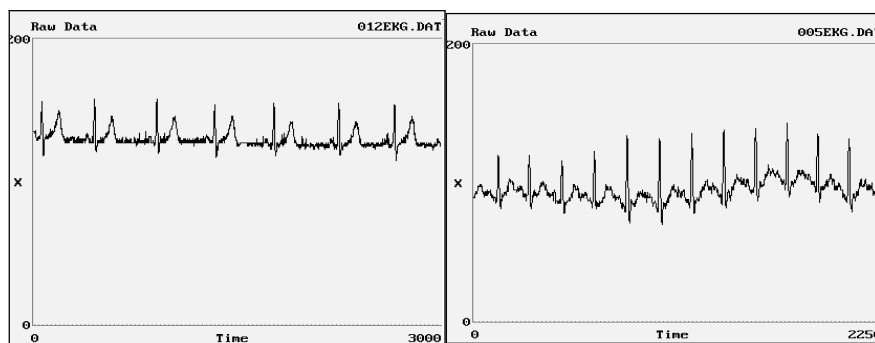


Figure 1 a-b. Normal ECG (a-left) and adrenaline loaded ECG (b-right)

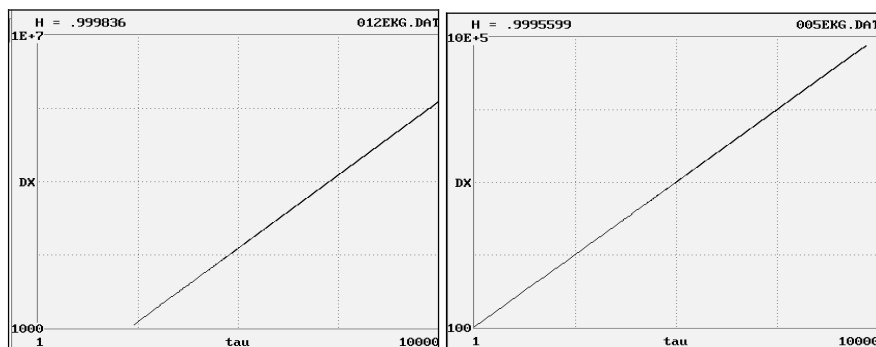


Figure 2 a-b. Hurst exponent versus time in normal (a-left) and adrenaline loaded subjects (b-right)

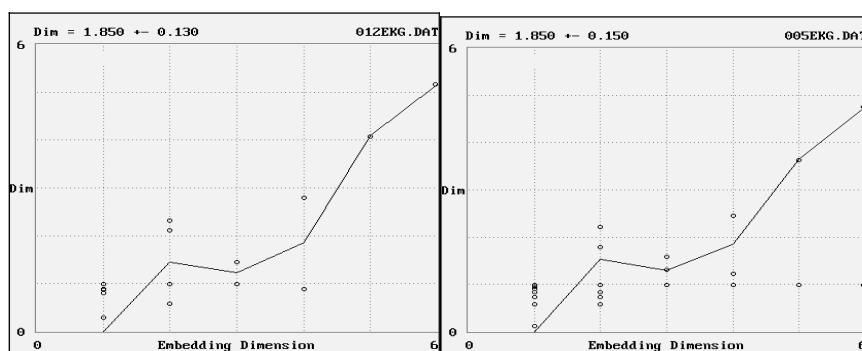


Figure 3 a-b. The capacity dimension versus embedding dimension in normal (a-left) and increased adrenaline subject (b-right)

Considering the limited precision of the computational test, the smoothed data have been also analyzed (every point is replaced by the averaged value between the point and its two closer neighbors). In figure 4 a, b the results obtained for ECG smoothed data series are represented. The differences between the two discussed situations are obvious now. The capacity dimension tends to saturate to higher value (1.652 in comparison to 1.112) in the case of higher adrenaline level (fig. 4 b) suggesting increased complexity degree in this group of subjects.

Averaged values for every ten individual values obtained in the physiological normal group and the high adrenaline one presented standard deviation of 8.56%. The application of the t-test revealed significant differences between the capacity dimension values of the tow lots of subjects accordingly to the significance level of 0.05. It seems that numerical smoothing was able to reveal the higher weight of the chaotic component within the adrenaline loaded subjects.

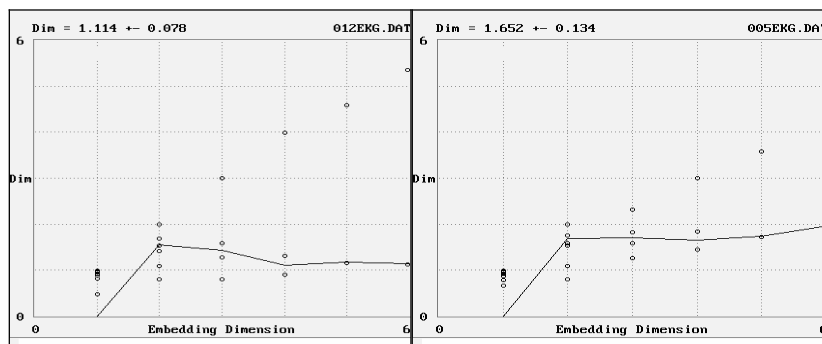


Figure 4 a-b. The capacity dimension versus embedding dimension for the smoothed data corresponding to normal (a-left) and adrenaline loaded subjects (b-right)

CONCLUSIONS

The stress hormone – the adrenaline, that is supposed to be significantly increased in the emotionally stressed subject group seems to be able to induce significant changes in the ECG data series. The capacity dimension was suitable for the evidence of the differences between physiological normal and stressed people.

REFERENCES

1. Reidbord, S.P. & Redington, D. J., 1992. *J. Nerv. Ment. Dis.* 180, 649-657
2. Sleight, J.W. & Donovan, J., 1999. *Br. J. Anaesth.*, 82, 666-671
3. Pomfrett, C.J.D., 1999. *Br. J. Anaesth.*, 82, 559-661
4. Fortrat, J.O., Yamamoto & Y., Hughson, R. L., 1997. *Biol. Cybern.* 77, 1-10
5. Lin, D. C. & Hughson, R. L., 2001. *Phys. Rev. Lett.*, 86, 1650-1653
6. Bernaola-Galvan, P., Ivanov, P., Amaral L., & Stanley, H. 2001. *Phys. Rev. Lett.*, 87, 168105-168109
7. Yoshikawa, Y. & Yasuda, Y., 2003. *Bulletin of Toyohashi Sozo College*, 7, 6378
8. Lass, J., 2002. PhD theses, TTU press
9. Thurner, S. Feurstein, M.C. & Teich, M.C., 1998. *Phys. Rev. Lett.*, 80 1544-1547
10. Creanga, D.E., Ursu, D., Gheorghiu, M. & Radu, C., 2000. *2nd European Symposium in Biomedical Engineering and Medical Physics*, Patras Grecia, Abstract book BME 18
11. Sprott, J. & Rowlands, G., 1994. *Chaos Data Analyzer*, American Institute of Physics, New York, USA
12. Creanga, D.E., In Dobrescu, R. & Vasilescu, C., Eds., *Interdisciplinary application of fractal and chaos theory*, Ed. Acad. Rom. 2004 Bucuresti, 274-287
13. Neacsu, I., Creanga, D. E. & Tufescu, Fl. M., 2005. *General Physiology and Biophysics*, in press

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THE EFFECT OF MAGNETIC LIQUIDS IN SOME TREE SEEDLINGS

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Keywords: chlorophyll, nucleic acids, ferrofluid, oak, poplar

Abstract: The seedlings of two tree species, the black poplar hybrid (*Populus canadensis* Moench.) and the pedunculate oak (*Quercus robur* L.), among the most important for the temperate region, were treated with various concentrations of oil-ferrofluid based on natural hydrocarbons. The experiment has revealed the ferrofluid influence on the assimilatory pigments as well as on the nucleic acids (spectral measurements) in young plantlets aged of 3 months. It was found that the levels of assimilatory pigments are generally diminished though the ratio chlorophyll a/chlorophyll b is generally enhanced for ferrofluid samples suggesting the seedlings sensitivity to the chemical and magnetic stimuli consistent with the ferrofluid addition. The LHC II system (Light Harvesting Complex II) sensitivity to external factors might be associated with the ferrofluid influence on the young seedlings photosynthesis.

INTRODUCTION

Since tree species from the Earth temperate regions are more and more threatened by pollution and atmosphere adversity in the last decades, this study was designed to reveal the influence of small aliquots of iron and hydrocarbons on the young seedlings of oak and black poplar hybrids. Beside its relative role of local pollutant, iron is a major constituent of soils with iron contents between 0.5 and 5 percent, depending on parent rocks and soil type. Despite its general abundance, iron concentrations in aerobic soil solutions are usually very low (due to the low solubility of iron oxides, oxyhydroxides, and hydroxides that limit dissolved iron concentrations, particularly in neutral or alkaline soils), so that the artificial increase of its concentration may represent a source of toxicity. It was shown that an efficient mechanism of iron acquisition by microorganisms and graminaceous plants involves the release of iron complexing compounds, called siderophores. Plants may benefit from the presence of some growth stimulatory bacteria since these microorganisms can produce iron complex combinations (under iron-limited conditions) called siderophores - that can be internalized by plant root cells. (1). The ability of the fungus *Rhizopus arrhizus* to produce chelators such as the so called siderophore rhizoferrin was searched by Yehuda et al. (2) who focused on the mechanisms by which some graminaceous species utilize iron from phytosiderophores. Sherker et al. (3) studied the plant-produced chelators called phytosiderophores that are excreted directly to the rhizosphere. Iron uptake by barley and corn plants grown in nutrient solution was found to run parallel to the diurnal rhythms of phytosiderophore releasing via an indirect mechanism of ligand exchange between the ferrated microbial siderophore and phytosiderophores, which are then taken up by the plant. In the experiment presented inhere, the controlled supply with hydrocarbon ferrofluid was chosen to study its effects upon the young seedlings.

MATERIALS AND METHODS

Since tree species from the Earth temperate regions are more and more threatened by pollution and atmosphere adversity in the last decades, this study was designed to reveal the influence of small aliquots of iron and hydrocarbons on the young seedlings of oak and black poplar hybrids. Beside its relative role of local pollutant, iron is a major constituent of soils with iron contents between 0.5 and 5 percent, depending on parent rocks and soil type. Despite its general abundance, iron concentrations in aerobic soil solutions are usually very low (due to the low solubility of iron oxides, oxyhydroxides, and hydroxides that limit dissolved iron concentrations, particularly in neutral or alkaline soils), so that the artificial increase of its concentration may represent a source of toxicity. It was shown that an efficient mechanism of iron acquisition by microorganisms and graminaceous plants involves the release of iron complexing compounds, called siderophores. Plants may benefit from the presence of some growth stimulatory bacteria since these microorganisms can produce iron complex combinations (under iron-limited conditions) called siderophores - that can be internalized by plant root cells. (1). The ability of the fungus *Rhizopus arrhizus* to produce chelators such as the so called siderophore rhizoferrin was searched by Yehuda et al. (2) who focused on the mechanisms by which some graminaceous species utilize iron from phytosiderophores. Sherker et al. (3) studied the plant-produced chelators called phytosiderophores that are excreted directly to the rhizosphere. Iron uptake by barley and corn plants grown in nutrient solution was found to run parallel to the diurnal rhythms of phytosiderophore releasing via an indirect mechanism of ligand exchange between the ferrated microbial siderophore and phytosiderophores, which are then taken up by the plant. In the experiment presented inhere, the controlled supply with hydrocarbon ferrofluid was chosen to study its effects upon the young seedlings.

RESULTS AND DISSCUSIONS

The average values of the chlorophyll a and b contents from the leaves of the pedunculate oak seedlings (*Quercus robur* L.) treated with ferrofluid aliquots are given in figure 1. One can see that, mainly, an inhibitory effect was recorded, except the sample treated with 20 microl/l, where the content of chlorophyll a is slightly increased in comparison to the control. The same thing is valuable also for the content of total carotenoid pigments (fig. 2).

As in the case of chlorophyll a, the concentration of 60 microl/l led to the most considerable diminution (about 40%) in comparison to the control. However, the biochemical parameter that is the most important for the plant physiology is the ratio chlorophyll a/b and this one is enhanced for the most ferrofluid concentrations tested in this experiment (fig. 3). So, in spite of the absolute values of chlorophylls and carotenes contents, the relative value of the main assimilatory pigment (chlorophyll a) to the chlorophyll b (a secondary pigment) indicates a positive influence of the ferrofluid upon the young seedlings of pedunculate oak.

In the study of young saplings of black poplar hybride (*Populus canadensis* Moench.) the

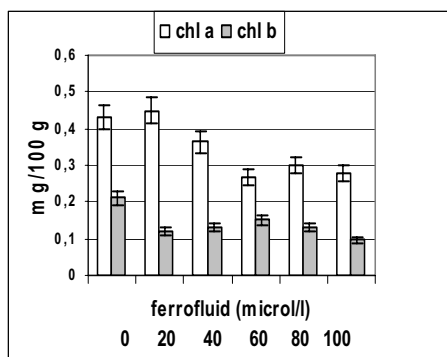


Fig. 1. The contents of chlorophyll a and chlorophyll b in oak seedlings

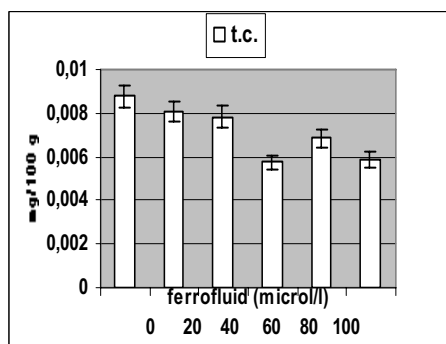


Fig. 2. Total carotenoid pigments in oak seedlings

results obtained for the spectrophotometric assay of photosynthetic pigments are presented in figures 4-5.

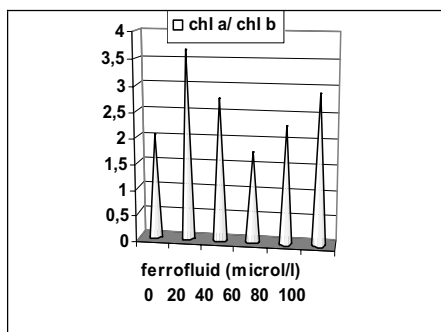


Fig. 3. The ratio chlorophyll a/chlorophyll b in oak seedlings

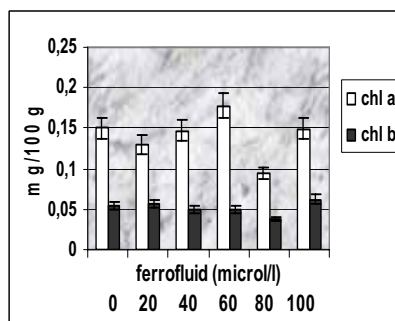


Fig. 4. The contents of chlorophyll a and b in black poplar hybrid

The absolute values of all three assimilatory pigments are lower (about twice) than for the other tree species presented above. The absolute values of chlorophyll a, chlorophyll b and carotenoid pigments are smaller or equal to that of the control sample except one ferrofluid concentration – in the case of the black poplar the concentration of 60 microl/l (figures 4-5)

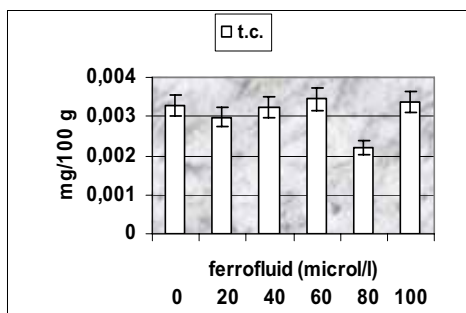


Fig. 5. The total carotenoid pigments in the black poplar hybrid

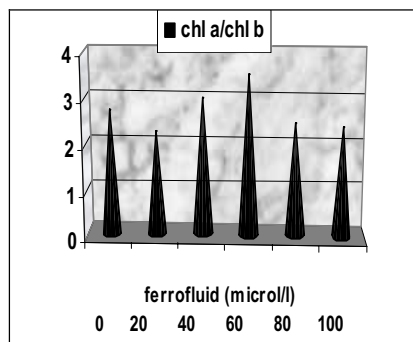


Fig. 6. The ratio chlorophyll a/b in the black poplar hybrid

The chlorophylls ratio (chlorophyll a/b) suggests a stimulatory effect for the concentrations of 40 and 60 microl/l (fig. 6). Considering that the ratio chlorophyll a/b is the most significant biological parameter that reflects the physiological response of the photosynthetic systems I and II, the authors might underline the stimulatory effect of small concentrations of ferrofluid in both tree species (though no distinct correlation between the ferrofluid concentration and the ratio chlorophyll a/b was emphasized). One might assume that the iron oxide supply could have a certain effect due to the iron itself as well as due to the magnetic properties of the iron oxide particles within the ferrofluid since most of them have dimensions comparable to the magnetization domains. The sensitivity of other young tree seedlings (black locust) to external factors of magnetic nature (microwaves) was discussed also in (4), where the putative damage of chlorophyll biosynthesis is assumed too. The situation of carotenoid pigments in oak seedlings has emphasized the inhibitory ferrofluid effect on the biosynthesis of the secondary assimilatory pigments. In the case of poplar seedlings the carotenoid pigment content was not significant modified by ferrofluid addition. Corroborating this issue with the smaller modification of the ratio chlorophyll a/b in poplar seedlings in comparison to the oak ones the authors assume the lower sensitivity of young poplar plants to ferrofluid administration in comparison to that of oaks of the same age. In fig.7 the comparative situation of the nucleic acid contents is presented for the two studied species. There is a significant inhibitory effect in the case of poplar seedlings suggesting that possible negative influences on the plant growth are expected, while for oaks no significant modifications have been noticed.

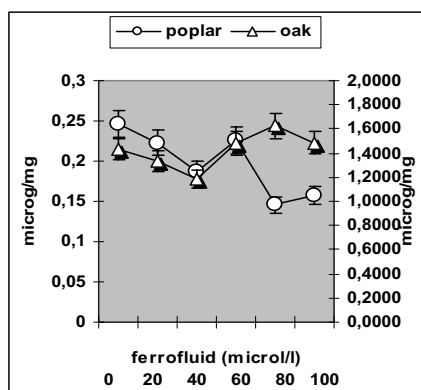


Fig. 7. Nucleic acid content in oak and poplar

CONCLUSIONS

We might say that ferrofluid supply represented a source of iron and, possibly, magnetic energy that influenced in different ways the young plantlets of the two tree species investigated inhere. The biosynthesis of chlorophyll is considerably stimulated in oaks (where the biosynthesis of nucleic acids is non-significantly affected) while in poplars the chlorophyll biosynthesis is only slightly stimulated and the nucleic acids content is diminished. Further investigations, deeper and more specific, are intended for the next stages of this research

REFERENCES

- (1) De Weger, L. A., Van Arendonk, J. J., Recourt, K., Van der Hofstad, G. A., Weisbeek, P. J., & Luhtenberg, B., 1988. *J. Bacteriol.* 170 (10), 4693–4698
- (2) Yehuda, Z., Shenker, M., Romheld, V., Marschner, R., Hadar, Y. & Chen Y., 2001. *Plant Physiology*, 112 (3) 1273-1280
- (3) Shenker, M., Fan, T. W. M. & Crowley, D.E., 2001. *Journal of Environmental Quality*, 30, 2091-2098
- (4) Sandu, D.D., Goiceanu, C., Creanga, I., Ispas, A., Miclaus, S. & Creanga, D.E., 2005. *Acta Biologica Hungarica*, 56 (1-2), 109-117.

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EFFECTS OF AN WEAK ELECTROMAGNETIC FIELD UPON RAT BLOOD PARAMETERS

ION V. NEACSU^{1*}, CALIN L. MANIU¹, LUCIAN G. HRITCU¹

Key words: electromagnetic field, haematy, leucocytes, erythrocyte parameters.

Abstract: Wistar rats were treated 5, 10 and 20 days with an constant electromagnetic field (EMF, 100Hz) 10 min. daily. It were determined: number of haematies and total leucocytes, haematocrit, hemoglobin, mean erythrocyte volume (MEV) and hemoglobin erythrocyte mean content (HEMC). It was realized a comparison between the results of treated and control lots. EMF induced an increase of haematies number, of haematocrit and hemoglobin correlated with an increase of HEMC and decrease of MEV. The total leucocytes number fluctuated: decrease at 5 and 20 days and increase at 10 days of treatment.

INTRODUCTION

The electromagnetic radiations is an environment factor with important impact on leaving beings especially in the last decades along with advanced technologies development.

En earlier series of researches had evidenced different biological effects of EMF, especially the high frequency and intensity (Zamfirescu et all. 2000). It were evidenced, at same the time, some specific effects of weak EMF upon animal (Jitariu, 1987) and vegetal organisms (Gheorghita, 1987; Corneanu, 1989) as well as microorganisms (Ailiesei, 1996).

In some earlier works we observed evident effects of weak EMF upon ion dynamics (Maniu and Neacsu, 2004) and enzymes activity (Maniu et all, 2004) from rat blood. In present paper we follow, in the same conditions, the EMF action on sanguine cells dynamics according to the treatment duration.

MATERIAL AND METHODS

Experiments were performed on six lots of Wistar rats: three control lots (untreated) and other three, treated with EMF (100Hz) generated by an Magnetodiaflux device. EMF was applied constantly 10 min. daily, 5, 10 and 20 days. The control lots were manipulated similar to the treated ones. After the each period, the animals were killed and the blood collected with anticoagulant. It was determined the number of haematies and total leucocytes by hemocytometric method, haematocrit by micro haematocrit techniques, hemoglobin by Sahli method, MEV and HEMC by specific mathematical relations (Neacsu and Cimpeanu, 1999). The obtained data were statistically appreciated (R. A. Fisher, 1925, J. Fowler, L. Cohen, P. Jarvis, 2000).

RESULTS AND DISCUSSIONS

The sanguine parameters of untreated animals (control lot), present a series of modifications – increasing and decreasing of the values depending on experimental duration, determined especially animal manipulation. For take off these modifications were established control lots for each experimental phase (5, 10 and 20 days).

At treated lot 5 days (fig.1 6) it was recorded in comparison with control low values of haematies number (97.56%), total leucocytes (83.65%), haematocrit (92.88%) and MEV (94.47%), and also increase values of hemoglobin (111.16%), correlated with a HEMC augmentation (114.42%).

After 10 days of treatment were obtained insignificant modifications of the values (fig.1 6): a decrease of haematies (97.44%), haematocrit (92.40%) and MEV (94.66%) and an increase of hemoglobin (101.36%), HEMC (104.45%) and total leucocytes (103.49%).

At 20 days, the treatment induce some different modification (fig.1 6): a increased values of haematies (125.33%), haematocrit (119.38%) and hemoglobin (104.29%) and decreased values of total leucocytes (83.96%), MEV (95.14%) and HEMC (82.29%).

The obtained data highlights a series of characteristic effects of EMF action on rat sanguine parameters depending on parameters type and treatment duration. Thus, EMF had a different influence on haematies and leucocytes dynamic number. A such an effect can be determined by a selective action of EMF on haematopoietic processes and the cell maturation rhythm and on sanguine reservoir. Same selective action of EMF was observed on other sanguine components: ions, proteins, leukocytary formula (Jitariu, 1987, Maniu and Neacsu, 2004).

During the experiment, haematocrit modifications (fig.3) follow the haematies number fluctuations (fig.1). Hemoglobin (g/%) record an increase values (fig.4) according to increasing of HEMC values (fig.6), indicating a better level of respiratory performances of haematies as a result of EMF action. The increasing of hemoglobin values (g/%) takes places especially by an increase of HEMC and less by an increase of haematies number. This effect assume a hemoglobin synthesis stimulation by EMF.

MEV recorded low values, possible owing to an effect of EMF on membrane permeability with a lost of the cellular water and a cellular volume reducing. This effect is accompanied by the HEMC increase with preserve of cellular respiratory capacity.

CONCLUSIONS

EMF influence determines number modifications of haematies and leucocytes as well as the erythrocytary parameters. Number of haematies record a decrease after 5 and 10 days, followed by an increase at 20 days. Leukocyte dynamics is inconstant: a decrease at 5 and 20 days and an increase at 10 days. The haematocrit, MEV and HEMC record specific modification under EMF.

REFERENCES

- Ailiesei O., 1996, *Elemente de magnetobiologie*, Ed. Univ. “Alexandru Ioan Cuza” Iași,
Corneanu G. (sub red.), 1989, *Elemente de radiobiologie vegetală*, Ed. Ceres, București.
Fisher R. A., 1925, *Statistical Method for Research Workers*, Ed. Oliver and Boyd, London.
Fowler J., Cohen L., Jarvis P., 2000, *Practical Statistics for Field Biology*, Second Edition, Ed. John Wiley & Sons, New York.
Gheorghita G. (sub red.), 1987, *Radiobiologie vegetală*, Ed. Academiei, R. S. Romania, București.
Jitariu P. (coord.), 1987, *Acțiunea câmpurilor magnetice și electromagnetice asupra organismelor animale*, Ed. Academiei, R.S. Romania, București.
Maniu C., Neacșu I., Ungureanu E., Hrițcu L., Tiron A., 2004, *Sci. Ann. Univ. Agr. Sci. Vet. Med. „Ion Ionescu de la Brad”*, Iași, Tom XLVII (in press.)
Maniu C., Neacsu I., 2004, *Anal. St. Univ. „Alexandru Ioan Cuza” Iași – Genet. și Biol. Molec.*, Tome V, 302 305.
Neacșu I., Cîmpeanu C.S., 1999, *Biologie celulară – lucrări practice*, Ed. Univ., „Alexandru Ioan Cuza”, Iași.
Zamfirescu M., Sajin Gh., Rusu I., Sajin Maria, Kovacs Eugenia, 2000, *Efecte biologice ale radiațiilor electromagnetice de radiofrecvență și microunde*, Ed. Medicală, București.

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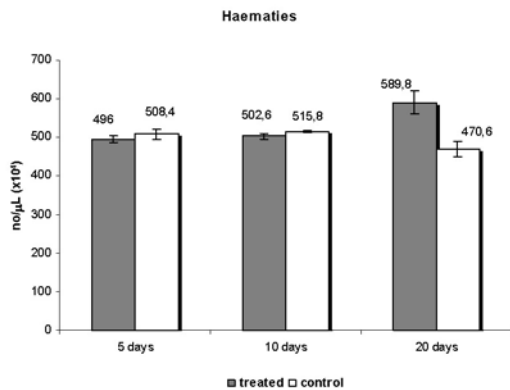


Fig. 1

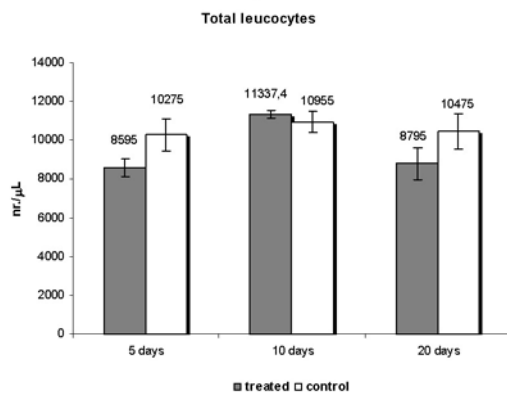


Fig. 2

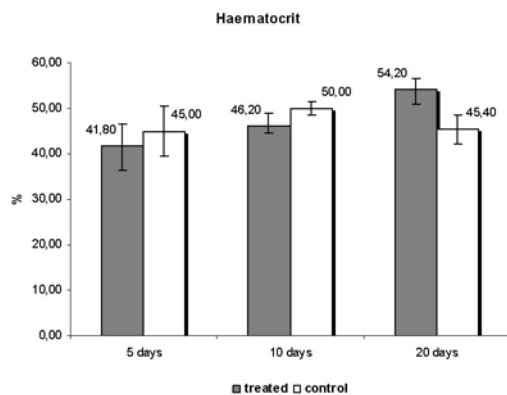


Fig. 3

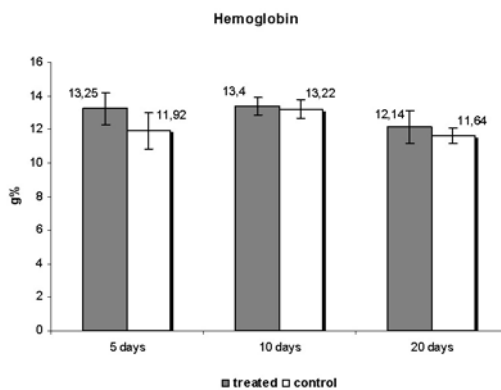


Fig. 4

Mean Erythrocyte Volume (MEV)

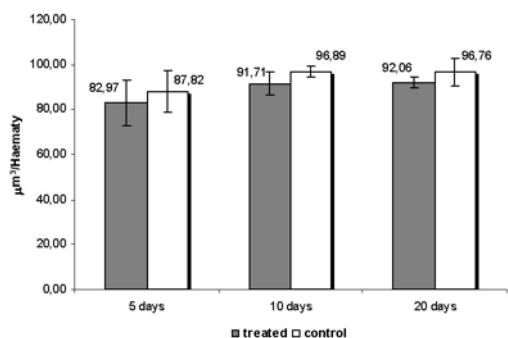


Fig. 5

Hemoglobin Erythrocyte Mean Content

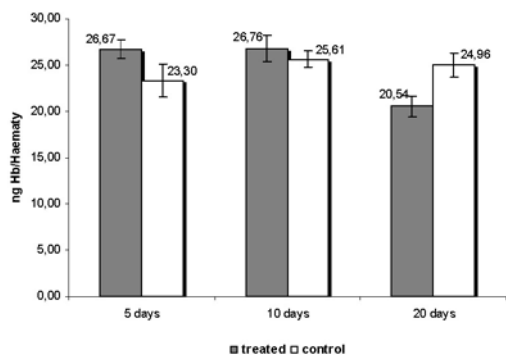


Fig. 6

THE EFFECTS OF ACUTE AND CHRONIC STRESS ON ERYTHROCYTE DYNAMIC IN COMBINATION WITH β -ADRENERGIC RECEPTORS BLOCKADE IN RATS

LUCIAN HRITCU^{*1}, VASILE HEFCO¹, ION NEACSU¹, CALIN MANIU¹

Key words: propranolol, acute stress, chronic stress, peripheral blood erythrocyte

Abstract: 3 consecutive days propranolol hydrochloride administration (5 mg/kg b.w., subcutaneous injections) under acute and chronic stress conditions causes changes of peripheral erythrocyte distribution in rats. The effects of acute stress and its combination with β -adrenergic receptor blockade on erythrocyte dynamic were more pregnant beside the effects of chronic stress and its combination with β -adrenergic receptor blockade, respectively. β -adrenergic mechanisms were shown to be involved in regulation of erythrocyte dynamic in acute and chronic stress response.

INTRODUCTION

Adrenergic compounds are extremely important factors in neuroendocrinal regulation of the function of the immune system in the norm and in the case of different pathological conditions. With such processes like stress, the adrenergic mechanisms may play a key role in the changes of the immune system (Khlosov et al., 1993; Spengler et al., 1994), in the redistribution of T lymphocytes (Zimin et al., 1985) and in the regulation of medullar hemopoiesis (Goldberg et al., 1991). Concurrently, β - and α -adrenoreceptors are expressed on the cells of the immune system and on the erythrocyte system. The aim of this work was to study changes in the erythrocyte dynamic under acute and chronic stress in combination with the β -adrenergic receptor blockade in rats.

MATERIAL AND METHODS

Male Wistar rats weighing $200\text{g} \pm 25\text{g}$ at the beginning of experiments were used. They were fed and allowed to drink water ad libitum.

The rats of the first two groups were treated with propranolol hydrochloride (5 mg/kg b.w., subcutaneous injections) and were subjected to an acute stress (intermittent session of footshock; 0,5 mA, during 60s (5s shock, 5s pause)) after 1 and 3 days from the moment of the propranolol administration. Control groups were subjected to the same conditions and were treated with saline solution.

The rats of the third group were treated with propranolol hydrochloride (5 mg/kg b.w., subcutaneous injections) and were subjected to an chronic stress (intermittent session of footshock; 0,5 mA, during 60s (5s shock, 5s pause)) during of 3 consecutive days from the moment of the propranolol administration. Control group were subjected to the same conditions without the propranolol administration and were treated with saline solution.

Four days after the drug administration, rats were killed by decapitation. We used a Coulter Counter Beckman for determination of total number of erythrocytes, hematocrit (HCT) and hemoglobin (HB).

Statistical analysis

Results were expressed as mean \pm S.E.M. The results were analyzed statistically by means of the Student's "t" test. $p < 0.05$ was taken as the criterion for significance.

RESULTS AND DISCUSSIONS

1. Effects of acute and chronic stress and β -adrenoreceptor blockade on the erythrocyte dynamic

The experimental data are shown in Fig 1. The total number of erythrocyte decrease significantly in the control group after 3 days under the acute stress influence, as well as in the control group under the chronic stress influence. In the groups treated with propranolol the total number of erythrocyte increase significantly only after 3 days under the acute stress influence, and decrease in the chronic stress condition.

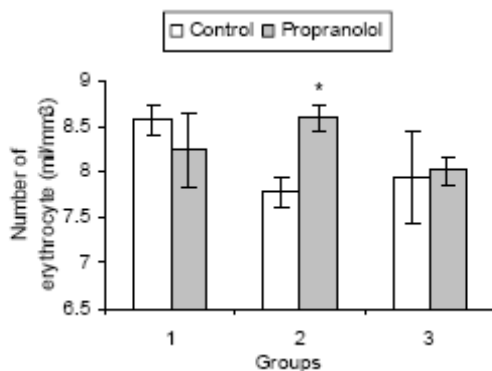


Fig 1. Changes of the total number of erythrocyte after 1 and 3 days of propranolol administration under acute (groups 1, 2) and chronic (group 3) stress. Values are means \pm SEM (n = 6 per group). *p<0.05 vs. Control.

2. Effects of acute and chronic stress and β -adrenoreceptor blockade on the hemoglobin (HGB) quantity.

The experimental data are shown in Fig 2. Propranolol treatment enhances the quantity of hemoglobin after 3 days under acute stress exposure (groups 2). Acute stress exposure after 1 day of propranolol treatment induced no significant variation of hemoglobin quantity (groups 1). The same effect was observed under 3 consecutive days chronic stress exposure in groups 3 treated with propranolol.

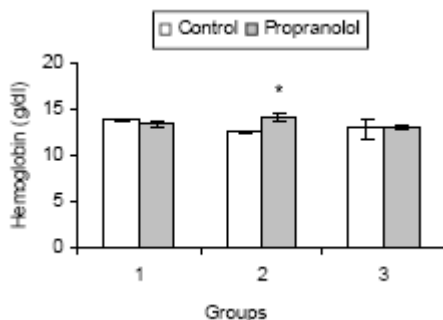


Fig 2. Hemoglobin quantity after 1 (groups1), 3 (group2) of acute stress exposure and 3 consecutive days (groups 3) of chronic stress exposure in combination with β -adrenoreceptor blockade. Values are means \pm SEM (n = 6 per group). *p<0.05 vs. Control

3. Effects of acute and chronic stress and β -adrenoreceptor blockade on hematocrit (Ht) values.

The experimental data are shown in Fig 3. Acute stress exposure after 3 days of propranolol treatment induced a significant variation on the hematocrit value. Acute stress exposure after 1 day of propranolol treatment and chronic stress exposure after 3 days of propranolol treatment induced no significant variation on the hematocrit value.

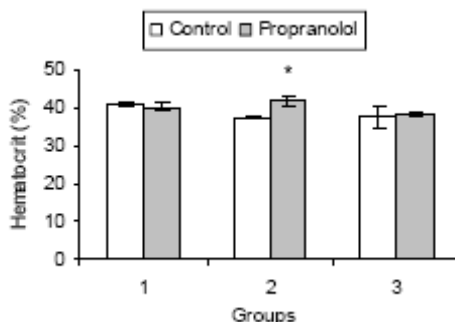


Fig 3. Variation of hematocrit value after 1 (groups1), 3 (groups 2) of acute stress exposure and 3 consecutive days (groups 3) of chronic stress exposure in combination with β -adrenoreceptor blockade. Values are means \pm SEM (n = 6 per group). *p<0.05 vs. Control

Our results showed that adrenergic receptor blockade with propranolol enhances the total number of erythrocyte, the quantity of hemoglobin and the hematocrit under acute stress influences and a no significant influences under chronic stress conditions. These data confirmed those from the literature concerning the influence of blockade of β -adrenoreceptors and acute and chronic stress on medullar hemopoiesis (Goldberg et al., 1991; Shilov et al., 2001).

Adrenergic compounds are extremely important factors in neuroendocrinal regulation of the function of the immune system in the norm and in the case of different pathological conditions. With such processes like stress, the adrenergic mechanisms may play a key role in the changes of the immune system (Khlusov et al., 1993; Spengler et al., 1994), in the redistribution of T lymphocytes (Zimin et al., 1985) and in the regulation of medullar hemopoiesis (Goldberg et al., 1991). Concurrently, β - and α -adrenoreceptors are expressed on the cells of the immune system and on the erythrocyte system that indicate the important role of β -adrenoreceptor mechanisms in the regulation of erythrocyte dynamic.

CONCLUSIONS

On the whole the obtained data indicate the important role of β -adrenoreceptor mechanisms in the regulation of erythrocyte dynamic.

REFERENCES

1. Goldberg, E.D., Dygai, A.M., Bogdashin, I.V., Sherbostoev E. Yu., 1991- Role of humoral factors in the regulation of hemopoiesis under stress. *Bull. Exp. Biol. Med*, 112, (7), 15-17.
2. Khlusov, I.A., Dygai, A.M., Goldberg, E.D., 1993 - Adrenergic regulation of interleukin production by bone marrow cells under conditions of immobilized stress. *Bull. Exp. Biol. Med*, 116, (12), 570-572.
3. Shilov, J.I., Gein, S.V., Chereshev, V.A., 2001 - Influence of blockade of β -adrenoreceptors and acute stress on antibody formation, delayed type of hypersensitivity, phagocytic cell activity in local immune response. *Russian Journal of Immunology*, 6 (3), 302-308.
4. Spengler, R.N., Chensue, S.W., Giacherio D.A., Blenk, N., 1994 - Endogenous norepinephrine regulates tumor necrosis factor-alpha production from macrophages in vitro. *J. Immunol.*, 152 (6), 3024-3031.

5. Zimin, Yu., I., Sukhikh, G.T., Nalivaiko, E.S., 1985 - Action of surgery stress on the state of cellular immunity. *Vestnik AMS USSR*, 8, 30-34.

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THE BEHAVIOUR OF THE MEMBRANARY $\text{Na}^+ - \text{K}^+ - \text{ATP-ase}$ OF NEOPLASTIC CELLS TO THE IN VITRO CYTOSTATIC TREATMENT WITH BIOSYNTHESIS OR SEMISYNTHESIS FUROSTANOLIC-GLYCOSIDES

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Key words: furostanolic glycoside cytostatics; neoplastic cell cultures; $\text{Na}^+ - \text{K}^+ - \text{ATP-ase}$ activity; membrenary ionic transport; mechanism of action.

Abstract: The in vitro short lasting antitumoral treatment of the HEp-2p and HeLa human tumoral cells with some active cytostatic furostanolic-glycoside biopreparations has induced an inhibitory impact upon $\text{Na}^+ - \text{K}^+$ -depending electrogenic pump. These furostanolic glycoside agents interferes, probably, direct or indirect with the membrane $\text{Na}^+ - \text{K}^+ - \text{ase}$ activity, either by their binding of the enzyme macromolecule or by perturbing of the energetical metabolism which leads to a decreased level of the ATP biosynthesis.

INTRODUCTION

A lot of biologically active compounds are glycosides. Sometimes, the glycosidic residue is crucial for their activity, in other cases glycosylation only improves pharmacokinetic parameters. Recent developments in molecular glycobiology brought better understanding to the aglycone versus glycoside activities, and made possible to develop new, more active or more effective glycodrugs based on these findings (Kren and Martinkova, 2001).

Our previous preclinical studies – performed on experimental models adequate to the in vitro investigation on neoplastic cell cultures – were relevant for the appreciation of some original biopreparations of furostanolic glycoside type, extracted from *Digitalis purpurea* leaves, as potential cytostatic drugs with possible biomedical significance (Gherghel et al., 2004).

The pharmacological characterization of a new biosynthesis, semisynthesis or synthesis product as chemotherapeutic agent is assured not only by in vitro and in vivo highlighting, confirmation and quantification of the pharmacodynamic effect, but also by data regarding the action mechanism at cellular, subcellular and molecular level implied in the overall expression of the specific pharmacological property (Calabresi and Parks, 1985; Goodman and Gilman, 1985; Chiricuță, 1988; Boyd, 1989; Phillips et al., 1990; Bissery and Chabot, 1991; DeVita, 1991; Stroescu, 1998; Lyden et al., 2001; Sheetala and Prabhavathi, 2001; Weinstein, 2001; Habeck, 2002; Wong, 2002).

Consequently, in the light of the above affirmations, the extending and thoroughgoing of the research have been required in order to establish the probably action mechanism involved in inducing of the in vitro antitumoral effect. Thus, in a first step, we have decided to investigate the reactivity of the cancerous cell's membrane phenomena in the conditions in the in vitro treatment with some glycosidic cytostatics.

The purpose of the present paper, which includes results of a preliminary research, is the analysis of the interference of B1 and E5 cytostatic agents of furostanolic glycoside nature with $\text{Na}^+ - \text{K}^+ - \text{ATP-ase}$ activity from the membrane level of the HEp-2p and HeLa tumoral cells.

MATERIALS AND METHODS

The active cytostatic compounds of furostanolic glycoside nature which were used in the in vitro experiments have been the following:

- B1, a biosynthesis product, extracted and specifically purified from leaves of *Digitalis purpurea*;
- E5, which is a semisynthesis product, derived from the former by partial oxidation until to obtaining of some aldehydic groups.

The biological material used in the in vitro investigations was represented by the control and treated HEp-2p and HeLa cellular cultures of human neoplastic origin (laryngeal carcinoma and cervix carcinosarcoma, respectively).

The test tubes have been inoculated with 1×10^5 tumoral cells in Eagles' MEM growing medium supplemented with 10% calf serum, they being incubated at 37°C for a period of 72 hours.

After 72 hours of cultures development, when the monolayer stage was attained, the initial medium was replaced with a medium containing one from the two furostanolic glycoside biopreparations in a dose of 5 mg/ml. The cultures were incubated again at 36.5–37°C for 180 minutes in the presence of the drugs.

After this short in vitro antitumoral treatment, the medium was discarded from the test tubes. The layer of tumoral cells was washed with PBS and then subjected to the biochemical determination steps of membrane enzyme implied in the transmembranary active transport of Na^+ and K^+ ions.

The activity of these cations –depending electrogenic pump was assessed by the spectrophotometrical quantitative analysis of inorganic phosphate (mg P_i /g of cell protein) from the cell homogenate, after Ca^{2+} -ATP-ase blocking with EDTA and ATP hydrolysis by Na^+ - K^+ -ATP-ase action (Artenie and Tănase, 1981).

Five tubes of cultures have been employed for each culture type, the results being analyzed statistically by means of Student' „t” test (Snedecor, 1968).

RESULTS AND DISCUSSIONS

In a first test we have investigated the effect of B1 and E5 active cytostatic furostanolic-glycoside biopreparations upon the membranary Na^+ - K^+ -ATP-ase activity from the HEp-2p tumoral cells which has been expressed by quantitative and percentage values.

Table 1. The behaviour of the Na^+ - K^+ -ATP-ase (mg P_i /g protein) from the membranes of the HEp-2p neoplastic cells submitted to the short treatment with B1 and E5 cytostatic agents, in a dose of 5 mg/ml. Figures in brackets indicate the number of experimental cultures for each type.

Culture types	$\bar{X} \pm \text{ES}$	p
Control	56.90 ± 3.95 (5)	
B1	21.45 ± 1.80 (5)	<0.001
E5	26.10 ± 2.05 (5)	<0.001

It can be seen, in Table 1, that the in vitro short antitumoral treatment of the 72 hours old HEp-2p cellular cultures has induced a statistically significant decrease of the inorganic phosphate contents from the membranes of the treated neoplastic cells, comparatively with the control level.

Another in vitro experiment has been performed in the same conditions for the appreciation of the membrane Na^+ - K^+ -ATP-ase reactivity to the cytostatic impact of the bioactive agents of glycosidic nature upon HeLa cell cultures.

Table 2. The membrane Na^+ - K^+ -ATP-ase activity (mg P_i /g protein) in the HeLa tumoral cells treated with 5 mg/ml of B1 and E5, comparatively with the control cultures. Figures in brackets indicate the number of experimental cultures for each type.

Culture types	$\bar{X} \pm \text{ES}$	p
Control	55.77 ± 4.05 (5)	–
B1	23.40 ± 1.90 (5)	<0.001
E5	33.15 ± 2.47 (5)	<0.002

From Table 2, it can be observed that the inorganic phosphate concentrations have again presented an important diminution as compared to the control value.

The reduced contents of the inorganic phosphate, released by enzymatic hydrolysis of the ATP, have revealed lower degrees of the activity of the Na^+ - K^+ -depending membrane electrogenic pump in the case of HEp-2p and HeLa tumoral cells submitted to the in vitro short cytostatic treatment.

Thus, it can be highlighted, from the following figures and in comparison with 100% control values, that the membranary Na^+ - K^+ -ATP-ase activity reaches intensities of 37.7% (B1) and 45.9% (E5), respectively, in the case of the HEp-2p treated neoplastic cells, as well as of 41.9% (B1) and respectively 59.4% (E5) in the case of the HeLa treated cancerous cultures.

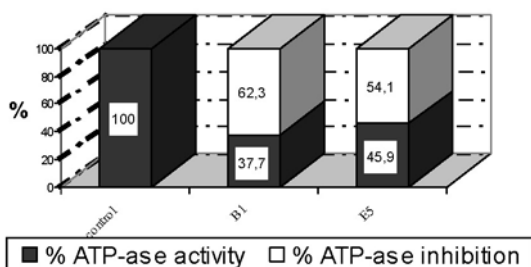


Fig. 1. Modulation of the $\text{Na}^+\text{-K}^+$ -depending electrogenic pump's activity (%) from the HEp-2p tumoral cells submitted to the furostanolic glycoside treatment, in comparison with the control cultures.

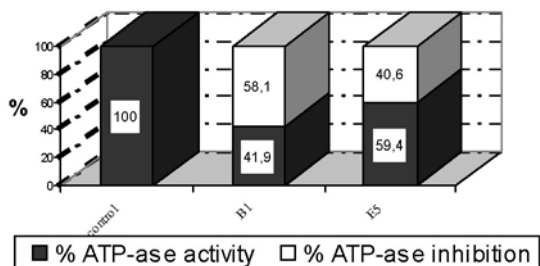


Fig. 2. Variations (%) of the membranary $\text{Na}^+\text{-K}^+$ -ATP-ase activity at HeLa cancerous cells treated with the glycosidic cytostatic agents, comparatively with the control one.

The various *in vitro* testing systems with tumoral cell cultures have practical importance both in the selection of potential oncochemotherapeutic agents of diverse chemical nature and in the understanding of the action mechanism responsible for therapeutic impact. The tumoral cell cultures are compatible and useful experimental models for preliminary appreciation of the mechanism implied in inducing a pharmacodynamic effect of an active biological agent (Leiter et al., 1965; Bissery and Boyd, 1989; Chabot, 1991; Phillips et al., 1991; Lyden, 2001; Habeck, 2002; Wong, 2002).

The animal eukaryotic cells contain self regulation and self control mechanisms which maintain the cell homeostatic state, they being the target of the biologically active substances. Generally speaking, the activation of molecular mechanisms of the cellular functional regulation is dependent on the transformation of the extracellular information in an action of cellular response. In this condition, the starting molecular event is logically localized at the level of the environment-cell interface, meaning in the cellular membranes. After this primary interaction between an agent and a cell membrane, there takes place the transfer and traducing of the extracellular signal. Consequently, the intracellular mechanisms of control and the activity of the enzymatic systems will be influenced. These specific modulations would stimulate and inhibit the different metabolic processes which will exteriorize by global pharmacodynamic effect (Benga 1985; Karp, 1996; Alberts et al., 1998; Stroescu, 1998; Cruce, 1999).

The lack of poise between the structural components of the tumoral cell membranes, the decrease of the membranary fluidity, the modification of the packing degree of the membrane overmolecular structures, the different topographical location and activity of the membrane ATP-ases are functionally expressed by perturbation of the membranary permeability. The modification of the ionic fluxes leads to the appearance of the transmineralization phenomenon.

This specific feature of the neoplastic cells consists in an abnormal distribution of the ions (Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- etc.) correlated with other ionic ratios in extra- and intracellular compartments and with a decrease of the membrane resting potential. Among other membranary peculiarities of the tumoral cells, it is important to mention the powerful enhancement of the activity of the Na^+ - K^+ -depending electrogenic pump (Benga, 1979; Chaubal and Firket, 1979; Binggeli and Cameron, 1980; Bianchi et al., 1986; Rusu et al., 1988; Bannasch et al., 1998; Cruce, 1999; Olbe, 1999; Miron, 2000; Owens, 2001; Wong, 2002).

This informational context justifies our objective of investigating of the effect of the B1 and E5 cytostatic agents of biosynthesis and semisynthesis upon the neoplastic cell membrane phenomena and to appreciate our experimental results in order to explain the possible mechanism of inducing their in vitro antitumoral impact.

Thus, in the present study we have followed the effect of the furostanolic-glycoside cytostatics upon the membranary Na^+ - K^+ -depending ATP-ase activity of the HEP-2p and HeLa tumoral cells.

The experimental results, registered after short lasting treatment of HEP-2p and respectively HeLa cells with the B1 or E5 antitumoral agents, have highlighted inferior amplitudes of the membrane Na^+ - K^+ -ATP-ase activity, comparatively with the one of control. Therefore, the furostanolic-glycoside biopreparations have induced an inhibitory impact upon the membrane electrogenic pump of Na^+ - K^+ . The intensity of the depressing effect was assessed at 62.3% (HEP-2p) and 58.1% (HeLa) for the B1, as well as at 54.1% (HEP-2p) and 40.6% (HeLa) in the case of the E5.

The decreased levels of the ATP enzymatic hydrolysis don't assure the energetical needs for the active transmembranary fluxes of Na^+ and K^+ cations, which are also perturbed.

Consequently, new extra- and intracellular ionic ratios will be established. These will modify both optimal conditions for the diverse intracellular enzymatic systems' activity and the unfolding of the metabolic events. Finally, these disturbances induced by the glycosidic compounds could represent the molecular substratum of their cytostatic activity.

The inhibitory effect upon membranary Na^+ - K^+ -ATP-ase activity can be the consequence of a direct binding of the furostanolic-glycoside substances with the electrogenic pump molecule. This appreciation is a plausible hypothesis if we remind that ouabain – a membranotropic modulator – blocks the active transmembranary transport of the Na^+ and K^+ cations by coupling with the macromolecule of the membrane enzymes (Baker et al., 1980; Churchill and Churchill, 1980; Dawsen and Smith, 1986; Tran and Farley, 1986), therefore, with the same cellular substratum.

Certainly, we can't exclude the idea that the inhibitory impact of B1 and E5 -glycosidic agents upon the activity of the Na^+ - K^+ -ATP-ase could be the secondary result of another possible interaction with the intracellular receptors. This kind of interactions could be followed by alteration of the energetical metabolism, with negative consequences both on level of ATP biosynthesis and on membranary Na^+ - K^+ -depending ATP-ase pump activity.

In this moment, the analysis of the experimental results allows us to suggest that the main mechanism probably involved in the expression of the cytostatic effect seems to be one of membranary type, the furostanolic glycoside agents would interact with one from the specific membrane receptors of the tumoral cells.

In future studies we will investigate other aspects of the in vitro interference of the glycoside antitumoral drugs with the membrane and metabolic processes of the neoplastic cells in order to elucidate the mechanism responsible for their pharmacodynamic property.

CONCLUSIONS

The short lasting in vitro antitumoral treatment of the HEP-2p and HeLa human cancerous cells with the active cytostatic biopreparations of furostanolic glycoside nature have induced an inhibitory impact upon $\text{Na}^+ - \text{K}^+$ -depending electrogenic pump.

The decreased ATP enzymatic hydrolysis has negative consequences upon the active membrenary transport of Na^+ and K^+ , as well as on cell metabolic processes.

The similar sense and different amplitude of the membrane reactivity of the HEP-2p and HeLa cultures highlights the various sensibilities of the tumoral cells to the B1 and E5 action.

In this preliminary step of the research, the results indicate that the most probably mechanism involved in the expression of the antitumoral impact of the biosynthetic and semisynthetic glycoside compounds seems to be one of membrenary type.

It can be possible that B1 and E5 agents to interacts with the membrane $\text{Na}^+ - \text{K}^+ - \text{ATP}$ -ase, their binding of the enzyme macromolecule partially blocking the electrogenic pump of Na^+ and K^+ and perturbing – by the consequences upon the active membrenary transport of ions – the metabolic events of the tumoral cells.

REFERENCES

1. Alberts, B., Bray, O., Johnsons, A., 1998. *Essential Cell Biology*, Garland Publ. Inc. N.Y., London, 193-553.
2. Arteni, V., Tănase, Elvira, 1981. *Practicum de biochimie generală*, Ed. Universității „Al. I. Cuza”, Iași, 128-133.
3. Backer, T., Andrew, J., Neville, M., 1980. *J. Membr. Biol.*, 52, 141-146.
4. Bannasch, P., Kanduc, D., Papa, S., Tager, J.M., 1998. *Cell growth and oncogenesis*, Birkhäuser Verlag, Basel–Boston–Berlin.
5. Benga, G., 1979. *Biologia moleculară a membranelor cu aplicații medicale*, Ed. Dacia, Cluj-Napoca, 195-213; 273-281; 250-257.
6. Benga, G., 1985. *Biologie celulară și moleculară*, Ed. Dacia, Cluj-Napoca, 47-130; 209-277.
7. Bianchi, G., Carafoli, E., Scarpa A., 1986. *Membrane pathology*, New York Academy of Sciences, Vol. 488, 430-491.
8. Binggeli, R., Cameron, I. L., 1980. *Cancer Res.*, 40, 1830-1835.
9. Bissery, M.C., Chabot, G.G., 1991. *Bull. Cancer (Paris)*, 78, 587-602.
10. Boyd, M.R., 1989. *Cancer: Princ. Pract. Oncol. Updates*, 3, 1-12.
11. Calabresi, P., Parks, R.E. Jr., 1985. *The Pharmacological Basis of Therapeutics*, Gilman A.G., Goodman L.S., Rall Th. W., Murad F., Eds. N.Y., Mac Millan Publishing Company, 1240-1308.
12. Chaubal, K., Firket, H., 1979. *Sciences Soc. Biol.*, 173, 627-631.
13. Chiricuță, I., 1988. *Cancerologie vol. I și II*, Ed. Med. București.
14. Churchill, M. C., Churchill, P.C., 1980. *J. Physiol.(London)*, 300, 105-114.
15. Cruce, M., 1999. *Biologie celulară și moleculară*, Ed. Aius, Craiova, 14-25; 33-56, 141-161; 193-300.
16. Dawson, W., Smith, T., 1986. *Biochim. Biophys. Acta*, 860, 293-300.
17. DeVita, V.T. Jr., 1991. *Cancer: Principles and Practice of Oncology*, Third Edition, De Vita Jr. et al., (Eds.), Philadelphia, Lippincott, 276-300.
18. Gherghel Daniela, Rotinberg P., Iurea Dorina, Kintea P., 2004. *Lucrările celui de-al X-lea Simpozion de Microbiologie și Biotehnologie*, Iași, Ed. Corso, 231–236.

19. Goodman, L., Gilman, A., 1985. *The Pharmacological Basis of Therapeutics*, Macmillan Publishing Company, New York, 1240-1308.
20. Habeck, M., 2002. *Drug Discovery Today*, 7, 635–637.
21. Karp, G., 1996. *Cell and Molecular Biology*, John Wiley and Sons, INC., N.Y., Brisbane, Toronto, 694-725.
22. Kren V., Martinkova L., 2001. *Current Medicinal Chemistry*, 8, 1313-1338.
23. Leiter, J., Abbott, D.J., Schepartz, S.A., 1965. *Cancer Res.*, 25, 20-35.
24. Lyden, D., Hu, Z., Caren, A., Kresty, L.A., 2001. *Drug Discovery Today*, 6, 1252–1254.
25. Miron, L., 2000. *Oncologie generală*, Ed. Egal, Bacău, 9-131.
26. Olbe, L., 1999. *Proton pump inhibitors*, Parnham, M., Bruinvels, J., Eds., Birkhäuser Verlag, Basel-Boston-Berlin, 3-173.
27. Owens, J., 2001. *Drug Discovery Today*, 6, 1203–1206.
28. Phillips, R.M., Bibby, M.C., Double, J.A., Loadman, P.M., 1991. *Int. J. Cell Cloning*, 9, 144-154.
29. Rusu, V., Baran, T., Brănișteanu, D., D., 1988. *Biomembrane și patologie*, Ed. Medicală, București, Vol. I, 63-87; 395-435.
30. Seethala, R., Prabhavathi F., 2001. *Drugs Pharm. Sci.*, 114, 5–520.
31. Snedecor, G.W., 1968. *Metode statistice aplicate în agricultură și biologie*, Ed. Did. Ped., București.
32. Stroescu, V., 1998. *Bazele farmacologice ale practicii medicale*, Ed. Med. București.
33. Tran, M., Farley, R., 1986. *Biochim. Biophys. Acta*, 860, 9-15.
34. Weinstein, J., 2001. *Drug Discovery Today*, 6, 1145–1248.
35. Wong, J.M.Y., 2002. *Drug Discovery Today*, 7, 1072–1073.

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THE REACTIVITY OF THE METABOLIC PROCESSES OF THE HEP-2p TUMORAL CELLS TO THE ACTION OF SOME ACTIVE CYTOSTATIC BIOPREPARATIONS OF POLYPHENOLIC NATURE

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Key words: polyphenolic cytostatic agents; tumoral cells; intermediary metabolism; nucleic acids biochemistry; mechanism of anticancerous action.

Abstract: The in vitro cytostatic treatment of the HEP-2p tumoral cell cultures with some autochthonous original polyphenolic biopreparations has conditioned the perturbation of the glucidic, lipidic and proteic intermediary metabolism processes and of the nucleic acids biochemistry.

The metabolic profile of the treated cells seems to be of catabolic type, being outlined by enhancement of the glicogenolysis, glycolysis, lipolysis and proteolysis, of intensification of intracellular consumption of the glucose, lactic acid, free fatty acids and aminoacids, of inhibitory effect upon nucleic acids biosynthesis. These metabolic events were appreciated on the basis of the reduced contents of glycogen, glucose, lactic acid, total lipids, free fatty acids, soluble and unsoluble proteins, DNA and RNA biomolecules. The new tumoral cell metabolic behaviour induced by polyphenolic cytostatics – analyzed in comparison with that of the control untreated tumoral cells – can be consequence of an interaction between the bioactive agents either with the membrane receptors or with intracellular receptors.

INTRODUCTION

The morphological, structural, physiological, genetical, biochemical, biophysical and antigenic features of the tumoral cells – although assure yet their relative invulnerability – provide the numerous targets for chemotherapy, immunotherapy, genic therapy and biochemical therapy of the malignant diseases (Benga, 1985; Bianchi et al., 1986; Chiricuță, 1988; Rusu et al., 1988; Stroescu, 1998; Miron, 2000; Owens, 2001).

Despite the fact that there has been continuous progress in cancer diagnosis and treatment as a result of recent discoveries in cellular and molecular oncobiology, structural and functional genomics, pharmacogenomics and toxicogenomics, proteomics and metabolomics, antineoplastic therapy is still of little effectiveness (Karp, 1996; Cruce, 1999; Lyden et al., 2001; Weinstein, 2001; Adams, 2002; Anderson et al., 2002; Habeck, 2002; Wong, 2002).

One of the most significant objectives of contemporary studies in pathology consists in improving the efficacy of means to control the carcinogenesis. In the fight against cancerous diseases, chemotherapy holds pride of place, but it is still of small effectiveness, a fact explained especially by its negative impact on the normal cells of the organism under neoplasm aggression and by the development of a resistance phenomenon of the tumoral cells to the cytostatic drugs action. Consequently, for the improvement of the oncochemotherapy there are necessary the extending and thoroughgoing researches for: the discovery and design of new oncolytic agents that should specifically target the tumoral cells; the identification of new therapeutic ways of action upon carcinogenesis process; the conceiving of new strategies and programs of anticancerous chemotherapy; the use of different drug monithorized delivery and transport systems; the discovery of agents which can potentiate the antitumoral effect of the oncochemoterapeutic drugs (Leiter et al., 1965; DeVita, 1991; Stroescu, 1998; Weinstein, 2001).

The identification of a new antitumoral agent and its introduction in clinical practice – the main purpose of the screening chemotherapeutic programs – are the result of some complex preclinical and clinical pharmacological investigations according to appropriate experimental patterns, which use various testing biological systems having different degrees of reactivity (Leiter et al., 1965; Jungstand et al., 1971; Boyd, 1989; Bissery and Chabot, 1991; Phillips et al., 1991; Stroescu, 1998; Seethala et al., 2001).

Our previous preclinical studies – performed on experimental models adequate to the in vitro and in vivo pharmacodynamic investigation both on neoplastic cell cultures and on animals with different tumoral systems – were relevant for the appreciation of some autochthonous, original biopreparations of polyphenolic type, extracted from phyto mass, as potential cytostatic drugs with possible biomedical significance (Rotinberg et al., 1998; Rotinberg et al., 2000; Rotinberg et al., 2000).

In the light of the above affirmations, supplementary researches have been required in order to enlarge our data base necessary both for the confirmation of the cytostatic property of the natural vegetable polyphenolic biopreparations and

for the establishment of their action mechanism at cellular, subcellular and molecular level involved in the global expression of the antitumoral pharmacodynamic effect.

Thus, the purpose of the present paper is to investigate the metabolic behaviour of the HEP-2p tumoral cells in the conditions of the in vitro cytostatic treatment with POLYAS I and POLYAS II autochthonous polyphenolic extracts.

MATERIALS AND METHODS

The aromatic extracts of polyphenolic type, which were used in the in vitro experiments, have been the following:

– POLYAS I, representing a total polyphenolic biopreparation separated and purified from a crude alkaline extract obtained from the October harvested leaves of *Asclepias syriaca* after the removal of the hemicellulosic structures and the readjustment of the pH at 7.0-7.1;

– POLYAS II, which is a biopreparation similar to the former presenting also a readjusted pH (7.0-7.1). However, unlike POLYAS I, it contains no waxes, latex, alcohols, fatty acids and terpenoids that have all been removed from its composition by a pre-extraction with cyclohexane.

The chemical compounds remaining in the supernatants after the exclusion of the above mentioned substances are of a phenolic nature. Polyphenol concentration was determined with a spectrophotometer and the total content was expressed in terms of gallic acid (Kren and Martinkova, 2001). The polyphenolic biopreparations with a total polyphenolic content of 12.0% and 15.0%, respectively, have been obtained by dissolving some known quantities of dry substance – resulted from the evaporation of the final supernatants – in appropriate volumes of bidistilled water. Stock solution concentrations were established.

The biological material used in the in vitro investigations was represented by the control and treated HEP-2p cellular cultures of human neoplastic origin (laryngeal carcinoma). The test tubes have been inoculated with 1×10^5 tumoral cells in Eagles' MEM growing medium supplemented with 10% calf serum, they being incubated at 37°C for a period of 72 hours of culture development. When the monolayer stage was attained, the initial medium was replaced with a medium containing one of the two polyphenolic biopreparations in a dose of 10 mg/ml. The cultures were incubated again at 36.5–37°C for 180 minutes in the presence of the drugs.

At the end of this short in vitro antitumoral treatment, the medium was discarded from the test tubes. The layer of tumoral cells was washed with PBS and then subjected to the steps of obtaining of the cell clarified lyzates. Adequate aliquots were used for the biochemical determination of some metabolic indices (Artenie and Tănase, 1981): glycogen (G), glucose (g) and lactic acid (L.A.); total lipids (T.L.) and free fatty acids (F.F.A); soluble (S.P.), unsoluble (U.P) and total proteins (T.P.); deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and total nucleic acids (TNA).

Five tubes of cultures have been employed for each culture type, the results being analyzed statistically by means of Student' „t” test (Snedecor, 1968).

RESULTS AND DISCUSSIONS

In a first step of the research, we have followed the reactivity of the glucidic intermediary metabolism of the HEP-2p tumoral cells submitted to the short cytostatic treatment with the POLYAS I and POLYAS II natural polyphenolic biopreparations. The sense and the intensity of the metabolic processes have been expressed by the quantitative values of some glucidic biochemical parameters: glycogen, glucose and lactic acid.

It can be seen, in Table 1, that the in vitro short antitumoral treatment of the 72 hours old HEP-2p cell cultures has induced statistically significant decreases of the glycogen, glucose and lactic acid contents, as compared to the control level.

Table 1. The effect of active cytostatic polyphenolic biopreparations, in dose of 5 mg/ml upon the contents of glycogen, glucose and lactic acid (mg/g cellular mass), from HEP-2p tumoral cell cultures of 72 hours, submitted to the in vitro short antitumoral treatment. Figures in brackets indicate the number of experimental cultures for each type.

Experimental group	Glycogen		Glucose		Lactic acid	
	X ± SE	p	X ± SE	p	X ± SE	p
Control	30.5 ± 1.3 (5)	–	4.50 ± 0.21 (5)	–	1.53 ± 0.05 (5)	–
POLYAS I	23.5 ± 1.4 (5)	<0.01	4.05 ± 0.25 (5)	N.S.	0.92 ± 0.03 (5)	<0.001
POLYAS II	22.0 ± 1.1 (5)	<0.01	3.85 ± 0.18 (5)	<0.05	0.80 ± 0.03 (5)	<0.001

From the Figure 1, it is observed that the amplitude of the quantitative diminutions reaches – in comparison with 100% control value – percentage levels of: 23%, 10% and respectively 39.9% for glycogen, glucose and respectively lactic acid in the case of the HEP-2p treated cells with POLYAS I, as well as of 27.9%, 14.5% and respectively 47.7% in the case of neoplastic cells submitted to the action of POLYAS II.

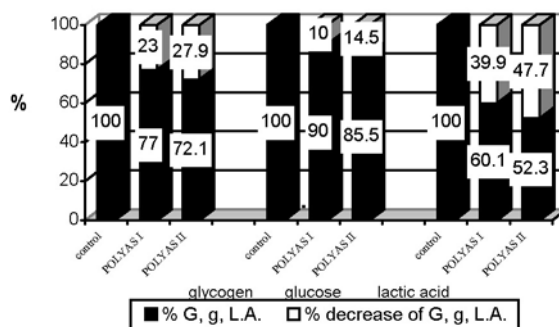


Fig. 1. Percentage variation of the glycogen, glucose and lactic acids concentrations induced by the in vitro short cytostatic polyphenolic treatment of the HEP-2p neoplastic cells.

These quantitative and procentual variations of the glucidic biochemical indices reveal the modulations of the cellular metabolic events. Thus, it can be highlighted an intensification of the glycogenolysis, glycolysis and the intracellular consumption of the glucose and lactic acid.

Another intermediary metabolism which was investigated is the lipidic one, the pattern of unfolding of the biochemical processes in the tumoral cells treated with the polyphenolic cytostatic extracts being illustrated by some parameters: total lipids and free fatty acids (Table 2 and Figure 2).

Table 2. Total lipids and free fatty acids concentrations (mg/g cellular mass) of the HEP-2p tumoral cells incubated with POLYAS I or POLYAS II (5 mg/ml) polyphenolic biopreparations. Figures in brackets indicate the number of experimental cultures for each type.

Experimental group	Total lipids		Free fatty acids	
	X ± SE	p	X ± SE	p
Control	20.56 ± 1.20 (5)	–	6.05 ± 0.35 (5)	–
POLYAS I	16.50 ± 0.90 (5)	<0.05	4.79 ± 0.22 (5)	<0.02
POLYAS II	15.54 ± 0.85 (5)	<0.01	4.32 ± 0.20 (5)	<0.01

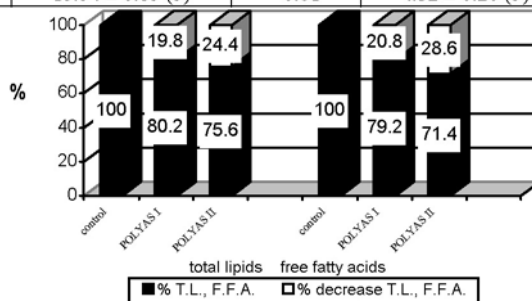


Fig. 2. The sense and the amplitude of the lipidic metabolism modulation, in the malignant HEP-2p cells, by the active cytostatic polyphenolic agents.

In vitro short time incubation of the HEP-2p with POLYAS I and POLYAS II cytostatic extracts has conditioned – as can be observed from Table 2 and Figure 2 – the perturbation of the

lipidic metabolism processes which were materialized by intracellular depletions of the lipidic reserves. Thus, as compared with the control values, the contents of the total lipids and free fatty acids have registered significant quantitative and procentual decreases. The variations of the lipidic parameters – of negative sense and moderate degrees – have emphasized the intensification of the intracellular lipolysis and metabolic utilization of the free fatty acids.

The study of the intermediary metabolism of the HEp-2p tumoral cells, submitted to the action of the vegetable polyphenolic biopreparations, was extended by the investigation of the protidic metabolism biochemistry to the cytostatic treatment. The reactivity of the metabolic events was analysed on the basis of the soluble proteins, unsoluble proteins and total proteins variations evidenced in comparison to the control values, these being inserted in Table 3 and Figure 3.

Table 3. Contents of the soluble, unsoluble and total proteins (mg/g cellular mass), of the 72 hours HEp-2p tumoral cells cultures, incubated for 3 hours with the cytostatic polyphenolic biopreparations (5 mg/ ml). Figures in brackets indicate the number of experimental cultures for each type.

Experimental group	Soluble proteins		Unsoluble proteins		Total proteins	
	X \pm SE	p	X \pm SE	p	X \pm SE	p
Control	45.06 \pm 2.1 (5)	–	23.15 \pm 2.1 (5)	–	68.21 \pm 2.8 (5)	–
POLYAS I	34.02 \pm 1.7 (5)	<0.01	14.00 \pm 1.7 (5)	<0.01	48.02 \pm 2.6 (5)	<0.001
POLYAS II	30.10 \pm 1.1 (5)	<0.001	10.49 \pm 1.5 (5)	<0.002	40.59 \pm 2.1 (5)	<0.001

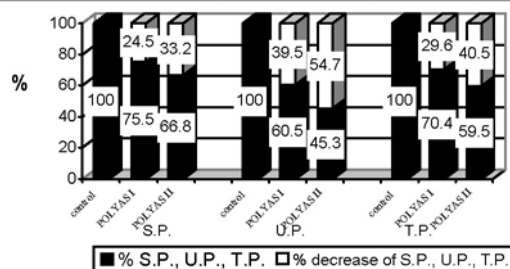


Fig. 3. Percentage variation of the soluble, unsoluble and total proteins concentrations induced by the in vitro short cytostatic polyphenolic treatment of the HEp-2p neoplastic cells.

It also can be seen that the HEp-2p cellular cultures treated with the POLYAS I or POLYAS II active cytostatic agents have been characterized, as compared to control, by significantly reduced contents of the soluble, unsoluble and respectively total proteins, which reach levels of: 24.5%, 33.5% and respectively 29.6% in the case of POLYAS I and 33.2%, 54.7% and respectively 40.5% in the case of POLYAS II. It is confirmed the inhibitory impact of the polyphenolic biopreparations upon the proteinsynthesis.

In order to obtain supplementary information about the interference of polyphenolic extracts with the tumoral cell metabolism we proposed ourselves to investigate some aspects of nucleic acids metabolism in the HEp-2p cells in the presence of cytostatic agents of polyphenolic nature.

The cytophysiologic behaviour of the nucleic acids, in the HEp-2p malignant cells submitted to the cytostatic treatment with the biologically active polyphenolic extracts, can be appreciated from the direction and intensity of display of the metabolic processes illustrated by the data included in Table 4 and Figure 4.

Table 4. Deoxyribonucleic acid, ribonucleic acid and total nucleic acids concentrations (mg/g cellular mass) of the HEP-2p tumoral cells incubated with POLYAS I or POLYAS II (5 mg/ml) polyphenolic biopreparations. Figures in brackets indicate the number of experimental cultures for each type.

Experimental group	DNA		RNA		TNA	
	X \pm SE	p	X \pm SE	p	X \pm SE	p
Control	2.19 \pm 0.095 (5)	–	2.26 \pm 0.100 (5)	–	4.45 \pm 0.28 (5)	–
POLYAS I	1.76 \pm 0.065 (5)	<0.01	1.81 \pm 0.075 (5)	<0.01	3.57 \pm 0.22 (5)	<0.05
POLYAS II	1.66 \pm 0.070 (5)	<0.01	1.71 \pm 0.065 (5)	<0.002	3.37 \pm 0.15 (5)	<0.01

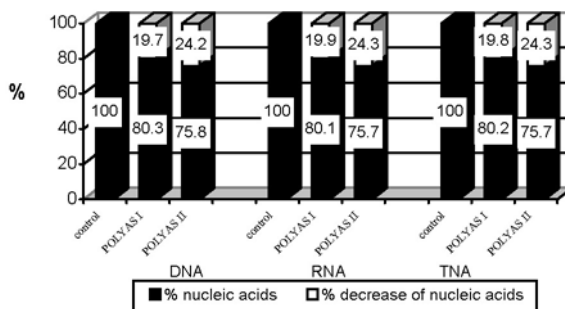


Fig. 4. The sense and the amplitude of the nucleic acids metabolism modulation, in the malignant HEP-2p cells, by the active cytostatic polyphenolic agents.

Once again, the experimental results have highlighted significant smaller amounts of DNA, RNA and respectively TNA in comparison with the control values, registered on untreated HEP-2p cultures. Thus, an interaction between the polyphenolic cytostatic agents and the metabolic events of the nucleic acids, it can be assumed, this materializing itself in an inhibitory impact (of about 20% for POLYAS I and 24% for POLYAS II) upon biosynthesis of the nucleic biomolecules.

The numerous, various and profound structural alterations (of the plasmatic membrane; glycocalix; extracellular matrix; cytoskeleton; cytoplasm; nucleus; nucleoli; endoplasmic reticulum; Golgi apparatus; mitochondria; peroxisomes; centrosome; lysosomes; cell topochemistry; enzymatic and isoenzymatic biomolecules) and citophysiological perturbations (of the membrane permeability and transport; cell signaling; transmission and expression of genetic information; energy conversion; cell metabolism; sorting and transport of the biomolecules in intracellular compartment; cell motility; intercellular and cell–matrix adhesion; cell proliferation; molecular regulation mechanisms) of the cellular, subcellular and molecular components of the dedifferentiated tumoral cells induced by erroneous functioning of the cellular genetic apparatus of selfregulation and control, turn the cancerous cells – apparently primitive and vulnerable – into a type of vigorous and viable cell, full of vitality and relative resistance to the chemical, physical and biological factors, this transformed cell being characterized by another homeostatic level (Benga, 1985; Bianchi et al., 1986; Chiricuță, 1988; Karp, 1996; Alberts et al., 1998; Stroescu, 1998; Cruce, 1999; Miron, 2000).

One of the most important features of the neoplastic cell is strongly connected to the qualitative and quantitative modifications of the cellular metabolism processes (Bustamante et al., 1981; Chiricuță, 1988; Bagetto, 1992; Gonzales et al., 1993; Mathupala et al., 1995; Karp, 1996; Bannasch et al., 1998; Cruce, 1999; Miron, 2000). Generally speaking, in comparison with the corresponding normal cell, the tumoral cell presents:

- intracellularly increased concentrations of proteins, aminoacids and nucleosides, due to: the intensified transmembranary transport of these biomolecules; augmented activity degree of the protein synthase kynases; amplified proteinsynthesis and nucleoside biosynthesis; swiching of the catabolic reactions of aminoacids and nucleosides in an anabolic pathway of synthesis of the polyaminoacids and polinucleosides (proteins, enzymes, DNA, RNA);

- reduced contents of glycogen, glucose correlated to intracellular lactic acid accumulations, conditioned by: exaggerate, uncontrolled intensification of the hexokinase, phosphofructokinase, piruvatkinase, ATP-ase activity; glycolysis; intracellular quantitative increasing of glucose and other hexoses with membranary determination; depressing of the key gluconeogenesis enzymes activity;

- intracellular augmented amounts of some tumoral lipids (desmosterol, cholesterol, triglycerides) and fatty acids due to: the changed membrane permeability; the quantitative and qualitative modification of the key opposite enzymes of the isoenzyme patterns and of the metabolic pathway.

The biochemical unbalance of the glucidic, lipidic and protidic metabolism and of the nucleic acids metabolism is the result of the reschedule of the corresponding genetic expression in tumoral cell.

However, the structural and functional peculiarities of the tumoral cell assure at the same time the targets of the previously mentioned factors within the frame-work of the different kinds of antineoplastic therapy. Among these is the cytostatic chemotherapy, which allows interactions drugs–cancerous cells and, therefore, antitumoral effect struggle.

In the light of the above information we will discuss and interpret the results we obtained in the study of the metabolic behaviour of the human HEP-2p neoplastic cells submitted to the in vitro cytostatic treatment with two bioactive autochthonous vegetable polyphenolic extracts, POLYAS I and POLYAS II.

The comparative analysis of our data, in relation to the control metabolic profile of the untreated HEP-2p cultures, highlights quantitative variations – always of negative sense and different amplitudes – of some glucidic, lipidic and protidic biomolecules and of the nucleic macromolecules. Thus, there were assessed reduced intracellular contents of glycogen, glucose and lactic acid, soluble and insoluble proteins, aminoacids, total lipids and free fatty acids, DNA and RNA. Therefore, we can appreciate that the polyphenolic cytostatics accent the glycogenolysis, activate the lipolysis and proteolysis, inhibit the nucleic macromolecule biosynthesis and intensify the intracellular metabolic consumption of the glucose, lactic acid, free fatty acids and aminoacids biomolecules.

Certainly, the intracellular utilization pathway is not represented by anabolic reactions of synthesis of the glucidic, lipidic, protidic and nucleic compounds, but it is probably assured by energogenetic catabolic reactions, which use the glucose, lactic acid, aminoacids and free fatty acids as fuels. Therefore, it is possible for the polyphenolic structures to stimulate the energetic metabolism of the HEP-2p tumoral cells. Thus, we must to prove this hypothesis, as soon is possible, by the investigation of the effect of the polyphenolic cytostatics upon cellular respiration of the HEP-2p tumoral cultures.

Finally, it can be revealed that the antitumoral polyphenolic agents condition a new lack of poise between the two sides of the cell metabolism, inducing an inhibitory impact upon the glucidic, lipidic, protidic and nucleic metabolism and an exacerbate stimulatory effect upon the exergonic metabolic reactions. These metabolic consequences – incompatible with the tumoral

cell life – are induced by polyphenolic perturbation of the activity of the disordered genetic apparatus and of the diverse enzymatic systems involved in catalyzing the biochemical reactions.

CONCLUSIONS

The antitumoral polyphenolic biopreparations influence negatively the development of the metabolic processes in the HEP-2p tumoral cells. The multitude of the metabolic effects can be the consequence of interactions of the polyphenolic structures either with the cell membrane receptors or with the intracellular ones. The bulk of the present results globalizes the behavioural spectrum of the HEP-2p tumoral cells to the action of the vegetable polyphenolic biopreparations confirming their cytostatic property.

REFERENCES

1. Adams, J., 2002. *Trends Mol. Med.*, 8, 49–54.
2. Alberts, B., Bray, O., Johnsons, A., 1998. *Essential Cell Biology*, Garland Publ. Inc. N.Y., London, 193–553.
3. Anderson, S., Chiplin, J., 2002. *Drug Disc. Today*, 7, 105–107.
4. Arteni, V., Tănase, Elvira, 1981. *Practicum de biochimie generală*, Ed. Universității „Al. I. Cuza”, Iași, 128–133
5. Bagetto, L., G., 1992. *Biochemistry*, 74, 959–974.
6. Bannasch, P., Kanduc, D., Papa, S., Tager, J.M., 1998. *Cell growth and oncogenesis*, Birkhäuser Verlag, Basel–Boston–Berlin.
7. Benga, G., 1985. *Biologie celulară și moleculară*, Ed. Dacia, Cluj-Napoca, 47–130; 209–277.
8. Bianchi, G., Carafoli, E., Scarpa A., 1986. *Membrane pathology*, New York Academy of Sciences, Vol. 488, 430–491.
9. Bissery, M.C., Chabot, G.G., 1991. *Bull. Cancer (Paris)*, 78, 587–602.
10. Boyd, M.R., 1989. *Cancer: Princ. Pract. Oncol. Updates*, 3, 1–12.
11. Bustamante, E., Morris, H., Pedersen, P., 1981. *The Journal of Biological Chemistry*, 256, 8699–8704.
12. Chiricuță, I., 1988. *Cancerologie* vol. I și II, Ed. Med. București.
13. Cruce, M., 1999. *Biologie celulară și moleculară*, Ed. Aius, Craiova, 14–25; 33–56, 141–161; 193–300.
14. DeVita, V.T. Jr., 1991. *Cancer: Principles and Practice of Oncology*, Third Edition, De Vita Jr. et al., (Eds.), Philadelphia, Lippincott, 276–300.
15. Gonzales-Mateos, F., Gomez, Maria-Esther, Garcia-Salguero, Leticia, Sanches Valentina, Aragon, J., 1993. *The Journal of Biological Chemistry*, 268, 7809–7817.
16. Habeck, M., 2002. *Drug Discovery Today*, 7, 635–637.
17. Jungstandt, von W., Guntchew, W., Wohlrabe, K., 1971. *Arzneim.–Forsch.*, 21, 404–410.
18. Karp, G., 1996. *Cell and Molecular Biology*, John Wiley and Sons, INC., N.Y., Brisbane, Toronto, 694–725.
19. Kren V., Martinkova L., 2001. *Current Medicinal Chemistry*, 8, 1313–1338.
20. Leiter, J., Abbott, D.J., Schepartz, S.A., 1965., *Cancer Res.*, 25, 20–35.
21. Lyden, D., Hu, Z., Caren, A., Kresty, L.A., 2001. *Drug Discovery Today*, 6, 1252–1254.
22. Mathupala, S., Rempel Annette, Pedersen, P., 1995. *The Journal of Biological Chemistry*, 270, 16918–16925.
23. Miron, L., 2000. *Oncologie generală*, Ed. Egal, Bacău, 9–131.

24. Owens, J., 2001. *Drug Discovery Today*, 6, 1203–1206.
25. Phillips, R.M., Bibby, M.C., Double, J.A., Loadman, P.M., 1991. *Int. J. Cell Cloning*, 9, 144–154.
26. Rotinberg, P., Nuță, Violeta, Kelemen, Smaranda, Petrașincu, Doina, Rotinberg, Hellen, 1998. *Romanian Journal of Physiology*, 35, 91–98.
27. Rotinberg, P., Kelemen, Smaranda, Grănescu, Mihaela, Rotinberg, Hellen, Nuță, Violeta, 2000. *Romanian Journal of Physiology*, 37, 91–103.
28. Rotinberg, P., Kelemen, Smaranda, Grănescu, Mihaela, Rotinberg, Hellen, Nuță, Violeta, 2000. *Romanian Journal of Physiology*, 37, 105–118.
29. Rusu, V., Baran, T., Brănișteanu, D., D., 1988. *Biomembrane și patologie*, Ed. Medicală, București, Vol. I, 63–87; 395–435.
30. Seethala, R., Prabhavathi F., 2001. *Drugs Pharm. Sci.*, 114, 5–520.
31. Snedecor, G.W., 1968. *Metode statistice aplicate în agricultură și biologie*, Ed. Did. Ped., București.
32. Stroescu, V., 1998. *Bazele farmacologice ale practicii medicale*, Ed. Med. București.
33. Weinstein, J., 2001. *Drug Discovery Today*, 6, 1145–1248.
34. Wong, J.M.Y., 2002. *Drug Discovery Today*, 7, 1072–1073.

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VARIATIONS OF IMMUNOGLOBULINS IN THE SERUM OF ADOLESCENTS OF PUBERTY AGE

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Key words: immunoglobulins, puberty, humoral specific immunity

Abstract: The study was performed on 13 schoolboys and 13 schoolgirls at puberty age. The immunoglobulins (Ig G, Ig A and Ig M) concentrations were determined at rest conditions in the morning serum. The rest values of immunoglobulins are significantly higher in the schoolgirls than the values in the schoolboys of the same age (Ig G 13.3 ± 1.36 g/L vs. 9.45 ± 1.69 g/L $p < 0.001$; Ig A 2.76 ± 0.31 g/L vs. 1.96 ± 0.63 g/L, $p < 0.001$ and Ig M 2.96 ± 0.43 g/L vs. 1.72 ± 0.62 g/L, $p < 0.001$). These values are significantly lower than the values determined in adults of both sexes. Simultaneously was determined the same parameters in the children of pre-puberty age. These determinations reveal concentrations situated much under the levels found in youths of puberty age, the differences being significant between them. The onset of puberty has effects not only on genital system, on sexual secondary features, on the growth and the development of the organism, but also on the immune system in the studied cases on specific humoral immunity.

INTRODUCTION

The puberty is a complex and polymorphous phenomenon and, in the view of a lot of specialists, it was contradictory or at least un-uniformly interpreted. (Berg et al., 1989; Cotuna., 1997; Cotuna et al., 1998; Cotuna, 2004; Kroop et al., 1976; Cotuna and Neacsu, 2003).

Some specialists consider that the puberty restricts to one moment - the first menstrual flow for girls and the first pollution for boys. In the present view, the puberty is a step of variable duration. During this step, primary sexual features become similar to that of adults by accelerating development process. The sexual secondary features have very important place in the body structure (Cotuna, 1997). Simultaneously, significant modifications appear in the process of growth and development. A series of investigations were performed on the sportsmen of different ages. (Green et al.1981, Lewicki et al., 1987, Cotuna and Neacsu, 2003, Cotuna, 2004).

Few investigations approached the puberty age influence on immune function of the organism, more exact on the existent differences between young and adult organisms (Cotuna and Neacsu, 2003). We did not yet find references regarding the ontogenesis of immunoglobulin synthesis in human and animal organism.

Starting from this general view, we give in this paper our results about the influence of puberty onset on immune function of the organism, namely of specific humoral immunity (the producing of the three types of immunoglobulins - IgG, IgA and IgM).

MATERIAL AND METHODS

The determinations of the three types of immunoglobulin concentration (IgG, IgA and IgM) in the puberty age children were made by nephelometric technique.

The blood samples (3 – 4 mL) were taken in the morning, without anticoagulating agent. The separation of serum was made by centrifugation 10 minutes at 30000 r.p.m.

It was demonstrated that serum proteins quantification by immunoprecipitation in liquid phase is well correlated with the determinations by Mancini radial immunodiffusion and have a good reproducibility. The immunoprecipitation in liquid phase with final nephelometric point was performed by Orion Diagnostica Turbox test.

The determinations were carried out on two equally groups of puberty age children (totally 26 persons), clinically healthy and also, on 10 pre-puberty children. All investigations were performed after the most three hours from blood sampling. Student “t” test was applied for the statistical analysis of the obtained data.

RESULTS AND DISCUSSIONS

The mean values of serum immunoglobulin concentrations in healthy adults are: IgG = 10.31 ± 0.48 g/L; IgA = 2.32 ± 0.22 g/L and IgM = 1.05 ± 0.11 g/L (Nieman, 1989), considered as reference data.

In the table 1 there are presented our results for puberty age subjects comparatively with this reference data.

Table 1. The serum immunoglobulin titre (Mean \pm SE).

AD=adults, PUB=puberals

Author	n	IgG g/L	IgA g/L	IgM g/L
Nieman D.C.(AD)	9	10.31 \pm 0.48	2.32 \pm 0.22	1.05 \pm 0.11
Cotuna D. (PUB)	26	11.38 \pm 0.46	2.36 \pm 0.09	2.34 \pm 0.10

The determinations in a group of 26 individuals (girls and boys) revealed the following mean values: IgG = 11.38 \pm 0.46 g/L; IgA = 2.36 \pm 0.09 g/L; IgM = 2.34 \pm 0.10 g/L

The mean values of serum immunoglobulins concentration registered in two sexes are different in the moment of puberty installation (table 2, a and b).

Table 2 a..The variations of the immunoglobulin titre depending on sex :

Parameter	n	IgG g/L	IgA g/L	IgM g/L
Girls (G)	13	13.31 \pm 0.14	2.76 \pm 0.08	2.96 \pm 0.12
Boys (B)	13	9.45 \pm 0.46	1.96 \pm 0.17	1.72 \pm 0.17
Mean (G + B)	26	11.38 \pm 0.46	2.36 \pm 0.09	2.34 \pm 0.10

b..Relations between sexes and statistical analysis:

Parameter	Mean \pm SE	t	p
IgG	Girls 13.31 \pm 0.14	5.43	< 0.001
	Boys 9.45 \pm 0.46		
IgA	Girls 2.76 \pm 0.08	4.06	< 0.001
	Boys 1.96 \pm 0.17		
IgM	Girls 2.96 \pm 0.12	4.70	< 0.001
	Boys 1.72 \pm 0.17		

For establish if during individual development appears modifications of the titre of these parameters, the determinations in a group of 10 pre-puberty youths were performed. The mean values of these determinations are presented in the table 3.

The comparative study of reference data (Nieman et al.,1998) of normal concentration values of serum immunoglobulins for IgG = 7.16 –16 g/L, IgA = 0.70 - 4.00 g/L and IgM = 0.40 - 2.30 g/L and the results of our investigations in puberty individuals reveals that our data are situated in the middle area of the parameters variation. Our results can be compared also with data established by Nieman D.C. et al (1989) in healthy adults for IgG and IgA, but they are in a strong discordance for IgM, the differences being very significant. In the present stage of knowledge it is difficult to explain the high values of IgM in the puberty age individuals comparative with those of adults obtained by Nieman D.C. et al. (1989).

By statistical analysis of these data it is possible to infer that in puberty age the immune system, more exact humoural specific immunity, is perfectly developed in the same measure as in adults. There are few data about ontogenesis of immunoglobulins and a long period dominates the view that was not identified immunoglobulin synthesis in human fetus (Cotuna,1997; Cotuna et al., 1998, Cotuna, 2004). Our comparative investigations in two groups of puberty and pre-puberty age children reveal that in pre-puberty age subject there are much lower values than in puberty subject, the differences being significant (Table 3). Looking at the variations of

immunoglobulin titre separately registered in the two sexes (Table 2) we find superior mean values in the girls comparatively with the boys for all three types immunoglobulins : IgG, IgA, and IgM. The statistical analysis (Student) reveals that all differences are very significant.

Table 3 The immunoglobulins variations in the children of pre-puberty age (up) and comparative values of puberty and pre-puberty age youths (down). Mean \pm SE;

P : puberty age, PP: pre-puberty age; t and p: Student “t” test

The sex	IgG	IgA	IgM
Girls	8.01 \pm 0.34	0.96 \pm 0.06	0.93 \pm 0.11(NS)
Boys	8.03 \pm 0.41	0.91 \pm 0.07	0.92 \pm 0.08 (NS)
Girls and Boys	8.02 \pm 0.37	0.93 \pm 0.07	0.92 \pm 0.08

Comparative values:

Parameter	Baseline	t	p
IgG (g/L)	P : 11.38 \pm 0.29 PP : 8.01 \pm 0.02	4.43	<0.001
IgA (g/L)	P : 2.36 \pm 0.05 PP: 0.93 \pm 0.05	3.95	<0.001
IgM (g/L)	P : 2.34 \pm 0.10 PP: 0.92 \pm 0.05	9.98	<0.001

During puberty modifications, the two sexes differentiate gradually. Complex phenomena of puberty developmet appear and are generally closed earlier in girls than in the boys; the same situation are also in the case of immune activity.

Concerning the pre-puberty period, the differences of immunoglobulin concentration in the girls and in the boys are not significant (Table 3). This convincing proves that till 10 – 11 year age there are not clear differences between girls and boys of equal age. After age 11 – 12 years the rhythm of the processes of growth and development of the girls is higher than in the boys, due to “neuro-endocrine storm” which carry on during this period. This phenomenon has evident consequences in immune status on the titre of serum immunoglobulin (Table 3), the Student test showing very significant differences (<0.001).

Additional investigations are still necessary for a complete elucidation of the mechanisms governing such phenomena and their correlations with other processes in this field, too.

CONCLUSIONS

The puberty onset has important effects not only on the growth and the development of the human organism, but also on the immune system

The IgG, IgA and IgM immunoglobulin values obtained in two groups of adolescents – of pre-puberty and puberty age – reveal significant differences of values , that of pre-puberty age subjects being much lower than that of puberty ones.

Also, the mean immunoglobulin values in the girl are superior comparatively with the values of boys for all three types of immunoglobulin.

Mean level of IgG and IgA immunoglobulins of the adolescents is similar with that of adults subjects, but IgM records higher values.

REFERENCES

1. Berg A., Northoff H., Keul J., (1989), , in: Böning D. Brauman K.M., Busse M.W., Maasen N., Schmit W. (Hrsg): *Sport Rettung oder Risiko für die Gesundheit*, Deutscher Asztverlag, Köln, 659 -665.
2. Cotuna D., (1997), *Imunitate si sport*, Ed. Mirton, Timisoara
3. Cotuna D., Pilat L., Cotuna C., Albu C., Mera E., (1998), in: 3rd International Symposium Interdisciplinary Regional Research (Hungary, Romania, Yugoslavia), 573-576
4. Cotuna D., Neacsu I., (2003), *Studii si Comunicari* Complexul Muzeal de Stiintele Naturii “Ion Borcea” Bacau, 277-279.
5. Cotuna D. ,(2004), *Anal. St. Univ. "Al. I. Cuza" Iasi – Genetica si Biol. Molec., tom V*, 306-309.
6. Green R.L. Kaplan S.S., Rubin B.S. (1981) *Ann. Alergy*, 47, 73-75.
7. Kroop J., Fuchs K., Weicher H., (1976) in *Seren von Leistung Sportlern, Sportarzt und Sportmed*, 27, 124-126
8. Lewicki R., Tchorzewski H., Denys A., (1987), *Ann. Sports. Med.* 3, 1-4.
9. Niemann D.C., Tann S.A., Lee J.W., and Berk S., (1989), *Int. J. Sports Med.*, 124-128.

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SOME ASPECTS ON THE SOIL–PLANT RELATION IN *NEPETA NEPETELLA*

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Keywords: redox, *Nepeta*, essential oils

Abstract: The possible occurrence of a redox-mediation within the soil–plant relation, characterizing the soil from the vicinity of the (approximately 20) plants as well the herbaceous mass, through a global biochemical parameter, the rH, has been checked. At the same time, the content of etheric oils has been estimated refractometrically. A certain dependence of soil's rH (characterized by a considerable variability) has been observed, for both the tissular rH and the content of etheric oils. As previously observed for other biochemical parameters, this dependence is of compensatory-type, that is, according to derivative I of the direct dependence, is manifested at the level of biomass accumulation.

INTRODUCTION

Nepeta nepetella var. *Amethystina* (fem. *Labiatae*) has been investigated as to its content of etheric oils – up to 1% (the composition of which is the following: geraniol 25%, nerol 23%, geraniol acetate and citronelal 18%, limonen 10%, citral, terpineol) –, largely utilized in both alimentary and cosmetic industries. Involving here being biosynthesis of an active principle, the present research has been approached comparatively with the case of the poppy. In agreement with the observations of several others authors, an extremely large diversity of capsules' morphine content has been noticed, situated between 0 and about 1%, that could be correlated with soil's redox character [1]; on the same occasion, a large diversity of “offers” – from a redox point of view – from the part of the soil, was observed, expressed by rH values both favourable and unfavourable to the plant, at extremely low spatial differences. This is the reason that challenged the authors to investigate a possible ecological organism–environment relation, as evidenced in *Nepeta nepetella*.

MATERIALS AND METHODS

Within an experimental culture of *Nepeta nepetella*, a surface of 15 m² has been delimited, out of which 20 pairs of reference samples (from plant's acrian part and, respectively, from the soil neighbouring it) have been taken over. The moment of taking over selected has been the flowering time (with a view to possible future correlations among different species, a characteristic/defining/unique moment, once known that, during its ontogenesis, the plant is characterized by a complex evolution of its redox characteristics [2]) – the same time as the one of other investigations [3, 4]. The obtained samples have been processed in a differentiated manner, according to their destination. Considering that, actually, an ecological plant–soil relation was actually looked for, the obtaining of some comparable data between the abiotic and the biotic environment has been followed, both segments being therefore to be described/characterized by an unique parameter, namely plant's tissular and, respectively, soil's rH. Implicitly, both the soil and the plant have been frozen up to the determination of their rH. Part of the plant has been immediately subjected to the extraction of the etheric oils with 45 v/v ethylic alcohol, in a 1 g plant to 10 mL solvent ratio, for one week. Further on, the hydroalcoholic extract containing etheric oils has been globally characterized, through refractometric measurements. Determination of the rH was made by a previously mentioned electrometric method, by means of a computerized system of data acquisition. The results obtained are listed in Table 1.

RESULTS AND DISCUSSION

The data of Table 1 may be interpreted in several ways.

Consequently, a first correlation may be established between the tissular and soil's rH (Fig. 1). More precisely, this is characterized by a slope in M – to be detailed in the following – being defined – at least in this point of the investigation – as a dependence of compensatory type of tissular rH on that of the soil.

Table 1

Sample	rH		n
	soil	Plant	
1	22.93	27.40	0.13611
2	30.82	18.73	0.13617
3	32.36	20.77	0.13616
4	29.20	27.795	0.13616
5	31.03	12.47	0.13621
6	30.49	16.975	0.13614
7	32.26	31.38	0.13612
8	32.615	7.005	0.13617
9	32.76	34.89	0.13616
10	32.205	13.86	0.13620
11	30.34	11.19	0.13640
12	30.95	23.595	0.13618
13	36.14	10.90	0.13640
14	26.715	23.62	0.13616
15	28.90	36.355	0.13616
16	33.80	28.745	0.13615
17	34.06	20.83	0.13613
18	34.33	24.86	0.13616
19	32.17	20.08	0.13627
20	32.98	23.75	0.13613

In a similar manner there may be defined, too, the dependence of the content in volatile oils (that may be correlated with the refraction index, n) of the plant, on the soil's rH (Fig. 2).

In other words, both markers – of biochemical nature –, obey their own type of dependence, according to the sufficiently numerous previous data [1, 3, 4, 6].

As to the slope in M, some previously reported aspects [7] should be once again mentioned, namely:

“An organism's reaction to the modification of the environmental rH differs from one level to another. More precisely, at the level at which the primary – such as, nuclear – impact takes place is of the Gaussian type while, at the immediately higher – in this case, cellular – one, it assumes the slope in M – that is, derivative I of a “module” function, resulting from the response of the inferior hierarchical system, to which a continuously positive slope is attributed (Fig. 3: 1, 3 and 2, respectively). Consequently, reaction (1) is defined as a direct one, while (3) is a compensating reaction.

One should be therefore tempted to correlate the biochemical aspect with the biological one through derivation, such as the evolutive shifting from an inferior (integrated) to a superior (integrating) level, as might urge one to do the comparison between the rough biomass, generally, and the refinement of the reserve substance. The same aspect may be discussed between the individual and, respectively, populational level.”

If an attempt is made at evidencing the dependence of the content in volatile oils on the tissular rH, a slope similar to that plotted in figure 4 will result. As one may observe, the process of volatile oils' accumulation is characterized by the existence of an optimum, that is, the highest amounts are biosynthesized in the plant in which, as a result of the interaction with the soil and with its own rH, acquire a tissular rH situated at reducing values, of about 13, namely the one

representing the minimum of figure 1 – a characteristic parameter of the species, as shown elsewhere [6] –, which are plants grown on soils with a relatively oxidative rH – i.e., favourable to them [6] – of about 32.

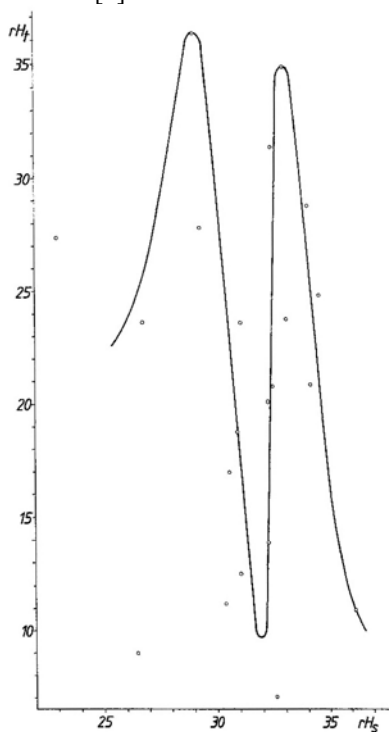


Fig. 1

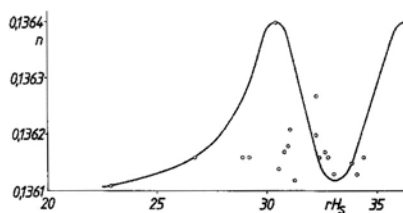


Fig. 2

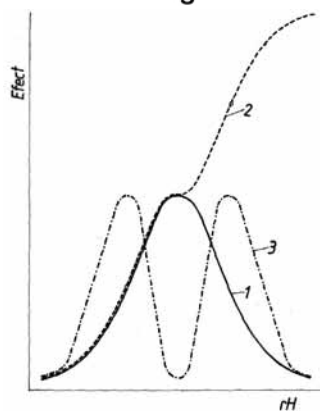


Fig. 3

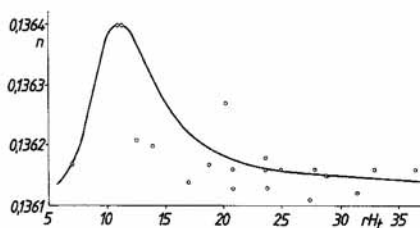


Fig. 4

CONCLUSIONS

The biosynthesis of etheric oils, in *Nepeta nepetella*, depends on the value of plant's tissular rH, its maximum occurring at a rH value around 13. In its turn, it depends on soil's rH, being characteristic to plants grown on soils with rH values of about 32.

REFERENCES

1. Zănoagă C. V., Păun Cristina, Bârleanu Tatiana, 1997, *Studii și cercetări științifice*, Univ. Bacău, Biol., serie nouă, 2, 53-57
2. Zănoagă C. V., *Comunicări și referate*, 1997, Muzeul județean de științele naturii Prahova, Ploiești, 44-49
3. Zănoagă C. V., Zănoagă Mădălina, 2002, *Simpozionul național "Diversitate biologică – biotehnologii – dezvoltare socio-economică"*, Cluj-Napoca, 5-6 dec 2002, 12-13
4. Zănoagă C. V., Zănoagă Mădălina, 2002, *The Second International Conference on Ecological Chemistry*. Abstract book, Chișinău, Oct 11-12, 2002, 197-199
5. Zănoagă C. V., Neacșu I., Zănoagă Mădălina, 1988, *St. cerc. biochim.*, 31, 1, 53-58
6. Duca G., Zănoagă C. V., Duca Maria, Gladchi Viorica, 2001, *Procese redox în mediul ambiant*, Ed. Universității de stat din Moldova, Chișinău
7. Zănoagă C. V., Lungu-Dodu D., Păun Cristina, 1995, *Bul. Soc. Nat. Biol. Cel.*, 23, 130

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A POPULATIONAL RESEARCH REGARDING THE FREQUENCY AND TRANSMISSION OF AB0 BLOOD GROUPS IN THE ROMANIAN REGION BÎRLAD

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IULIANA CSILLA I. BĂRA¹**

Keywords: AB0 blood groups, frequency, populational genetics.

Abstract: Part of a larger study regarding the genetic polymorphisms present in the human population of Romania, we have studied the frequency and transmission of AB0 blood groups in the Birlad region. We have investigated all the children born in Birlad (and resident in the region of birth) during a given year. AB0 blood groups were determined by the Blood Transfusion Centre of Birlad. The obtained frequencies (group 0 = 34,36% group A = 40,28%, group B = 17,46%, group AB = 7,90%) and sex ratios were concordant with previous values obtained for the romanian population. We are discussing various reasons for the slight regional differences of the AB0 blood group frequencies registered between different romanian regions. One of the most important causes of variation could be the materno-fetal incompatibility regarding AB0 blood groups. After performing a thorough family inquiry, our research proved a very limited importance of this mechanism as a generator of populational variability.

INTRODUCTION

AB0 blood groups are ones of the most studied pure inherited traits. Due to their monogenic determinism, AB0 blood groups are fulfilling all the criteria for the optimal genetic study: high frequency, easy to be determine and analysed statistically (Tudose et al., 2000).

Part of a larger study regarding the genetic polymorphisms present in the human population of Romania, we have studied the frequency and transmission of AB0 blood groups in the Birlad region. The motivation of the study resides in the need of a centralised and widespread research covering all Romanian regions. There are also medical and forensic reasons to study thoroughly this subject, such as transfusions, certification of identity, paternity and filiation. The well known correlation between AB0 worldwide distribution in human populations and the biological history of some infectious diseases will be investigated in a future study of ecogenetics, a major concern of the authors.

Our research focused mainly on two directions: the frequency of AB0 blood groups in Birlad region and the study of the hereditary transmission of this monogenic trait in various families. We have also kept in mind and observed when encountered other topics, such as materno-fetal incompatibility, paternity and filiation problems.

MATERIALS AND METHODS

We have investigated a group of 355 teenagers born in Birlad between 1982 - 1985 (and resident in the region of birth) gathered in the same school unit. AB0 blood groups were determined by the Blood Transfusion Centre of Birlad which staff kindly provided us the results. Data were systematised into tables and frequencies were calculated and statistically investigated. We are mentioning the difficulties encountered because of the low addressability for blood group typing of the local population after 1990.

We have conducted family inquiries, we have gathered the data into genetic consultation sheets and we have drawn pedigrees.

RESULTS AND DISCUSSIONS

The frequency of AB0 blood group in the studied population (teenagers born in Birlad between 1982 - 1985 and resident in the region of birth, gathered in the same school unit), n = 355, lead to the following result (fig. 1):

- Group 0 - 34,36%
- Group A - 40,28%
- Group B - 17,46%
- Group AB - 7,90%

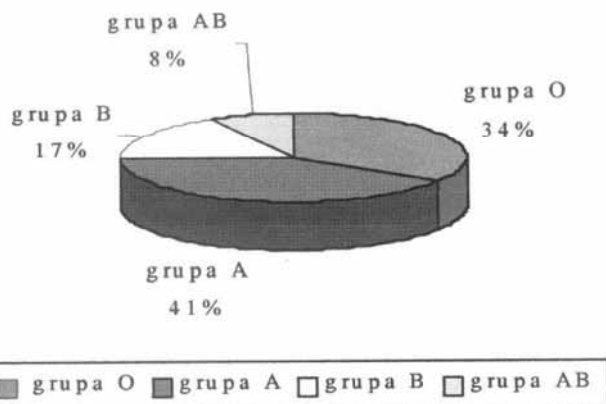


Figure 1: The frequencies of AB0 blood groups obtained in Birlad county

The obtained frequencies and sex ratios were concordant with previous values obtained for the romanian population: groups 0 and B are more frequent in men, while groups A and AB in women.

We also discussed various reasons for the slight regional differences of the AB0 blood group frequencies registered between different romanian regions. One of the most important causes of variation could be the materno-fetal incompatibility regarding AB0 blood groups.

After a thorough analysis of the consultation sheets (fig. 2) and of the drawn pedigrees (fig. 3) our research proved a very limited importance of this mechanism as a generator of populational variability.

Fișa nr.17

Subiectul	Gradul de rudenie	Grupa sanguină	Născut(ă) în	Locuiește în
A.C. ♀		AII	Birlad Jud.Vaslui	Birlad Jud.Vaslui
Frate		O I	Birlad Jud.Vaslui	Birlad Jud.Vaslui
Mama		O I	Com Băcani Jud.Vaslui	Birlad Jud.Vaslui
Mătușa de pe mamă		O I	Com Băcani Jud.Vaslui	Birlad Jud.Vaslui
Unchi de pe mamă		O I	Com Băcani Jud.Vaslui	Birlad Jud.Vaslui
Unchi de pe mamă		O I	Com Băcani Jud.Vaslui	Birlad Jud.Vaslui
Tata		AII	Com Banca Jud.Vaslui	Birlad Jud.Vaslui
Mătușa de pe tata		AII	Com Banca Jud.Vaslui	Constanța Jud.Constanța
Mătușa de pe tata		AII	Com Banca Jud.Vaslui	Com Fedelești Jud.Vaslui
Unchi de pe tata		AII	Com Banca Jud.Vaslui	Com Băcești Jud.Botoșani
Bunica de pe mamă		O I	Com Băcani Jud.Vaslui	Birlad Jud.Vaslui
Bunicul de pe mamă		O I	Com Băcani Jud.Vaslui	Birlad Jud.Vaslui
Bunica de pe tata		AII	Com Banca Jud.Vaslui	Com Banca Jud.Vaslui
Bunicul de pe tata		AII	Com Banca Jud.Vaslui	Com Banca Jud.Vaslui

Figure 2. Exemple of a genetic consultation sheet

The decreased importance of the materno-fetal incompatibility resulted in unions of type 0 x A, 0 x B, 0 x AB might be influenced also by the reduced number of offspring per family.

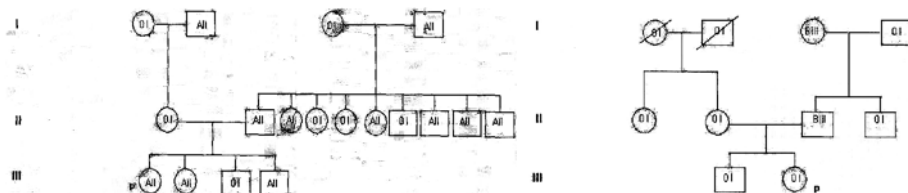


Figure 3: Examples of pedigrees used in our study

CONCLUSIONS

The frequency of AB0 blood group in the studied population (355 teenagers born in Bîrlad between 1982 - 1985 and resident in the region of birth, gathered in the same school unit), $n = 355$, lead to the following result: Group 0 - 34,36%, Group A - 40,28%, Group B - 17,46% and Group AB - 7,90%

The obtained frequencies and sex ratios were concordant with previous values obtained for the romanian population: groups 0 and B are more frequent in men, while groups A and AB in women.

We also discussed various reasons for the slight regional differences of the AB0 blood group frequencies registered between different rumanian regions. One of the most important causes of variation could be the materno-fetal incompatibility regarding AB0 blood groups.

After a thorough analysis of the consultation sheets and of the drawn pedigrees our research proved a very limited importance of this mechanism as a generator of populational variability. The decreased importance of the materno-fetal incompatibility resulted in unions of type 0 x A, 0 x B, 0 x AB might be influenced also by the reduced number of offspring per family.

REFERENCES

- Covic M., Sandulovici I., Ștefănescu D., 2004. Tratat de genetică medicală. Ed. Polirom, Iași.
 Stine J., 1999. The new human genetics. Wilkins and sons, New York
 Tudose C., Maniu M., Maniu C.L., 2000. Genetica umana, Ed. Corson, Iași.

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ECOGENETICS AND PHARMACOGENETICS: THE IMPORTANCE OF GENETIC POLYMORPHISMS IN THE VARIABILITY OF ORGANISMS RESPONSE TO ENVIRONMENTAL FACTORS

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Keywords: ecogenetics, genetic polymorphisms, pharmacogenetics, genomics.

Abstract: Genetics and genomics are certain to have a large impact in drug development and proper pharmaceutical treatment of subgroups of patients with many specific diseases. We should be able to increase the therapeutic margin for many agents. Genetic variation will also be important in refining estimates of risk from all kinds of environmental agents and in choosing more effective and more cost-effective risk reduction strategies. The linkage of information about genetic variation and information about environmental, nutritional, behavioral, metabolic, medical, and healthcare factors will be necessary to interpret the variation in clinical and public health terms. However, there is a great risk that present efforts to protect confidentiality and privacy of individual genetic information may make such research infeasible. In the present paper we expose some general considerations about the importance of the borderline disciplines which are studying the cited aspects (ecogenetics, pharmacogenetics and pharmacogenomics), emphasising the importance of human populations genome polymorphisms affecting drug efficiency and producing adverse reactions; eventually we expose the most recent trends in pharmacogenomics related to the subject.

INTRODUCTION

George Brewer of the University of Michigan introduced the term "eco-genetics" in 1971, and many others helped develop this field (Motulsky G., 2002). As described in many papers, there are striking examples of genetic variation in responses to foods, food additives, alcohol, cigarette smoking, and other agents, as well as pharmaceuticals. Various state regulatory agencies are now increasingly interested in research data about variation in susceptibility within highly heterogeneous human populations, to improve the basis for health protection and to replace arbitrary, generally extremely conservative safety factors and related assumptions in risk estimates (Omenn A.G., 2001). This approach aims to overcome the predominant regulatory strategy of dealing with one chemical at a time, in one environmental medium (air, water, food, soil), and each health risk (cancer, birth defects, liver toxicity) in isolation. During the past decade there were issued numerous recommendations for each of the various agencies that regulate chemical hazards and reinforced strategies for risk communication.

One of the major challenges arising from studies of polymorphic genetic variation with particular cancers or other diseases is interpreting the inconsistency of associations reported; such as an experience with multiple ancillary studies in the lung cancer chemoprevention trial, CARET (Omenn G.A., 2001). Partly this problem reflects ethnic differences in gene frequencies and marked heterogeneity of the causes of common diseases. However, some combinations of P450 and glutathione S-transferase variants, or combinations of N-acetyltransferases and smoking history, shed light on what will surely become a general phenomenon: that we must investigate an array of relevant genes and the interacting environmental factors, not just single genes in isolation, to understand the predispositions to common diseases. The new technologies presented surely will facilitate such research and permit its application in medicine, public health, and environmental policy as we moved into the new millennium.

Even it is a branch of ecogenetics, pharmacogenetics is older and much "richer"; the origin and development of pharmacogenetics are traced with emphasis on early hints by Garrod, Haldane, and later by RJ Williams. The field was delineated by Motulsky in 1957 and described as pharmacogenetics by Vogel in 1959. Kalow's monograph (1962) definitely established the discipline. Resemblance of identical twins in drug metabolism as compared with non identical twins (Vesell, 1970) established the general importance of polygenic inheritance in disposal of many drugs. Ecogenetics was defined by Brewer in 1971 as dealing with genetic variation affecting the response to any environmental agents with emphasis on xenobiotics. More recent developments have broadened pharmacogenetic approaches to include novel genomic techniques with introduction of the term pharmacogenomics in the 1990's (Motulsky, 2002).

Genetic and genomic approaches (toxicogenetics and toxicogenomics) are also being applied in the "environmental genome project". The interaction of genetic variation with dietary factors led to the field of Nutritional ecogenetics (Nutrigenomics) which relates the role of genetics to nutritional requirements and nutrition-mediated susceptibility to chronic disease. The total promise of pharmacogenomics is often overstated. The field is likely to have an impact on choice of drug therapy and avoidance of adverse events but is unlikely to lead to a revolution in therapeutics. Aspects of pharmacogenomic approaches and its applications including problems of premature commercialization are discussed.

ECOGENETICS: THE ROLE OF GENETICS IN ENVIRONMENTAL RISK ASSESSMENT AND RISK MANAGEMENT

The differences noted in what concerns the efficacy of some therapeutically agents in normal humans and their risk of toxicity are determined by the presence of many allele which code for enzymes distinct in their metabolic activities. These constitutions are referred to as genetic polymorphisms.

Ecogenetics is dealing with the role of such genetic polymorphisms in the variability of organisms response to environmental factors. The result of such variability is a genetic vulnerability (susceptibility) in front of the environmental aggression. Ecogenetical diseases are produced by the interaction of genetic vulnerability and environment aggression.

In accordance with the environmental agents involved, one may describe the following domains:

- infections ecogenetics
- nutritional ecogenetics
- physical ecogenets
- chemical ecogenetics (including the most developed branch pharmacogenetics).

Knowledge from pharmacology and toxicology can be linked on a mechanistic basis to anticipate the polymorphic biotransformation enzymes and polymorphic receptors and other sites of action that would be relevant to new drugs and to environmentally encountered chemicals. An example is the metabolism of benzo(a)pyrene, a pro-carcinogen of the polycyclic aromatic hydrocarbon class of compounds common in combustion effluents and cigarette smoke. Benzo(a)pyrene is activated successively by cytochrome P450s and mitochondrial epoxide hydrolase to the benzo(a)pyrene-9, 10-diol-epoxide, the potent carcinogenic intermediate; several pathways serve to detoxify the carcinogenic derivatives (Omenn G.A., 2001).

Variation in susceptibility to chemical, infectious, and physical agents encountered in the workplace and in other environments increasingly is being recognized as an important variable in environmental and occupational medicine and environmental risk management.

PHARMACOGENETICS AND PHARMACOGENOMICS: HUMAN GENOME POLYMORPHISMS AND THEIR EFFECT ON THE RESPONSE TO DRUGS

It is well known in the medical practice that some pharmacological agents are more effective fore some humans in comparison to other. The individual variation of the response to drugs is a very important clinical problem; interindividual differences extend from the absence of the response to a specific pharmacological agent, till the sudden apparition of an adverse reaction.

The clinical consequences can vary from simple to severe symptoms, even exitus. A study performed in United Kingdom suggests that one in fifteen hospitalisations is due to adverse reactions to drugs; another study performed in USA estimates that 100,000 patients die and another 2.2 million are affected because of adverse reactions to drugs (Wolf et al., 2000).

Numerous factors, including genetics, affect drug metabolism and thus alter the bioavailability of therapeutic drugs. The best studied metabolizing enzymes are the cytochrome P450 (CYP450) isoenzymes, the N-acetyl transferase (NAT) isoenzymes, the UDP-glucuronosyl transferases, and the methyl transferases. Of these enzymes, the CYP450s are very important because they metabolize drugs into products that are readily excreted into the urine and faeces. In humans, six different forms of CYP450 (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) are largely responsible for eliminating drugs.

The rate of metabolism by several of the cytochrome CYP450 enzyme subfamilies varies, due to genetically-determined polymorphisms in all populations studied. Recent research using phenotyping and genotyping techniques has reflected the interest and importance of these pharmacogenetic factors in determining drug responses. Some of the metabolizing enzymes such as CYP1A1, 1A2, 2A6, 2C9, 2C19, 2D6, 2E1, NAT1, NAT2 and NQO1 exhibit genetic polymorphism and alter responses to drugs.

These metabolic polymorphisms are determined by gender (e.g. CYP1A2) and racial/ethnic origin. Increased CYP1A activity (an enzyme catalysing a phase I oxidation reaction), coupled with slow acetylation (a phase II conjugation reaction), resulted in less myelosuppression from the active metabolites of the drug amonafide.

Because every individual represents a combination of drug-metabolizer phenotypes, given the large number of enzymes involved in drug metabolism, it is apparent that some individuals are likely to have unusual reactions to drugs, or to combination of drugs, due to the coincident occurrence of multiple genetic defects in drug-metabolizing enzymes. Such an alignment of genotypes, particularly when coupled with polymorphisms in drug receptors, is likely to constitute part of the mechanism for the so-called 'idiosyncratic' drug reactions.

Although no evidence to date suggests the CYP3A4 isoenzyme exhibits genetic polymorphism, in recent years there has been much discussion about the 3A4 system because of life-threatening arrhythmic side-effects that can occur as result of enzyme inhibition and accumulation of the antihistamines terfenadine, astemizole and cisapride. Terfenadine has been removed from the market because of its serious cardiovascular drug interactions.

Concerning CYP2C9, recent data suggest that patients who require low doses of warfarin (1.5 mg/day) carry point mutations (alleles CYP2C9*2 and CYP2C9*3) at the gene coding for CYP2C9 (which could occur at a frequency of 21% in the general population). These patients metabolized warfarin poorly, and responded to small doses of the drug with greater lengthening of the prothrombin time and higher international normalized ratio (INR) values than did carriers of the wild-type allele CYP2C9*1. Genetically determined high-responders to warfarin had bleeding complications four times more commonly than did a control group stabilized on larger doses of the drug. Knowledge of carriage of the hyper-responsiveness alleles of CYP2C9*2 and CYP2C9*3 might help the clinician to decide against the use of warfarin (in favour of other coumarin derivatives such as phenprocoumon and acenocoumarol, the metabolism of which is less influenced by CYP2C9), particularly in high-risk elderly patients (Meyer U.A., 2000).

In addition to variation in drug metabolism or pharmacokinetics, the genetic variations in receptor function (and thereby pharmacodynamic effects) are important. Subtle differences in the sequences of receptor subtypes for dopamine, serotonin and catecholamines may result in individual differences in behavior and drug responses. Overall, a highly complex picture emerges in which genetic variation in both pharmacodynamic and pharmacokinetic factors contributes to drug responses. Some patients do not respond to a given drug because it is not processed efficiently; other patients do not respond because the disease gene defects or its pathway is not targeted by the drug (Tudose and Patraș, 2003).

Great progress has been made in understanding the molecular genetics of acetylation as well as the clinical consequences of being a rapid or slow acetylator. Inborn errors (several different alleles) at the hepatic arylamine N-acetyltransferase-2 (NAT2) locus are responsible for the traditional acetylator polymorphism. Rapid and slow acetylators reflect the genetically determined variation in the elimination of xenobiotics, as well as in NAT2 activity in the liver and other tissues. The human NAT2 gene contains an 870 bp intronless protein-coding region To

date, one allele with a code for fast acetylation (wild-type) and several mutated alleles with codes for impaired acetylation activity have been discovered. Of all the NAT2 allelic variants that had been identified, three (NAT*5, NAT*6 and NAT*7) account for majority of the slow NAT2 acetylator genotype in White subjects. N-acetylation status seems to be associated with several kinds of diseases, such as colon cancer, rheumatoid arthritis, and systemic lupus erythematosus (Farlow C.A., 1996).

The independent genetic feature as a rate of acetylation was shown to be related to the immunological system dysfunction. It may be one of the factors that makes an individual susceptible to the development of an atopic disease, and one study showed that up to 80% of individuals with chronic allergic rhinitis had a slow acetylation phenotype. A recent study which assessed the influence of NAT2 polymorphism on the risk of development of atopic disease also suggests that the risk of development of atopic diseases was five-fold greater for homozygous slow acetylators compared to healthy subjects, and that slow acetylation genotype may be an important factor of individual susceptibility to atopic diseases. This group of patients may also be at increased risk of adverse reactions after using drugs which are mainly metabolized by acetylation reaction. Among them, the mechanism of hypersensitivity to sulfonamides typical for slow acetylators seems to be of particular importance (Kallow W., 1997).

Consideration of the genetic characteristics leads to population segmentation into groups, the slow metabolizers (having a slow metabolism) and fast metabolizers (having a normal metabolism). For example, in some Asian populations the incidence of poor metabolizers of the gastrointestinal drug omeprazole (due to polymorphism in CYP2C19) is 15–23%, compared to 2.5–6% in Caucasians. In individuals with a poor-metabolizer genotype for CYP2C19, the therapeutic efficacy of omeprazole (a proton-pump inhibitor widely used as acid inhibitory agent for the treatment of upper gastrointestinal diseases and metabolized by CYP2C19) may be increased. In patients with a poor-metabolizer phenotype or genotype of CYP2C19, the area under the plasma concentration-time curve of omeprazole is markedly increased, and the clinical effect of omeprazole is greater. Acid secretion in patients with a poor metabolizer status of CYP2C19 who are undergoing an omeprazole therapy is therefore assumed to be more strongly inhibited than those with the extensive metabolizer status. Cure rates for *Helicobacter pylori* were noted to be 28.6%, 60% and 100% in the rapid-, intermediate-, and poor-metabolizer groups, respectively (Wolf et al., 2000).

The results of the genotyping test for CYP2C19 seem to predict the cure of *Helicobacter pylori* infection and peptic ulcer in patients who receive dual therapy with omeprazole and amoxicillin. A recent study designed to determine whether the effects of omeprazole on intragastric pH depends on CYP2C19 genotype status confirmed that after omeprazole administration, significant differences in mean intragastric pH values and plasma levels of gastrin, omeprazole and its metabolites were observed among the three groups of volunteers (homozygous extensive metabolizers, heterozygous extensive metabolizers and poor metabolizers), whereas no significant differences in these parameters were observed with the placebo administration. Both the individual omeprazole AUC and mean intragastric pH values were greater in the poor metabolizer group compared with those in the homozygous extensive metabolizer and heterozygous extensive metabolizer groups. The results confirmed that the effects of omeprazole on intragastric pH significantly depends on CYP2C19 genotype status, and also suggest that the genotyping test of CYP2C19 may be useful for an optimal prescription of omeprazole (Levy A.R., 1993).

Low metabolic activity of the CYP2D6 enzymes is inherited as an autosomal recessive gene and although CYP2D6 represents only about 1.5% of the total liver enzymes, it is involved in the metabolism of a number of commonly used drugs. There are now more than 20 identified variant CYP2D6 alleles which contribute to the variation in CYP2D6 metabolism. The most common allelic variations associated with poor-metabolizers in Caucasians are CYP2D6*4 (75%), *3 (5%) and the gene deletion *5 (15%). For drugs in which CYP2D6 plays a predominant role in metabolism, poor-metabolizers will have high plasma concentrations and report the most severe adverse reactions (Meyer U.A., 2000).

Studies in Caucasian extensive-metabolizers and poor-metabolizers have uniformly demonstrated a 2- to 5-fold difference in the capacity to metabolize CYP2D6 substrates, such as antidepressants and neuroleptics. On the other hand, non-Westerners (Asians and Indians) may require lower doses of several classes of psychotropics that are metabolized by CYP2D6 (e.g. conventional neuroleptics and tricyclic antidepressants) than do Westerners. The poor-metabolizers lack this enzyme as a result of an autosomal recessively transmitted defect in its expression. When drugs are converted to an active metabolite by 2D6 (e.g. conversion of codeine to morphine), the drug may be ineffective in poor-metabolizers. Although significant interactions between 2D6-metabolized drugs with the well-known inducers rifampin and antiepileptics have been described, specific inducers of 2D6 have yet to be clearly identified. Administration of dextromethorphan followed by measurement of O-demethylated metabolite excretion in urine is an accurate and non-invasive way of phenotyping individuals as either extensive-metabolizers or poor-metabolizers for 2D6 activity (Levy A.R., 1993).

Many opioid analgesics are activated by CYP2D6, rendering the 2–10% of the population who are homozygous for non-functional CYP2D6 mutant alleles relatively resistant to opioid analgesic effects. It is thus not surprising that there is remarkable interindividual variability in the adequacy of pain relief when uniform doses of codeine are used (Mungiu et al., 2000).

Thiopurine methyltransferase (TPMT) is a cytosolic enzyme that catalyses the S-methylation of aromatic and heterocyclic sulfhydryl compounds, including the thiopurine drugs 6-mercaptopurine (6-MP) and 6-thioguanine. Thiopurines are used to treat patients with neoplasia and autoimmune disease as well as recipients of transplanted organs. The TPMT genetic polymorphism may represent a striking example of the potential clinical importance of pharmacogenetic variation in expression of a drug-metabolizing enzyme. Individuals with genetically very low levels of TPMT activity are at a greatly increased risk for potentially life-threatening toxicity when exposed to standard doses of thiopurines, while those with very high levels of this enzyme activity may be undertreated with the same dosages of these drugs (Katz D.A., 2002).

Recent genetic data suggest that the active gene for the TPMT enzyme is ~34 kb in length, consists of 10 exons and has been localized to chromosome band 6p22.3. The wild-type allele for high TPMT activity has been designated TPMT*1, and to date eight variants for very low TPMT activity have been reported (Kallow W., 1997). The most common of these in Caucasians, TPMT*3A, represents 55–70% of all variant alleles for very low activity. TPMT*3A contains two point mutations, G460A and A719G, resulting in Ala154Thr and Tyr240Cys amino acid substitutions, respectively. However, because of the clinical significance of inherited variation in levels of TPMT activity, characterization of as many variant alleles responsible for very low TPMT activity as possible will be necessary so that DNA-based diagnostic tests can be compared with the phenotypic test presently used to individualize therapy with thiopurine drugs. The

ultimate aim is to minimize toxicity and improve the therapeutic efficacy of this important class of pharmacotherapeutic treatments (Levy A.R., 1993).

Bronchodilator responsiveness to β_2 -adrenergic receptor agonists in patients with asthma varies considerably and several missense mutations in the coding region of the β_2 -adrenergic receptor gene have been identified. Farlow C.A., 1996 Among the general population (including patients with asthma), β_2 -adrenergic receptor alleles are distributed in the following approximate proportions: homozygous Arg (Arg16/Arg16), 15%; heterozygous (Arg16/Gly16) 38%; homozygous Gly 16 (Gly 16/Gly 16), 45%; homozygous Gln27 (Gln27/Gln27), 26%; heterozygous (Gln27/Glu27), 49%; and homozygous Glu27 (Glu27/Glu27), 22%. The Gly6 allele has been associated with enhanced agonist-promoted β_2 -receptor down-regulation, whereas the Glu27 allele showed minimal down-regulation compared with the Arg16 and Gln27 alleles. Although asthma is primarily an inflammatory disease of the airways, mutations in the β_2 -adrenergic receptor may be risk factors in certain asthma phenotypes.

The variation in cytochrome drug-metabolizing genes that correlates with patients' adverse response or non-response in clinical trials need to be considered. This information could be used to stratify clinical trials, leading to higher efficacy and limiting adverse reactions (Kuivenhoven A.S., 1998).

Ultimately, detailed information about each patient's genetic variants relevant to drug treatments might eliminate the use of ineffective or even dangerous treatments. Prognosis of patients will be more informed, because more precise information on the aetiology of the illness, its pathophysiology and the effectiveness of therapeutic interventions will be available. Thus, the incorporation of pharmacogenetic information into trials as early as possible is recommended and appears very useful for effective drug development (Ruano-Ravina et al., 2002).

ETHICAL AND LEGAL IMPLICATIONS

Much of the excitement surrounding pharmacogenomics stems from the possibility of improving the safety and efficacy of drug interventions. Additionally, by conducting clinical studies in genetically homogeneous populations, it should be possible to use smaller, faster, and cheaper clinical trials. There is even the possibility that certain drugs that have failed clinical trials in broad populations could be "rescued" and demonstrated to be safe and effective when used only by individuals with certain genotypes.

Along with the mixture of hype and hope, the reality is that pharmacogenomics presents a number of challenges from an ethical, legal, and policy standpoint. Among these challenges are 1) the ethical, economic, and policy implications of market segmentation, 2) the ethical and social issues surrounding research in pharmacogenomics, including the generation of sensitive genetic information, and 3) the political challenge of ensuring equal access to beneficial pharmaceutical products developed through pharmacogenomics .

Most of the pharmacogenomic research is currently at the preclinical stage. Both at this stage and the later clinical research stage, an important, but generally unexplored, issue is whether the target population is supportive of the research. In particular, it is important to consider 1) whether individuals are willing to participate in research by donating biological samples and sharing medical records with investigators, 2) whether individuals are willing to undergo genetic testing as part of the research process, 3) whether individuals have suspicions about the medical research establishment, 4) whether concerns about privacy and confidentiality will cause individuals to decline to participate in research, and 5) whether individuals are concerned about the morality of research into human genetic variation. Although these concerns are certainly common to all

clinical trials, they warrant mention here because of the unique concerns centering around genetics in general (Omenn G.A., 2001).

As the research proceeds to the clinical stage, it will be important to develop inclusion and exclusion criteria based on genotype. One important issue is the ethics of including random or "nonmatched" controls in the studies (i.e., individuals whose genotypes do not suggest favorable responses). Informed consent will also be a major concern, including how researchers inform potential participants about the possible economic and social consequences of the research, including possible group-based harms. In this regard, the idea of community consultation before performing research in discrete ethnic groups has been debated in the literature.

From a policy perspective, as pharmaceutical companies segment the market, it may become economically impossible to pursue drug development for individuals with rare genotypes. Consequently, some governmental subsidies akin to those under the Orphan Drug Act may be necessary to encourage the development of "small market" drugs (Omenn G.A., 2001).

Over the next several years, as pharmacogenomic based medications become available, will managed care plans include them in their formularies? Most companies can be expected to undertake a detailed cost-benefit analysis to determine whether the incremental benefits are worth the incremental costs. Even if they are cost-effective, it remains to be seen whether the costs will be borne by consumers or third-party payers and how increased pharmaceutical costs will affect access to health care in general.

Finally, whenever the standard of care in medicine changes there is an increased possibility of liability for those providers who fail to meet the new standard of care. For physicians, the range of possible liability issues includes the failure to order the appropriate genetic tests or to interpret them and explain them to patients properly, the duty to warn patients of possible genotype-specific side effects of medications, and the possible issue of failure to warn at-risk relatives. To meet this heightened standard of care it will be necessary to include instruction in pharmacogenomics in schools of medicine, nursing, pharmacy, and other health care fields, as well as to include new developments in continuing education courses. Pharmacogenomics is a very promising avenue of research, but we must be careful to make sure that there are no unintended social consequences from introducing this technology (Omenn G.A., 2001).

CONCLUSIONS

Building upon the historical approaches taken in the field of eco- and pharmacogenetics, the information resource gained from the completion of the Human Genome Project, coupled with the development of high-throughput technologies, the development of sophisticated analytical tools, and the identification of relatively specific *in vivo* phenotypic markers, provides the potential for this field to make rapid advances.

New gene targets for therapeutic intervention only provide a starting point in the long and difficult process of drug discovery. However, genomics will have an important impact in the later stages of drug development, especially in providing an understanding of the molecular nature of diseases and of the responses, both desirable and adverse to drugs.

Modern genetics will bring about significant improvements in the provision and practice of healthcare by redefining disease and targeting treatment. It will also lead to the discovery of novel targets and effective treatments and the provision of more effective preventative healthcare.

The therapeutic industry will soon be entering a time when solutions to therapeutic problems can be targeted to the individual. Using knowledge of gene functions and commercially available genomics tools, a genomics consumer will be able to employ focused, high-speed technologies

that will produce an individualized treatment in a short period of time. This is a fundamental change in research and clinical medicine.

REFERENCES

1. Farlow C.A. , 1996. *Ann NY Acad Sci*, 802:101 –110.
2. Kuivenhoven A.S., 1998. *N Engl J Med*, 338: 86-89.
3. Kalow W. , 1997. *Pharmacological Reviews*, 49,4: 369-380.
4. Katz D.A. , 2002. *Drug Information Journal*, 36:751-761.
5. Levy A.R. ,1993. *National Pharmaceutical Council*, 1: 1-21.
6. Meyer U.A. , 2000. *Lancet*, 356 (9242):1667-71.
7. Motulsky AG., 2002. *Med Secoli*. 14(3):683-705.
8. Mungiu O.C., Paveliu F., Paveliu S. , 2000. *Farmacologie biochimică – o abordare interdisciplinară*, Ed. Infomedica, București.
9. Omenn G., 2001. *JASP29* (2):611-614
10. Ruano-Ravina A., Figueiras A, Barros-Dios J.M. , 2002. *Drug information Journal*, 36: 725-726.
11. Tudose C., Patraș Xenia, 2003. *Juventostomatologia*, Supliment al Revistei de Medicină Stomatologică, 1:324-331.
12. Wolf C.R., Smith G., Smith R.L., 2000. *BMJ*, 320: 987-990.

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DOWN SYNDROME IN BACĂU COUNTY

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Keywords: Down syndrome

Abstract: According to a study on a lot of 27,623 children from Bacău county 23 cases of Down syndrome were found – 7 girls and 16 boys. This syndrome has been found at children between 2 months and 10 years old. These cases are coming from the rural environment – 16 cases and from the urban environment – 7 cases. Among the children with Down syndrome, who had also congenital malformations of the heart, it has been noticed that 3 deceases occurred at boys under the age of 1 year.

INTRODUCTION

Down syndrome or trisomy 21 syndrome is the most frequent and well – known chromosomal disease. The incidence of trisomy 21 has been estimated at 1/650 – 1/704 newborn babies (according to BRAY et. al., 1998), and the frequency of the products of conception having this trisomy is increased to 1/200 from which $\frac{3}{4}$ are eliminated as spontaneous miscarriages. The disease is more frequent on male children, the sex ratio is 3 boys to 2 girls (COVIC, 2004). Down syndrome can be clinically diagnosed in the neonatal period or at the newborn children, because of the important abnormalities which, event that they can vary from patient to patient, all realize a phenotypical feature which has led to an improper term of “mongols”. Because of the seriousness of this disease, of the “position” of handicap that the affected persons have and also of social implication of this matter – keeping alive a child with Down syndrome is estimated around 250,000\$ (CEBOTARI et. al., 1998) so a great importance must be granted to the prevention of this syndrome by genetic advice and examination. To realize this is very important to study the cases which are in the analysed area.

MATERIAL AND METHODS

During 1998 – 2003, it has been made researches on a lot of 27,623 subjects represented by children with ages between a couple of weeks and 16 years old from the County Hospital of Peditry Bacău. In order to discover these cases, it has been realized the clinical examination of the patients who have presented 6 features (COVIC, 2004) from 10 features of Down syndrome (MAXIMILIAN, 1996). Depending of the mother’s age – over 35 years old (HUNTER, 2001) and the presence/absence of such cases in the target family (other children born with this disease) for a certainty diagnosis will be ruled some genetical tests the cariotype of the person (BURKE, 2002). The results of these investigations are shown in the tables 1 – 5 and figure 1.

RESULTS AND COMMENTS

After the research that has been made in the County Hospital of Peditry Bacău, we found and then there were confirmed and reported 23 cases of Down syndrome (table 1).

Table 1. The frequency of cases of Down syndrome

Year	Number of subjects	Cases of Down syndrome	The frequency of cases (‰)
1998	5183	2	0,385
1999	4878	3	0,615
2000	4819	3	0,622
2001	3919	7	1,786
2002	4413	-	-
2003	4411	8	1,813
1998 – 2003	27623	23	0,832

From these cases, a number of 7 are girls and 16 are boys, confirming in this way the information from medical literature which shows that the cases with Down syndrome are more frequent at boys (table 2).

The most cases are recognized at birth or immediately after birth, but there are a lot of cases recognized later because of the neglect of the child's parents or due to their ignorance. In what regards the distribution of the cases of Down syndrome according to the group of ages when this disease was confirmed and recognized, we noticed that the group of age with the most cases of Down syndrome was 0-1 year (figure 1). These children, because of their phenotypical feature, are under the suspicion of the existence of this disease.

Table 2 The distribution and the frequency of cases of Down syndrome on sexes

Year	Cases of Down syndrome	The number and the frequency of cases (%)			
		girls		boys	
1998	2	1	50	1	50
1999	3	1	33,33	2	66,67
2000	3	1	33,33	2	66,67
2001	7	3	42,86	4	57,14
2002	-	-	-	-	-
2003	8	1	12,5	7	87,5
1998 – 2003	23	7	30,43	16	69,57

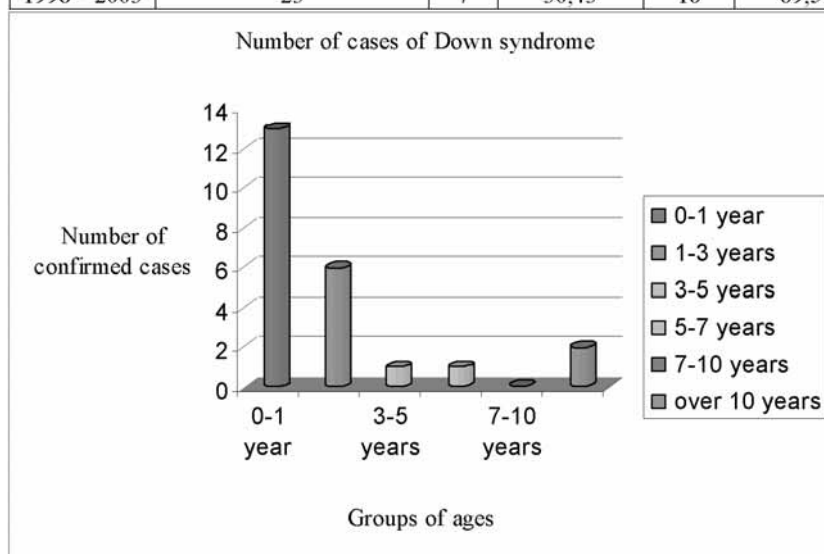


Figure 1 The distribution of Down syndrome according to the ages of children

According to the information from table 3, the 23 cases of Down syndrome found, are classified on groups of ages as following: 0-1 year – 13 cases; 1-3 years – 5 cases; 3-5 years and over 10 years – 2 cases and 5-7 years – one case. It is noticed that regarding group of ages 7-10 years there are attending school and so the incidence of being discovered is increased.

Table 3 The distribution and the frequency of Down syndrome according to the groups of ages

Year	Number of cases	Groups of ages and their frequency (%)						
		0-1 year	1-3 years	3-5 years	5-7 years	7-10 years	over 10 years	
1998	2	1	50	1	50	-	-	-
1999	3	2	66,67	1	33,33	-	-	-
2000	3	1	33,33	1	33,33	1	33,34	-
2001	7	4	57,14	2	28,57	-	-	-
2002	-	-	-	-	-	-	-	-
2003	8	5	62,5	-	1	12,5	1	12,5
1998 – 2003	23	13	56,52	5	21,74	2	8,7	1

The cases of Down syndrome are coming from older parents (especially the mother), have many brothers and sisters, the low level of culture and education of the parents, even if they discover that their child is abnormal (malformations, troubles of reception and speak or mental retardation) and then the parents abandon the children to their grandparents and they didn't put the child in a recovery institution. Also, it is a very serious fact that the child is in impossibility of having medical examination and advice, recovering and mentenance treatment.

According to the table 4, it is noticed that most cases of Down syndrome are coming from the rural environment – 16 cases and from the urban environment are only 7 cases, from which 3 cases are from Bacău city. So, the distribution of the found and confirmed cases with Down syndrome in rural environment is the following: 4 cases in Traian; 2 cases in Nicolae Bălcescu and one case in Mărgineni, Luizi – Călugăra, Parincea, Răcăciuni; Sănduleni; Păncești, Negri, Secuieni, Lipova and Brusturoasa. The distribution in urban environment is the following: 3 cases in Bacău; 3 cases in Buhuși and one case in Comănești.

Table 4 The frequency of Down syndrome in the rural and urban environment

Year	Cases of Down syndrome	The number and the frequency of cases (%)					
		Urban environment				Rural environment	
		Bacău	Other cities				
1998	2	-	-	-	-	2	100
1999	3	-	-	-	-	3	100
2000	3	-	-	-	-	3	100
2001	7	1	14,29	4	57,14	2	28,57
2002	-	-	-	-	-	-	-
2003	8	2	25	-	-	6	75
1998 – 2003	23	3	13,04	4	17,4	16	69,56

During 1998 – 2003, there were noticed 3 deceased children with Down syndrome (table 5). The deceases are coming only from the boys under one year, so they died at short time after they were born because of the existence of a congenital malformations of the heart which went to inevitable death. The deceased children are coming from the rural environment – Negri, Luizi – Călugăra and one from the urban environment – Buhuși. These children didn't leave hospital after their birth, being monitorised and investigated till their death occurred.

Table 5 The frequency of deceases in Down syndrome

Year	Cases of Down syndrome	The number and the frequency of cases (%)					
		Urban environment				Rural environment	
		Bacău	Other cities				
1998	2	-	-	-	-	2	100
1999	3	-	-	-	-	3	100
2000	3	-	-	-	-	3	100
2001	7	1	14,29	4	57,14	2	28,57
2002	-	-	-	-	-	-	-
2003	8	2	25	-	-	6	75
1998 – 2003	23	3	13,04	4	17,4	16	69,56

The cases with Down syndrome which have been found, have also a lot of other complications which deteriorate the evolution such as: congenital malformations of the cord – 12 cases; congenital megacolon – one case, malformation of the diaphragm – one case; congenital sprain of the hip – one case and different stages of mental retardation, from which 2 of them very serious ones.

CONCLUSIONS

During 1998 – 2003 in Bacău county has been found, confirmed and reported a number of 23 cases of Down syndrome among the children between 2 months and 10 years old.

The cases of Down syndrome are distributed on sexes as following: 7 girls and 16 boys who have a severe or a less severe mental retardation.

From all the cases found with Down syndrome, it has been noticed a number of 3 deceases of some children who had congenital malformations of the cord, they were boys of small age coming from both rural and urban environment.

Taking care of the children with Down syndrome raises many problems, both affective and social ones. It is necessary a continuous psychological and adequate support of the parents in order that they can deal with the problems faced in bringing up such a child. So for this reason is very important the realizing of a prenatal diagnosis in order to inform the parents what have they must face and to give them the genetic advice in these circumstances.

REFERENCES

1. BRAY, I., WRIGHT, D.E., DAVIES, C., HOOK, E.B., 1998. *Prenatal Diag.*, Joint estimation of Down syndrome risk and ascertainment rates: A meta-analzsis of nine published data sets, 18, 9 – 20.
2. BURKE, W., 2002. Genetic testing, *N Engl J Med*, 347, 1867 – 1875.
3. CEBOTARI, L., COTORCEA, V., GROPPA, S., 1998. *Genetica generală și umană. Biopsia corionului în diagnosticul prenatal a patologiilor cromozomale*, F.E.P. Tipografia Centrală, Chișinău, 53 – 55.
4. COVIC, M., 1992. *Genetica umană*, Iași, 86 – 94.
5. COVIC, M., ȘTEFĂNESCU, D., SANDOVICI, I., 2004. *Genetica medicală*, Polirom, Iași, 607.
6. HUNTER, A.G., 2001. *Management of Genetic Syndromes*, Ed. Cassidy SB, Allanson JE, Wiley-Liss, New York, 103 – 129.
7. MAXIMILIAN, C., IOAN, D.M., 1986. *Genetica medicală*, Ed. Medicală, București, 512.
8. MAXIMILIAN, C., POENARU MOTOC, L., BEMBEA, M., 1996. *Genetica clinică*, Ed. Pan – Publishing House, București, 144.

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HAEMOPHILIA IN BACĂU COUNTY

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Keywords: haemophilia

Abstract: It has been made a research by comparison of the cases of haemophilia at infants from Bacău county. During 1998 – 2003 6 cases of haemophilia were discovered amongst a lot of 27,623 subjects that have been investigated. The 6 cases of haemophilia found and confirmed are distributed as it follows: 3 cases of haemophilia type A, 2 cases haemophilia type B and one case of vascular haemophilia. These cases are children of age over 10 years old and who come mostly from the urban environment.

INTRODUCTION

Haemophilia is a trouble of coagulation due to some deficiencies of the VIII factor and/or IX and XI factors of the coagulation. The factors of coagulation – plasmatic proteins – interfere and obstruct the wound in the case of an affected blood vessel. Any abnormality and the quality or quantity of these factors leads to haemophilia. The following types of haemophilia are known:

- Haemophilia type A is a trouble in coagulation due to the chromosome X and a deficiency of the VIII plasmatic factor. Its incidence is 1/5,000 – 10,000 of male newborns and on female newborns are very rare cases;
- Haemophilia type B is a trouble in coagulation due to the chromosome X and a deficiency of the IX plasmatic factor. It is from 5 to 10 times less frequent then the other form (WALTER – ROȘIANU, 1986);
- Haemophilia type C is a trouble in coagulation due to the deficiency of the XI plasmatic factor and it can be transmitted autosomal recessive;
- Vascular haemophilia (VON WILLEBRAND disease) is due to the abnormalities of the VIII factor, adding a slight deficiency of the IX/XI factors. Its transmission is autosomally dominant (GEORMĂNEANU, 1986).

MATERIAL AND METHODES

During 1965 – 1975 in Bacău county were discovered 18 cases of haemophilia from which 16 haemophilia type A and 2 cases haemophilia type B from a lot of 60,000 subjects. In order to discover these cases it has been applied the clinical examination in order to explore: the plasmatic and thrombocytic factors, blood investigations – haemoleukogram and the determination of the blood group, testing the sight for distinguishing the colours, radiological investigations regarding the bone system, but also doing the family investigation in order to realise the genealogical tree (ANTON, 1975).

After 30 years, during 1998 – 2003 a number of 6 cases of haemophilia were found, by investigation a lot of 27,623 subjects represented by children with ages between a couple of days and 16 years old. These patients have been discovered after they have suffered some injuries and the paraclinical examination has confirmed the existence of haemophilia. The diagnosis of certainty was confirmed after the determination of the VIII or IX factor of coagulation (ȘTEFĂNESCU, 2004). After studying the background (pedigree) of the family, the existence of some maternal relatives who had different types of haemophilia was detected.

RESULTS AND COMMENTS

The 6 cases of haemophilia discovered during 1998 – 2003 (table 1) are distributed as following: 3 haemophilia type A, 2 haemophilia type B and one case of vascular haemophilia.

Table 1 - The frequency of haemophilia in Bacau county

Year	Number of subjects	Cases of haemophilia	The frequency haemophilia (‰)
1998	5183	-	-
1999	4878	1	0,205
2000	4819	2	0,415
2001	3919	1	0,255
2002	4413	-	-
2003	4411	2	0,453
1998 – 2003	27623	6	0,217

The ratio between haemophilia type A and haemophilia type B is 3/2 and in medical literature the ratio is 10/1 (BOWEN, 2002). In the researches made during 1965 – 1975 were discovered 18 cases of haemophilia from a lot of 60,000 subjects in a period of 10 years. The increased number of cases is due to the legislation from that period which has forbidden the interruption of pregnancy and the impossibility of making general genetic tests for confirming the disease. So, the ratio between haemophilia type A and haemophilia B was 8/1 (16 cases type A/2 cases type B) and one case of vascular haemophilia.

From 6 cases of haemophilia, 5 cases are boys and only one case is a girl of 11 years old from Bacău who has vascular haemophilia (table 2). The diagnosis of this girl was put after a metroragy, then she was sent to “Fundeni” Hospital București. The disease has all three characteristics: the deficiency of the VIII factor, low number of thrombocytes and the defect of the capillary vessels. The girl had her skin and the mucous membranes bleeding when she was slightly wounded, on epistaxis and metroragy.

Table 2 - The distribution and the frequency of the cases of haemophilia on sexes

Year	Cases of haemophilia	The number and the frequency of cases (%)			
		girls		boys	
1998	-	-	-	-	-
1999	1	-	-	1	100
2000	2	-	-	2	100
2001	1	-	-	1	100
2002	-	-	-	-	-
2003	2	1	50	1	50
1998 – 2003	6	1	16,66	5	83,34

The cases of haemophilia are composed of children of the age over 10 years old (table 3). These children were discovered after they have suffered some injuries or removed teeth when it was noticed that the time of bleeding was increased (RUSEN, 2004) more than it would be in normal conditions.

Table 3 - The distribution and the frequency of haemophilia according to the groups of ages

Year	Number of cases	Groups of ages and their frequency (%)									
		0-1 year	1-3 years	3-5 years	5-7 years	7-10 years	over 10 years				
1998	-	-	-	-	-	-	-	-	-	-	-
1999	1	-	-	-	-	-	-	-	1	100	
2000	2	-	-	-	-	-	-	-	2	100	
2001	1	-	-	-	-	-	-	-	1	100	
2002	-	-	-	-	-	-	-	-	-	-	
2003	2	-	-	-	-	-	-	-	2	100	
1998 – 2003	6	-	-	-	-	-	-	-	6	100	

In the researches made during 1965 – 1975, the children with haemophilia were of different ages as following:

- the group 0-1 year – one case;
- the group 1-3 years – 3 cases;
- the group 3-5 years – 4 cases;
- the group 5-7 years – 3 cases;
- the group 7-10 years – 5 cases;
- the group over 10 years – 2 cases (ANTON, 1975).

At the same time, the children with haemophilia were from urban environment: Comănești – 6 cases and Bacău – 2 cases. In the rural environment, the distribution of the cases with haemophilia type A was the following: Urechești – 3 cases and Huruiesti, Plopana, Colonești, Zemeș and Solonț – one case. The cases with haemophilia type B were in: Onești town – one case (urban environment) and Sânduleni – one case (rural environment).

In the researches made during 1998 – 2003, the children with haemophilia are coming especially from the urban environment – 4 cases from which 3 are from Bacău city and one case from Moinești town. In the rural environment were 2 cases: one case in Filipești and the other from Agăș. The cases with haemophilia type A are coming from Moinești – one case and Bacău – 2 cases. The cases with haemophilia type B are coming from Filipești and Agăș both with one case and vascular haemophilia from Bacău city (table 4).

Table 4 - The frequency of the cases of haemophilia in rural and urban environment

Year	The number and the frequency of cases (%)					
	Urban environment		Rural environment		Total cases	
	Bacău	Other cities				
1998	-	-	-	-	-	-
1999	1	100	-	-	-	1
2000	-	-	1	50	1	2
2001	1	100	-	-	-	1
2002	-	-	-	-	-	-
2003	1	50	-	-	1	2
1998 – 2003	3	50	1	16,67	2	33,33

In 4 of 6 cases of confirmed haemophilia some complications appeared due to the bleeding with different locations (table 5). Three cases with complications are represented by the boys with haemophilia type A and type B who have haemarthrosis and blood accumulation on the right knee, the left elbow, the right arm and anaemia post haemorrhage. Another case with complication is of the girl with vascular haemophilia. When this case was discovered, the girl had metrorragy and anaemia due to this haemorrhage.

Table 5 - The frequency of cases of haemophilia with complications

Year	Cases of haemophilia	The number and the frequency of cases with complications (%)	
1998	-	-	-
1999	1	-	-
2000	2	1	50
2001	1	1	100
2002	-	-	-
2003	2	2	100
1998 - 2003	6	4	66,66

In medical literature, the severe forms contain cephalic haematoma or extended haemorrhage at the level of the navel cordon (SANDOVICI, 2004). The children with haemophilia during 1965 – 1975 had many bruises, haemarthrosis, epistaxis, bleeding gums and faeces mixed with blood.

At present, there weren't registered any deceases among the children with haemophilia. Thirty years ago there were 2 deceases among the patients of less then one year old who had meningocerebral haemorrhage and urine with blood (ANTON, 1975).

Nowadays, the haemophilia doesn't cause victims anymore, because of the treatment which consist of a transfusion of blood which has embodied the missing factor of the coagulation of blood, also of the increased possibilities of liver transplant and genetic therapy (RAICU G., 2004), but in the same time of the increased possibilities of genetic examination and advice.

CONCLUSIONS

The researches in the cases of haemophilia at infants from Bacău county in two different periods of time revealed the following:

During 1965 – 1975 were discovered 18 cases of haemophilia more than 6 cases in 1998 – 2003.

Compared to the previuos period, in present the frequency of the cases of haemophilia type B was increased, also it has been discovered a case of vascular haemophilia. All these cases have a positive family pedigree.

The children with haemophilia are over 10 years old and the most of them are boys; only one case is represented by a girl of 11 years old who has vascular haemophilia, case that has been confirmed by "Fundeni" Hospital București.

The discovered cases are coming from the urban environment, from which Bacău city has 50% from the cases of infants with haemophilia.

Even if the actual progress of science allows that the result and the recovery to be made immediately after the local treatment and transfusions of blood which has embodied the missing factor of the coagulation of blood, it also must pay attention to the prevention of this disease based on the examination and specific advice.

REFERENCES

1. ANTON, D.D., 1975. *Considerații clinico – epidemiologice, genetice și biologice asupra hemofiliei în județul Bacău. Dispensarizarea bolnavilor. Teză de doctorat. UMF, Iași, 180.*
2. BOWEN, D.J., 2002. Haemophilia A and haemophilia B: molecular insights. *J Clin Pathol*, 55: 1-18.
3. COVIC, M., ȘTEFĂNESCU, D., SANDOVICI, I., 2004. *Genetica medicală. Polirom, Iași, 607.*
4. MAXIMILIAN, C., POENARU MOȚOC, L., BEMBEA, M., 1996. *Genetica clinică*, Ed. Pan – Publishing House, București, 144.
5. RAICU, P., 1997. *Genetica generală și umană. Humanitas, București, 357.*
6. RAICU, G., 2004. *Viața medicală. Aspecte etice, legale și sociale ale testărilor genetice.* 23, 6.
7. ROȘIANU – WALTER, A., GEORMĂNEANU, M., 1986. *Boli ereditare în pediatrie. Ed. Medicală, București, 324.*
8. RUSEN, L., 2004. *Viața medicală. Când trebuie să ne temem de hemoragia neașteptată*, 37, 5.
9. ȘTEFĂNESCU, D.T., CĂLIN, G.A., ȘTEFĂNESCU, F.C., 1998. *Genetica medicală. Progrese recente. Ed. Tehnică, București, 192.*

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MORPHOGENETICAL AND HISTOLOGICAL STUDIES OF „IN VITRO” ANTHOR CULTURES OF *BRASSICA OLERACEA* L.

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Key words: cabbage, anther, callus, regeneration

Abstract. The anthers were inoculated on 8 hormonal variants of MS (Murashige-Skoog, 1962) medium and on one variant of B5 (Gamborg, 1968) slightly changed medium formula. The main morphogenetic reaction was callus formation. The callus's consistency and colour differed depending on the hormonal variant. The friable callus for all the tested genotypes had caulogenetic capacity. Its passage on differentiation media comprising cytokinins led to new shoots formation, the intensity of this phenomenon varied with the genotype and with the initial medium that provided the respective callus.

The histological studies of calluses or of anther shoots pointed out callus structure and the numerous histogenetic stages due to growth regulators, as well as in vitro regenerated shoots' structure.

INTRODUCTION

Brassica oleracea L is a vegetable species belonging to the *Cruciferae* family. The ancestor of today cabbage is *B. oleracea* L. ssp. *silvestris*, a species that provided a great number of varieties and forms over the centuries.

Brassica oleracea L. is appreciated from the economical viewpoint due to its high nourishing value occupying an important role in human diet and also for its therapeutic effects.

It is well – known that cabbage juice's content rich in sulphur, chlorine, calcium, iodine, iron and vitamins is indicated to cure duodenal ulcer, constipation, skin rashes, also in the diet of patients suffering from osteoporosis and anemia, and its combination with carrot juice offers an excellent vitamin and mineral salts source.

Of all the numerous varieties of *Brassica oleracea* L. we mention the most known and used world wide: white cabbage (variety *capitata*, form *alba* Lam.), broccoli (variety *botrytis* L., subvariety *cymosa* Lam.) and cauliflower (variety *botrytis* L., subvariety *cauliflora* Alef.).

During our investigations we observed the morphogenetic reaction of the in vitro cultured anthers belonging to several genotypes of the a small number of plants were provided (with different levels of ploidy) by anther culture of several species of *Brassica* (Keller și colab., 1984, Dias, J., C., 1999), but the most efficient method was microspore culture (Adamus, A., Samek, L., 2003). This method favoured the regeneration by microspore culture of *B. rapa*, *B. campestris* (Keller et al., 1975; Keller and Armstrong, 1979), *B. chinensis* (Chung et al., 1977) and *B. pekinensis* (Teng and Kuo, 1977).

MATERIAL AND METHODS

As biological material we used in our research floral buds from the inflorescences harvested from the Vegetable Research Centre of Bacau. We tested 12 genotypes of white cabbage (Z2; Z2-12; RM1; G37; 2TC – 19; TRM1; TRM2; DE; BR-4; BCO-7-6; BCO-7-10, BCO-076), 4 genotypes of broccoli (BR-312-3; BR-312-5; BR-11-2; BR-S) and one of cauliflower (CT-Bc).

The sterilisation of biological material was proceeded by emerging it into mercury chloride (solution 0.1 ‰) followed by repeated rinses with sterile distilled water.

Subsequently the anthers were excised from the floral buds and inoculated on many hormonal variants of the culture media. 8 of them comprised the basal MS medium (Murashige-Skoog, 1962) and only one contained the basal B5m (Gamborg, 1968) modified by Lillo and Shamin, 1983 (table 1)

Table 1. Hormonic formulii to prepare the initiation media

Nr.	Hormonic formulii	Basal medium	Growth regulators (mg/l)					
			IAA	IBA	NAA	2,4-D	BAP	KIN
1.	BB ₂	MS	-	0,1	-	-	1	-
2.	BD	MS	-	-	-	0,5	1	-
3.	ND	MS	-	-	0,1	0,1	-	-
4.	BAD	MS	0,1	-	-	0,5	1	-
5.	KD	MS	-	-	-	1	-	1
6.	BDN	MS	-	-	0,1	0,02	0,3	-
7.	BN	MS	-	-	0,1	-	0,5	-
8.	BA	MS	0,1	-	-	-	1	-
9.	ND	B ₅	-	-	0,1	0,1	-	-

After inoculation, the anthers were kept in darkness for 6 days at 35°C, then placed in an in vitro culture room (photoperiod of 16 hours), at a temperature of 23°C.

The general reaction of the initiation media was callus formation, which was then transferred on media to induce caulogenesis (table 2).

Table 2. Nourishing media used for differentiation

Nr.	Basal medium	BAP mg/l	KIN mg/l	GA3 mg/l
1.	MS	0,5	-	-
2.	MS	1	-	-
3.	MS	2	-	-
4.	MS	0,5	0,5	-
5.	MS	1	-	0,1

The shoots obtained were passed on hormone-free MS to produce roots.

Neoplantlet accommodation to septic media was accomplished in a hydroponic system. Afterwards they were transferred in pots containing a mixture of soil and perlite.

For histological analysis of calluses or anther shoots of the three varieties of *B. oleracea* species we used microscopic preparations of vegetal material included in paraffin. Microscope analysis was meant to establish callus structure and the varied histogenetic aspects appeared under the influence growth regulators and also the in vitro provided shoot structure.

RESULTS AND DISCUSSIONS

After being kept in darkness and at a high temperature for six days, most part of the anthers provided a small-sized, quite friable, white callus. The anthers were passed to light and two weeks after their inoculation on the initial media the anther callus had a different evolution depending on the the hormonic variants and especially on the genotype. The three varieties of *Brassica* formed two types of callus (of different consistency): friable and compact, that belonged to 2 categories: green and cream coloured.

The morphogenetic reaction of anthers from the 12 genotypes of white cabbage inoculated on induction media varied with the genotype and also with the hormonic content of the nourishing medium. Most frequently a friable, green or light green callus appeared and it had the highest regenerative capacity, providing shoots either directly on the induction medium or at the moment of its transfer on a medium formula comprising only cytokinins (fig. 1). This type of callus proliferated highly, particularly on the initial variants: BDN and BN, and those passed on BD (BAP – 0,5 mg/l, 2,4-D – 0,5 mg/l) and BAD (BAP – 1 mg/l, IAA – 0,1 mg/l, 2,4 – D – 0,5 mg/l) had a medium proliferation speed. Regarding the genotypes, the anthers of genotypes RM1 and Z2-12 were the most efficient to provide and proliferate the caulogenetic callus and the anthers of BCO-076 displayed a low capacity to produce this androgenetic structure (graphic nr. 1).

Generally, placing the friable green callus on the differentiation media (regardless the genotype) provided shoot regeneration (fig 2). This phenomenon's intensity varied, the highest shooting percentage was obtained on B2 variant (2 mg/l BAP), followed by BK (1 mg/l BAP and 1 mg/l kinetine).

Compact green callus of hard or semi-hard consistency, was provided mostly by the anthers inoculated on BD, BA, KD and BAD, but the proliferation speed was not high. The genotypes DE, Z2-12, BCO-7-6 and 2TC19 manifested a good caulogenetic reaction on the above-mentioned medium variants. The meristematic centres were rendered evident on anther callus produced on BA and KD, but the transfer of this type of callus on differentiation media did not lead to shoot regeneration; the callus maintained its consistency. At contact surface between medium (rich in cytokinins) and callus, the latter generated long roots endowed with secondary branches, covered by absorbent hairs.

Similarly to the case of friable green callus, a quite great number of anthers provided a friable cream, whitish-cream or cream-yellowish callus. Although on some hormonal variants this type of callus proliferated intensely and it manifested a low caulogenetic capacity (though it was transferred on the same medium formula to differentiate). Root formation was induced on BA, ND, KD and BAD on the same type of callus, with short roots grouped in tufts.

A small number of inoculated anthers generated a hard cream callus, characterised by an average and low proliferation speed, with an ability to produce just roots. It was noticed on the anthers placed on the following medium variants: ND (genotypes: DE, TRM2), BAD (genotypes: BCO-076, BCO-7-10, Z2), KD (genotypes: RM1, BR-4, G37) and BA (genotypes: Z2-12, Z2). Transferring this type of callus on media comprising only cytokinins resulted in its low proliferation, sometimes accompanied by roots with an intense growth on the medium surface and also inside the culture medium.

Of all 12 genotypes of white cabbage, a small number of anthers from the genotypes Z2-12 and TRM1 provided plantlets directly on the initiation media, on KD, BN and ND (B5m) , (fig. 3).

Anther reaction for broccoli (4 genotypes) was callus formation (the same types): friable, green or cream coloured, and compact, also green and cream. Regarding the callusogenetic capacity, the anthers from the genotypes BR-312-3 and BR-312-5 proved to be the most efficient.

The friable green callus was provided only on two hormonal variants of the initiation media: BN and BDN (fig 4). This callus was characterised by high proliferation speed for the majority of genotypes. Most of the calluses had meristematic centres, the most numerous on BDN (graphic nr. 2).

Shoot regeneration from these androgenetic structures was evident on the differentiation medium B2.

The compact, green callus appeared less frequently than in the case of white cabbage. The anthers of genotypes BR-312-3 and BR-312-5 of broccoli placed on medium variant BAD generated a compact, small-sized, green callus. The same kind of callus, but with a higher proliferation, was provided by the anthers of BR-11-2 on BDN medium, on this callus being also rendered meristematic centres, but caulogenesis couldn't be induced, not even when the calluses were transferred on differentiation media.

The friable, cream callus was also characterised by an intense proliferation, being produced by a great number of anthers belonging to BR-312-3 and BR-312-5. It mainly displayed rhizogenetic capacity, a process that was visibly increased after the transfer on media comprising only cytokinin-like hormones. The cytokinins from the differentiation media also favoured the regeneration of a small number of shoots derived from the friable cream callus.

As for the subvariety cauliflora, the hormonal combination of the 9 medium formula provided the same two types of callus on anthers. In the case of cauliflower, the best proliferation speed and caulogenetic capacity was also rendered evident for the friable green callus (fig. 5). The

hormonic variants for this callus's induction in a high percentage were: BDN and BN (graphic nr.3).

Anther inoculation on media comprising only auxins (ND with 0,1 mg/l NAA and 0,1 mg/l 2,4-D) favoured a foamy, high-proliferative, cream callus that proved to be rhizogenetic. In the case of the same hormone combination (ND), but with a basal medium modified – B5, the reaction was formation of friable, low-proliferative, cream callus with roots and shoots (some shoots having inflorescences).

The transfer of friable cream callus on organogenetic media proved its caulogenetic capacity, only the callus formed on ND formula (B5m) generated a greater shoot number. We also noticed that in the case of friable green callus provided by cauliflower anthers the presence of a high amount of cytokinin (2 mg/l) in the differentiation media favoured the regeneration of a greater shoot number.

The aspect of the in vitro regenerated shoots was normal, each stem having 4 to 6 leaves with an alternate disposition. A small percentage was represented by individuals with chlorophyllian deficiency, albino or even shoots with leaf malformations (conrescent, very large and crisp leaves), but they did not survive (fig.6, 7).

Shoot accommodation to ex vitro medium was accomplished in a hydroponic system and it took about 2 weeks, the survival rate was of 80%. After accommodation the neoplantlets were transferred to soil pots (fig. 8).

Histoanatomical studies pointed out the presence of histogenetic structures that provide vascular elements and also of some organogenetic structures (either caulogenetic or rhizogenetic) within androgenetic calluses (fig. 9, 10, 11, 12, 13, 14).

The shoots generated by anther callus displayed an almost normal structure.

CONCLUSIONS

The main morphogenetic reaction was callus formation, the newly formed callus having a consistency and colour that differed with the genotype and with the hormonal balance in the nourishing media.

Of all the four categories of callus that appeared, the friable green callus had the displayed the highest regenerative capacity.

The best proliferation speed of this type of callus was noticed on BDN, BN and BD media.

The friable cream callus (that had the lower regenerative capacity), proliferated intensely on BAD, BA and KD variants.

The most intense callusogenetic reaction was observed at genotypes RM1, DE, Z2-12, BCO-7-10, BCO-076 (for *capitata* variety) and BR-312-3, BR-312-5 (subvariety *cymosa*)

The phenomenon of shoot regeneration from androgenetic calluses was best represented on differentiation medium comprising 2 mg/l BAP

Genotypes TRM1 and Z2-12 provided plants from anthers on the following media: KD, BN and ND (B5m)

Histo-anatomical studies made on anther callus evinced the presence of some histogenetic and organogenetic structures.

REFERENCES

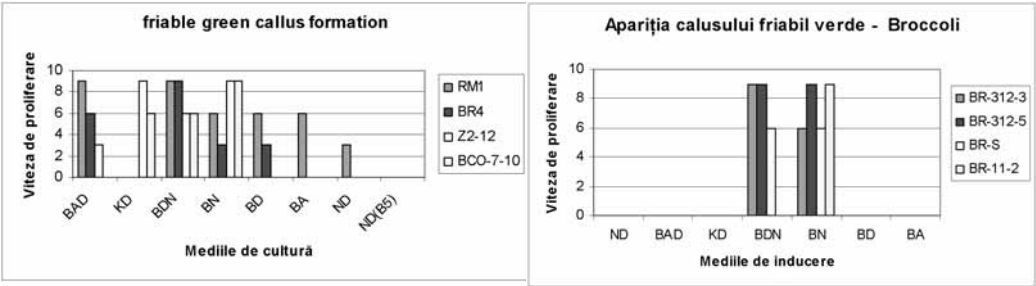
1. ADAMUS, A., SAMEK, L., 2003. *Increasing the efficiency of white cabbage (Brassica oleracea L., var. capitata) androgenic embryos production.*

2. [www.biotech.univ.gda.pl/impresy/IAPTC/sessions/sess_6.html]
3. CROCOMO, J., O., 1981. *Plant tissue culture methods and applications in agriculture*. Acad. Press, New York, London, Toronto, 359.
4. DIAS, J., C., 1999. Effect of activated charcoal on Brassica oleracea microspore culture embryogenesis. *Euphytica* 108, Kluwer Academic Publishers, p. 65-69.
5. JAHNE, A., LORZ, H., 1995. Cereal microspore culture, *Plant Sci.*, 109 (1), 1 - 12.
6. KELLER, W., A., 1984. *Anther culture of Brassica. Cell Culture and Somatic Cell. Genetics of Plant*, Academic Press. Inc, vol 1, p. 302 – 307.
7. STAN, N., MUNTEANU, N, 2001. *Legumicultură* , VOL. II, Ed. Ion Ionescu de la Brad, Iași, p.158 – 177.

1 – Universitatea din Bacau, Calea Mărășești, 157, Bacău – 600115, tel. 0234/571012,

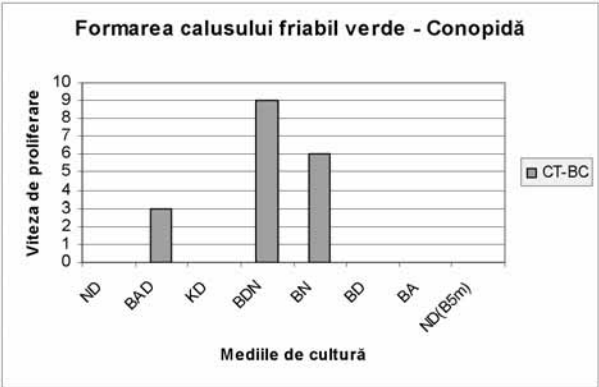
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Graphic 1 – Generation and proliferation of friable green callus from white cabbage anthers

Graphic 2 – Proliferation of friable green callus from the anthers of broccoli



Graphic 3– Friable green callus formation from cauliflower anthers



Fig. 1 Green friable callus from anthers- Cabbage

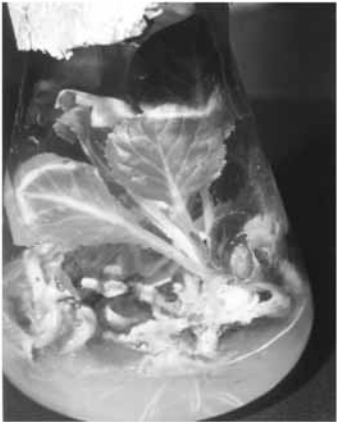


Fig. 2. Shoots regenerated on differentiation medium



Fig. 3. Neoplantlets generated from anthers of white cabbage (Z₂-12)



Fig 4. Green friable callus from anthers- Broccoli

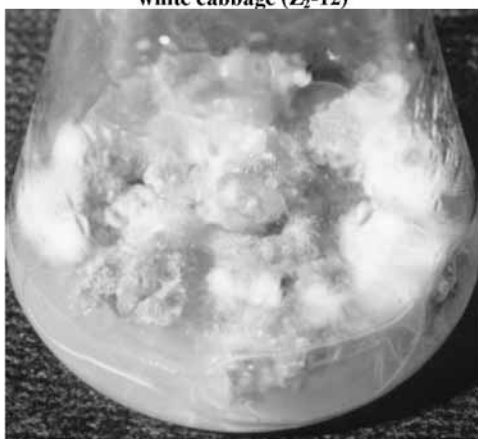


Fig. 5. Green friable callus from anthers – Cauliflower



Fig.6. Shoots with chlorophyllian deficiency

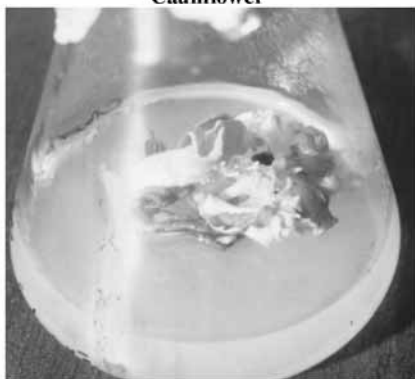


Fig. 7. Abnormally developed shoots



Fig. 8. Accommodated plants in soil pots

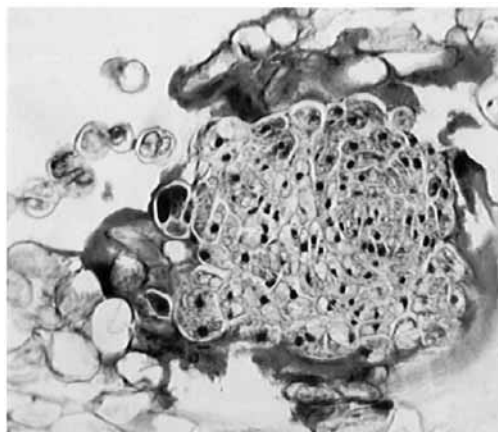


Fig. 9 – Meristematic centre

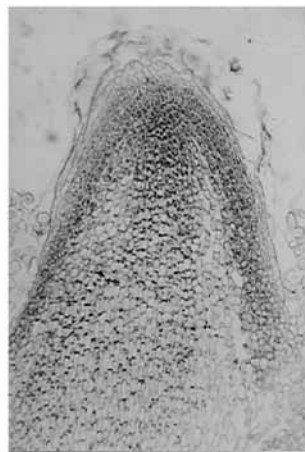


Fig. 10 – Root

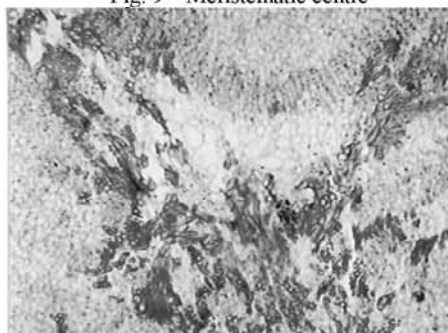


Fig. 11 – Tracheides from callus

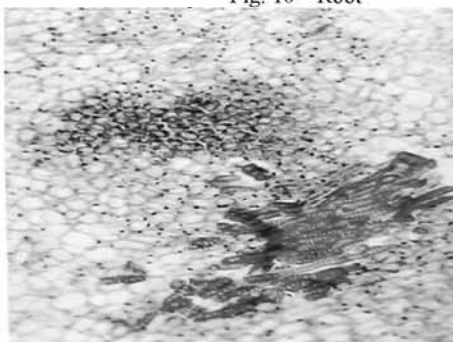


Fig. 12 – Tracheides and meristematic centre from
callus

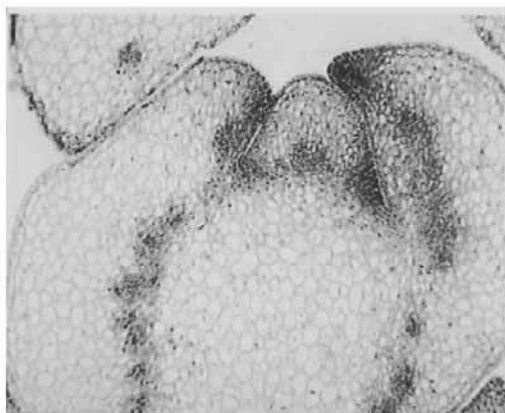


Fig. 13 – Axillary bud

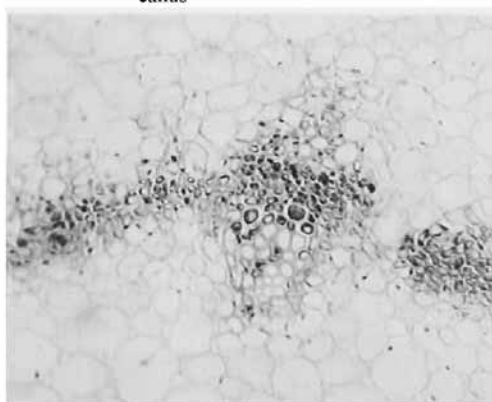


Fig. 14 – Conductive fascicle

INVESTIGATIONS ON THE IN VITRO MORPHOGENETIC REACTION OF *MELISSA OFFICINALIS* L. SPECIES

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Keywords: lemon balm, micropropagation, morphogenetic reaction

Abstract: The paper presents some preliminary results concerning the in vitro initiation of *Melissa officinalis* species and the morphogenetic reaction of some explants on several hormonal formulæ of the basal Murashige-Skoog medium. We recommended some medium formulæ that are effective for this species' micropropagation as well as the appropriate accommodation to septic conditions of the neoplantlets obtained in vitro and for the regenerants' transfer to field.

INTRODUCTION

Melissa officinalis L. is a herbaceous, perennial plant of the *Lamiaceae* family, a native of the northern Mediterranean region. It is a xeromesophitic, moderate thermophile plant that is spread in sunny and also a bit shady places and is resistant to drought. It is sensitive to low temperatures, requiring mild winters. Its development is favourable on argillaceous earth and also on sandy, loamy ground. On vegetable soil its content of essential oils is lower. The aerial part of plant comprises 0.05 to 0.15% of volatile oil (that contains citronellal, citral, geraniol, linalool), polyphenols, tannins (3 to 6%), mucilages (12%), bitter substances etc. The seeds contain fat oil made up of linolenic, linoleic, oleic, palmitic and stearic acids (1-3, 5-8,10). The main action of its active principles, especially of volatile oil of *Melissa officinalis* is spasmolytic and sedative, recommended for gastro - intestinal spasms and cardiac neurosis. They are also known for an antiseptic, sedative, carminative, choleric, mild laxative, stomachic, cicatrizing, galactagogue and insecticide, (2,3,6,8,10).

Considering this species economical importance we intended to find out some information considering its in vitro behaviour, the reaction of several explants on varied hormonal formulæ, the possibility of identifying an effective micropropagation technology and the isolation of possible valuable genotypes. This kind of investigations were done by by others authors too, (4,7,9).

MATERIAL AND METHODS

The explant source for in vitro cultures initiation at *Melissa officinalis* were some individuals brought from Chalkidiki (Greece), grown in soil pots, in laboratory. We used shoot tips that were sterilized with a chloramine-T solution (5%) for 25 minutes, subsequently they were rinsed twice with sterile water and inoculated on hormone-free Murashige-Skoog (1962) medium or on MS supplemented with BAP (0.2-0.5 mg/l) or with kinetin (1 mg/l) and NAA (0.5 mg/l). The sterile neoplantlets obtained were used to test the morphogenetic reaction of different types of explants (nodes, shoot tips, internode, root and leaf fragments) on a series of hormonal formulæ of MS medium. Cultures were grown in Erlenmeyer flasks of 100 ml (B type). Saccharose (25 g/l) was the carbon source and agar was used to solidify the nutritive medium. The inoculated flasks were incubated in a half-climatized culture room within the 'Stejarul' Research Centre from Piatra Neamt (temperature was 23-25°C, light – about 2500 lux, continuous illumination). The neoplantlets provided on certain hormonal formulæ were accommodated to septic conditions in a hydroponic system. The results are presented in table no.1 and figures 1-8.

RESULTS AND DISCUSSIONS

Our observations at the moment of in vitro culture initiation of *Melissa officinalis* species evinced the fact that including the plant in this culture system does not cause any particular problem and the use of chloramine -T (5% solution, for 25-30 minutes) to sterilize the explants proved to be effective. The use of basal hormone-free MS medium as well as MS supplemented with small amounts of BAP (0.2-0.5 mg/l) but especially with kinetin (1 mg/l) and NAA (0.5 mg/l) favoured the process of shoot development and generation of neoplantlets that are then

used to test the morphogenetic reaction of varied explants of lemon balm on different hormonal formulæ of MS.

Nodes and shoot tips were most frequently used. When these explants were inoculated on basal MS, their reaction was neopantlet generation, that generally have up to three basal shoots, rather long internodes (between 2 and 4 in about 3 weeks), well developed leaves and long roots with secondary branches. Nodes and shoot tips inoculated on MS supplemented with cytokinines (BAP) provided neopantlets characterized by vigorous multiple shoots of varied dimensions (fig. 1), quite thick stems and dark green leaves; sometimes a compact, small-sized callus is generated and it turns green at the contact surface between the explants and the culture medium. In other cases long roots with secondary branches appear between 2 successive transfers (about 30 days) or roots are provided only sporadically. The multiple shooting is enhanced on a culture medium containing kinetine (0.5 mg/l) and BAP (0.5 mg/l) but the growth rate is slower, root formation being also less intense.

In case of an MS medium supplemented with auxins the use of IAA (2 mg/l) and of nodes and shoot tips as explants favour either neopantlet generation or just roots, the reaction of explants being a slow one. On a culture medium supplemented with NAA (2 mg/l) a friable, low proliferating cream callus appeared, other times it provided neopantlets bearing shoots with long internodes and seldom long roots, but more frequently short and numerous roots growing from the node that contacts medium. Nodes and shoot tips placed on MS comprising IBA (2 mg/l) led to neopantlet formation and they were more vigorous than on media containing IAA and NAA, the newly formed shoots having long internodes and the roots in a greater number, some of them more stronger and with secondary branches. The medium variant with 2 mg/l IBA seems to be appropriate to obtain vigorous neopantlets in order to multiply some valuable genotypes of this species.

During our research a special attention was offered to nodes and shoot tips on media comprising combinations of auxins and cytokinines (fig. 2, 3, 4), or cytokinines and giberellins. It was ascertained that the best reaction of these explants was obtained on a medium supplemented with kinetine (1 mg/l) and NAA (0.5-1 mg) that favoured the generation of neopantlets with a high speed growth, bearing strong stems and roots, sometimes much larger leaves, very long roots with many secondary branches (fig. 4). This hormonal formula is the most recommended for neopantlet formation in order to micropropagate lemon balm. The MS variant containing BAP (1 mg/l) and IAA (0.5 mg/l) favoured the generation of neopantlets with shoots and shorter internodes, smaller leaves sometimes arranged feather – like from the node, thinner stems, frail, long and whitish roots (fig. 2). In the case of an MS medium containing BAP (1 mg/l) and IBA (0.5 mg/l) the reaction of shoot tips and nodes was weaker, meaning that the newly generated neopantlets were feeble and small-sized, bearing green-yellowish leaves, poor root formation was observed (as a rule secondary roots are fewer or even absent)(fig. 3). On an MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) the neopantlets were more vigorous than on the previous formula but less developed than on KN, IB and N; shoots were smaller-sized, leaves a bit yellowish, root formation less represented. Multiple shooting phenomenon was obviously stimulated on MS medium comprising BAP (1 mg/l) and giberellic acid (0.5 mg/l), shoots were small – sized, with reduced green-yellowish leaves and root formation did not occur. We ascertained that the reaction of explants and the strength of the biological material obtained on varied hormonal formulæ clearly depended on the hormones within the nutritive medium and also on the vigour of the neopantlets used as explant source.

We used some hormonal variants to test the reaction of other types of explants. We observed that the inoculation of internode fragments on MS supplemented with 2 mg/l 2,4-D (fig. 5) provided a compact, green-whitish callus with a medium proliferation speed, mostly formed at the ends of the fragments (conferring them a bar bells shape). If 2,4-D (0.5 mg/l) is combined with BAP (1 mg/l) internodes reacted more intensely generating a compact, green-whitish callus on the entire explant surface, especially at the ends where cell proliferation is much more intense. Root fragments had a similar reaction on this medium formula providing a cream-greenish callus (fig. 6), but its proliferation speed was lower than that of the internode fragments. Leaves reaction on the previous hormonal formula was also similar to the mentioned one. On a medium comprising 2,4-D (2 mg/l) leaves generated a hard, small-dimension whitish callus in the petiole region. 2,4-D (0.5 mg/l) associated with BAP (1 mg/l) assured the proliferation of a compact, light green callus on the entire surface of the limb that contacts the nutritive medium (particularly in the petiole region), with a medium cell proliferation speed.

Within our research we also tried to test the reaction of the varied origin callus tissue on other hormonal formula of MS medium. We observed, for instance, that by transferring the internode callus on media supplemented with 0.2-0.5 mg/l BAP it continued to proliferate slowly, turning cream and friable with an obvious tendency to degenerate (it turns brown on large areas). On certain regions it seldom provided short thick thorny roots. Internode callus transferred on MS medium supplemented with BAP and GA suffered the same reaction. Leaf callus on a medium comprising BAP (0.5 mg/l) had a better proliferation, maintained its consistency and colour, sporadically generating roots at the surface of the nutritive medium depending on the vigour of the neoplantlets used as explant source.

Caulogenesis was absent in both cases on the tested media formula. The neoplantlets obtained on the varied media formula were accommodated to septic environment in a hydroponic system (fig. 7). Due to their thin leaves plants frequently suffer water losses, this being the reason why their accommodation is not easy to accomplish, the room requiring a more humid atmosphere and a lower temperature (about 20°C) and major thermic changes must be avoided. The previously mentioned conditions permitted a more facile accommodation period and diminished losses of biological material.

During the spring of 2004 we transferred some dozens of in vitro obtained regenerants (fig. 8) on a plot belonging to ‘Stejarul’ Research Centre in Piatra Neamt. Generally those regenerants are quite frail, their leaves being very thin and that is why they stagnate for a long period of time after their transfer to field, but subsequently they resume growth processes. It is for a fact that the agro-meteorological conditions (lower temperatures and abundant rain) of the research region during the spring of 2004 were not the most appropriate ones for plant breeding. Nevertheless the regenerants developed well until September 2004 and certainly they will surpass the winter season without significant losses.

CONCLUSIONS

In vitro culture initiation of *Melissa officinalis* can be accomplished beginning with shoot tips drawn from field-grown or greenhouse plants, sterilised in a chloramine-T solution (5%) and inoculated on basal MS medium or on MS supplemented with kinetin and NAA.

The explants (nodes and shoot tips) provided neoplantlets on the majority of the hormonal formula tested (comprising cytokinins, auxins or combinations of those two types of growth regulators). The best reaction was obtained on MS medium supplemented with kinetin and NAA, followed by IBA, NAA and even on hormone-free MS.

The addition of BAP and GA to the nutritive medium favoured multiple shooting and inhibited root formation of the new shoots that originate in nodes and shoot tips.

Internode, leaf and root fragments provided callus on media comprising 2,4-D. Callus formation process was more intense if 2,4-D was associated with BAP. The callus was compact, green in the case of internodes and leaves and cream for roots. Its proliferation from internode fragments was more intense.

The callus provided by internodes and leaves on MS supplemented with BAP or with BAP and GA maintained its characteristics, assured its proliferation and sporadically induced root formation on its surface. Caulogenesis was absent on the tested media formulæ and generally the callus tended to degenerate on large areas.

In order to micropropagate *Melissa officinalis* we recommend the use of MS medium with kinetine and NAA, followed by those supplemented with IBA, NAA and even of basal MS. After a period of growth stagnation, field-grown regenerants resumed their growth, stems and leaves became more vigorous compared to their aspect during the accommodation period, with high survival chances the next unfavourable season.

REFERENCES

1. APROTOSOAIE, C., 2005, *Cercetări privind acțiunea unor pesticide aplicate în cultura plantelor medicinale: aspecte morfologice și biochimice*. Teză de doctorat. UMF Gr.T.Popa, Iasi, 377p.
2. BODEA, C., 1982, *Tratat de biochimie vegetala. 2. Compoziția chimică a principalelor plante de cultură*. Ed. Acad. RSR, Bucuresti, 4, 243 – 246.
3. CIULEI, I., GRIGORESCU, E., STANESCU, U., 1993, *Plante medicinale, fitochimie si fitoterapie*, Ed. Medicala, Bucuresti, 2, 87 – 90.
4. MESZAROS, A., BELLON, A., PINTER, E., HORVATH, G., 1999, *Plant cell, tissue and organ culture*, 57, 149 – 152.
5. PARVU, C., 2000, *Universul plantelor. Mica enciclopedie*. Ed. Enciclopedica, Bucuresti, 567
6. PAUN, E., MIHALEA, A., DUMITRESCU, A., VERZEA, M., COSOCARIU, O., 1988, *Tratat de plante medicinale si aromatice cultivate*. Ed. Acad. RSR, Bucuresti, 2, 74 – 79.
7. SCHULTZE, W., HOSE, S., ABOU-MANDOUR, A., CZYGAN, F.C., 1993, *Biotechnology in agriculture and forestry*. Springer-Verlag, Berlin, Heidelberg, 24, 242 – 268.
8. STANESCU, U., MIRON, A., HANCIANU, M., APROTOSOAIE, C., 2002, *Bazele farmaceutice si clinice ale fitoterapiei*. Ed. Gr.T.Popa, Iasi, 1, 360p.
9. TAVARES, A.C., PIMENTA, M.C., GONCALVES, M.T., 1996, *Plant. Cell Rep.*, 15, 441 – 444.
10. TITA, I., 2003, *Botanica farmaceutica*. Ed. did. si ped., Bucuresti, 801.

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Table nr. 1 – The morphogenetic reaction of some lemon balm explants on varied hormonal formula of the Murashige-Skoog medium

Var.	Explant	Medium formula	Growth regulators (mg/l)							Morphogenetic reaction and proliferation speed
			BAP	GA	IAA	IBA	NAA	KIN	2,4-D	
1	Nodes and shoot tips	A			2,0					Neopiantlets (+), roots (+); slow reaction
2	"	B	0,2-1,0							Neopiantlets with multiple shoots (++) and vigorous leaves, strong roots (+++) with secondary branches
3	"	BA	1,0		0,5					Neopiantlets with shorter internodes (++), leaves with a feather-like disposition, thin, long (++) whitish roots; multiple shooting (+) rarely
4	"	BB	0,5			0,5				Neopiantlets (+) with small shoots and yellowish leaves; root formation (+) less represented
5		BK	1,0					0,5		Neopiantlets (++) with multiple shoots (+++), shoots of different sizes, root formation (++) less intense
6		BG	1,0	0,5						Multiple shooting (+++), small shoots with small yellowish leaves, absence of root formation
7	"	BN	1,0				0,5			Neopiantlets (++) with 1 shoot per node, shoots of varied dimensions, leaves a bit yellow, root formation (++) less intense
8	"	IB				2,0				Well developed neopiantlets (+++), long internodes, strong main root (+++) and numerous rather long secondary branches
9	"	N					2,0			Neopiantlets (++) with long shoots and internodes; roots (++) mostly short ones and surrounding the node, but also long roots with secondary branches; sometimes a thin basal layer of friable callus provided by nodes
10	"	KN					0,5	1,0		Neopiantlets (+++) with strong shoots and roots, internodes and thick roots; large dark green leaves
11	"	MS								Neopiantlets (++) with vigorous shoots, long internodes, sometimes multiple shoots at basal node, strong root with secondary branches
12	Internode	BD	1,0						0,5	Compact callus (+++), green on the entire explant surface, mostly at the ends
14	Leaves	BD	1,0						0,5	Compact callus (++), light green colour, on the whole surface of the leaves in contact with the nutritive medium
16	"	D							2,0	Compact, whitish callus (+), generated particularly in the petiole region
17	Roots	BD	1,0						0,5	Compact callus (++), cream-greenish colour on entire explant surface
18	Internode callus	BG	1,0	0,5						Proliferative callus (++) that maintains its characteristics but does not differentiate
19	Leaf callus	B	0,5							Callus with a slow proliferation (++) sporadically providing thorn-like roots on its surface (+)

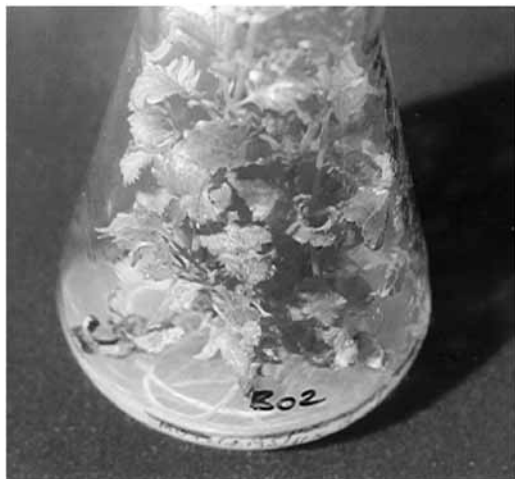


Fig.1. Neoplantlets generated on MS+0.2 mg/l BAP



Fig. 2. Neoplantlets generated on MS+1 mg/l BAP+0.5 mg/l IAA

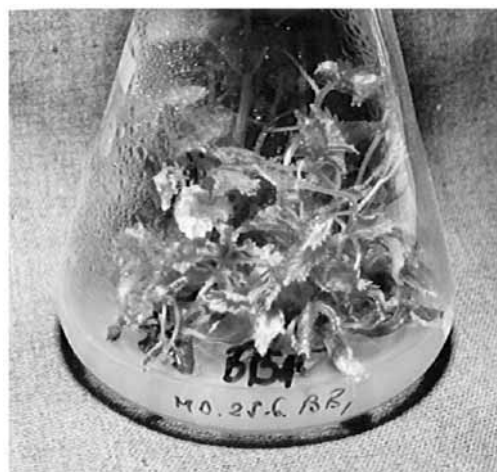


Fig.3. Neoplantlets generated on MS+0.5 mg/l BAP+0.5 mg/l IBA



Fig.4. . Neoplantlets provided on MS+1 mg/l kinetine+0.5 mg/l NAA

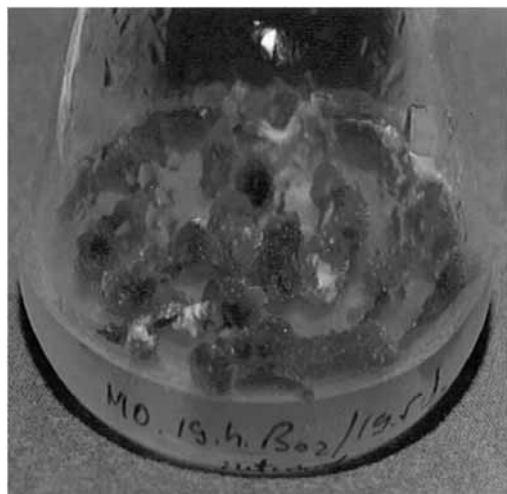


Fig.5. Callus from internodes on MS+2 mg/l 2.4-D



Fig.6. Callus originating from roots on MS+1 mg/l BAP+0.5 mg/l 2.4-D



Fig.7. Neoplantlet accommodation to septic environments (hydroponic system)



Fig.8. First year regenerants in field conditions

THE ACTION OF UV RADIATION ON MITOTIC INDEX AND MITOTIC DIVISION PHASES AT *PHASEOLUS VULGARIS* L.

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Key words: *Phaseolus vulgaris*, cultivars, UV-B, radiation, mitosis.

Abstract: In this work, damaging effects of UV radiations on bean *Phaseolus vulgaris* L. plantule root tips were investigated. Our study proves that by bean plants, the decrease of cell division frequency appears to be part of protection mechanism against especially the short waved UV radiation, with variations depending on cultivar.

INTRODUCTION

It is known that due to the increased UV radiation level on earth surface in the last decades of the 20th century, plants as sessile organisms had to develop different protective mechanism to adapt to the changing climatic conditions. Depending on duration, intensity or quality of light irradiation (including UV radiations), plants are able to react by inhibitions of developmental or growing processes (Whitelam and Millar, 1998; Batschauer 1999). The aim of the study was to investigate and compare the changes (occured either due to damaging effect or like beeing part of protective mechanism against inducing possible damages) in the mitotic division at the level of meristematic root tips of six romanian cultivars of *Phaseolus vulgaris* L. as an answer of UV irradiation.

MATERIAL AND METHODS

Biological material: *Phaseolus vulgaris* L., 6 romanian cultivars: Avans, Ardeleana, Star, Ami, Diva, Vera. Seeds were obtained from Podu Iloaie Seed Center.

Mutagenical agent: UV radiation with different wavelength.

Light sources as described by Surugiu and Maniu, 2002.

Filters: WG360, WG320, WG305, WG275; Q (Schott and Gen., Mainz, Germany), with 50% transmission for the given wavelength and cutting off the shorter wavelengths.

Working steps: Seeds germination: for each cultivar, 20 seeds for each experimental variant were sawn in Vermiculite, in 9/9 cm transparent plastic boxes and than placed in dark at 25°C. When seedlings root lengh was about 1,00-1,5cm, irradiation treatment was applied for 10 seedlings for each variant, in plastic boxes covered with different cutt-off filters. Experimental variants for each cultivar depending of UV irradiation: control, WG360, WG320, WG305, WG275, Q, for different time periods: 0,5h; 1,5h; 3h. For control corresponding to each experimental variant, 10 seedlings were kept in dark, corresponding time periods.

After irradiation, roots were prelucrured by Feulgen method for cytogenetical studies. All determinations were performed accordind literature protocols (Tudose et al., 1991; Tudose et al., 1996).

RESULTS AND DISCUSSIONS

As can be observed in Fig.1., for 0,5h irradiation durate, at the experimental variants including UV-C and UV-B in addition to UV-A (WG275; Q), for each of the 6 cultivars the cell division frequency descrease comparing with the dark controle and also comparing with the WG360 variant (considered as contole for potential damaging short wave UV like UV-B and UV-C) containing just non damaging long wave UV (UV-A).

Regarding cell division frequency for irradiation variants including just UV-B as potential damaging radiation in addition to non damaging UV-A (WG320; WG305), it can be observed that comparing with dark controle it can be noticed a descrease for all the six cultivars. Comparing with UV-B controle (WG360), it can be observed that excepting Ardeleana cultivar where frequency increase with a non signifiant low percent (0,43%), for all the other cultivars cell division frequency descrease.

For 1,5h irradiation durrate, as shown in Fig.2., in the case of UV-A, UV-B and UV-C irradiation, (WG275; Q), the mitotic index decrease comparing with dark contole for all six cultivars and comparing with UV-B controle (WG360) decrease for all cultivars excepting Diva where for WG275 variant it can be noticed an non signifiant increase (0,23%).

For irradiation variants including just UV-B in addition to non damaging UV-A (WG320; WG305), it can be observed that comparing with either dark or UV-B controle it can be noticed a decrease for Ardeleana, Star, Diva and Vera in the frequency of cells division. For Avans by the WG320 variant the mitotic index is practically the same with the one for WG360 (higher with 0,09%), and for Ami the cell division frequency increase in the case of WG320 variant with 1,39% comparing with dark controle and with 1,86% comparing with WG360.

As shown in Fig.3., for 3h irradiation durate, at the experimental variants including potential damaging UV (UV-C, UV-B) in addition to UV-A (WG275; Q), for each of the six cultivars the cell division frequency decrease comparing with the dark controle and also comparing with the WG360 variant .

For WG320 and WG305 variants, excepting Avans where mitotic index is for WG305 higher with 0,84%than the UV-B controle index and Diva where for WG320 the value is with 0,77% increased comparing with dark controle, for all the other experimental variants, the cell division frequency decrease compared either with dark or with UV-B controle.

In corelations with mitotic index it can be noticed (Fig.4., Fig.5., Fig.6., Fig.7., Fig.8., Fig.9.) that regarding the distribution of frequency on different mitotic division phases, for all the experimental variants, the highest percent belongs to prophases. An inhibition occures at anaphase level. The increase of telophase percent compared with the percent of the coresponding anaphases can be explained with a delay in begining a new cell cicle for the new formed cells from the previous cell cicle.

CONCLUSIONS

For each investigated cultivar, not depending of irradiation duration, the cell division frequency was strongly inhibited in the case of irradiation with low waved UV, beeing either plant protective mechanism or damage consequence.

In correlation with mitotical index values, it can be observed that an inhibition of cell division frequency occures at anaphase level.

Regarding the distribution on phases of cell division frequency, for all variants, it can be observed that the UV irradiation had no influence, comparing with controle.

It is no obviusse difference between investigated six bean cultivars regarding the effects of UV radiation at mitotic cell division level.

REFERENCES

1. Batschauer, A., 1999. Light perception in higher plant. In *Cellular & Molecular life Sciences*55, p: 153-165.
2. Surugiu, C.I., Maniu, M., 2002. *Analele st. UNIV. „Al.I.Cuza“* T III, serie noua, p:151-155.
3. Tudose, I.Gh., Pricop, M., Tudose, M., 1991. *Analele st. UNIV. „Al.I.Cuza“* T XXXVII, s.II a, p:129-136.
4. Tudose, I.Gh., Bara,I., Tudose, M., Pricop, M., Tudose, Cr., 1996. *Analele st. UNIV. „Al.I.Cuza“* T XLII, s.II a, p: 95-99.

5. Whitelam, G.C., Millar, A.J., 1998. Light regulation and biological clocks. *In Annual Plant Reviews Vol I. Sheffield Academic Press*, Ed by M. Anderson, J.A.. Roberts, p: 331-359.

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Fig.1. MI variation for each cultivar, depending of irradiation time, for 0,6h.

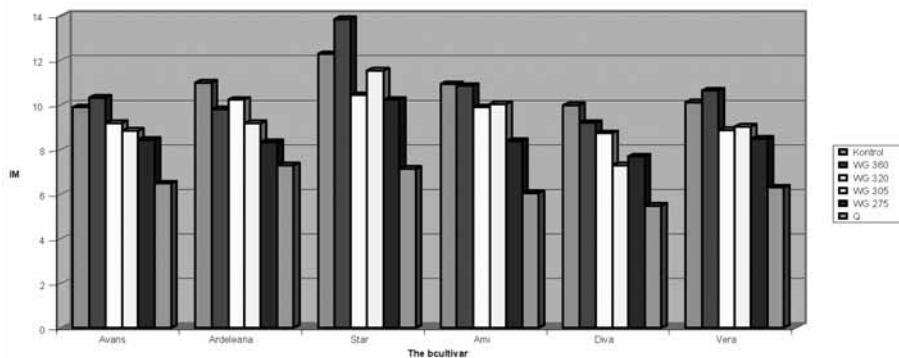


Fig. 2. MI variations for different cultivars, depending on irradiation variant for 1,5h

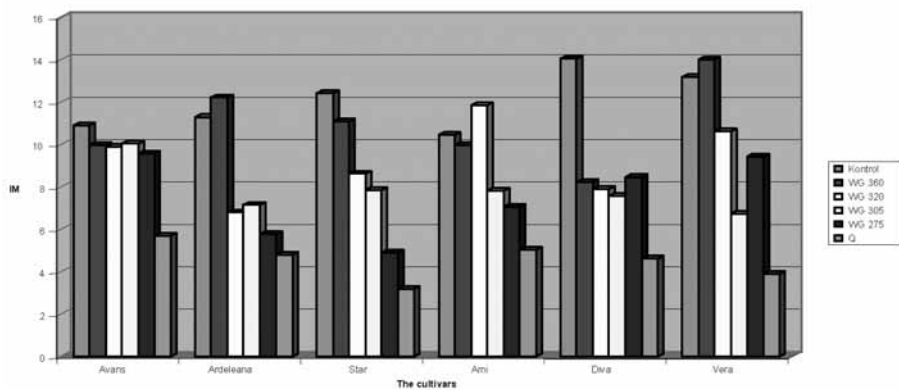


Fig. 3. MI variation for each cultivar, depending on irradiation variant, for 3h

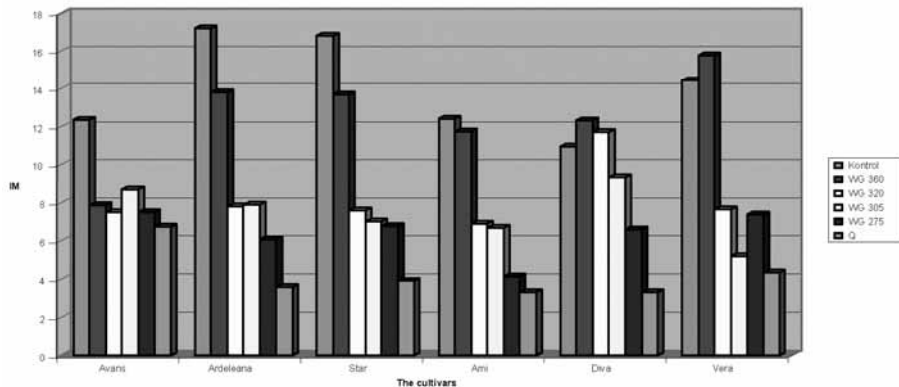


Fig.4. The percentage of division phases, depending on irradiation period, at Avans cultivar

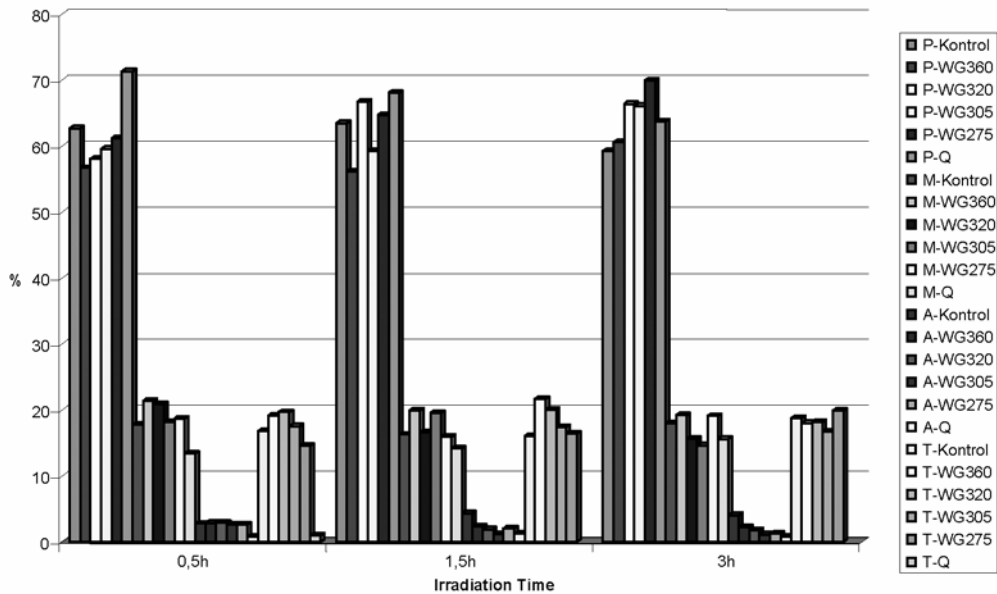


Fig.5. The percentage of division phases, depending on irradiation period, at Ardeleana cultivar

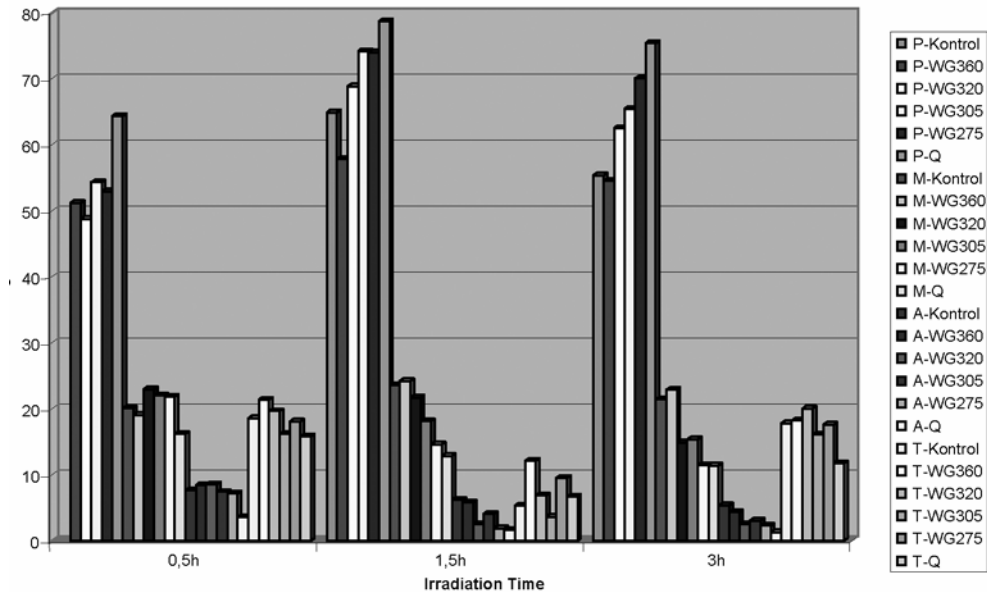


Fig. 6. The percentage of division phases, depending on irradiation period, at Star cultivar

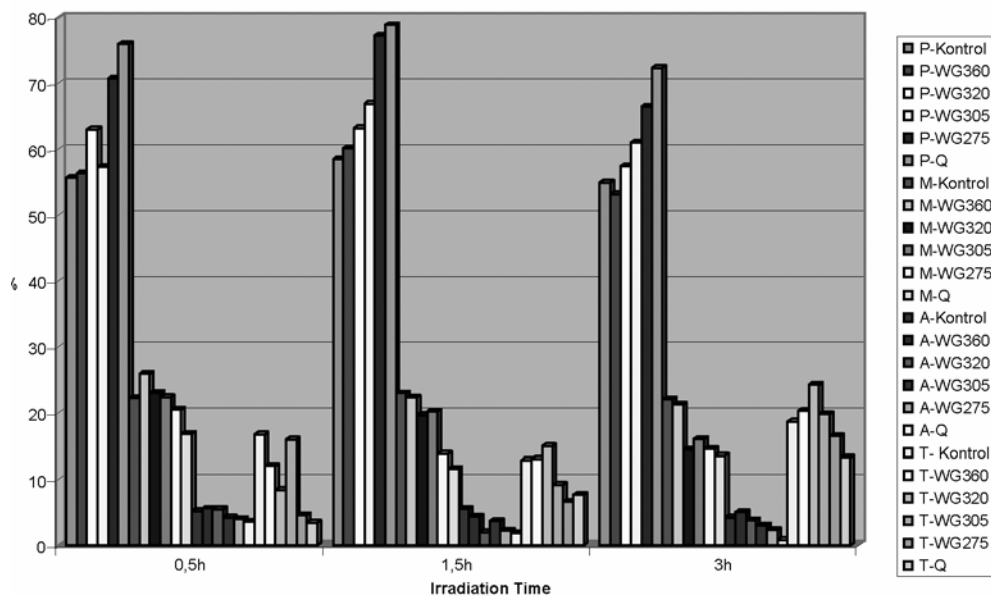


Fig. 7. The percentage of division phases, depending on irradiation time, at Ami cultivar

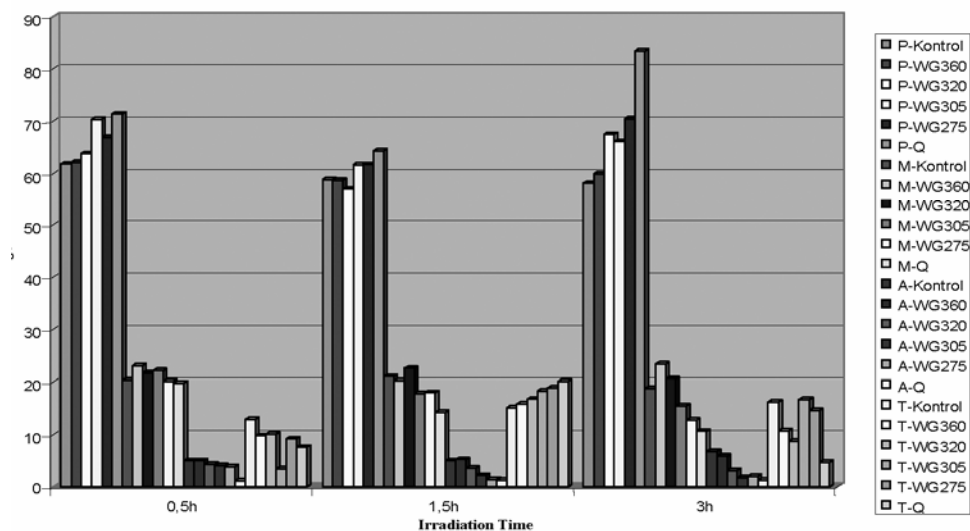


Fig. 8. The percentage of division phases, depending on irradiation time, at Diva cultivar.

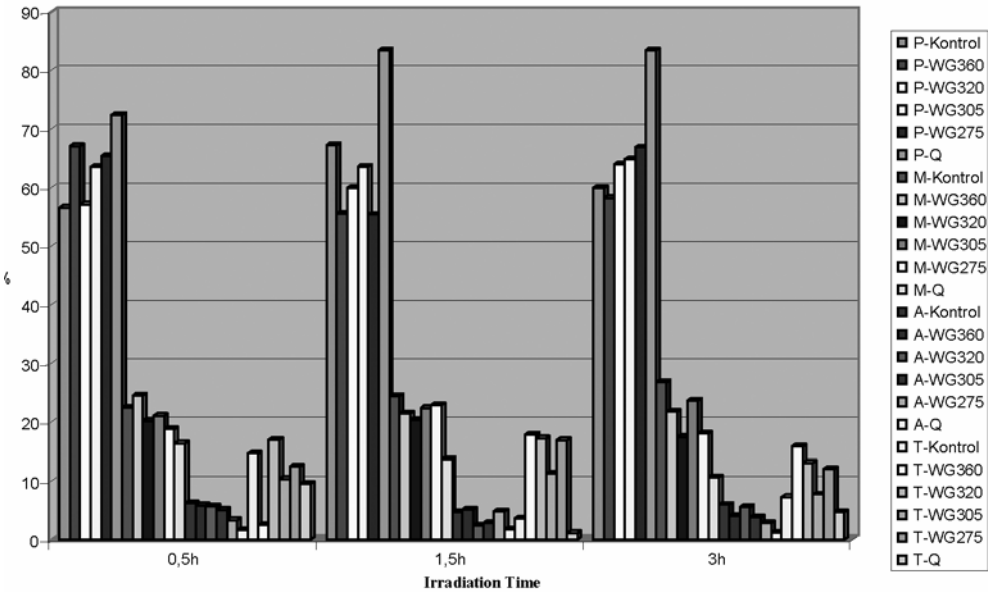
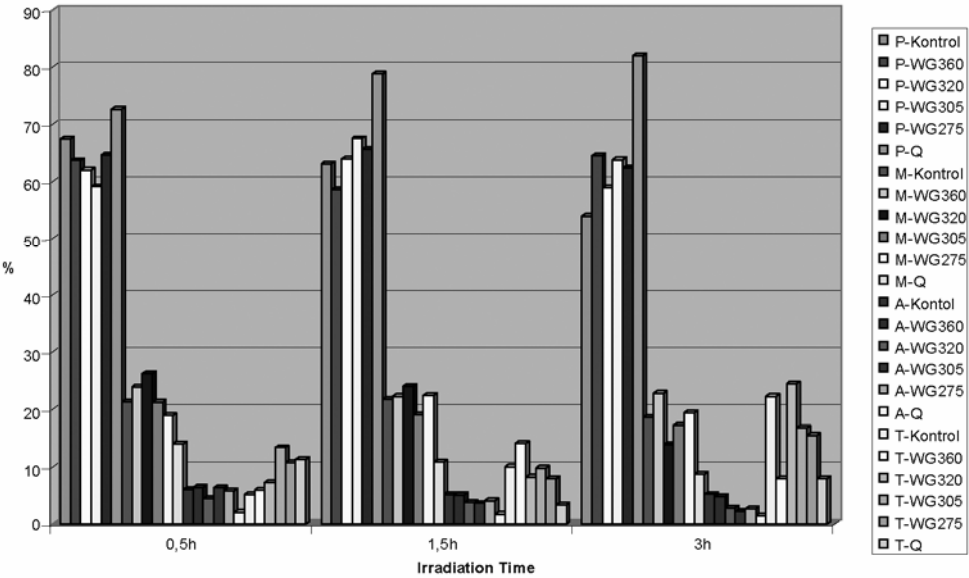


Fig.9. The percentage of division phases, depending on irradiation period, at Vera cultivar



THE VARIATION OF NUCLEIC ACIDS CONTENT AFTER SIMAZIN TREATMENT ON *VICIA SATIVA* L.

ODETTA GRAMA-ȚIGĂNAȘ¹, CSILLA IULIANA BĂRA¹, ION I. BĂRA^{1*}

Key words: *Vicia sativa* L., nucleic acids, simazin.

Abstract: Simazin has in certain conditions stimulatory effects on nucleic acids biosynthesis. The biosynthesis and mitotic division stimulation suggest the possibility to use simazin like growing and germination stimulator.

INTRODUCTION

The simazin action mechanism on vegetal organism, has as target the photosynthesis inhibition, and can have secundar effects. The plants which are tolerant at simazin action, metabolize it in hidroxy-simazin and conjugated amino-acids (Sarpe 1987, 1976).

MATERIAL AND METHODS

The quantitative measurements of nucleic acids were made after Spirin method (1958) readapted for vegetal tissues.

RESULTS AND DISCUSSION

Plants productivity depends of the intensity of metabolic process which takes places on whole vegetation period, being hereditarily regulated. Nucleic acids actively participate at different molecular process like those of proteins and chlorophyll synthesis.

For *Vicia Sativa* L., the 6 hours treatment with simazin induced the increase of total nucleic acids quantity. The most intense stimulation was noticed by 0,1% concentration treatment variant (78,927 mg/g). The increase of nucleic acids quantity was induced also by DNA also by RNA. Decrease of quantity of RNA, DNA and total nucleic acids were noticed for 1% simazin concentration (RNA- 11,163 mg/g; DNA- 45,250 mg/g; total nucleic acids- 56,423 mg/g comparing with RNA 12,808 mg/g; DNA 51,773 mg/g; and 64,581 mg/g for total nucleic acids for controle).

For 12 hours treatment, it could be noticed an increase of total nucleic acids content comparing with controle, the maximal value being reached by 0,1% simazin concentration treatment (69,446 mg/g) which decrease to 53,717 mg/g by 1% simazin concentration. For both, treatment or controle variant, higher quantity values were reached by DNA. For 12 hours treatment time, the total nucleic acids content increase especially due to RNA, and for 6 hours treatment, due both RNA and DNA.

For 24 hours treatment time, the functional activity of hereditary apparatus in *Vicia Sativa* L. embryonic roots (fig.3), excepting the 0,01% treatment variant, subjects to a general rule: the diminution of total nucleic acids quantity concomitantly with the increase of herbicide concentration. The lowest quantity of total nucleic acids (30,277 mg/g) was noticed by 1% treatment variant, comparing with 57,921 mg/g by controle. The decrease of nucleic acids quantity was made by the decrease of DNA quantity, while the RNA quantity increased by 0,01%; 0,1%; 0,25%; and 0,5% treatment variant comparing with control.

For the variants treated with high concentrations (0,75% and 1%), the nucleic acids quantity decrease because of DNA and also RNA. The decrease of DNA quantity and increase of RNA quantity, suggest that at that time, in root tips cells by *Vicia sativa* L., the protein biosynthesis was more intense and the DNA autoreplication weaker.

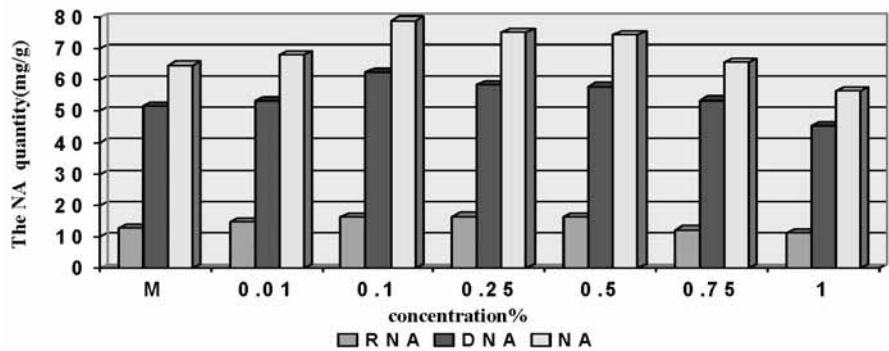


Figure 1. The variability of nucleic acids quantity under treatment of simazin (6 h) at *Vicia sativa* L species.

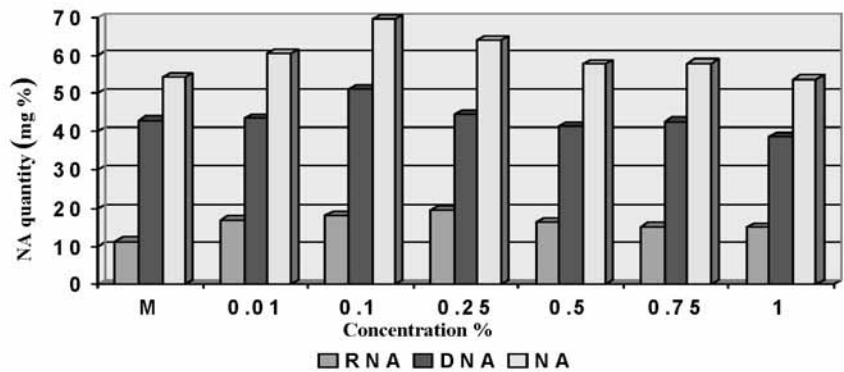


Figure 2. The variability of nucleic acids quantity under treatment of simazin (12 h) at *Vicia sativa* L species.

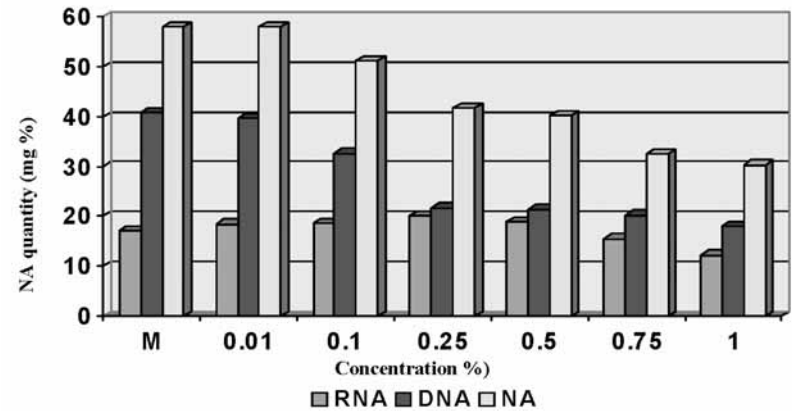


Figure 3. The variability of nucleic acids quantity under treatment of simazin (24 h) at *Vicia sativa* L species.

CONCLUSIONS

By *Vicia sativa* L., simazin has in certain conditions stimulatory effects in nucleic acids biosynthese.

The total nucleic acids quantity increase also because DNA also because RNA.

The maximal stimulation of biosynthese was obtained for 0,1% simazin concentration for 6 hours and 12 hours.

The prolonged treatment, for 24 hours, strongly disturb the hereditary apparatus function.

The biosynthese and mitotic division stimulation suggest the possibility to use simazin like growing and germination stimulator.

Simazin, used in low concentrations do not represent danger for legumes cultures.

BIBLIOGRAPHY

1. ARTENIE VI., 1991. *Biochimie*. Editura Universității “Alexandru Ioan Cuza”-Iași.
2. BERCA M., 1985. *Relațiile dintre erbicide și mediul înconjurător*. Editura Ceres, București, p.100-103.
3. COMĂNIȚĂ E., ȘOLDEA C., DUMITRESCU C., 1986. *Chimia și tehnologia pesticidelor*. Editura Tehnică, București, p. 13-17, 398-402.
4. GRANITOV I.I., 1980, *Aktual'nye voprosy issledovaniya pestitsidov i rastitel'nykh resursov*. GPI, Taschkent, p.64.
5. SPIRIN A. S., 1958. Spektrofotometricheskoe opredilenie summarnovo kalichestva nucleinovykh kislot. *Biohimiya*, 23: 656-662.
6. ȘARPE N., 1987. *Combaterea integrată a buruienilor din culturile agricole*. Editura Ceres, București, p. 46-47, 163-164.
7. ȘARPE N., CIORLĂUȘ A., GHINEA L., VLĂDUȚU I., 1976. *Ierbicidele principiile și practica combaterii buruienilor*. Editura Ceres, București, p. 65-161.

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THE IMPACT OF UVB-B TREATMENT ON THE VARIATION OF NUCLEIC ACIDS CONTENT BY *VICIA SATIVA* L.

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Key words: *Vicia sativa* L., UV-B, nucleic acids, RNA, DNA.

Abstract: The ultraviolet radiations UV-B administrated on *Vicia Sativa* L. inhibit the nucleic acids biosynthesis. It can be concluded that it appears a plant defense reaction against UV-B. The nucleic acids quantity decrease directly proportional with irradiation time. Under the ultraviolet irradiation, the quantity decrease either for DNA or for RNA.

INTRODUCTION

The UV-B radiation (280-315nm) is part of the invisible solar spectrum. The stratospheric ozone layer from the upper Earth atmosphere absorbs short waved 290nm UV radiation (the potential damaging ones). The UV-C radiation is completely absorbed by ozone and atmospheric oxygen, the UV-B radiation is partially absorbed by ozone, and the UV-A radiation is just in a small amount absorbed (Tosserams, 1996; Frederic et. al., 1989). The stratospheric ozone depletion has like consequence the increase of UV-B radiation level which reach the Earth surface (Caldwell et. al., 1995).

MATERIALS AND METHODS

The experiment consists in UV irradiations, for different time periods, on *Vicia Sativa* L L, being in early ontogenetic stages, followed by biochemical tests (the content in nucleic acids).

25 mg vegetal tissue, were grinded in 2 ml perchloric acid (HClO₄-0,2N) and kept at 4°C till next day. After samples centrifugation for 15 minutes at 3000 rpm, pellet was resuspended in 2ml mixture ethanol 70%:glacial perchloric acid. After a new centrifugation in the same conditions, pellet was treated with 2 ml mixture ethanol 96%: ethyl ether 3:1. After lipids elimination (10 minutes on water bath at 50°C), samples were centrifugated 15 minutes at 3000 rpm. 5 ml perchloric acid 1N was added. Next day, from supernatant was determined RNA and from pellet DNA. For DNA dosing pellet was resuspended in 5 ml perchloric acid 0,5N, samples being boiled in a reflux refrigerator for 30 minutes. After centrifugation extinction were read by spectrophotometer, in UV light at 270nm and 290nm, reported to controle sample (perchloric acid 1N for RNA and perchloric acid 0,5N for DNA).

Calculation of DNA and RNA quantity was made after the formula:

$$RNA = \frac{5315 \times \Delta}{m} \times 0,1 \qquad DNA = \frac{5525 \times \Delta}{m} \times 0,25$$

Delta (Δ) represents the difference between extinctions read by 270nm and by 290nm, made with UV/VIS- Jasko, X-530. Values were expressed in mg/g fresh tissue.

RESULTS AND DISCUSSIONS

After the UV-A treatment (370nm), considered like controle for UV-B, applied 12 hours on *Vicia Sativa* L L, it was observed the decrease of total nucleic acids quantity comparing with dark controle, due to decrease of DNA content (to 55,462 mg/g from 73,757 mg/g). The RNA quantity increase comparing with controle (to 18,311 mg/g from 11,734 mg/g).

For 24 hours irradiation time, the nucleic acids quantity dramatically decreased to 21,626 mg/g. The decrease was again due to DNA which reached a very low value (7,718 mg/g DNA comparing with 67,175 mg/g by controle).

The drastically inhibition of biosynthesis after 24 hours recovers at 48 hours and can be appreciated like a plant defense reaction against UV, for repairing the lesions at molecular levels.

For 12 hours irradiation with UV-B (305nm) could be observed a decrease of nucleic acids level (71,441 mg/g) comparing with controle (85,491 mg/g), again due to decrease of DNA

level. The RNA level increase to double value comparing with controle (20,621 mg/g instead 11,734 mg/g). We can conclude that UV-B aplied for 12 hours on seedlings descrease DNA synthesys, but stimulate RNA activity. For 24 hours treatment with UV-B, the nucleic acids quantity continue to descrease comparing to controle, due to DNA and RNA, same effect being observed also for UV-B controle.

For 48 hours irradiation time, the nucleic acids quantity increase, a very interesting fact which we can not explain yet.

For 12 hours irradiation time with UV-B (295nm), the nucleic acids quantity do not suffer quantitative changes, having close values to the controle(84,797 mg/g comparing with 85,491 mg/g). It can be seen (Fig.3) RNA descrease (9,756 mg/g comparing to 11,734 mg/g for dark controle) and a DNA increase (75,042 mg/g,comparing with 73,757 mg/g).

For 24 hours irradiation with UV-B 295nm, for *Vicia Sativa* L, it could be noticed a light descrease of nucleic acids descrease in fresh vegetal tissue. The descrease was also due to RNA quantity descrease (to 15,565 mg/g from 17,839 mg/g by controle), and also due to DNA (to 63,529 mg/g from 67, 175 mg/g).

For 48 hours irradiation time the nucleic acids quantity dramatically descrease (52,887 mg/g comparing to 84,700 mg/g). This time the descrease was also due to RNA(10,272 mg/g comparing to 16,259 mg/g),also DNA (42,615 mg/g comparing to 68,441 mg/g for controle).

Analising this data, we can conclude that UV-B with 295nm wavelength,do not have a significant impact in first 24 hours, but for 48 hours irradiation time it can be noticed an inhibiting effect.

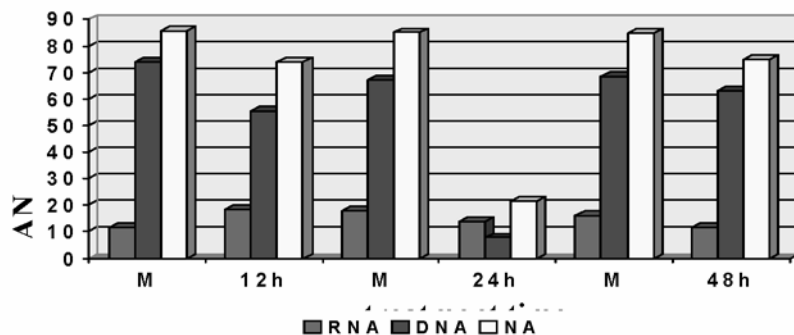


Figure 1. The variability of nucleic acids quantity under action of UV-B ($\lambda=370$ nm) at *Vicia sativa* L.species (mg/g fresh tissue).

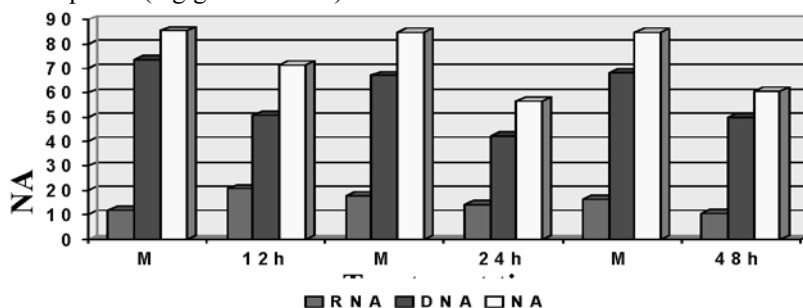


Figure 2 The variability of nucleic acids quantity under action of UV-B ($\lambda=305$ nm) at *Vicia sativa* L.species (mg/g fresh tissue).

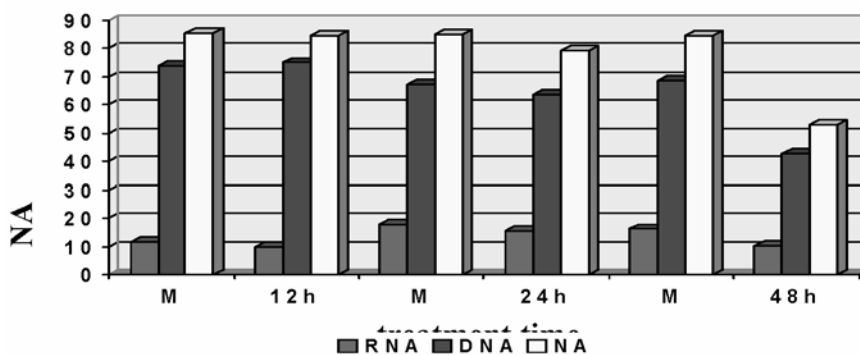


Figure 3. The variability of nucleic acids quantity under action of UV-B ($\lambda = 295$ nm) at *Vicia sativa* L. species (mg/g fresh tissue).

CONCLUSIONS

The ultraviolet radiations UV-B with 305nm and UV-A with 370 nm wavelength administrated for 24 hours on *Vicia Sativa* L. inhibit the nucleic acids biosynthesis, but inhibition disappears in the case of 48 hours irradiation.

It can be concluded that it appears a plant defense reaction against UV-B, having as result the repair of lesions at molecular level.

The nucleic acids quantity decrease directly proportional with irradiation time for UV-B 295nm.

Under the ultraviolet irradiation, the quantity decreases either for DNA or for RNA.

BIBLIOGRAPHY

1. CALDWELL M., M., TERAMURA A., H., TEVINI M., et. al., 1995, Effects of increased solar ultraviolet radiation of terrestrial plants. *Ambio* 24: 166-173
2. DUCA M., SAUCA E., BUDEANU O., GURĂU D., 1998, Dinamica ontogenetică a acizilor nucleici la diferite genotipuri de *Helianthus annuus* L. *Genetica și ameliorarea plantelor și animalelor în R.M.*, Chișinău, 60-62.
3. DUBININ N. P., 1966, *Genetica moleculară și acțiunea radiațiilor asupra eredității*, Editura Științifică, București, 236-261.
4. FREDERIC J., SNELL H., HAYWOOD E., 1989, Solar Ultraviolet Radiation at the earth's surface. *Photochemistry and Photobiology*, Pergamon Press, 50: 443-450.
5. SPIRIN A. S., 1958, Spektrofotometricheskoe opredelenie summarnovo kalichestva nucleinovykh kislot. *Biohimiya*, 23: 656-662.
6. TOSSERAMS M., ANTONIO PAIS DE SA, JELTE ROSEMA, 1995, the effect of solar UV radiation on four plant species occurring in coastal grassland vegetation in the Netherlands. *Physiologia Plantarum* 97: 731-739.

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THE EFFECTS OF SOME CHEMICAL COMPOUNDS ABOUT THE INDIVIDUALS OF *DROSOPHILA MELANOGASTER*

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Key words: *Drosophila melanogaster*, lead acetate, E vitamin, potassium permanganate, caffeine, urea, phenol, nicotine, benefic triangle.

Abstract: There were registered different effects of the actions of some chemical substances on *Drosophila melanogaster* individuals. We concluded that low concentrations of substances stimulated the proliferation and high concentrations of those substances inhibiting this process. Both the concentration values which had stimulating effects and the substances which had inhibiting effects are specific for each used substances.

INTRODUCTION

It is agreed that the xenobiotic factors, especially substances of synthesis, have dangerous effects for living systems, inducing a decrease of viability and proliferation, encouraging the appearance of ills, decreasing the longevity etc. Frequently, at certain concentrations, some compounds are inconsistent with life. But there are situations when very dangerous substances, administered in low concentration, have a stimulating effect. We believe that this aspect is very interesting and that the investigations about it may supply very important and interesting theoretical and practical conclusions.

MATERIAL AND METHODS

The investigations were effectuated with lead acetate, K_2MnO_4 , caffeine, urea, phenol, nicotine, and E vitamin, administered in different concentrations on *Drosophila melanogaster* individuals, from Suceveni, Kutî, Iași, Onești, Ungheni and Catranăc populations. The investigations endorsed to estimate the action of mentioned substances, in different concentrations, about *Drosophila melanogaster* individuals from natural populations. For each substance it was established 3-4 concentrations which assured the reasonable individuals number survive. It was assured, for each substance, desired concentration in culture medium. After that, in each tube, were introduced 2 males and 2 females. The descent was analyzed for number of off springs in each tube, during three generations. All data were compared with control and statistical processed.

RESULTS AND DISCUSSIONS

The obtained results pointed out some effects induced by mentioned compounds, depending of their concentration and duration of treatment. We shall present on a synthesis of obtained results which presents, in our opinion, a great theoretical and practical importance.

After the treatment with mentioned substances we have observed that all compounds, in certain concentrations (small concentrations only) have a stimulating effect about number of *Drosophila melanogaster* resulted individuals. The stimulating effect, in first generation, has appeared for all substances (used in this experiment). In the second generation, under the same treatment, only under urea and E vitamin treatment it was registered an increase of individuals number per tube. In the third generation the individuals number increased only in the case of E vitamin administration.

If, on abscissa (x axis), put the concentrations of substance and on ordinate (y axis) put the numbers of individuals (appeared under substance influence), will obtain a curve with dynamics of individuals number, depending of the substance concentration.

So, if we draw a parallel to x axe through point corresponding to the number of individuals from control variant, on y axe, will see that the obtained line intersect the curve and divide the graphic in two parts – above one and below one. The above zone has the aspect of a triangle

(ABC) for which we propose the name beneficial triangle of substance action, because this part correspond to the bigger values registered for individuals number, in variants of treatment comparative with control variant. So, we may assert that every substance has a beneficial effect upon individual system, when is administered in a low concentration.

Thus, inside of benefic triangle architecture, we found three concentration values named as followed:

a) the concentration which corresponding to CAB angle named **minim concentration with zero effect** - this low value did not generated an effect on population number;

b) the concentration which corresponding to ABC angle named **maxim concentration with stimulator effect** - this value corresponding to the maxim level for stimulator action of chemical substances on population number;

c) the concentration which corresponding to BCA angle named **maxim concentration with zero effect** - this value corresponding to an inhibiting effect of the chemical substances which reducing the population number until the control value. This concentration representing a superior limit of benefic triangle of a chemical substance.

The chemical substances which we investigated, in some specific concentrations, had benefic effects on living cells, individuals and, after that, on population. However, the persistence of this effect, as a consequence of this treatment in the next generations, is specific for each investigated substance. For many times, the benefic effect appearing just in first generation (for example, lead acetate, potassium permanganate, caffeine, phenol and nicotine).

Therefore, we realized a chemical substances classification depending on toxicity degree (the presence and persistence of benefic triangle during many generations).

For the analysis of benefic triangle evolution inside of investigated chemical substances, we observed that during the administration of a same substance quantity in nutritive medium, this is lower from a generation to the next one until disappearance. This facts suggesting us that the chemical substances would be accumulated inside of cells from a generation to the next one. The substance quantity growing up until, after a time, it is breaking the normal biochemical reactions from inside. Therefore, inside of a further generation even a low substance concentration had a noxiousness influence. The persistence of benefic triangle depending on cells needs for this substance, metabolism speed of it, and certainly another features.

The knowledge concerning the benefic triangle properties for those substances at population level is very useful, especially because of manipulation possibilities of populations through growing of individual numbers with a high variety. Because it is very well known that there not exist two similar individuals from genetic point of view, the growing of individual variability generate high efficiency of selection.

Also, those knowledge concerning to substances benefic effects are useful for agriculture (for chemical control of pests). We have to evidencing than some substances, in specific concentrations, could have either a lethal action for some species, or a stimulator effects generating a high growing of individual number for another species.

But, the most useful for us are the laws of benefic triangle of chemical substances action used in ecology domain. Many specialists practice a diminution of polluting substances through their spreading on large surfaces. Therefore, we could do an evaluation on their impact on the environment. After that, we try to establish a correct intervention in critical situation through efficient methods, and we can avoid ecological catastrophes with important consequences.

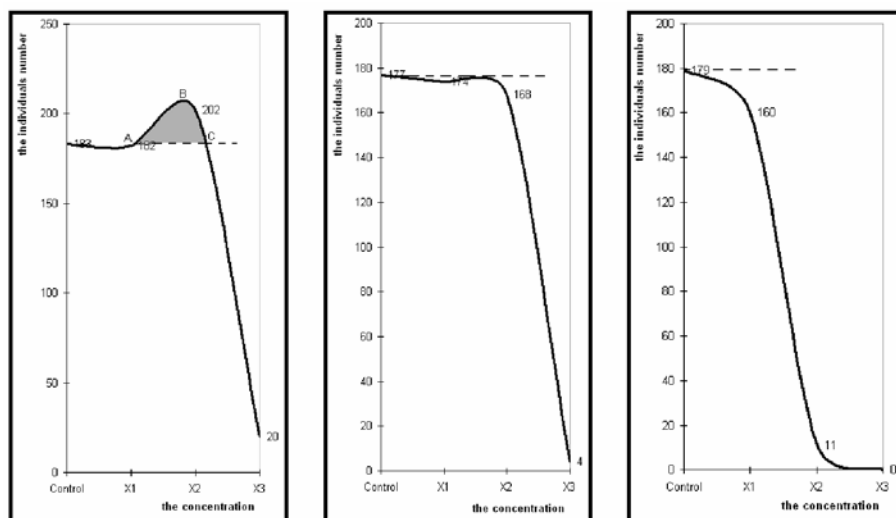


Figure 1. Diagram of individuals number dynamics as result of the lead acetate treatment ($((\text{CH}_3\text{COO})_2\text{Pb} \cdot 3\text{H}_2\text{O})$) applied on three successive generations (a – first generation; b - second generation; c - third generation).

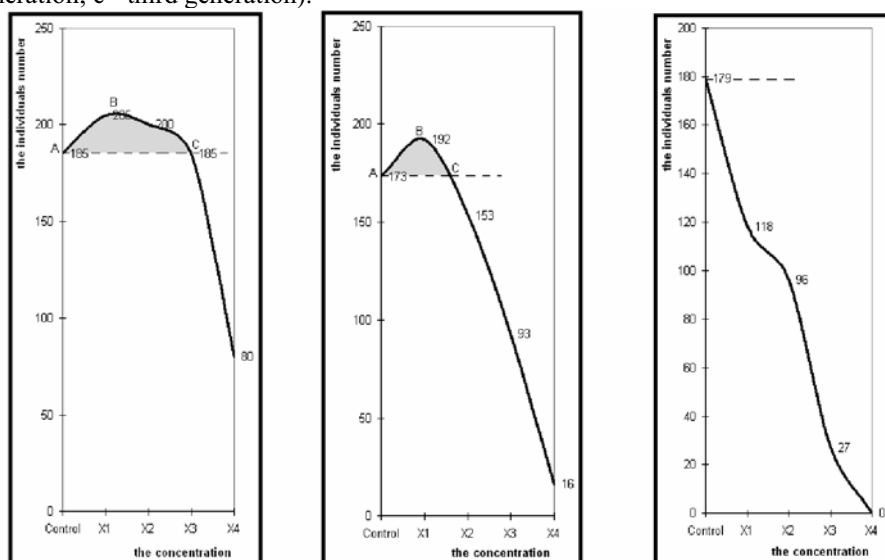


Figure 2. Diagram of individuals number dynamics as result of the urea treatment applied on three successive generations (a – first generation; b - second generation; c - third generation).

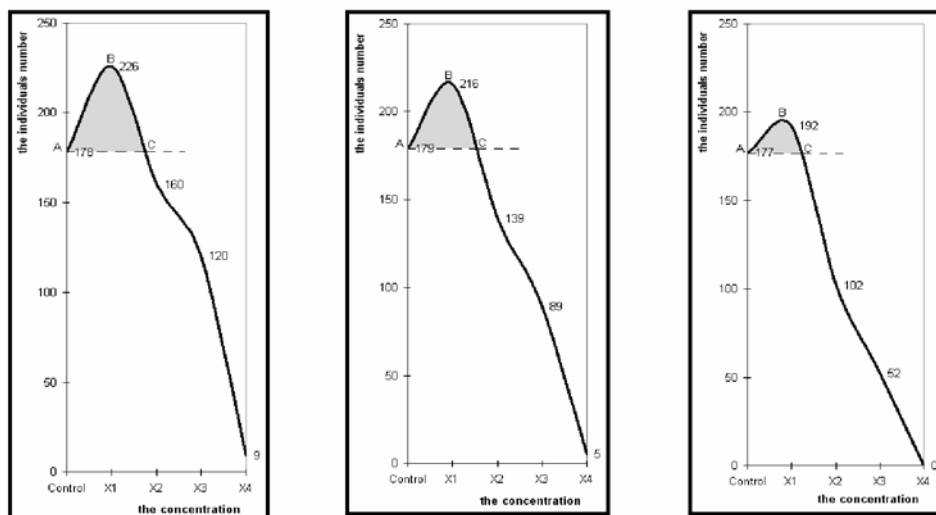


Figure 3. Diagram of individuals number dynamics as result of the E vitamin treatment applied on three successive generations (a – first generation; b - second generation; c – third generation).

CONCLUSIONS

We were able to point out the effect of different compounds about the *Drosophila melanogaster* individuals number dynamics in successive generations.

We concluded that low concentrations of substances stimulated the increase of individuals number and high concentrations induced a decrease of individuals number.

Both the concentration values which had stimulating effects and the substances which had inhibiting effects are specific for each used substances.

BIBLIOGRAPHY

1. Ashburner, M., and Wright, T. R. F., 1989. *The Genetics and Biology of Drosophila*. Vol. 2b, Academic Press, London, New York, San Francisco. 500-504.
2. Băra, I. I., 1999. *Genetica*. Ed. Corson, Iași.
3. Beilharz, R. G., and Nitter, G., 1998. The missing E: the role of the environment in evolution and animal breeding. *Zeitschrift für Tierzucht und Zuchtungsbiologie* 115: 439-453.
4. Chapman, R. F., 1998. *The Insects, Structure and Function*, chapter 22, Vision, Cambridge University Press.
5. Charlesworth, B., Charlesworth, D., and Morgan, M. T., 1990. Genetic loads and estimates of mutation rates in very inbred plant populations. *Nature* 347: 380-382.
6. Crow, J. F., and Simmons, M. J., 1983. The mutation load in *Drosophila*, pp. 1–35 in *The Genetics and Biology of Drosophila*, Vol. 3c, edited by M. Ashburner, H. L. Carson and J. N. Thompson. Academic Press, London.

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THE TESTING OF CAFFEIN “IN VITRO” REACTION ON *CYMBIDIUM HYBRIDUM* PROTOCOLORMS SUBCULTURED ON ESPECIALLY BRIDGE OF FILTERED PAPER

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Keywords: protocorms, orchids, *Cymbidium*, caffein, paper bridge.

Abstract: The *Cymbidium hybridum* protocorms, in vitroculture regime, on *Murashige-Skoog* liquid medium, were maintained on especially filtered paper bridges, which had responded different, depending on the introduced quantity of caffein on the cultures medium. After 90 days of vitroculture, the maximal number of protocorms, and the *fresh* and *dry* biomass of these, was registered at the variants of culture medium with caffein in a 0,001% concentration. On the lowest or highest concentration of 0,001% caffein, we find out a progressive decrease of the protocorms vitality.

INTRODUCTION

There are numerous compounds which can influence negative the biostructures of the vegetal cell, in this can be engaged the antifuzoriale substances or c-mitotic agents, which can inhibit the performance of the division spindle, the chromatoclastic substances or chromosomal “poisons”, called as radiomimetic substances or alkylates agents, which produce the chromosomes fragmentation, or the stathmodieretic substances which can stop cytodieresis, by inhibiting the fragmoplast differentiation (**Constantinescu**, 1958, 1961, 1965). Based on the studies with a fundamental character in this domain, **Apostol & Niculescu** (1970) were made a series of experiments on experimental models, with the purpose of the investigation of those sensitivity at the action of caffein on this, and of the alkylates agents administrated in low concentrations, same as the stathmodieretic effect of xanthine methyl derivatives. The Romanian school of experimental vegetal cytology, on the researches made from **Constantinescu** et. al (1958, 1961, 1965), becoming the merit to establishing that the methylic derivatives of xanthine, especially of caffein, in M/1000 concentrations, can sensitize the vegetal cells at the action of alkylic agents, condition in which the alterations produced in these, can be identical, till identity with the mitotic figures caused by this substances, or by the ionized radiations, at the culture. Researches in this domain were made by **Kihlman** (1965, 1971 a & b, 1972) which had explained the chromatoclastic action of purinici derivatives, especially of etoxycaffeine.

The investigations of various groups of researchers, has established the correlation between the chemical structure and the stathmodieretic activity in derivatives series of the theobromine, theophylline and caffein, and on the other hand can clear up the mechanism in which the purinic alkaloids and some derivatives can sensitizing the meristematic cells at the alkylic cytostatic actions. These researches were made with photonic microscope and with electronic microscope, too.

Our studies in the present work, represent a confirmation of the performed studies by **Constantinescu** at all, by using *Cymbidium hybridum* protocorms as an experimental sample, with the purpose of analysing the effect of this on their growth and morphogenesis level, cultivated on liquid medium which contained various concentrations of caffein.

MATERIAL AND METHOD

The *Cymbidium hybridum* protocorms were derived from the vitrophytobase of Laboratory of Biotechnology, University of Oradea, maintained on a basic medium (BM) *Murashige-Skoog* (1962) (MS), classic, without glicine, IAA, agar-agar and phytohormones, but with PP, B₆ and B₁ vitamins in a concentration of 1 mg/l. Periodically, at approximate 3 month, it was possible the cloning of these protocorms by subculture.

In this performed experiments, the *Cymbidium* protocorms were cultivated on the same type of MS medium culture, by adding various quantity of caffein, resulting the next medium cultures:

- V₀ – basic medium (BM), without caffein;
- V₁ – BM-MS with 0,0001% caffein;
- V₂ – BM-MS with 0,001% caffein;
- V₃ – BM-MS with 0,01% caffein;
- V₄ – BM-MS with 0,1% caffein;

After the pH adjustment at 5,7, this was distributed in thermoresistant glass recipients (ampicillin type), with 70 mm height and 25 mm diameter (interior). Before inserting the culture medium in the recipient, was proceed to a positioning of a filter paper bridge, with cross form, for sustain the protocorms (**Blidar**, 2004), assuring an inserted “platform” on the liquid medium surface, being 2-3 mm height of those level. In each bottle was introduced 5 ml culture medium, this assuring a liquid column with 13-14 mm height.

For the aseptization of the recipients with medium, the phials were obturated with hydrophilic cotton plug, after this there was autoclaved at 121°C temperature (which correspondent to 1 atm. pressure), for 20 minutes. After the cooling of these, in the perfect aseptic condition (at the box with laminar flux sterile air), was proceed to the protocorms separation

from the donor glomerules, derived from the vitrophytobase of laboratory, for inoculations using only green protocorms (the proof of there viability), they having the some characteristics looking their form and diameter. In each bottle, centered on the deck from the filtered paper, were located only one protocorm.

After inoculation, the obturation of the bottles was made with transparent foil, from polyethylene, preliminarily sterilized, for 15 minute, with 70° ethanol.

The incubation and growth of the cultures, was accomplished through exposure bottles on shelves, to white fluorescent light, with an intensity of 1700 of lucs, the tubes of neon be seated to a distance of 33 cm of vitrocultures, the photoperiod has corresponded to 18 of 24 hours light; temperature in the growth room oscillated between 26° C (the day) and 22° C (the night).

To an interval of 30 of days, respectively to 30, 60 and 90 days from inoculation, were performed prolusion matter the general appearance of vitrocultures, analysing the evolution of three parameters: *the number of neoformed protocorms* from the initially protocorms, the *fresh weight* and the *dry weight* of the biomass at these protocorms.

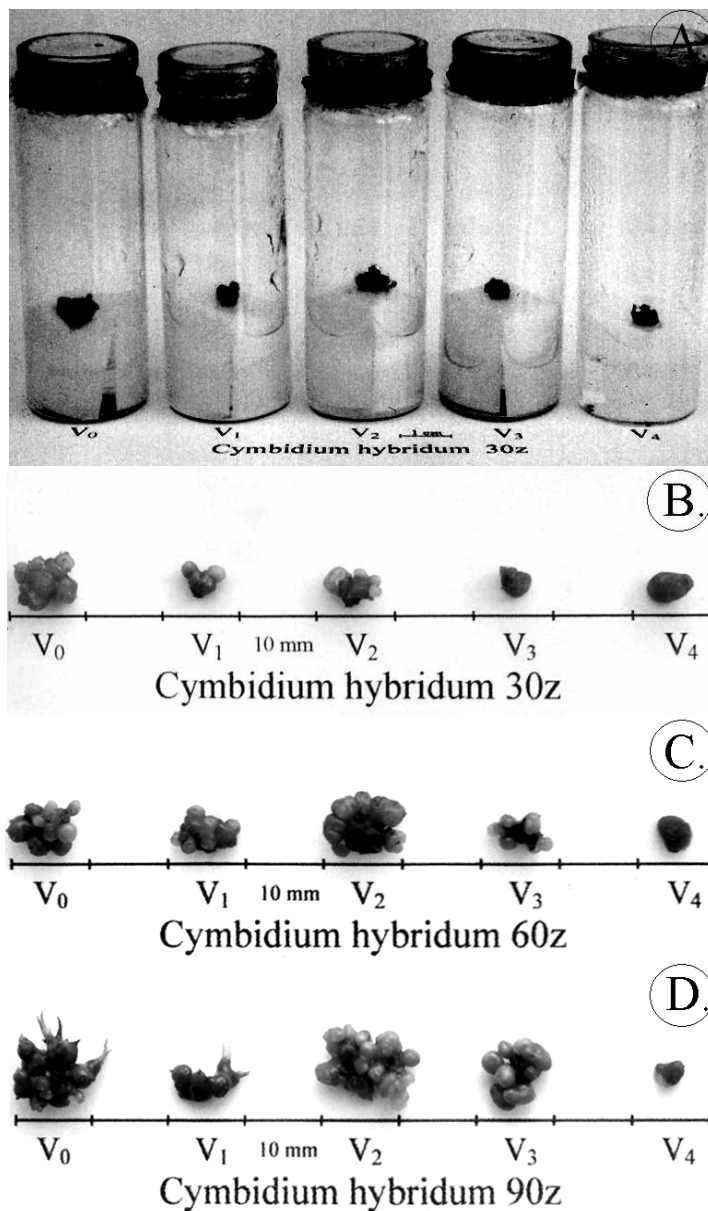
For each of these parameters, the values registered at 30 days of vitroculture, on the witness medium – without caffen (V₀), were considered reference value (as 100%), to this reference value retrospect all the other values of the respective parameter, on the period of 90 days from inoculation.

RESULT AND DISCUSSIONS

The most illustrative appearance, concerning the reactivity of differentiated protocorms, respectively the resulted vitrocultures from these, on five variants of culture medium, made the object of the photos from Drawing 1, and histograms from Figure 1.

The performed prolusions at 30 days from inoculation: most higher values in matter the *number of protocorms* regenerated to the level of each inocul, but the *dry cumulated substances* of these, they were registered to witness variant of average (V₀), without caffen (fig. 1, A & C); with all these, most significant accumulations of *fresh substance* were marked on variant of average with a content of 0,001% caffen (V₂), a closed value to this, was register on check average, the quantitative report among V₂ and V₀ being only 1,09: 1 (fig. 1, B). We mention the fact that in the conditions of a presences of a higher concentrations of caffen on the culture medium, respectively of 0,01%, but mostly 0,1% (variants V₃ and V₄), we remarked a strong inhibition on the multiplication and growth of the *Cymbidium* protocorms, the values at all three parameters take in step, were inferior against the witness, direct proportional phenomenon with the growing of the concentration of caffen from the culture medium (fig. 1, A & C, Drawing 1, A & B).

In behind the performed determinations at 60 days of vitroculture, we ascertained an increasing of the *number of protocorms* of *Cymbidium*, as much to variant V₂ (BM with 0,001% caffen), and to the variant V₃ (BM with 0,01% caffen), the values of this parameter being of 1,21 either, respectively of 1,05 either else bigger than the values registered to the witness lot (fig. 1, A). Instead, just like at 30 days from inoculation, the values of studied gravimetric parameters (*fresh and dry substance*), to this date, they maintained to the maximum value to variant of check average (fig. 1, B & C). From among the variants of medium with caffen, most good reactivity was registered on the medium V₂ (MB with 0,001% caffen), as much the appearance, and the number of neoprotocorms generated from the initially protocorm, but concerning the fresh and dry weight, remarking a stimulation of multiplication and growth of these (Drawing 1, C).



Drawing 1. Comparative appearance concerning the reactivity of the *Cymbidium hybridum* protocorms, cultivated on basic medium *Murashige-Skoog* (1962), where: V₀ – witness lot deprived of caffeine; V₁ – medium with 0,0001% caffeine; V₂ – medium with 0,001% caffeine; V₃ – medium with 0,01% caffeine; V₄ – medium with 0,1% caffeine, to 30 days (A & B), 60 days (C) and to 90 days (D) from the inoculation of unique protocorm per bottle.

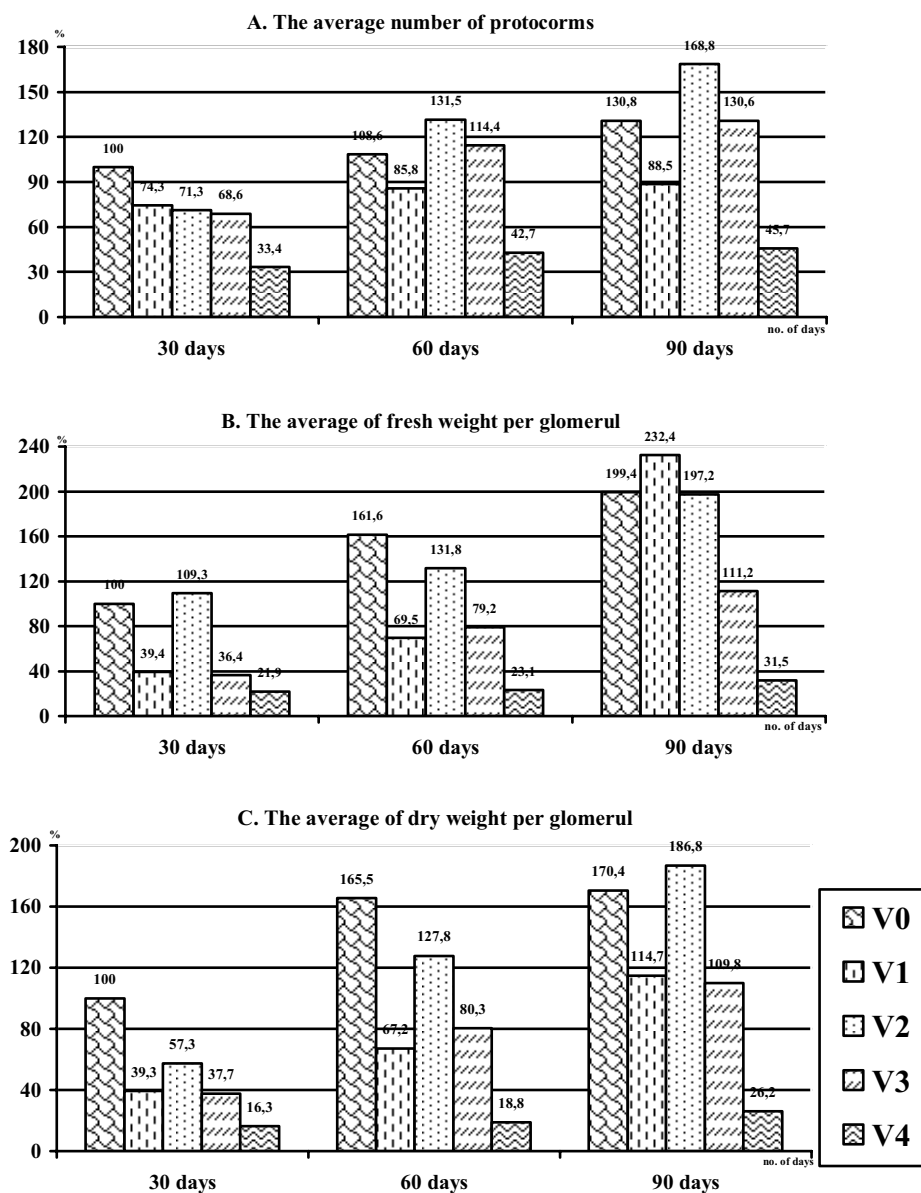


Figure 1. Dynamically growth of vitroculture of *Cymbidium hybridum* protocorm, for 90 days, on filter paper bridges, positioned on average of liquid culture medium *Murashige-Skoog* (1962), with addition of caffeine (where V₁ - BM with content of 0,0001% caffeine; V₂ – BM with content of 0,001% caffeine; V₃ – BM with content of 0,1% caffeine; V₄ – BM with content of 0,1% caffeine); the percentage reports were made taking as reference values (100%) the medium number of protocorms, the *fresh weight* and *dry weight* of these per glomerules, generated from initially protocorms of vitrocultures, after the first 30 days from the initiation of the experiments.

To final experimental period, respectively *at 90 days from inoculation*, the digital determinations sets off an amplification a the proliferation of cultivated protocorms on medium V₂ (BM with a content of 0,001% caffeine), the report of the *number of neoprotocorms* – against of the registered values to witness (V₀ – BM deprives the caffeine) – amplifying from 1,21:1 to 60 of days, to 1,29:1 to this last experimental development (fig. 1, A). We remarked, however, a strong growth of *fresh weight* to *Cymbidium* protocorms on the medium with a content of 0,0001% in caffeine (V₁), forward slip even the gravimetric values putted in evidence to the performed cultures on witness medium (V₀), the reported values being of 1,16:1 (fig. 1, B). Therefore, not but that the *number of protocorms* regenerated on variant of medium V₂ were most raised, from the all experimental series, numbers that illustrates that the *fresh weight* of generated glomerules in the variant of medium V₁, were superior to medium V₂. Concerning the *dry substance*, expectedly, the bigger values brands to medium variant with a content of 0,001% in caffeine (V₂), the report against the same parameter registered on the witness, being of 1, 09:1. Most thin proliferation were observed to protocorms cultivated on culture medium V₄ with 0,1% caffeine, where we distinguished a strong inhibition, in report with the witness, the values registered being inferior of 2,86 either concerning the *number of neoprotocorms*, respectively of 6,33 times and 6,5 either for the *fresh substance* and the *dry substance* (fig. 1, B & C and Drawing 1, D).

The performed biometric analysis about inoculs of *Cymbidium hybridum*, cultivates on liquid medium with different concentrations in caffeine, supported on especially filter paper bridges in sight avoiding hypoxias, we distinguished the following ultimate conclusions:

CONCLUSIONS

1. The bigger *number of neoprotocorms* generated at the surface of the inocul, as much at 60 days, but chiefly at 90 days of vitrocultures, it was registered to variant with 0,001% caffeine (V₂). Concerning this biometric parameter, good result was reflected on the witness variant of average, too, without caffeine, but they were inferior against the results registered to variant with 0,001% caffeine on the medium. On these motives, in sight obtain of a big number of neoprotocorms, after 60 days of vitroculture, we recommend the usage of liquid culture medium *Murashige-Skoog* (1962), with concentrations in caffeine of 0,001%. The presence of a concentration of 0,1% caffeine on the culture medium, it was proved to be inhibitory on the multiplication and growth of the *Cymbidium hybridum* protocorms, which conducted to the installation of a premature senescence on these level, phenomenon selling of with the necrosis of these, as far back as first 30 days from the initiation of the experiments.

2. The resulted *fresh weight* of the total amount protocorms per bottle, were higher on the variant of average with 0,0001% caffeine (V₁), values followed by the results obtained on the protocorms generated on the witness lot (medium MS without caffeine). As a succession of the proliferation of protocorms, as much below the appearance number, same as the volume of these, to variant of average V₂ (MS with 0,001% caffeine), the *fresh weight* registered per glomerules were all high, the values obtained was situated immediately below the values registered on variant V₀. Same as in the case *number of protocorms*, most thin result concerning the *fresh weight* of these, was marked to protocorms cultivated on the medium of culture with a contain of 0,1% caffeine.

3. The most significant accumulation of *dry substance*, therefore the most good evolution of the protocorms *Cymbidium hybridum*, cultivated on liquid culture medium *Murashige-Skoog* (1962), on filter paper bridges, putted in evidence in the conditions of using a concentration in

caffeine of 0,001%, as the addition to this medium, which fact is explained through that on this variant of medium, has generated the higher number of neoprotocorms, what bring us to recommend the including of this compound as growth regulator on the culture medium of fated for micropropagation of the protocorms at this plant species. On the contrary, the presence on the culture medium a concentration of 0,1% caffeine, caused a premature necrosis to these protocorms.

REFERENCES

- Apostol, I., 1970, *Celula vegetală reactiv biologic în controlul medicamentului. Studiul acțiunii alcaloizilor purinici și a unor noi derivați de sinteză*. Autoreferatul tezei de doctorat, Fac. Farmacie, IMF București;
- Blidar, C.F., 2004, *Evoluția protocormilor de Cymbidium hybridum cultivați „in vitro” pe medii lichide, pe punți din hârtie de filtru, în funcție de sezonul de inoculare*. În: *Lucrările celui de al XII-lea Simpozion Național de Cultură de Țesuturi și Celule Vegetale „Fiziopatologia celulei vegetale în regim de vitrocultură”*, editori: Cachiță, C. D., Ardelean Aurel, Fati Vasile, 5 iunie 2003, Jibou, Ed. Daya, Satu-Mare, 213-227;
- Constantinescu, D. G., Radian, V., 1958, *Corelația dintre structura chimică și activitatea statmodieretică*, *Lucrările Conferinței Naționale de Farmacie*, București, p. 545;
- Constantinescu, D. G., Retezeanu, M., Constantinescu, M., Stoenescu, V., 1961, *Action de quelques nouveaux 8-dérivés de la caféine sur la cellule végétale*, C.R. Acad. Sci. Paris, 253, p. 176;
- Constantinescu, D. G., Apostol, I., Cosma, D., 1965, *Action de la 1β-oxypropil-théobromine sur les racines de Blé*, C.R. Acad. Sci. Paris, 261, p. 3657-3659;
- Murashige, T., Skoog, F., 1962, *A revised medium for rapid growth bioassays with tobacco tissue cultures*. *Physiol. Plant.*, 15, 473-497.
- Kihlman, B. A., 1965, *Effects of chromosome-breaking purine derivatives on nucleic acid synthesis and on the levels of adenosine 5'-triphosphate and deoxyadenosine 5'-triphosphate in bean root tips*, *Mutat Res.* 1965 Jun;2(3):274-86;
- Kihlman, B. A., Sturelids, Norlen, K., Tidriks, D., 1971a, *Caffeine, caffeine derivatives and chromosomal aberrations. II. Different responses of Allium root tips and Chinese hamster cells to treatments with caffeine, 8-ethoxycaffeine and 6-methylcoumarin*, *Hereditas*, 69 (1), 35-50.
- Kihlman, B. A., Norlen, K., Sturelid, S., Karlsson, M. B., Kronborg, D., 1971b, *Caffeine, caffeine derivatives and chromosomal aberrations. III. The ATP-dependence of the production of chromosomal aberrations by 8-ethoxycaffeine in Chinese hamster cells at 17 degrees C.*, *Hereditas*, 69 (2), 323-325;
- Kihlman, B. A., 1972, *Caffeine, caffeine derivatives and chromosomal-aberrations .V. Influence of temperature and concentration on induced aberration frequency in Vicia faba*, *Hereditas-Genetiskt Arkiv* 71(1):101-&;

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ACQUIREMENT OF TRANSGENIC COTTON (*GOSSYPIMUM HIRSUTUM* L.) RESISTANT TO HERBICIDE AND INSECT USING GLYPHOSATE-TOLERANT *aroAM12* GENE AS A SELECTABLE MARKER

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Keywords: transgenic cotton, glyphosate, Bt, selectable marker

Abstract: A new binary vector, pAM12-S1m, harboring the *aroAM12* gene encoding for 5-enolpyruvyl- shikimate-3-phosphate synthase (EPSPS) and a synthetic recombinant *BtS1m* toxin gene consisting of 331 N-terminal amino acids of CryIAC and 284 C-terminal amino acids of CryIAB has been constructed. The truncated *aroAM12* gene, which was obtained through gene shuffling technology, was ligated with a transit sequence of *Arabidopsis* EPSPS and expressed in cotton plants driven by cauliflower mosaic virus 35S (CaMV35S) promoter. The chimeric *BtS1m* toxin gene was fused with DNA sequence encoding PR1b secretory signal peptide and expressed under the control of 2E-35S promoter and “Ω” translation enhancer sequence derived from tobacco mosaic virus. The mutant EPSPS of *aroAM12* gene product conferring highly resistant to glyphosate, the active ingredient in herbicide Roundup®, was used as a dominant selectable marker for cotton plant transformation. The genes were introduced into commercial cultivar Zhongmian12 of cotton (*Gossypium hirsutum* L.) by *Agrobacterium*-mediated transformation. The transformants were directly selected on medium supplemented with 80 μmol/L glyphosate. In this research, 40 regenerative cotton plantlets were obtained through screening. Integration of *aroAM12* and *BtS1m* genes was confirmed by PCR and Southern blot, the results indicated that all the 40 plants possessed the *aroAM12* gene, 28 of which possessed both the *aroAM12* and *BtS1m* genes. Expression of both the genes was established by Western blots. Insect bioassay and glyphosate resistance assay indicated that the transgenic cotton plants obtained were highly resistant to glyphosate and insect. The results of glyphosate resistance and insect bioassay of T₁ generation showed that the numbers of resistance and sensitive phenotypes showed Mendelian segregation ratio.

INTRODUCTION

Cotton, a global crop, is a very important source of fiber, feed and edible oil. However, due to the weeds and insects damages, the yield and quality of cotton decrease seriously. So, the development of cultivars resistant to herbicide and insect is of tremendously importance to cotton production throughout the world. The tools of gene engineering technology have made it possible to develop cotton that are resistant to glyphosate and insect.

At present, several classes of herbicides are used for broad-spectrum weed control, one such herbicide is glyphosate, the active ingredient in Roundup herbicide. Glyphosate is a broad-spectrum, nonselective herbicide, it is immobilized by the soil and readily degraded by soil microorganisms, so it can't persist in nature. On the other hand, it is also proved that glyphosate has extremely low toxicity to animals. Glyphosate specifically binds to and blocks the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EPSPS), an enzyme of the aromatic amino acid biosynthetic pathway. EPSPS catalyzes the reaction of shikimate-3-phosphate and phosphoenolpyruvate to form 5-enolpyruvylshikimate-3-phosphate and phosphate. The reaction takes place in the chloroplast (Comai et al, 1985). In the past, the tactics to achieving glyphosate resistant crops were: (I) to introduce the EPSPS gene (*aroA*) which has an overexpression in the plants to compete the inhibition of glyphosate ; (II) to carry out site-directed mutagenesis to obtain none or decreased affinity for glyphosate. The research of glyphosate-tolerant crops has been pursued since the 1980's. So far, there are variety of glyphosate resistant crops appeared (Della-Cioppa et al, 1986; Padgett, et al, 1995; Debbi et al, 1996; Marie Mannerlöf, et al, 1997). In our research, the glyphosate- resistant gene, *aroAM12*, which is obtained through the gene shuffling technology (He et al., 2002), was transferred into cotton.

In addition, another important trait concerning cotton, the insect -resistance, has been extensively carried out. Transgenic cotton producing insecticidal toxins from *Bacillus thuringiensis* (Bt) are widely used for insect control. (Firozabady et al., 1989; EL-Hiatemy et al, 1990; Debbie et al., 1996). The *Bt* gene we introduced is *BtS1m*, which is a chimeric gene with its production consisting of 331 N-terminal amino acids of CryIAC and 284 C-terminal amino acids of CryIAB.

The objective of this research was to reconstruct a high efficient binary plant expression vector harboring the *aroAM12* and *BtS1m* genes that confer resistance to glyphosate and insect, respectively. The genes were transferred to

commercial cultivar Zhongmian12 utilizing *Agrobacterium*-mediated transformation method, and as we know, it is the first report to use *aroAM12* as a selective marker gene to screen off the transformants of cotton plants.

MATERIALS AND METHODS

Plasmid constructs

A 1.3 kb DNA fragment carrying the *aroAM12* gene was obtained by gene shuffling technology. Another 0.22 kb DNA fragment, ASP (*Arabidopsis* signal peptide), was attached to *aroAM12*. This recombinant fragment was cloned into a plant expression vector, pCambia1300, that included a cauliflower mosaic viral 35S promoter (CaMV35S), Nos (polyA) addition signal. The coding region of *aroAM12* gene is flanked by a MCS (multicloning site) at the 5' end and a *Xho*I site at the 3' end. The above cloning resulted in the plasmid pGAT1300. The plasmid pBtS1m harboring the *BtS1m* gene was received from Tian Y.C, Institute of Microbiology, CASB, China. The chimeric *BtS1m* gene were inserted between promoter of the 2E-35S(CaMV35S promoter supplemented with four repeats of the enhancer domain to stimulate the transcription of *BtS1m* gene in plants and nopaline synthase (Nos) terminator sequences. The 5' end of *BtS1m* gene was fused to the untransformed Ω leader sequence of tobacco mosaic virus to enhance the translation of mRNAs. The coding region of *BtS1m* cassette is flanked by a *Hind*III site at the 5' end and a *Xba*I site at the 3' end. Both the pGAT1300 and pBtS1m plasmid DNA were digested with *Hind*III and *Xba*I. A plant expression vector, pCM12-S1m, was constructed by assembling the *aroAM12* and *BtS1m* cassette (Fig.1).

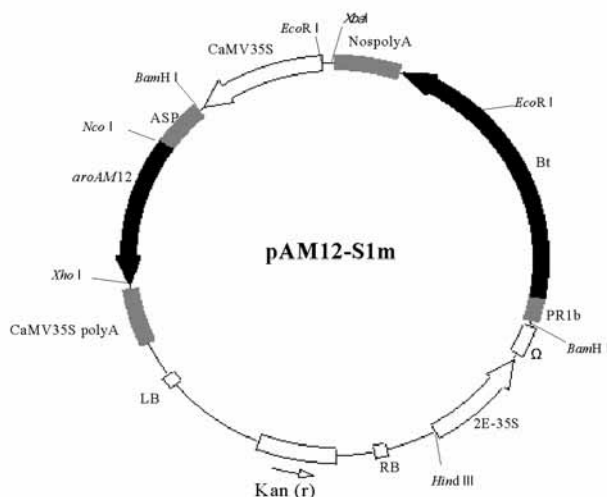


Fig.1. Construction of a high efficient expression binary vector pCM12-S1m with *aroAM12* and *BtS1m* genes

Bacterial strain and vector

Agrobacterium strain LBA4404 carrying the binary plasmid pCM12-S1m, with *aroAM12* as a selective marker, was used as the vector system for cotton transformation and regeneration experiments.

Plant materials

Seeds of *Gossypium hirsutum* L. cv. Zhongmian12 were obtained from the Cotton Research Institute, CAAS, and Anyang, Henan Province.

Seed germination

Seeds were sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 5 minutes, rinsed subsequently with sterilized deionized water at least 4 times. The seeds were germinated aseptically on MS medium at 28°C. The pH of the medium was adjusted to 5.8–6.2 prior to autoclaving at 121°C for 20 min.

Transformation and cotton regeneration

Hypocotyl sections (0.3–0.5 cm) taken from 5-day-old seedlings were directly used to be infected with LBA4404 culture (A600 0.4–0.6). The hypocotyls were immersed in the bacterial suspension for approximately 7 min and

subsequently blotted dry on the sterile filter paper. Then they were co-cultivated on MS₀ media supplemented with 0.1mg/L 2,4-D, 0.1mg/L KT and 200mg/L Acetosyringone (pH 5.0 ,solidified with 0.2% Gelrite) under dark conditions for 48 hours at 28±2°C. After co-culture, the hypocotyls were rinsed thoroughly, blotted dry, then, transferred to MS₁ medium (same as MS₀, except that acetosyringone was taken out, pH 5.8-6.2) containing 400mg/L cefotaxime and 0.91g/L MgCl₂ and incubated at 28±2°C, under a 16 hours photoperiod. After 7 days cultivation, the explants were transferred to MS₂ medium (same as MS₁, except that glyphosate was added) containing 80μM glyphosate to induce the callus formation. After two subcultures on MS₂, the glyphosate-resistant calli were transferred to the proliferation medium (MS₀ +0.91g/L MgCl₂). Again after several subcultures, the calli were transferred to MSB medium (MS saults,B5-organics, 1g/L asparagines, 2g/L glutamine, 3% glucose, pH 5.8-6.2,solidified with 0.25% Gelrite), cultured on this medium under a 16 hours photoperiod at 28±2°C, with monthly subculture until somatic embryos appeared, germinated, rooted. , then the individual plantlets were transferred to soil and kept under high humidity for two weeks. At last, they were transferred to large pot with fertile soil and grown to maturity in the greenhouse.

PCR Analysis

Total DNA was extracted from the leaves of cotton plants utilizing cetyl-trimethyl ammonium bromide (CTAB) method as described in ref [9]. Transgenic plantlets were checked by PCR with *aroAM12* specific primers (5'-CATGCCATGG AATCCCTG ACG TTA CA A-3'forward, and 5'-CGCGGATCCTTAGCAGGCTACTCATTC-3',reverse) and *BtS1m* specific primers (5'-TATCCCATTGTTCGCAGTCC-3'forward, and 5'-CAT CACGACTCAAGTTGTTA-3',reverse). PCR reaction was performed in 20μL volume under the following conditions: 31 cycles of 94°C for 1 min (denaturation), 54°C for 1.5 min (annealing), and 72°C for 1 min (extension). Cycles were preceded by denaturation for 5 min at 94°C and a final extension at 72°C for 10 min. PCR products were electrophoresed on 0.8% agarose gel.

Southern blot Analysis

The procedures for Southern blot hybridization analysis were according to Sambrook et al (1989). Total genomic DNA from transgenic and untransformed plants were used for Southern blot, 15μL of DNA per sample was thoroughly digested with EcoRI,separated on 0.8% agarose gel, then transferred to a nylon membrane (HybondTM-N+,Amersham) by capillary action. The blot was prehybridized and subsequently hybridized with *aroAM12* gene probe and *BtS1m* gene probe, respectively. Labeling of the probe with ³²P was done by Hexal Label PlusTM DNA Labeling kit (MBI Fermentas, USA) and purified with CENTRI.SPIN Columns (PRINCETON). Following hybridization , the membranes were washed properly, then exposed 4 days to Kodak X-film at -70°C for autoradiography.

Western blot Analysis

Approximately 200 mg fresh leaves of cotton plants were quickly ground to a fine powder in liquid nitrogen. The powder was homogenized in 200μL extraction buffer (100m mol/L Tris-HCl, pH 8.0 ,5mmol/L MgCl₂, 2% SDS,10% glycerol, 5% β-mercaptoethanol), boiled for 10min. The sample was centrifuged and the supernatant collected. Protein concentrations were determined by using the Bradford assay, with BSA as the protein standard. 10% SDS-PAGE was performed to separate the proteins, subsequently transferred to HybondTM-P PVDF membrane (Amershan) by using the Semaphor transphor unit (Amershan). After transferring, the membrane was incubated with antibodies against the 48 kDa EPSPS (1: 250) and the polyclonal antibodies against the 68 kDa CryAc protein (1: 300), respectively. The western blot was performed following the Western Blot Kit BCIP/NBT System (Kirkegaard & Perry Laboratories, USA).

Herbicide resistance assays

Transgenic and untransformed plantlets were treated with 0.5% Roundup® (370g/L), once every 7 days, when the plants were approximately 40cm tall, and scored for phytotoxic effects after three weeks.

Insect Bioassays

The leaves with leafstalk of young seedling plants of transgenic lines and non-transgenic cultivars (negative control) were picked at the same seedling ages. They were placed on moist paper in Petri dishes with tight-fitting lids. Five second-instar larvae of *H.assulta* were distributed in each leaf. After three days in culture, the leaf damage index and the number of surviving larvae were recorded. Treatment was replicated 4 times.

Herbicide resistance and Insect Bioassays in T₁ generation

Selfed seeds collected from T₀ transgenic plants were germinated, and T₁ progenies were subjected to the Roundup® test and insect bioassays. Untransformed Zhongmian12 were used as comparable controls in both assays. The plants which have lateral root growth (growing on MS media containing 25 μmol/L glyphosate) were scored as resistant, while the plants, which have no appearance of lateral root, were recorded as susceptible.

RESULTS AND DISCUSSIONS

Transformation of cotton

Cotton hypocotyls explants were transformed with *Agrobacterium* strain LBA4404 harboring the binary plasmid pCM12-S1m containing the *aroAM12* and *BtS1m* genes. The regenerated putative plantlets were screened by PCR with primers against the *aroAM12* and *BtS1m* genes, respectively, for the presence of the T-DNA inserts. The results were described as in Table 1.

Table1 Transformation frequency calculated as obtained transgenic plantlet/total number of explants

Cultivar	Explants	Transgenic plantlets*	Transformation frequency (%)
Zhongmian12	1500	40	2.67

*Plant number of PCR positive with *aroAM12* primers.

In the 40 plantlets examined, all have the predicted 1.2 kb fragment by using the *aroAM12* specific primers, untransformed plant DNA, as expected, showed no products. Only 28 plantlets generated a predicted 0.96 kb PCR product by using the *BtS1m* specific primers, the left and untransformed ones had no PCR products. The results indicated that during the T-DNA integration to plant genome, approximately 25% *BtS1m* gene were lost. Fig.2 was the part PCR results of regenerated plantlets.

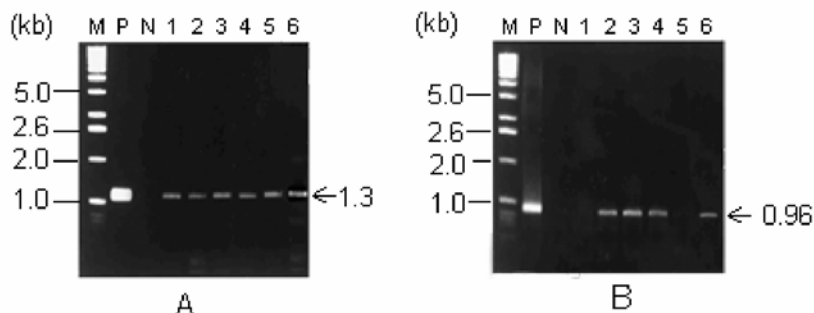


Fig. 2. PCR analysis of putative transformants and untransformed plant using two primer sets:

(A): *aroAM12* gene specific and (B) *BtS1m* gene specific. Lane M, 1kb ladder ; lane P, pCM12-BtS1m plasmid DNA ; lane N, untransformed plant DNA ; lanes 1-6, putative transformants: M12-Bt/a, M12-Bt/ b , M12-Bt/c M12-Bt/d , M12-Bt/e and M12-Bt/f.

Southern blot analysis

The *aroAM12* gene PCR positive plantlets were further analyzed with Southern hybridization to confirm the site-specific integration of T-DNA and to establish inserts number. Total DNA from each random transgenic and untransformed cottons were digested with *EcoRI* , and two blots were made from these samples. The blot shown in Fig.3B was probed with ^{32}P primer-labeled 1.3kb size *aroAM12* DNA fragment. Single band was present in lanes 1, 2,5,6; two bands were detected in lanes 3,4. No hybridization signal could be detected for the untransformed plant DNA, as expected. According to the distribution of *EcoRI* sites on the T-DNA region and the hybridization pattern, we can confirmed that the copy number of inserts were equal to the number of bands. The number of inserts in the transformants varied from one to two copies. The blot shown in Fig.3C was probed with ^{32}P primer-labeled 0.96kb DNA fragment; two, three, three and

two bands were detected in lane 2,3,4,6, respectively. Single band of approximately 1.0 kb size fragment were detected in all the 4 lanes. As can be seen in Fig.3A, there were two *EcoRI* sites in the T-DNA region, one flanks at the 5' of *aroAM12* gene, the other is located inside the *BtS1m* gene. The putative transformant in lane 1, 5 and the control plant showed no hybridizing fragment.

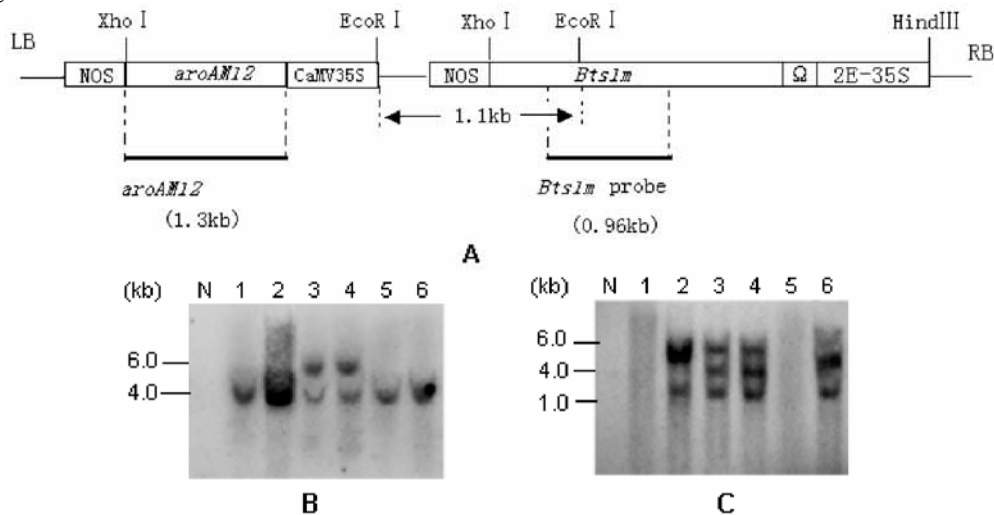


Fig.3. Southern blot analysis of DNA isolated from leaves of independent transformants and untransformed control.

A, map of the T-DNA region of the pCM12-S1m; B, Total genomic DNA of transformants was digested with *EcoRI* and hybridized with *aroAM12* DNA fragment. LaneN: untransformed plant; Lanes1-6: M12-Bt/a, M12-Bt/b, M12-Bt/c, M12-Bt/d, M12-Bt/e, M12-Bt/f; C, Total genomic DNA of transformants was digested with *EcoRI* and hybridized with *BtS1m* DNA fragment. Lanes1-6: M12-Bt/a, M12-Bt/b, M12-Bt/c, M12-Bt/d, M12-Bt/e, M12-Bt/f; Lane7: untransformed plant.

Immunoblot Analysis

To test whether the *aroAM12* mRNA and *BtS1m* mRNA were properly translated; total soluble protein obtained from transformed and untransformed leaves was subjected to Western blot analysis. The blot shown in Fig. 4A was probed with anti-EPSP synthase serum. In the six random selected transgenic plants, as well as the positive control (EPSP synthase protein, purified from an *E.coli* overproducing strain, in preparation), a band of 48 kDa size was detected with different band intensity. The blot shown in Fig. 4B was probed with anti-CryIAc serum. In lanes 2,3,4,6, bands of approximately 68 kDa size were detected, co-migrated with the positive control (CryIAc protein, in preparation), while the putative plantlet in lanes 1,5, as well as the untransformed plant, no visible signals were detected. This result coincided with the southern blot mentioned above.

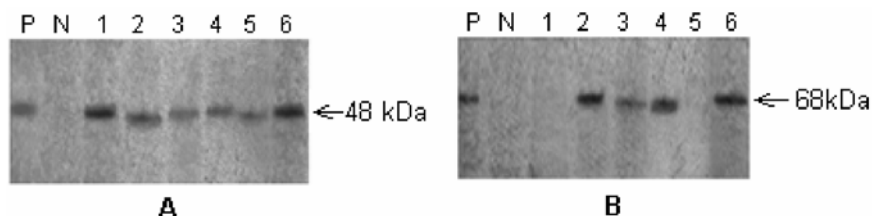


Fig.4. Western blot analysis of total proteins extracted from transformants and untransformed cotton plant.

(a) The EPSPS protein of *aroAM12* gene product was detected using a polyclonal antibody serum, Lane P: EPSPS protein, positive control; Lane N, untransformed cotton plant; Lanes 1-6: M12-Bt/a, M12-Bt/b, M12-Bt/c, M12-Bt/d, M12-Bt/e, M12-Bt/f. (b) The CryIAC protein of *BtS1m* gene product was detected using a polyclonal antibody serum. Lane P: CryIAC protein, positive control; Lane N, untransformed cotton plant; Lanes 1-6: M12-Bt/a, M12-Bt/b, M12-Bt/c, M12-Bt/d, M12-Bt/e, M12-Bt/f.

Insect Bioassay of T₀ transgenic cotton

Transgenic cotton plants obtained by transformation with the pCM12-BtS1m constructs and untransformed cotton were tested for insect tolerance by feeding leaves to neonate larvae of the bollworm (*H. assulta*). Larvae fed on transgenic plant leaves suffered from severe deleterious effects; surviving insects suffered reduction in body weight. Larvae fed on untransformed leaves, however, grew well. The detail results were shown in Fig.5 and Table 2



Fig.5. Insect bioassays.

The leaves of transgenic and untransformed plantlets were infested with *H. assulta* of second instar larvae at three days. TG, transgenic plant; CK, untransformed control.

Table 2 Effects of transgenic cotton on survival and development of *H. assulta* of second instar larvae at three-day infestation.

Number of plantlet	Mortality (%) 3days	Survival weight (mg) 3days	Leaf damage
1	0	5.50±0.40	++++
2	82.5	0.70±0.25	+
3	73.0	0.85±0.20	++
4	86.0	0.67±0.28	+
5	0	5.52±0.30	++++
6	82.5	0.71±0.25	+
CK	0	5.58±0.40	++++

Herbicide resistance of T₀ transgenic cotton

To discern the extent of protection afforded by the *aroAM12* gene, resistance of plantlets to different dosage of glyphosate were tested. In greenhouse assay, all regenerated cotton plantlets exhibited tolerance to glyphosate. A typical cotton is shown in Fig.6, from which we could observe that after three times spraying, the transgenic cotton plant grew well, while the control one were completely shriveled and dry.



Fig.6. Glyphosate resistance in transgenic and untransformed cotton plant.

The plantlets were sprayed with 0.5% Roundup®. TG, transgenic cotton of M12-Bt/b line; CK, untransformed cotton plant.

Herbicide resistance and Insect Bioassays in T₁ generation

Resistance to Roundup® were assessed 12 days after growing in media containing 25 μmol/L glyphosate. Some T₁ progenies having lateral root growth showed significant resistant against glyphosate, while others have no lateral root appearance (Fig.7). In insect bioassay, some

progenies showed little damage caused by *H. assulta* of second instar larvae, while others showed severe damage. Both the herbicide resistance and insect assays on segregating progenies of T₁ showed good fit to the Mendelian ratio of three resistant: one susceptible (one inserts) or fifteen resistant: one susceptible to the glyphosate and insect (two inserts), respectively (Table3, 4).



Fig .7. Effect of glyphosate on lateral root development of T₁ seedlings
A, Seedlings with well-developed lateral roots; B, Seedlings with no lateral roots.

Table3 Segregation of glyphosate tolerance in T1 progenies

Progenies of Tranformants	No. of plants Resistant	No. of plants Sensitive	χ^2 *	P
1	50	30	5.33 (3:1)	0.010-0.025
2	53	27	2.74 (3:1)	0.050-0.100
3	72	8	1.41 (15:1)	0.100-0.250
4	71	9	2.00 (15:1)	0.100-0.250
5	54	26	2.05 (3:1)	0.100-0.250
6	52	28	3.51 (3:1)	0.050-0.100

* Uncorrected chi-square goodness-of-fit test for hypothesis of 3:1 or 15:1 segregation. None of the chi-square values are significant at the 95% confidence level.

Table4 Segregation of insect- resistance in T₁ progenies

Progenies of Tranformants	No. of plants Resistant	No. of plants Sensitive	χ^2	P
1	0	12	-	-
2	28	16	2.45 (3:1)	0.100-0.250
3	27	5	2.13 (15:1)	0.100-0.250
4	24	3	1.80 (15:1)	0.100-0.250
5	0	10	-	-
6	18	10	1.40 (3:1)	0.100-0.250

CONCLUSIONS

Analysis of cotton plants regenerated from *Agrobacterium*-inoculated hypocotyls confirmed the feasibility of transferring the *aroAM12* gene and *BtS1m* gene, conferring resistance to the broad-spectrum glyphosate and insects, respectively. We also demonstrated the use of *aroAM12* gene as a convenient selectable marker gene in plant transformation experiments. To the best of our knowledge, in previous studies, the commonly used selectable marker genes for cotton are antibiotic resistant gene such as *NPTII* and *HPT* genes (Satyavathi et al., 2002; Yue et al., 2002). Glyphosate-resistant cotton was also obtained (Debbie et al., 1996), but the CP4 EPSPS gene transferred was only as a commercial interest, not as a selective marker gene. Glyphosate selection has many advantages, compared to the antibiotic genes. First, glyphosate is readily degraded in soil; the EPSPS is present merely in plants and bacteria, not in animals. In terms of safety, there exists the possibility that the antibiotic resistant genes migrate to the microorganisms to lead to the invalidation of medical antibiotics. Second, a very low frequency of non-transgenic escapes happened during the whole transformation process. In our research, all the 40 plantlets selected were proved to be transgenic. The *aroAM12* gene we introduced was through gene shuffling technology; several amino acids were changed compared to the original EPSPS gene (*Salmonella typhimurium* and *Escherichia coli*), which caused the EPSP enzyme with an extremely high k_i for glyphosate and low k_m for the substrate PEP (He et al., 2002). The levels of tolerance depend on the expression level of *aroAM12* –encoded EPSP synthase. As to the *BtS1m* gene, the feeding assay revealed that the CryIAC protein was toxic to the larvae of *H. assulta*. It had been observed that the insect resistance varied among different plants, this is perhaps due to the activity of the transgene's promoter or the translation enhancer “ Ω ” fragment. It was also observed that in the whole transformation process, 25% *BtS1m* gene was lost during the integration of T-DNA. According to Tinland (Tinland, 1991), the T-DNA integrates into the plant genome from the right to the left border, so the DNA adjacent to the left border might be lost. While in this study, the *BtS1m* gene adjacent to the right border of T-DNA was lost, what caused the disagreement need further research. Based on the southern blot, we can make a conclusion that the T-DNA inserted into the genome at random by illegitimate recombination, so that the number of the inserted copies, their chromosomal location varies among the transformants. Together with the immunoblot, it seems that there is no direct correlation

between copy number and the level of gene expression, and sometimes there exist the inverse correlation. Gene silencing might cause this phenomenon (Vaucheret and Fagard, 2001).

REFERENCES

1. Comai, L, Facciotti, D, Hiatt, W.R, Thompson, G, Rose, R.E, Stalker, D.M. Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate, *Nature*, 1985, 317: 741-744.
2. Debbie, L.N, Kolacz, K.H., Robert, E, and Buehler, W. Glyphosate-tolerant cotton: genetic characterization and protein expression, *J.Agric.Food.Chem*, 1996,44: 1960-1966.
3. Della-Cioppa, G, Bauer, S.C, Klein, B.K, Shah, D.M, Fraley, R.T, Kishore, G. Translation of the precursor of 5-enolpyruvylshikimate-3-phosphate synthase into chloroplasts of higher plants in vitro, *Proc.Natl.Acad.Sci.USA*, 1986,83: 6873-6877.
4. El-Hiatemy, Y, El-Shihy, O.M, Sharaf, A.N. Transfer of delta-endotoxin gene into cotton protoplasts. *Bulletin of Faculty of Agriculture University of Cairo*, 1990,41: 717-728.
5. Firozabady, E, DeBoer, D.J, Mer, D.J. Transformation of cotton by *Agrobacterium tumefaciens* and regeneration of transgenic plants, *Plant Mol.Biol*, 1989,10: 105-116.
6. He,M , Zeng,H.Y , Xu ,P.L. Cloning and mutagenesis of the *aroA* gene, *Acta Scientiarum Naturalium Universitatis Sun Yat Seni*, 2002, 41: 76-79.
7. Marie Mannerlöf, Stig Tuveesson, Per Steen, Paul Tenning. Transgenic sugar beet tolerant to glyphosate, *Euphytica*, 1997, 94: 83-91.
8. Paterson, A.H, Brubaker, C.L, Wendel, J.F. A rapid method for extraction of cotton (*Gossypium* ssp.) genomic DNA suitable for RFLP or PCR analysis, *Plant Mol.Biol*, 1987,41: 122-127.
9. Padgett, S.R, Kolacz, K.H, Delannay, X, Re, D.B, LaVallee, B. J, Tinus, C.N. Development, Identification, and Characterization of a Glyphosate-Tolerant Soybean Line, *Crop Science*, 1995,35: 1451-1461.
10. Satyavathi, V.V, Prasad, V, Gita.B.L, Lakshmi, G. High efficiency transformation protocol for three Indian cotton varieties via *Agrobacterium tumefaciens*, *Plant Science*, 2002,162: 215-223.
11. Sambrook, J, Fritsch, E.F, Maniatis, T. *Molecular cloning: a laboratory manual*. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989.
12. Tinlind, B. The integration of T-DNA into plant genomes. *Trends in plant sciences*, 1991,1: 178-184.
13. Vaucheret, H, Fagard, M. Transcriptional gene silencing in plants: targets, inducers and regulators. *Trends in Genetics*, 2001, 17: 29-34.
14. Yue, J.X, Zhang, H.J, Zhang, L.H. Hygromycin Resistance as an efficient selectable marker for cotton stable transformation, *Cotton Science*, 2002,14: 195-199.

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CLONING AND COMPARISON OF HEMOGLOBIN α CHAIN CDNA SEQUENCES FROM FIVE SPECIES OF FISHES IN CYPRINIFORMES

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Key words: *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Carassius auratus*, *Misgurnus anguillicaudatus*, *Paramisgurnus dabryanus*, α globin gene

Abstract: The complete cDNA sequences encoding hemaglobin α chain subunit (or α globin) were cloned by RT-PCR amplification from five species of fishes, *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Carassius auratus* Linnaeus, *Misgurnus anguillicaudatus*, *Paramisgurnus dabryanus*. Each of the five α globin cDNA contains full length of open reading frame that encode a 143 amino acid residues of α globin. The sequence comparison revealed that the α globin sequences among the five species are significantly divergent although they are all classified in *Cypriniformes*.

INTRODUCTION

Farm-grown fishes are one of the main protein sources in human protein supply. It has growing economic importance as the wild fishes were subjected to more strict protection from decreasing population. Studies to improve productivity and quality of farm fishes have been focused on selection and breeding of fish strains and/or species suitable for varieties of aquaculture purposes. Recent advances in transgenic technologies provided a powerful tool for generating fish strain that may bring about economic value to aquaculture production.

A number of genes, such as growth hormone (GH) and anti-freeze protein (AFP), were targeted to generate transgenic fish by heterologous integration[1]. The growth hormone transgenic fish were shown having a faster growth rate, whereas the AFP transgenic fish had little phenotypic change in tolerating to low water temperature. Extensive study was carried to explore the molecular structure and biochemical property of AFP [2]. Another tempt of transgenic fish study was to transfer hemaglobin gene from the fish that tolerates to low oxygen content in water to the fish sensitive to low oxygen condition. To explore the underline mechanisms for the variation of hemoglobin property, we have cloned hemaglobin α chain cDNAs from five species of fishes, *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Carassius auratus*, *Misgurnus anguillicaudatus*, *Paramisgurnus dabryanus* and compared their sequence similarity and phylogenetic relationship.

MATERIAL AND METHODS

Material

Fish samples, *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Carassius auratus*, *Misgurnus anguillicaudatus*, *Paramisgurnus dabryanus* were purchased from Xinxiang farmers' market.

Preparation of total RNA

Total RNA was extracted from blood using Promega total RNA Isolation System according to the manufacturer's protocol. Briefly, 0.2-0.3ml blood were collected by tail amputation, and the blood cells were precipitated by centrifugation at 300g, rinsed with sterile 1×PBS, broken in lysis buffer by homogenization. The cell lysis suspension was extracted with 600μl of Phenol: chloroform: Isoamyl Alcohol after adding 60μl of Sodium Acetate (pH4.0). Total RNA was precipitated by centrifugation at 12000rpm and washed once before air-drying. The RNA pellet was dissolved in 30μl of Nuclease-free water.

RT-PCR

Using Promega Access RT-PCR System kits, PCR reaction were performed in a 50μl reaction volume containing 1μl total RNA, 50pmol / L of each primer, 1μl 10mmol / L dNTP mix, 5μl MgSO₄, 10μl 5×AMV / Tfl reaction buffer, 5U AMV Reverse Transcriptase, 5 U Tfl DNA Polymerase. Thermocycling program was set with 1 cycle of reverse transcription at 48°C for 45min, 40 cycles of denature at 94°C for 30s, annealing at 54°C for 1min, extension at 72°C for 2min. After the final cycles, an extension reaction was carried out at 72°C for 10 min.

Upper primer:

5' ATGAGTCT(G/C)(A/T)C(A/T/C/G/G)(G/C/A)(A/T/C/G/G)(A/C)(A/C/G)(A/T/C/G)GA 3'

Down primer:

5' TTATTA(T/G)C(T/G)GTA(T/C)TT(A/C/G)TCAG 3'

Cloning and analysis of PCR product

PCR products were detected by electrophoretic separation in a 1.5% agarose gel followed by ethidium-bromide (EB) staining. The bands of interests were purified using DNA Gel Extraction Kits. The PCR products were directly ligated into pUCm-T vectors, and transformed into JM109 competent cells. Positive clones were selected through X-Gal color indication. Plasmid DNA was prepared using alkaline lysis and sequenced using dideoxy chain termination method.

Analysis of α globin sequence

Sequence similarity searches were done using the BLAST algorithm in GenBank.

RESULTS AND DISCUSSION

Cloning and sequences of globin cDNA of five fish species

Using degenerate primers, we amplified by PCR ~430 base pairs of cDNAs (Fig. 1) encoding full length of hemoglobin α chain subunit from five species of fishes, *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Carassius auratus* Linnaeus, *Misgurnus anguillicaudatus*. Each of the α globin cDNAs was cloned into pUCm-T vector and sequenced throughout its entirety. The results shown that they all encode a globin with 143 amino acid residues (Fig. 2) (GenBank accession number: AF528156, AF528157, AF528197, AF528198, AF528199). Sequence comparison with the existing hemoglobin α chain protein confirmed their identity.

Comparison of sequence similarity

Sequence alignment analysis shown that more than 81% of the amino acid residues are identical among the five globins. The highest sequence similarity, ~90% homology, was observed between the two closest species, *Misgurnus anguillicaudatus* and, *Paramisgurnus dabryanus*. (Table 1). The amino acid residues equivalent to those of mammalian α globin that are involved in interacting with heme and oxygen binding were identified by the sequence comparison, which include aa60 (His), aa62 (Lys), aa88 (Leu), aa89 (His), aa93 (Leu) and aa95 (Val). Although most of them are well conserved a number of intriguing variations exist in *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Carassius auratus* Linnaeus. Despite of the overall similarity, the α globin sequences are significantly diverged among these five species of fishes considering the conservative nature of hemoglobin in vertebrates. To explore the relationship between amino acid sequence similarity and species differentiation, we compared the above α -globin sequences plus five other fish globin sequences deposited in GenBank database by phylogenetic analysis (Fig. 3).

Hemoglobin Genes have so far been analyzed in many vertebrates, but the study of hemoglobin genes in fishes have been limited, to date, to the protein level. Fish is primordial vertebrate and it has many specific characteristics adapted for variety physiological needs. Oxygen content in water is not only one of the most important restriction factor but also one of the most important conditions affecting the growth of fish. The mobility of fish sharply reduced in hypoxic condition. When oxygen content remained at low level for an extended period of time, many symptoms may appear because of the declining of food intake, mobility, disease - resistance, etc. However, variation in hemoglobin sequences among different species may render various tolerance levels to low oxygen content in water, which is attributed to changes of hemoglobin binding affinity and dissociation constant to O_2 and CO_2 . The study of fish hemoglobin may help to understand the molecular mechanisms that under line the tolerance to low oxygen content in water.

Globin is species-specific. Different globin has different amino acid and different affinity of oxygen. The Hb components in adult fish are complicated. For instance, the rainbow trout shows three major Hb forms (HbIV, HbI, and HbII); HbIV and HbII display a strong Bohr effect, whereas HbI is insensitive to pH (i.e., the Bohr effect is completely absent)[4]. Rund published

the first systematic analysis of the “white” blood of an Antarctic icefish, *Chaenocephalus aceratus*. Furthermore, the oxygen-carrying capacity of *C. aceratus* blood was approximately 10% that of two red-blooded notothenioids. Adults of the family *Nototheniidae* (Antarctic rockcods) generally possess a major hemoglobin, Hb1, and a second, minor hemoglobin, Hb2, that differ in their α chains ($\alpha 1$ and $\alpha 2$ respectively) [5]. The more phyletically derived harpagiferids and bathydraconids have single hemoglobin. The trend toward reduced hemoglobin multiplicity in the notothenioid suborder, which reaches its extreme in the icefishes, probably results from evolutionary loss or mutation to transcriptional inactivity of globin genes[6]. The five species in this paper belong to *Cypriniformes*. They have close relationship. But they have different insensitive to low oxygen content in water due to they linked to the need for dealing with a mutable environment or different habitats. The α globin is different not only in suffocate point which in *C. carpio*; *C. idellus*; *C. auratus* Linnaeus; *M. anguillicaudatus*; *P. dabryanus* is 0.34 ~ 0.3mg/L, 0.51 ~ 0.3mg/L, 0.13 ~ 0.11mg/L, 0.24mg/L, 0.16mg/L respectively but in amino acid sequence[7,8]. In *C. auratus* Linnaeus and *P. dabryanus* the suffocate point is close to each other but there are 25 differential amino acids. Q6, S22, I35, is special in *C. auratus* Linnaeus. D5, P6, G19, S48, is special in *P. dabryanus*. It is deduced that the change of these positions could enhance the affinity of oxygen. The suffocate point in *C. idellus* which specific amino acid is V65, A66, A79, R89, A112, A117 is lowest in five fishes. These amino acid may decrease or increase the affinity of oxygen. Tab1 shows that the similarity of α globin in five fishes is consistent with phylogenetic relationships from morphological. The result was discrepancy with previous study of β globin in our laboratory. This may be α globin experience less selective pressure.

The first case of transgenic fish was obtained in 1985 in China. Following the development of DNA cloning and recombinant technology, transgenic fish is likely to into the market first in China[9]. It is urgent for improved breeds of fish with traits of high quality, fast-growing and resistance to disadvantage environmental factor in cultivation industry. Therefore, more and more study on transgenic fish will be done in order to improve the quality and resistance. Increasing the capacity of resistance low oxygen content can improve not only the cultivation density but the affinity of oxygen and then boost economic benefit. So breeding trans-globin genes fish that can resist low oxygen content has enormous economic benefit and will open a broader vista.

REFERENCES

1. Krasnov A, Agren JJ, Pitaknen TI, Molsa H., 1999 - Transfer of growth hormone (GH) transgenes into Arctic charr. (*Salvelinus alpinus* L.) II. Nutrient partitioning in rapidly growing fish. *Genet Anal*, 15(3-5) : 99-105.
2. Garth L Fletcher, Choy L Hew, Peter L Dacies., 2001 - Antifreeze Proteins of Teleost Fishes. *Annu. Rev. Physiol*, 63 : 359-390.
3. Yoshizaki G., 1991 - Introduction of carp α -globin gene into rainbow trout. *JAP.SOC.SCI.FISH*, 57(5) : 819-824.
4. Barra, D. Petruzzelli, R. Bossa, F. Brunori, M., 1983 - Primary structure of hemoglobin from trout (*Salmo irideus*) amino acid sequence of the beta chain of trout HbI. *Biochim. Biophys. Acta*, 742(1) : 72-77.

5. Fago,A., D'Avino,R. Di Prisco,G., 1992 - The hemoglobins of *Notothenia angustata*, a temperate fish belonging to a family largely endemic to the Antarctic Ocean. *Eur.J.Biochem*, 210 : 963-970.
6. Ennio Cocca, Manoja Ratnayake-Lecamwasam, Sandra K. Parker, Laura Camardella, Maria Ciaramella, Guido di Prisco, H.William Detrich III., 1995 - Genomic remnants of α -globin genes in the hemoglobinless Antarctic icefishes. *Proc.Natl.Acad.Sci.USA*, 92 : 1817-1821.
7. Shi Quanfang., 1991 - *The physiology of Fishes* . Beijin : Agriculture publication, 86.
8. Zhao Zhenshan, Yin Jie, Gao Guiqin, Feng Yaoguo, Chen Wenhui., 1999 - Study of consumption oxygen rate and suffocate point in *Misgurnus anguillicaudatus* and *Paramisgurnus dabryanus*. *Acta Hydrobiologica Sinica*, 19 (1) : 2-3.
9. Zhu Zuoyan Zeng Zhiqiang., 2000 - Open a door for Transgenic fish to Market. *Biotechnology Information*, 1 : 1-6.
10. Vidal,G., Scheffler,B., Michel,A. and D'Surney,S.J., 2004 - Genomic and phylogenic comparisons of the alpha-globin and beta-globin intergenic sequences between zebra fish (*Danio rerio*) and six closely related *Cyprinindae* species. *JOURNAL DNA Cell Biol.* 23 (5), 325-334.

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Fig.1 The RT-PCR amplification result of total RNA in five fishes

1. 100bp ladder marker

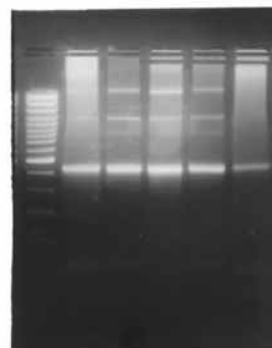
2. *Cyprinus carpio*

3. *Ctenopharyngodon idellus*

4. *Carassius auratus* Linnaeus

5. *Misgurnus anguillicaudatus*

6. *Paramisgurnus dabryanus*



1	M S L S A R D K A A U K A L W A K I S S K S D D I G A E A L	C. carpio
1	M S L S D P D K A U V K A L W A K I G S R A D E I G A E A L	C. auratus
1	M S L T A R D K A U V K A L W S K I S S K A D E I G A E A L	C. idella
1	M S L S A R D K S U V K A L W G K I S S R A D D I G A E A L	M. anguillicaudatus
1	M S L S E Q D K S A U K A H W S K I S S R S D D I G A E A L	P. dabryanus
31	G R M L T V Y P Q T K T Y F A D W A D L S P G S G P V K K H	C. carpio
31	G R M L T V Y P Q T K T Y F S H W S D L S P G S G P V K K H	C. auratus
31	G R M L T V Y P Q T K T Y F S H W A D L S P G S G P V K K H	C. idella
31	G R M L T V Y P Q T K T Y F S H W A D L S P G S A P V K K H	M. anguillicaudatus
31	G R M L X U Y P Q T K T Y F S D W A D L S P G S A P V K K H	P. dabryanus
61	G K V I M G A V G D A V S K I D D L V G G L A S L S E L H R	C. carpio
61	G K T I M G A V G D A V S K I D D L V G A L S S L S E L H A	C. auratus
61	G K V I V A A V G D A V S K I D D L A G G L A A L S E L R A	C. idella
61	G K T I M G A V G E A E S K I D E V T G S L A A L S E L H A	M. anguillicaudatus
61	G K T I M G A V G E A V S K I D D L T G A L S A L S E L H A	P. dabryanus
91	S K L R U D P A N F K I F A H N U I V U I G M L S P G D F P	C. carpio
91	F N U R I D P A N F K I L A L N U I V U I G M H F P G D F T	C. auratus
91	F K L R V D P A N F K I L A H N L I V U I A M L F P A D F S	C. idella
91	F K L R I D P A N F K I L A T N L I V U I G M L F P G D F S	M. anguillicaudatus
91	F K L R I D P A N F K I L A T N L I V U I G M L F P G D F S	P. dabryanus
121	P E V H M S V D K F F Q N L A L A L S D K Y R	C. carpio
121	P E V H M S V D K F F Q N L A L A L S D K Y R	C. auratus
121	P E V H V S V D K F F Q N L A L A L S D K Y R	C. idella
121	P E V D V S V D K F F Q N L A L A L S E K Y R	M. anguillicaudatus
121	P E V H V S V D K F F Q N L A L A L S E K Y R	P. dabryanus

Fig.2 The amino acid sequences of α -globin in five fishes

Tab.1 The similarity of α -globin amino acid sequence of five fishes

	<i>C.idellus</i>	<i>C.carpio</i>	<i>P.dabryanus</i>	<i>M.anguillicaudatus</i>	<i>C.auratus</i>
<i>C.idellus</i>	100%				
<i>C.carpio</i>	88.1%	100%			
<i>P.dabryanus</i>	83.2%	83.9%	100%		
<i>M.anguillicaudatus</i>	86.7%	85.3%	94.4%	100%	
<i>C.auratus</i>	86.0%	89.5%	86.7%	86.7%	100%

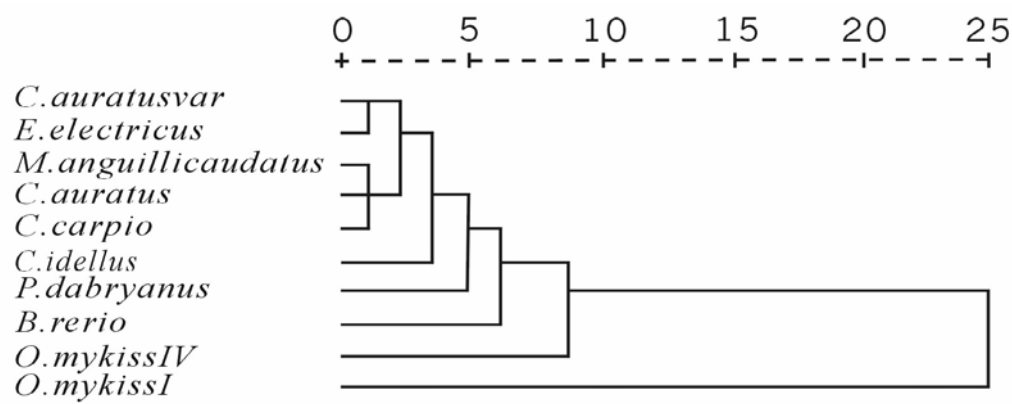


Fig.3 The dendrogram of ten fishes based on globin amino acid sequences similarity

EXPRESSION OF CHITINASE GENE IN TRANSGENIC RAPE PLANTS

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Key words: Rape, *Agrobacterium tumefaciens*, genetic transformation

Abstract : The hypocotyl and cotyledon of *Brassica napus* L. H165 and *Brassica juncea* DB3 were transformed with chitinase gene and herbicide-resistance gene by co-culture with *Agrobacterium tumefaciens* LBA4404, and rape plants were obtained which could grow on the medium containing herbicide. The PCR result showed that exotic genes were integrated in the genome of the rape. Further study was performed to determine the impact of temperature on the transgenic rate and the differentiation of explants.

INTRODUCTION

Rape is one of the most important oil crops. The fungal disease, such as *Pyrenopeziza brassica* Raw, *Leptosphaeria maculans*, *Cylindrosporium concentricum*, and especially *Rhizoctonia solani*, can largely reduce the production and quality of the oil seed [1,2]. In the past, selection of new resistant variety by traditional breeding and using chemical pesticide is the major measure to prevent fungal disease, but traditional breeding is not always effective, and the pesticide can cause large pollution to the environment. The advent of plant transgenic technology offered a new approach to obtain resistant crop variety.

Chitinase hydrolyses chitin existing in hyphae of fungal and prevents fungi from infecting plants and propagating inside plant tissues [3]. Furthermore, there are not any chitinase target in plant [4]. Hence, the chitinase gene was successfully transformed into tobacco [5], rice [6], and strawberry [7]. Some transgenic plants highly resistant to *Phoma lingam*, *Cylindrosporium concentricum* [8], *Rhizoctonia solani* [5] were also obtained. In this study, the chitinase gene was successfully introduced into rape plant, which may result in selection of plant lines resistant to fungal disease.

MATERIAL AND METHODS

MATERIAL

TYPE OF RAPE

B.napus winter rape H165 and *B.juncea* spring rape DB₃ were used as donor material.

MEDIUM

Bacteria-free seeding medium, tissue induction and differentiation medium used were based on GAO et al [9].

STRAINS AND PLASMID

The common virulent *Agrobacterium tumefaciens* LBA4404 carrying chitinase gene and bar gene, controlled by CaMV35S and Ubiquitin promoters, respectively.

METHODS

CULTURE OF AGROBACTERIUM TUMEFACIENS

A. Tumefaciens was grown from glycerol stock in MG/L liquid medium [10] supplemented with 100 mg/l kanamycin and 50mg/L streptecin. The culture was incubated overnight at 27–29°C with shaking (250 rpm) until log phase, then the cells were pelleted by centrifugation at 4.5 g for 10 min followed by resuspension in inoculation medium [9]. The inoculation density of *Agrobacterium* (OD₆₀₀) ranged from 1.0 to 2.0.

EXPLANT PREPARATION AND AGROBACTERIUM INFECTION

Seed sterilization, germination and *Agrobacterium* infection were based on GAO et al [9].

OBSERVATION BY SEM

Explants from hypocotyls and cotyledon were cultured on induction medium for 5, 9 and 11 days with light all day and night at 24±1°C, individually, and then they were proceeded to produce as described previously [11] and then observed by SEM. Those hypocotyls and cotyledon cultured on medium without inducing substances were as control.

EFFECT OF TEMPERATURE ON THE TRANSFORMATIONAL RATE OF RAPE

DB₃ explants were dipped in solution containing *Agrobacterium tumefaciens* for 5 min, and then were cultured in co-cultured medium at 19, 22, 25, 28 or 31°C under dark for 2 days before being cultured on selecting medium with 15mg/L PPT selectional pressure. Each treatment was composed of 100 explants and repeated for 3 times.

ROOTS AND PLANTS OF HERBICIDE –RESISTANT BUDS

The herbicide-resistant buds were sliced down and transferred to rooting medium after they had grown to 2-3cm, and then planted on bacteria-free soil in flower-basin at room temperature when 4-5 leaves appeared.

PCR assay

Genomic DNA samples were isolated from leaf tissues of putative transgenic plants as described by Dellaporta[12] and amplified by polymerase chain reaction. A left primer 5'-GCTCCACCTCCGATTA CTGC-3' and a right primer 5'-GCGTTGCCGTTGTTCTCCTC-3' were used to amplify an 438bp fragment of the chitinase gene. The PCR reactions were subjected to 30 cycles of: 4min pre-denatured at 94°C, 45s at 94°C, 45s at 45°C, and 105s prolonged at 72°C.

RESULTS AND DISCUSSIONS

Effect of temperature on transformation rate of rape

Highest fastness callus rate was found in rape co-cultured at 25°C. Transformation rate at 19°C, 22°C, or 33°C was apparently lower than that at 22°C and 25°C. Transformation rate of rape was affected significantly by temperature, and 22°C to 25°C were the most suitable transformation temperature range. Previously we had found 25°C was the most suitable transformation temperature to Maize, although Dillion[13] found there was no obvious difference on the transformation rate of tobacco between 19°C and 22°C. Hence, it might be concluded that both temperature and species have relationship with transformation rate.

Table1 Influence of temperature on *Agrobacterium tumefaciens* transformation

Material	temperature	explant	resistant callus				resistant callus	DUNCAN's
	(°C)	NO.	I	II	III	mean	frequency(%)	test
DB3	19	300	11	13	8	10.67	10.67	B
	22	290	17	22	22	20.33	21.03	A
	25	290	23	27	18	22.67	23.44	A
	28	300	12	16	11	13.00	13.00	B
	31	290	9	13	8	10.00	10.34	B

INDUCTION OF CALLUS ON COTYLEDON

Puffs appeared in the nick on hypocotyls after it was cultured on inducing medium for 5 days, and dark green trude appeared on day 9. By SEM, rape explant in slit was composed of cuticle and cortex. No obvious changes in morphorlogy and cell biology of blade after the cotyledon cultured on inducing medium for 2 days. However, on day 5 puffs appeared in the nick on the cotyledon, and attached medium turned brown and thicken, and cell turned much bigger in the nick of cotyledon, which showed cell dedifferentiation started. On day 9, active cell agglumulated into a cell mass. On day 11, mitotic cells spreaded all through to form separated cell mass, and scattered callus were visible (Figure3).

INDUCTION OF CALLUS ON HYPOCOTYLS

After culture for 5 days, puffs appeared on hypocotyls explant. On day 11 hypocotyls began to be thick, cutide slit, and light green, and tumor-shape tissue began to form in the nick. By SEM ,buldge appeared in the nick which means the dedifferentiation started. Active cell mitosis happened after 9 days of culture, and on day 11 dedifferentiation cell underwent mitosis rapidly to produce even-sized cells with long shape, and then cells turned to uneven-sized round or oval forms. Obvious changes of cells in the surface of the nick in hypocotyls were observed and

showed in figure 3-6. It is suggested that hypocotyls was dedifferentiated from the cells in folium tissue on the surface, and 400-600um or so from that of the nick (Figure 3).

TRANSGENIC PLANTS

As shown in Table 2, 22 fastness plants were screened out on PPT medium, 11 were positive assayed by PCR. Of these 11 ones, 6 were H165, 5 were DB₃(Figure 3). All of 22 plants came from hypocotyls explant.

Table 2 Result s of transformation

Variable	hypocotyls					cotyledon			
	1	2	3	4	5	1	2	3	4
NO. of explants	380	400	400	350	400	400	400	390	400
Forming shoots	18	25	11	20	18	8	5	6	2
transgenic plant	4	6	1	7	4	0	2	0	0
transformation									
frequency		1.14%					0.01%		

PCR ASSAY

Six of H165, and 5 of DB₃ were positive as in Figure 6. The size of the fragment amplified was the same with that of control, which suggested that target gene had been integrated in genome of rape (Figure 1).

EFFECT OF EXPLANT ON TRANSFORMATION OF RAPE

The development of reliable transformation protocols for recalcitrant species depends on the ability to deliver intact DNA molecules into the nuclear genome of regenerable cells and to recover fertile adult plants from tissue culture. The choice of starting material (explant) has proved to be crucial in successful *Agrobacterium*-mediated rape transformation. The regenerative way of bud differentiation of rape explant is very important to *Agrobacterium* infected and regeneration of transformation plant. It has been identified that transformation induced by *Agrobacterium* occurred mainly in cuticle cells of explant. It will be very difficult to get nice transgenic plant when the regenerative cells were from inner cells. Hypocotyl of *Brassica juncea* DB₃ has been found to be one of the nicest explant[14 15 16].

EFFECT OF TEMPERATURE ON TRANSFORMATION RATE

Temperature is believed to be a very important factor to affect. The transformation rate has an obvious relationship with the transportant talent of T-DNA . Fullner et al[17] ever suggested that 190C was helpful to T-DNA complex dissemble, as well as the most suitable temperature of the transportation of T-DNA . But the induction of Vir gene and the integration and steady expression of foreign gene fragment needed to be at 250C[18]. On the other hand, it was suggested that there was a most suitable temperature to be transformed by *Agrobacterium*, because cells in mitosis were liable to absorb exotic gene fragment[19]. Above 32°C, formative changes of Vir gene led to its unabled expression. Higher temperature also inhabited VirD₄, VirG, VirB₁ and Vir11, which were essential genes for the formation of T-DNA complex[20]. M.G.Salas et

al[18] suggested that at 25°C, exotic gene were much more liable to insert and integrant in plant cell and more effective in transformation.

REFERENCE

1. Rawlinson CJ , Muthyalu G , 1979 , Disease of winter oilseed rape : occurrence , effects and control , *Journal of Agricultural Science* , 93 : 593-606
2. Evans EJ , Gladders P , Davies JLM , Ellerton DR , Hardwick NV , et al , 1984 , Current status of diseases and disease control of winter oilseed rape in England , *Aspects of Applied Biology* , 6 : 323-324
3. Molano J, Polacheck I, Duran A, et al. 1979, An endochitinase from wheat germ. *The Journal of Biochemical Chemistry*, 10: 4901-4907.
4. Bell AA. 1981, Biochemical mechanisms of disease resistance. *Ann Rev. Plant Physiol*, 32: 21-81.
5. Brogile.K., Chet.I., Holliday.M., et al, 1991, Transgenic plants with enhanced resistance to the fungal pathogen *Rhizotonia solani*, *Science*, 254: 1194~1197
6. Lin.W., Anuratha.C.S., Datla.K., et al, 1995, Genetic engineering of rice for resistance to sheath blight, *Bio/ Technology*, 13: 686~691
7. ASAO.H., Nishizawa.Y., Arai.S., et al, 1997, Enhanced resistance against a fungal pathogen *Sphaerotheca humuli* in transgenic strawberry expression a rice chitinase gene, *Plant Biotechnology*, 14(3): 145~149
8. Rene Grison , Bruno GB , Michel S , Nicole L , et al , 1996 , Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene , *Nature Biotechnology* , 14 : 643-646
9. GAO Wu-jun, WANG Jing-xue, LU Long-dou, SUN Yi, DUAN Hong-ying, 2002 , Study on effects role of AgNO₃ in rape gene transformation, *Chinese Agricultural Science Bulletin*, 18 (4) : 43-45
10. Tingay S, McElroy D, Kalla R, Fieg S, Wang MB, Thornton S, Brettell R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J* 11:1369-1376
11. S. Jayasankar • B.R. Bondada • Z. Li • D.J. Gray 2002, A unique morphotype of grapevine somatic embryos exhibits accelerated germination and early plant development *Plant Cell Rep*, 20:907–911
12. Dellaporta SL , Wood J , Hicks JB , 1983 , A plant molecular DNA miniprep version : *J.Plant Mol Biol Reporter* 1 : 19-21
13. Dillen W, DeClercq J, Kapila J, Zambre M, Van Montagu M, Angenon G 1997 , The effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. *Plant J* 12:1459–1463
14. Fazekas GA, Sedmach PA, Palmer MV. 1986, Genetic and environmental effects on in vitro shoot regeneration from cotyledon explants of *Brassica juncea*. *Plant Cell Tissue Org. Cult.*, 6: 177-180.

15. Sharma KK, Bhojwaniss, Thorpe TA. 1990, Factors affecting high frequency differentiation of shoots and roots from cotyledon explants of *Brassica jncea*(L.)Czern. *Plant Sci*, 66: 247-253.
16. Mukhopadhyay.A , Arumugam.N , Nandkumar.PBA , Pradhan.AK , Gupta.V , Pental.D, 1992 , *Agrobacterium tumefaciens*-Mediated genetic Transformation of oilseed *Brassica campestris* : transformation frequency is strongly influenced by the mode of shoot regeneration , *Plant Cell Reports* , 11 : 506-513
17. Fullner KJ, Lara JC, Nester EW . 1996, Pilus assembly by *Agrobacterium* T-DNA transfer genes. *Science* , 273:1107–1109
18. M. G. Salas, S. H. Park, M. Srivatanakul and R. H. Smith. 2001, Temperature influence on stable T-DNA integration in plant cells. 20(8):701–705
19. Kudirka DT, Colburn SM, Hinchee MA, Wright MS . 1986, Interactions of *Agrobacterium tumefaciens* with soybean [*Glycine max* (L.) Merr.] leaf explants in tissue culture. *Can J Genet Cytol* , 28:808–817
20. Ishida,Y., Saito,H., Ohta,S. et al. 1996,High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens* . *Natural Biotechnol*, 14(6):745~750

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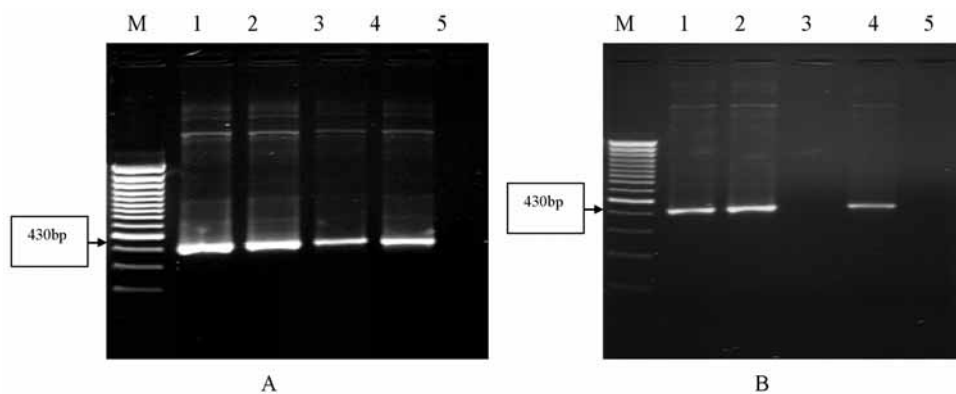


Fig.1 PCR assay of transgenic rape (A : DB₃ , B : H165)

M、 DNA Marker ; 1、 positive control ; 2 ~ 4、 transgenic plants ; 5、 negative control

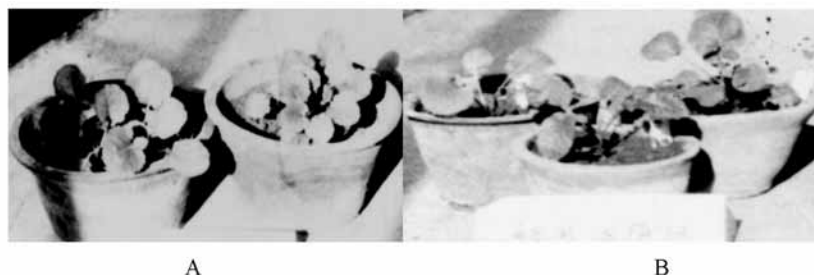


Fig.2 transgenic plants (A : DB₃ , B : H165)

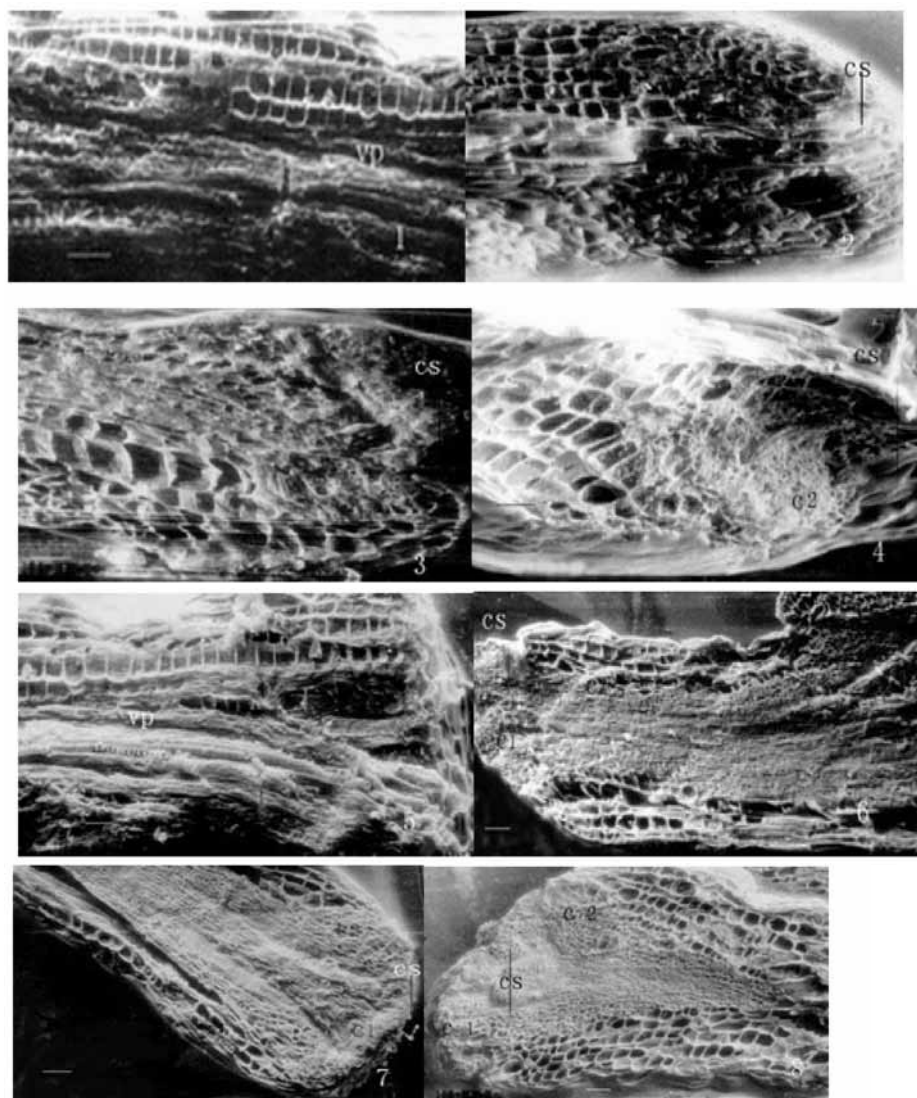


fig. 2 Histology of shoot bud differentiation from rape cotyledon and hypocotyl explants

1. cotyledon control
2. cotyledon 5d culture on induce callus medium
3. cotyledon 9d culture on induce callus medium
4. cotyledon 11d culture on induce callus medium
5. hypocotyl control
6. hypocotyl 5d culture on induce callus medium
7. hypocotyl 9d culture on induce callus medium
8. hypocotyl 11d culture on induce callus medium

note : Bar 1and 3. 200 μ m ; 2 and 5. 200 μ m ; 4. 150 μ m ; 6 , 7 and 8. 300 μ m

vp. vascular parenchyma ; cs. surface of cut ; c1. The first way of bud regeneration ; c2. The second way of bud regeneration

PRELIMINARY KARYOTYPE ANALYSIS IN MEMBERS OF *ASTERACEAE* FAMILY

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ANDREEA COSTEA³

Key words: *Callistephus chinensis*, *Rudbeckia hirta*, karyotype, *Asteraceae*

Abstract: chromosome's number were $2n=18$ on *Callistephus chinensis* (China aster) and $2n=36$ on *Rudbeckia hirta* (blackeyed susan). Until in *Callistephus chinensis* the chromosomes were found uniforms as dimension and morphology, in *Rudbeckia hirta* there are three morphological types.

INTRODUCTION

The *Asteraceae*, or *Composite* family, is one of the easiest to recognize. The inflorescence of the *Asteraceae* is so distinctive that the family was recognized as a distinct group early on and given a name, *Compositae*, that was so universally recognized that when the structure of family names was first formalized, it was agreed that *Compositae* would continue to be an accepted name. The *Asteraceae* is one of the four largest families, having about 1100 genera and 25,000 species. It occurs throughout the world, but has its greatest diversity in the semi-arid tropics. It is not abundant in tropical rain forests. Most of its members are shrubs or semi-shrubs, but most of our species are herbaceous. Supposed basic chromosome number of family is 9.



Fig. 1 *Callistephus chinensis* (left) and *Rudbeckia hirta* (right)

MATERIALS AND METHODS

The investigated species were China aster (*Callistephus chinensis*) and blackeyed susan (*Rudbeckia hirta*).

Germination of the seeds was performed into Petri dishes, on filter paper moistured with distilled water, at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$. When roots were reached 5 – 10mm in length, these were treated with 0.2% colchicines for 2 hours, at room temperature. Roots were kept for other two hours in distilled water. Fixation was done for about 16 hours in ethylic alcohol / acetic acid (3:1) mixture, at room temperature. For hydrolysis of vegetal material was used HCl 50% for 10min, at room temperature. The staining was realized with Carr reactive, according literature. The slides were prepared according squash method (1,2,3,4). Microscopy was carrying out using 100x objectives, with a Nikon Eclipse 600 light microscope. Photos were taken with a Nikon CoolPix 950 digital camera, at 1600x1200 dpi resolution. All images were processed with Adobe Photoshop software.

Homologous chromosome groups were settled in accordance with the rapport between long arm and short arm, the mitotic index, the difference between arms and with the relative length of the chromosomes.

RESULTS AND DISCUSSIONS

Callistephus chinensis

Chromosomes number in all analyzed metaphases were found to be $2n=18$. From the best metaphase (Fig.2) we settled 9 chromosomes pairs arranged into decrease order of their total length media. The total length media were found from $6.17\mu\text{m}$ (first chromosome pair) and $3.68\mu\text{m}$ (last pair). Variability limits were close enough that confirms the authenticity of homologues chromosomes pair's establishment. Centromeric index was between 49.45 (pair IX) and 40.45 (pair VIII). The 18 chromosomes from *Callistephus chinensis* were grouped in one morphological type: M.

Rudbeckia hirta

Chromosomes number in all analyzed metaphases were found to be $2n=36$, as in bibliography. From the best metaphase (Fig.3) we settled 18 chromosomes pairs arranged into decrease order of their total length media.

Total length of chromosomes was found between $3.48\mu\text{m}$ (pair I) and $1.81\mu\text{m}$ (pair XVIII). Relative length of the chromosomes pairs was from $7.45\mu\text{m}$ (pair I) to $3.79\mu\text{m}$ (pair XVIII). The 36 chromosomes from *Rudbeckia hirta* were grouped into two morphological types: M (pairs IX, XIII, XVI, XVII, XVIII), m (pairs I, II, III, IV, V, VI, VII, VIII, XI, XII, XIV, XV) and sm (pair X).

CONCLUSIONS

In the investigated species of *Asteraceae* chromosomes number was found to be $2n=18$ in *Callistephus chinensis* and $2n=36$ in *Rudbeckia hirta*.

On *Callistephus chinensis* we establish one morphological chromosomes type – M. The karyotype is symmetric and less evolved. In *Rudbeckia hirta* we have identified three chromosomes types (M, m and sm) and we consider that this specie has an evolved, asymmetric karyotype (2,4).

BIBLIOGRAPHY

1. Cîmpeanu M.M., Cîmpeanu C.S., Căpraru G., 2004 – Mitotic chromosomes studies in aromatic plants: 1. *Carum carvi* ($2n=20$), *Analele șt. Univ. "Al.I.Cuza" Iași, serie nouă, Tomul V*, 2004, 159-161
2. Cîmpeanu M.M., Căpraru G., Cîmpeanu C.S., Julian D., 2004 – Mitotic chromosomes studies in medicinal plants: 1. *Hippophae rhamnoides* ($2n=24$). *Analele șt. Univ. "Al.I.Cuza" Iași, serie nouă, Tomul V*, 2004, 166-168
3. Vorniceanu C., Băra I.I., Costică N., Cîmpeanu M.M., 2004 – The study of mitotic chromosomes at *Papaver somniferum* L. Species ($2n=22$). *Analele șt. Univ. "Al.I.Cuza" Iași, serie nouă, Tomul V*, 2004, 210-213
4. Căpraru G., Băra C.I., Băra I.I., Cîmpeanu M.M., Maxim E., 2004 – The characteristics of mitotic chromosomes at *Calendula officinalis* L. *Analele șt. Univ. "Al.I.Cuza" Iași, serie nouă, Tomul V*, 2004, 223-225

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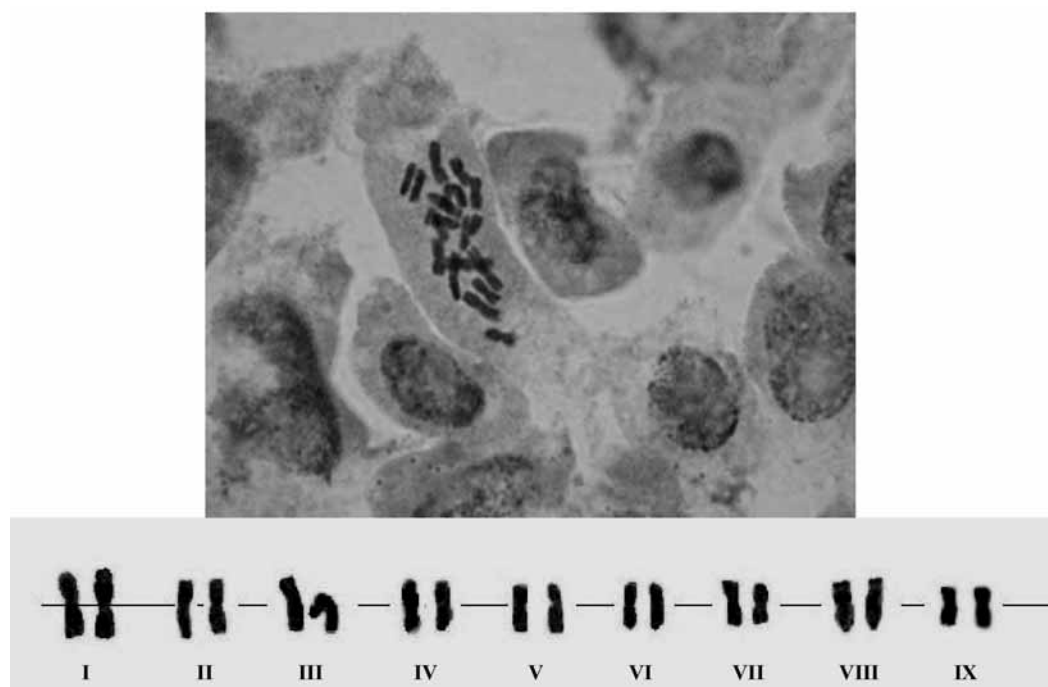


Fig. 2 Metaphase (up) and karyotype (down) in *Callistephus chinensis*

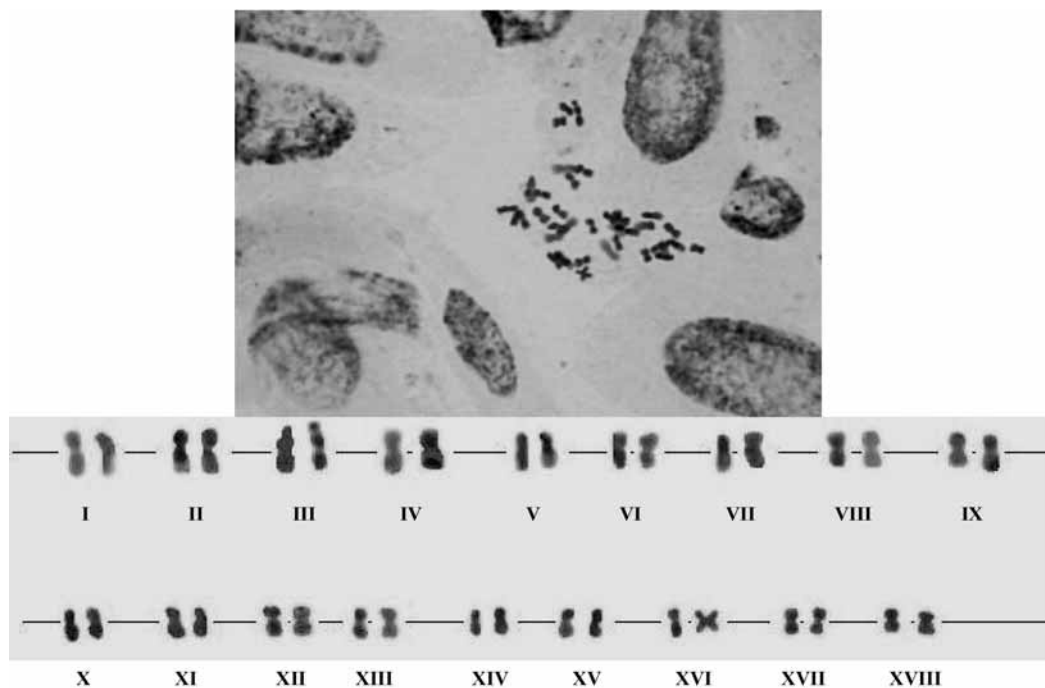


Fig. 3 Metaphase (up) and karyotype (down) in *Rudbekia hirta*

KARYOTYPE ANALYSIS IN *PHASEOLUS VULGARIS* L. CULTIVARS

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Key words: *Phaseolus vulgaris*, cultivars, karyotype

Abstract: Chromosome's number were $2n=22$ on all *Phaseolus* cultivars. All chromosomes were found morphological uniform, without satellites, and submetacentrics.

INTRODUCTION

Beans are one of the most ancient crops of the world. Together with maize and cassava, they have been a dominant staple in the low to mid-altitudes of the Americas for millennia. Beans (*Phaseolus spp.* L) are extremely diverse crops in terms of cultivation methods, uses, the range of environments to which they have been adapted, and morphological variability. They are found from sea level up to 3,000 metres above sea level, are cultivated in monoculture, in associations, or in rotations. Beans are consumed as mature grain, as immature seed, as well as a vegetable (both leaves and pods). Their genetic resources exist as a complex array of major and minor gene pools, races and intermediate types, with occasional introgression between wild ancestors and domesticated types. Beans are thus a crop that is adapted to many niches, both in agronomic and consumer preference terms. As fruit (pods) can be obtained in as little as two months, rotations are possible with other crops during short growing seasons. Short bush growth habits offer minimal competition and permit inter-planting with other species, for example, in reforestation projects or among fruit trees or coffee plantations during the early years until the main crop can be exploited. At the other extreme are aggressive climbers found at higher altitudes on subsistence farms where a few plants are maintained as a sort of insurance and are continually harvested for about six months. Over the past twenty years, beans have also been increasingly cultivated on a commercial scale, and are now offered in national, regional and international markets (1).

Diets of subsistence level farmers in Africa and Latin America often contain sufficient carbohydrates (through cassava, corn/maize, rice, wheat, etc), but are poor in proteins. Dietary proteins can take the form of scarce animal products (eggs, milk, meat, etc), but are usually derived from legumes (plants of the bean and pea family). Legumes are vital in agriculture as they form associations with bacteria that “fix-nitrogen” from the air. Effectively this amounts to internal fertilisation and is the main reason that legumes are richer in proteins than all other plants. Thousands of legume species exist but more common beans (*Phaseolus vulgaris* L.) are eaten than any other. In some countries such as Mexico and Brazil, and those around the Great Lakes in eastern Africa (Burundi, Rwanda, Kenya, Tanzania), beans are the primary source of protein in human diets. As half the grain legumes consumed worldwide are common beans, they represent the species of choice for the study of grain legume nutrition.

Phaseolus is the genus to which the common bean, *Phaseolus vulgaris* belongs. The genus *Phaseolus* contains 55 species, 5 of which have been domesticated. All *Phaseolus* species originate in Meso America and have been cultivated for thousands of years by pre-Columbian civilizations.

Previous classifications placed in this genus a number of other well known species that have now been removed to genus *Vigna*, sometimes necessitating a change of species name. For example, older literature refers to the Mung bean as *Phaseolus aureus*, whereas more modern sources classify it as *Vigna radiata* (1, 2, 4, 5).

MATERIALS AND METHODS

The investigated plants were *Phaseolus vulgaris* L. cultivars from Siret area: Seminole, Clujana, Saxa and Prelude.

Germination of the seeds was performed into Petri dishes, on filter paper moistured with distilled water, at $22 \pm 2^\circ\text{C}$. When roots were reached 5 – 10mm in length, these were treated with 0.2% colchicines for 2 hours, at room temperature. Roots were kept for other two hours in distilled water. Fixation was done for about 16 hours in ethylic alcohol / acetic acid (3:1) mixture, at room temperature. For hydrolysis of vegetal material was used HCl 50% for 10min, at room temperature. The staining was realized with Carr reactive, according literature (1, 2, 3). The slides were prepared according squash method. Microscopy was carrying out using 100x objectives, with a Nikon Eclipse 600 light microscope. Photos were taken with a Nikon CoolPix 950 digital camera, at 1600x1200 dpi resolution. All images were processed with Adobe Photoshop software.

Homologous chromosome groups were settled in accordance with the rapport between long arm and short arm, the difference between arms and with the relative length of the chromosomes.

RESULTS AND DISCUSSIONS

Seminole cultivar karyotype

In all investigated cultivars we found a chromosomal number of $2n=22$. In Seminole cultivar (Fig. 1) there are three morphological types: pairs I – III metacentrics, pair IV subtelocentrics and submetacentrics – pairs V – XI. The longest chromosomes were found to be chromosomes from first pair (3,84 μm and 3,76 μm), and the shortest the eleventh pair (1,96 μm and 1,94 μm).

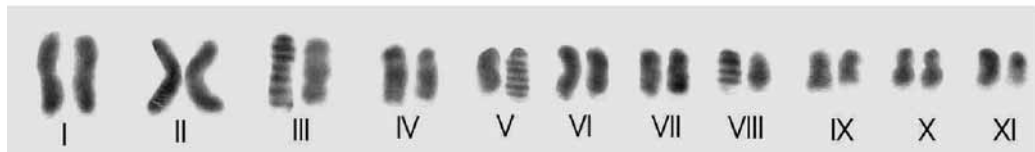
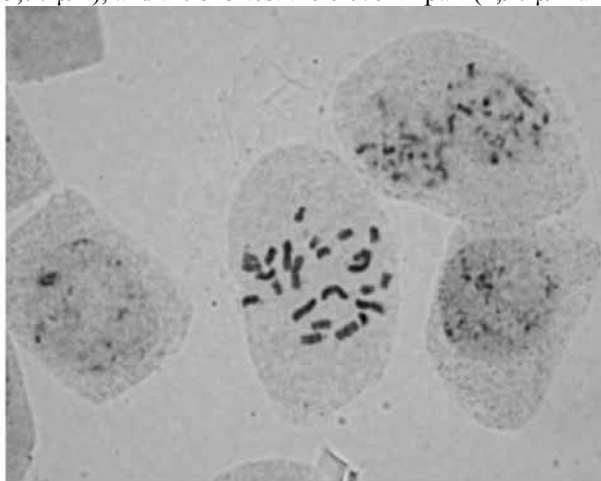


Fig. 1 Metaphase (up) and karyotype (down) in *Phaseolus vulgaris* L., Seminole cultivar

Clujana cultivar karyotype

In Clujana cultivar (Fig. 2) it isn't possible to establish morphological types prior to the uniformity and small dimensions of chromosomes. The longest chromosomes were found to be chromosomes from first pair (2 μm and 1.84 μm), and the shortest the eleventh pair (1.04 μm and 0.96 μm).

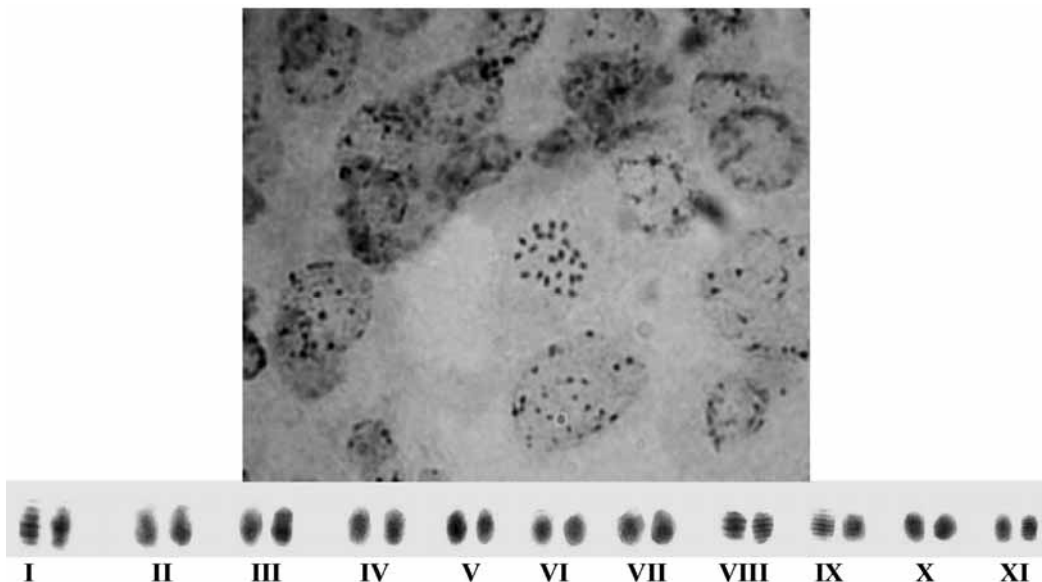


Fig. 2 Metaphase (up) and karyotype (down) in *Phaseolus vulgaris* L., Clujana cultivar

Saxa cultivar karyotype

In Saxa cultivar (Fig. 3) there are three pairs of metacentric chromosomes (I, IX and XI), and the all other pairs were found to be submetacentric.

The longest chromosomes were found to be chromosomes from first pair (2.84 μm and 2.38 μm), and the shortest the eleventh pair (1.22 μm and 1.21 μm).

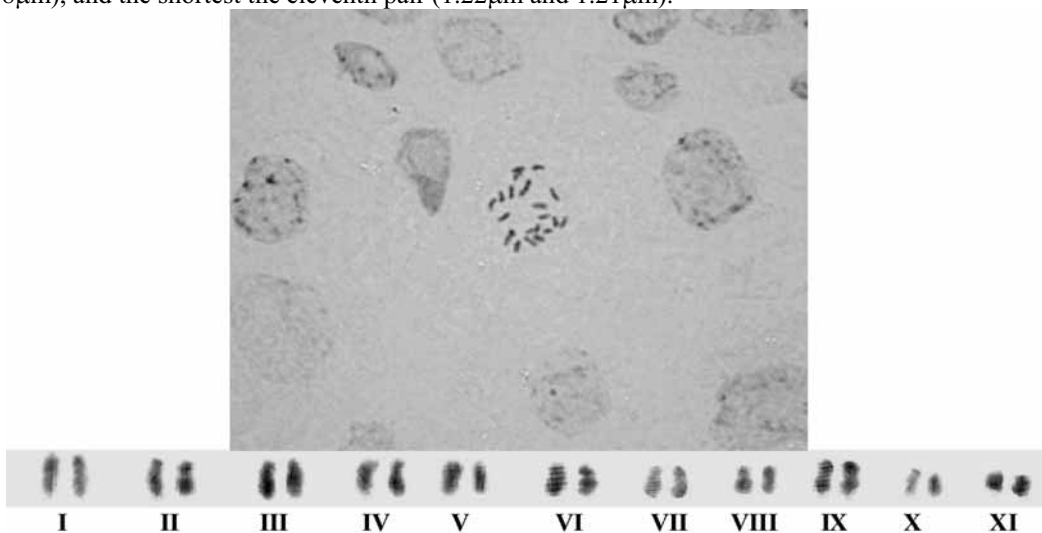


Fig. 3 Metaphase (up) and karyotype (down) in *Phaseolus vulgaris* L., Saxa cultivar

Prelude cultivar karyotype

In Prelude cultivar (Fig. 4) all chromosomes pairs were found to be extremely short, uniform, without satellites.

The longest chromosomes were found to be chromosomes from first pair (1.68 μ m and 1.64 μ m), and the shortest the eleventh pair (1.18 μ m and 1.14 μ m).

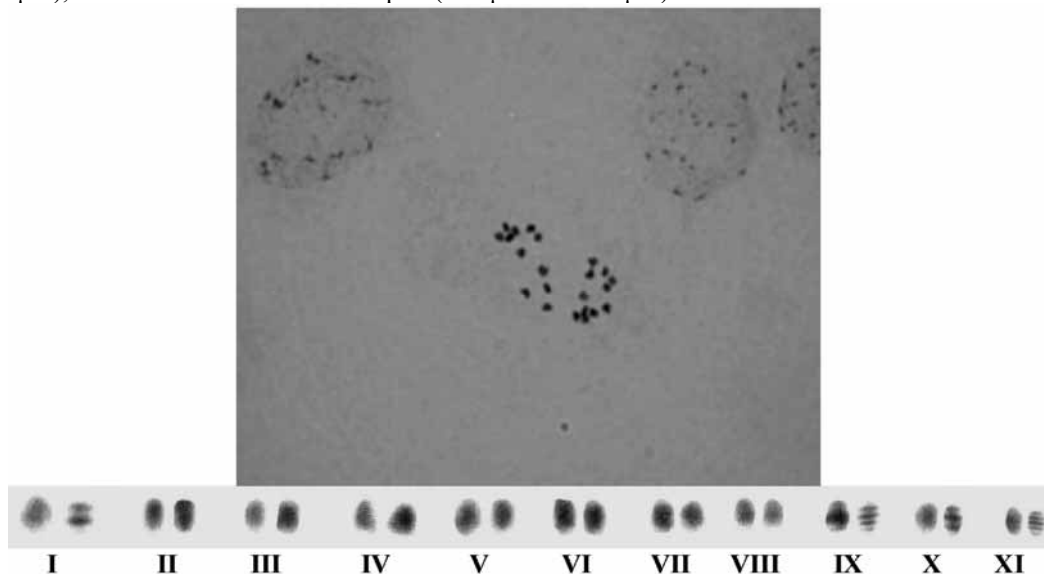


Fig. 4 Metaphase (up) and karyotype (down) in *Phaseolus vulgaris* L., Prelude cultivar

CONCLUSIONS

Chromosome number in all *Phaseolus vulgaris* L. cultivars investigated were $2n=22$ (4).

In Seminole cultivar, the main morphological type is submetacentric.

In Clujana and Prelude cultivars we found very short chromosomes, morphological uniform.

In Saxa cultivar, the main morphological type is submetacentric.

All investigated karyotypes were found to be not evolved.

BIBLIOGRAPHY

1. Colin M. S., 1967 - Origen de *Phaseolus vulgaris* L. (Frijol Común). *Agrociencia* 1(2): 99—109
 2. Jahan, B., Vahidy A.A., Ali S. I., 1994 - Chromosome numbers in some taxa of *Fabaceae* mostly native to Pakistan. *Annals of the Missouri Botanical Garden* 81: 792-799
 3. Kar, K., SenS., 1991 - A comparative karyological study of root and embryo tissue of a few genera of *Leguminosae*. *Cytologia* 56: 403-408
 4. Zheng, J., M. Nakata, H. Uchiyama, H. Morikawa, Tanaka R., 1991 - Giemsa C-banding patterns in several species of *Phaseolus* L. and *Vigna Savi*, *Fabaceae*. *Cytologia* 56: 459-466
- ***, 1958 – *Flora R. P. R.*, vol. VI, Ed. Acad. Române, București

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THE EFFECT OF THE TREATMENTS WITH GAMMA RADIATIONS ON THE CONTENT OF NUCLEIC ACIDS TO THE SPECIES OF *HYPERICUM PERFORATUM* L. AND *ECHINACEA PURPUREA* (L) MOENCH

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Keywords: gamma radiations, DNA, *Hypericum perforatum* L., *Echinacea purpurea* (L) Moench

Abstract: The gamma radiations determined to the species of *Echinacea purpurea* (L) Moench on increase of the quantity of DNA, comparatively to control and to the species of *Hypericum perforatum* (L.), the decrease of the quantity of DNA (excepting the 30 Gy dose which had a stimulative effect).

INTRODUCTION

The mechanism of action of the radiations is a very complex one and it is not still explained. Perhaps it is the most important chapter in radiobiology. Even if the acquired data in radiobiology have been some substantial ones for more than a century of investigations, there are still insufficient data about the effect of radiations on the control of the activity of genes.

The most important component part of the cell is the nucleus where we find the genetic material, which by its functions it represents a unique structure, so that the majority of the effects induced by the radiations in cells will have repercussions on it in some way or another.

The current researches focus on the use of the plant in the treatment of SIDA and of other types of cancer. *Echinacea purpurea* (L) Moench belongs to the family of *Asteraceae* and it is native to North America being intensively studied at present for its immunostimulatory effect.

Taking into consideration the world wide importance of this plant which in Romania was not known as a medicinal one, it was introduced in a culture, in Cluj, in 1982 by the researchers of the Faculty of Agronomy who established the adequate agrotechnics.

Our study intends to emphasize some effects induced by gamma radiations to the two species of medicinal herbs, *Hypericum perforatum* and *Echinacea purpurea* (L) Moench, namely as to the quantity of DNA.

We have referred to the possibility of the use of mutations induced in the improvement of the two species, directing it to the objectives of economic interest.

MATERIAL AND METHOD

There were used leaves of the 2nd year of vegetation plants, namely the period of fructification of the species of *Hypericum perforatum* L.

These plants were from the seeds obtained from the Agricultural and Zootechnic Researches Department of Secuieni, Neamț, harvested in 2001.

The mutagenic agent used was represented in the Nuclear Department of Chemical and Pharmaceutical Researches Institute, Bucharest (CPRI).

The doses of radiation were 10Gy, 30Gy, 50Gy, 75Gy, 100Gy, 120Gy and the power of irradiation was 5Gy / minute.

There were used 20 days old plantlets of the species of *Echinacea purpurea* (L) Moench, taken from the seeds obtained in the Agricultural and Zootechnic Researches Department of Secuieni, Neamț, harvested in 2003 and in Potatoes Institute, Brașov, harvested also in 2003.

The seeds were irradiated within the institute mentioned above and the doses of radiations were: 10Gy, 30Gy, 50Gy, 75Gy, 100Gy, 120Gy.

The power of the dose of gamma radiations was: 1,8k Gy/hour (measured by Fryck dosimeter and confirmed by ECB one).

The radiation was accomplished in normal conditions (pressure, temperature, humidity) of laboratory.

The germination was assured in Petri plates, on filter plates, on filter paper soaked in distilled water, at 24°C 20°C room temperature.

The purification of total DNA was realized with a SIGMA purification kit (Elite Plant Genomic DNA kit Type), according to the method recommended by the producer.

The establishment of the concentration of isolated genomic DNA was realized by the spectrophotometric method, to a wave s length of $\lambda \approx 280 \text{ nm}$.

The amounts of bichain DNA found on the basis of the exactions values were subsequently registered to $100 \mu\text{g}$ tissue.

RESULTS AND DISCUSSIONS

The doses of radiation used determined an increase of the DNA amount, comparatively to control, in the species of *Echinacea purpurea* (L) Moench.

The double amount of DNA, comparatively to control (8, 36 comparatively to 4, 275) was recorded to the dose of 75Gy, increased values of the DNA amount being recorded also to the doses of 10Gy, 30Gy and 100Gy.

It is possible that when the DNA amount doubles itself to be produced a phenomenon of polyploidy, which could be used in the improvement of the species.

Further researches about the DNA amount and also about some enzymes activity, using vegetal material from the plants obtained from conventional cultures are possible answers in this sense.

It was recorded an increase of the DNA amount, comparatively to control, to all the studied variants of Secuieni population which belongs to the species of *Echinacea purpurea* (L) Moench but this increase was not a very important one.

The maximum value of the DNA amount was recorded this time to the dose of 120Gy (9,255 comparatively to 6,33).

The increase of the DNA amount to the variants studied is possible to be associated with the phenomenon of aneuploidy. In this case, the improvement of the species could be hardly accomplished.

In conclusion, the gamma radiations determined an increase of the DNA amount, comparatively to control to all the studied variants of the two populations of the species of *Echinacea purpurea* (L) Moench.

The highest value of the DNA amount was recorded to Secuieni population, to the dose of 120 Gy, but a double amount of DNA, comparatively to control was recorded to the dose of 75Gy, to Braşov population (table 1 and figure 1).

The dose of 30Gy had a stimulative effect to the species of *Hypericum perforatum* (L), being recorded a maximum value, comparatively to control (15, 31 comparatively to 10,595).

The DNA amount was lower comparatively to control, to all the other doses of radiation, especially to the dose of 120 Gy (6,075 comparatively to 10,595). (table 2 and figure 2).

The data about the effect of gamma radiations on the quantity of nucleic acids was contradictory in the biological studies.

Javed (1976), quoted by Ghiorgă (2002) obtained a gradual decrease of the amount of nucleic acids, by a process of irradiation with 10 to 100 Gy doses to pepper, especially to the high doses of radiation.

But, in other investigations it was obtained the increase of the amount of nucleic acids. Selieva and Guschinov (1971), quoted by Ghiorgă (2002) obtained an increase of the amount of nucleic acids to alfalfa (*Medicago sativa*) and to trefoil (*Trifolium*), using 5 to 1000 Gy doses of radiation.

CONCLUSIONS

The doses of radiation determined the increase of the DNA amount comparatively to control, to both populations (Brașov and Secuieni ones), possibly because of the phenomenon of aneuploidy, the maximum value recorded being that of 120 Gy (9,255 to Secuieni population) and double DNA amount was recorded, comparatively to control, to the dose of 75 Gy (Brașov population), possibly because of the phenomenon of polyploidy.

The doses of irradiation used determined the decrease of the DNA amount, comparatively to control to the species of *Hypericum perforatum*, excepting the 30Gy dose which had a stimulative effect.

Both species behaved differently to the action of gamma radiations as to the DNA amount.

BIBLIOGRAPHY

1. Ghiorghită G.I. – 1987. *Radiobiologie vegetală*, Editura Academiei R.S.R., București, 52 – 56;
2. Ghiorghită G.I., Corneanu G. – 2002 *Radiobiologie*, Editura Alma Mater, Bacău, 78 – 80;
3. Stănescu Ursula, Hâncianu Monica, Miron Anca, Aprotosoia Clara – 2002. *Bazele farmaceutice, farmacologice și clinice ale fitoterapiei*, vol II, Ed. Gr.T.Popa – U.M.F. Iași, 216 – 221 ; 236 – 241.

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Table 1 The total ADN amount to the populations of the species of *Echinacea purpurea* (L.)

Origin zone	Control	10 Gy	30Gy	50Gy	75Gy	100Gy	120Gy
Brașov	4,275	8,075	7,67	5,425	8,36	7,205	5,09
Secuieni	6,33	7,545	7,56	7,44	7,49	7,365	9,255

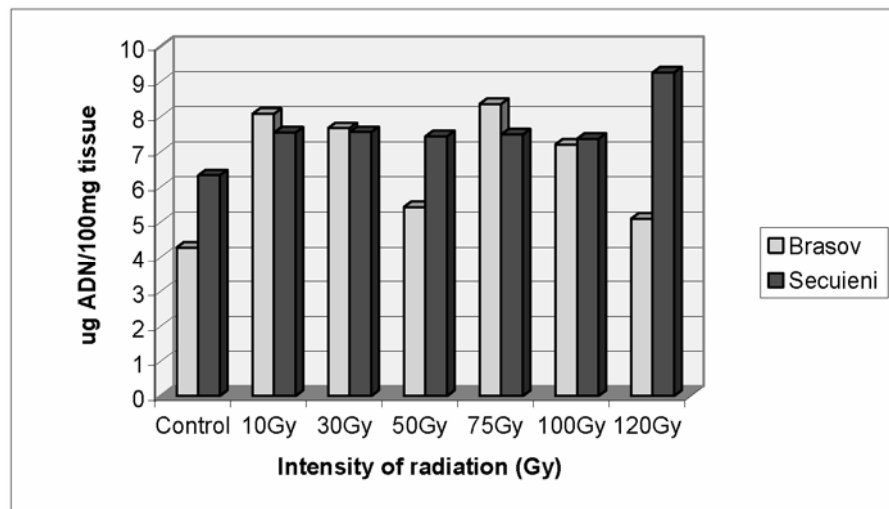


Figure 1 The Comparative Graphic Representation of the total ADN amount to the populations of the species of *Echinacea purpurea* (L.)

Table 2 The total ADN amount to the populations of *Hypericum perforatum* (L.)

Origin zone	Control	10 Gy	30Gy	50Gy	75Gy	100Gy	120Gy
Secuieni	10,595	9,25	15,31	7,61	9,025	8,845	6,075

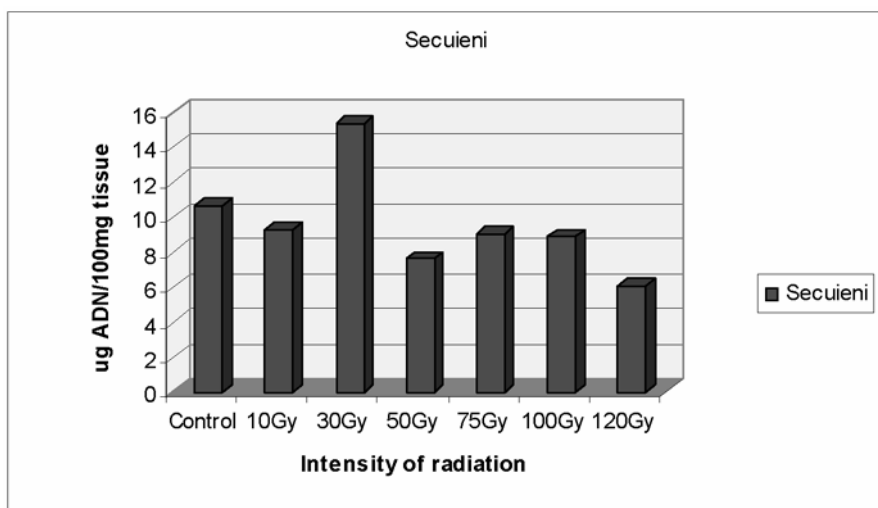


Figure 2 The total ADN to the population of the *Hypericum perforatum* (L.)

MITOTIC CHROMOSOMES STUDIES IN MEDICINAL PLANTS; L. *ECHINACEA PURPUREA* (L) MOENCH (2N = 22)

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Key words: metaphase, chromosomes, satellites, karyotype, *Echinacea purpurea* (L.) Moench.

Abstract: The metaphase contains 22 chromosomes. We have found 3 types of chromosomes. This species presents an evolved karyotype.

INTRODUCTION

The chromosomes number and type are very important to characterize a species.

Today, where we can observe an increasing interest in natural remedies, analysis of medicinal plants, also from a karyological point of view, represents an important goal of scientific research.

Our study intends to determine the karyotype of a medicinal plant – *Echinacea purpurea* (L.) Moench.

MATERIAL AND METHOD

Biological material: year old seeds belonging to the species of *Echinacea purpurea* (L.) Moench and harvested in 2003 in the Department of Agricultural and Zootechnic Researches, Secuieni – Neamț.

The germination has been assured in Petri plates, on filter paper, soaked in distilled water, at 24°C±2°C room temperature.

When the little roots reached 10-15mm height, they were harvested and it was being used a treatment with 0,2% colchicines, at room temperature, for 2 hours, after which they kept in distilled water for 2 hours. For hydrolysis it was used a 50% hydrochloric acid solution, for 10 minutes and a Carr reagent for coloring.

The solutions were done, using the squash method, the reading was made to the microscope with 20x object lens and they were photographed with the immersed 100x object lens, using the Nikon Eclipse 600 camera, and the digital camera Nikon Cool Pix, with a 1600 x 1200 dpi resolution.

The image was processed by the Adobe Photoshop programmer.

RESULTS AND DISCUSSIONS

The chromosomes have been grouped according to the ratio between the long arm and the short arm, to the centromeric index and to the difference between the stems / branches and the relative length of the chromosomes.

The number of chromosomes founded is $2n = 22$ in all the studied metaphases.

After the analysis of the best metaphase (fig. 1) with a minimum number of superposed chromosomes, there were established 11 pairs of chromosomes, which have been arranged by a process of reducing the average of their total length.

As we notice from table 1, the average of total length was between 5,94 μm (the 1st pair) and 3,34 μm (the 11th pair).

The limits of variability have been almost the same, which confirms the authenticity of the establishment of the pairs of homologues.

As it concerns the decreasing average of the total length from the first pair to the last one, it was on an unequal one. The highest value (0,92 μm) was recorded between the 1st and the 2nd pair while the lowest one (0,01 μm) was between the 2nd and the 3rd one.

The difference between the arm was between $0,73\ \mu m$ (the 2nd pair) and $1,99\ \mu m$ (the 8th pair). The ratio between the long arm the short arm and the criterion to establish the homologues were between $1,34\ \mu m$ (the 2nd pair) and $3,05\ \mu m$ (the 8th pair) and the centromeric index varied between $42,79\ \mu m$ (the 5th pair) and $75,12\ \mu m$ (the 8th one).

The relative length was between $12,24\ \mu m$ (the 1st pair) and $6,88\ \mu m$ (the 11th pair). At the 5th pair there have been noticed satellites whose dimensions were between $0,98 - 0,95\ \mu m$.

CONCLUSIONS

Chromosomal number in *Echinacea purpurea* (L) Moench is 22.

At the 5th pair there have been noticed satellites.

Considering the fact that we have found 3 types of chromosomes (median, submedian, subtelocentric) nr can say that this species presents an asymmetrical, evolved karyotype.

BIBLIOGRAPHY

1. Băra I.I., Cîmpeanu M. Mirela, 2003 – *Genetica*, Ed. Corson, Iași, 118 – 120;
2. Cîmpeanu Mirela, Maniu Marilena, Surugiu Iuliana, 2002 – *Genetica – metode de studiu*. Ed. Corson, Iași, 68 – 78; 86 – 98.
3. Cîmpeanu M. Mirela, Cîmpeanu S. Cristian, Căpraru Gabriela, 2004 – Mitotic chromosomes studies in aromatic plants; 1. *Carum carvi* ($2n = 20$) — *Analele științifice ale Universității Al.I. Cuza Iași (Serie nouă) – Genetică și biologie moleculară – Tomul V*, Ed. Universității Al.I.Cuza Iași, 159 – 163.
4. Mirela M. Cîmpeanu, Gabriela Căpraru, Cristian S. Cîmpeanu, Daniela Julan – 2004, Mitotic chromosomes studies in medicinal plants: 1. *Hippophae rhamnoides* ($2n=24$) — *Analele științifice ale Univ. Al.I.Cuza Iași (serie nouă) – Genetică și biologie moleculară – Tomul V* – ED. Univ. Al.I.Cuza Iași, 166 – 168.
5. Stebbins G.L., 1974. *Flowering Plants Evolution above Species Level*. Cambridge Massachusetts.

1) The Emergency Military Hospital, Iași

2) The Faculty of Sciences, Bacău

3) University “Al.I.Cuza”, Faculty of Biology, Iași.

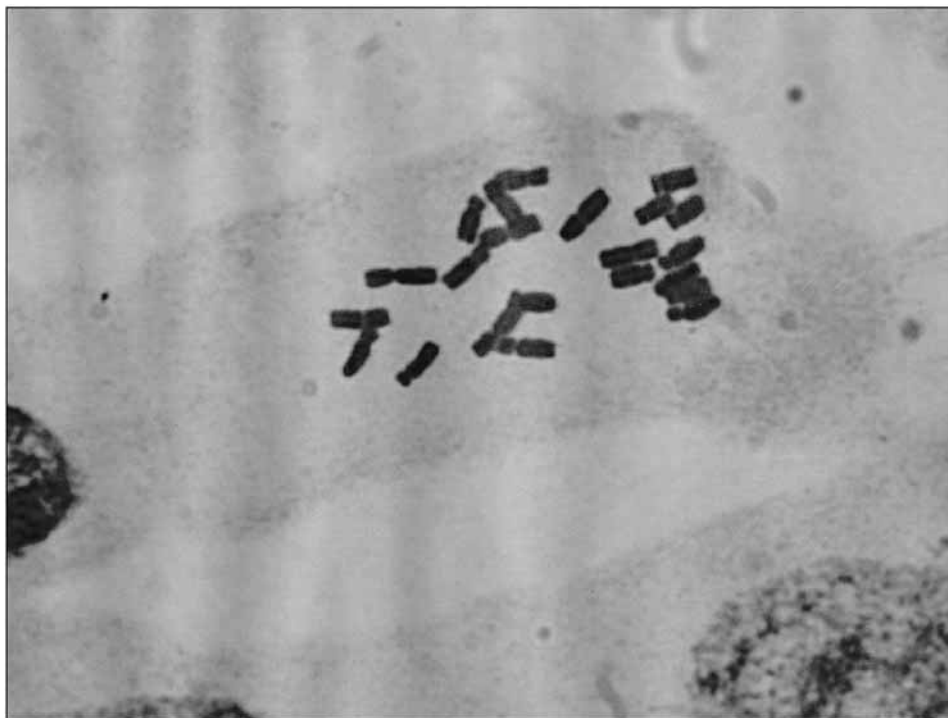


Figure 1 Metaphase at – *Echinacea purpurea* ($2n=22$)



Figure 2 Karyotype at *Echinacea purpurea* ($2n=22$)

Table 1 Chromosomes characteristics at *Echinacea purpurea* (2n=22)

Chromosomes	Pair	Type	Total length		Long arm		Short arm		Arms rapport A ₁ /A ₂	Arms sum	Arms difference (μ m)	Centro-metric index (%)	Relative length	Satellites	
			μ m	Variability limits (μ m)	μ m	Variability limits (μ m)	μ m	Variability limits (μ m)						μ m	Variability limits (μ m)
I	m	m	5.94	5.99-5.90	3.55	3.61-3.49	2.38	2.37-2.40	1.49	5.93	1.17	59.76	12.24		
II	m	m	5.02	5.09-4.96	2.86	2.92-2.80	2.13	2.16-2.10	1.34	4.99	0.73	56.97	10.35		
III	sm	sm	5.01	5.06-4.96	3.35	3.40-3.31	1.68	1.71-1.65	1.99	5.03	1.67	66.86	10.33		
IV	m	m	4.85	4.87-4.84	2.92	2.95-2.89	1.89	1.92-1.86	1.54	4.81	1.03	60.20	10.00		
V	sm	sm	4.58	4.75-4.42	1.96	2.04-1.89	1.66	1.77-1.56	1.81	3.62	0.30	42.79	9.44	0.93	0.98-0.95
VI	sm	sm	4.33	4.36-4.30	2.83	2.86-2.80	1.50	1.56-1.44	1.88	4.33	1.33	65.35	8.92		
VII	sm	sm	4.13	4.21-4.06	2.90	2.95-2.86	1.15	1.20-1.11	2.52	4.05	1.75	70.21	8.51		
VIII	sl	sl	3.94	4.00-3.88	2.96	3.01-2.92	0.97	0.99-0.96	3.05	3.93	1.99	75.12	8.12		
IX	sm	sm	3.77	3.85-3.70	2.68	2.71-2.65	0.99	1.05-0.93	1.83	3.67	1.69	71.08	7.77		
X	sm	sm	3.58	3.61-3.55	2.63	2.71-2.56	0.90	0.90-0.90	2.92	3.53	1.73	73.46	7.38		
XI	sm	sm	3.34	3.37-3.31	2.29	2.34-2.25	1.02	1.05-0.99	2.24	3.31	1.27	68.56	6.88		

*Chromosomes of satellites

THE INFLUENCE OF THE ELECTROMAGNETIC FIELD ON MITOSIS AND CATALASE ACTIVITY IN MAIZE CARYOPSES WITH DIFFERENT VIABILITIES AND AGES

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Keywords: alternant electromagnetic field (AEF), mitotic division, catalase activity

Abstract: Five maize caryopse samples, having various viabilities and storage periods, were subjected (as dry and imbibed seeds) to an alternating electromagnetic field of 6000 Hz, determining, in this conditions, the value of some mitosis indices and the catalase activity. At all studied samples, both the analysed mitosis indices (cells in division and polyploide cells) and the catalase activity have generally registered modifications, compared to blanks (unstimulated seeds), expressed through increase of the mentioned indices and of the enzyme activity, mainly when the electromagnetic stimulus was applied to the imbibed seeds.

INTRODUCTION

Many cytological, biochemical and physiological researches on reduced viabilities caryopses have evidenced some specific modifications to each domain, favourising, at the same time, some methods and proceedings development to increase the viability and vigour of those caryopses.

Within the framework of physical procedures there were initiated and developed stimulation techniques using a static or an alternant electromagnetic field.

Using an alternant electromagnetic field (AEF), the aim of this paper is to investigate some effects of this physical stimulus on the mitosis and catalase activity in maize caryopses having different ages and terms of storage.

MATERIALS AND METHODS

The researches were carried out on 5 samples of maize caryopses belonging of 5 maize landraces, stored in the Suceava Genebank, on different terms (between 1 and 17 years, under appropriate conditions of temperature and humidity (table nr.1). In these conditions, the sample P1, having the greatest germinative capacity (96%), has served as control sample.

Table 1. Features of used biological material

Caryopses Samples	Storage period (years)	Germination (%)	W.T.G (g)	Humidity(%)
P1	1	96	218	11,35
P2	10	78	220	11,40
P3	15	50	223	11,40
P4	15	65	220	11.45
P5	17	28	225	11,40

WTG = weight thousand grain; ; P1..P5 = caryopse samples.

We used 4 repetitions of maize caryopses(each of them containing 50 caryopses) and a standard device to apply the electromagnetic field (according to Alexander and Doijode, 1995).

The microscopical investigations were carried out after 72 hours of germination period on adventive roots, using the Carr method to investigate the mitosis and polyploid cells number.

The catalase activity was determined using the titrimetric method (according to Dumitru, 1967).

RESULTS AND DISCUSSIONS

The analysis of processed data evidences the number of the mitotic cells, polyploid cells and the catalase activity in all maize caryopses samples before the electromagnetic field action (table nr.2).

Table 2. The values of some mitotic indices and of the catalase activity in unstimulated biological material

Caryopses	Samples	P1	P2	P3	P4	P5
Indices	DC (‰)	120	95	60	84	28
	PC (‰)	8	18	25	15	34
	CA	20,75	23,32	16,14	12,09	15,94

DC = dividing cells; PC = polyploid cells; CA = catalase activity; P1..P5 = caryopse samples

As one can see, the mitotic cells number and the catalase activity have registered higher values in the caryopses samples with the highest germination percentages (P1 and P2). The caryopses samples with a reduced germination capacity (P3 and P5) were registered the highest oyploidy cells number.

The electromagnetic field action on the dried and imbibed caryopses has modified some mitotic indices in relation to the control samples (blanks) (table nr.3).

Table 3. The values of some mitotic indices under the influence of AEF treatment

Indices	DC-dc (‰)		PC-dc (‰)		DC-ic (‰)		PC-ic (‰)	
Samples	Cells nr.	% beside blank*	Cells nr.	% beside blank*	Cells nr.	% beside blank*	Cells nr.	% beside blank*
P1	138	115,00	9	111,11	144	120,00	16	200,00
P2	132	138,94	36	200,00	137	144,21	50	277,77
P3	93	155,00	67	268,00	110	183,33	77	308,00
P4	109	129,76	42	280,00	121	144,05	48	320,00
P5	49	175,00	162	476,47	80	285,71	189	555,88

DC-dc = dividing cells of dried caryopses; PC-dc = polyploid cells of dried caryopses; DC-ic = dividing cells of imbibed caryopses; PC-ic = polyploid cells of imbibed caryopses; * = blank (100%), index value without stimulation; P1..P5 = caryopse samples.

The mitotic cells number has registered the highest values (55 and 75%) in relation with blank in the lowest germination samples and the longest storage periods (P3 and P5). In high germination samples, the dividing cells number increase, beside blanks, has oscilated between 15% (P1) and 39% (P2). The sample P1 having highest germination capacity (stored under optimal conditions) has registered, as seen, the most reduced increasing of this index. As to polyplod cells number, the difference beside blanks (index value of unstimulated caryopses) has

oscilated between 11 and 376,5%, the most reduced germination sample P5 having the greatest value.

The greatest polyploidy cells number, as a result of the electromagnetic field action on old or/and low germination caryopses could be ascertain to division mechanism damage or cytokinesis inhibition, more frequent processes in aged cells.

Subjecting imbibed caryopses (ic) to electromagnetic field action, on can see that the both investigated indices have registered higher values beside dried caryopses. Thus, the dividing cells number (DC-ic) has had increases (beside blanks) between 20 and 185,7% and the polyploidy cells (PC-ic) between 100 and 455,9% - in this case the highest values being in reduced germination and long storage period samples as well.

The table nr.4 reproduces the maize caryopse catalase activity values, subjected (dried or imbibed) to electromagnetic field action. If on stimulated dried caryopses the catalase activity has registered a moderate increase (8,2 and 14% beside blanks) in two cases, in imbibed stimulated caryopses the activity of this enzyme has enhanced in 4 samples with values between 17 and 40%.

Table 4. Maize caryopse catalase activity values under the influence of AEF treatment

CS	P1		P2		P3		P4		P5	
SM	CA	% beside blank*	CA	% beside blank*	CA	% beside blank*	CA	% beside blank*	CA	% beside blank*
dc	22,45	108,19	21,57	92,49	15,05	93,25	13,78	113,98	15,07	94,54
ic	25,01	120,53	23,05	98,84	21,47	133,02	16,90	139,78	18,64	116,94

CS = caryopses samples; SM = stimulated material; CA = catalase activity; dc = dried caryopses; ic = imbibed caryopses; P1..P5 = caryopse samples.

The modification of the investigated mitotic indices and of the catalase activity under influence of alternating electromagnetic field, leads us to the idea of involving of physical stimulus on complex biochemical and physiological mechanisms and not only the elimination of inhibitions caused by tegument impermeability, deficiency in water, nutrients uptake a.s.o.

CONCLUSIONS

The mitotic division in the adventive roots meristem tips, belonging of 5 maize caryopses samples with different viabilities ages and germinative capacity, was modified under alternative electromagnetic field action with 6000 Hz frequency. The older maize caryopses samples with a low level of germination have registered the highest number of dividing and polyploid cells.

The influence of the alternative electromagnetic field manifested on the catalase activity, increasing the activity of the enzyme.

The electromagnetic signal increased the level of the mitotic indexes and the catalase activity too in the imbibed caryopses samples.

REFERENCES

1. Acatrinei, G., 1986 - "Cercetarea procesului de diviziune celulară sub influența substanțelor fiziologic active extrase din *Calendula officinalis* L. și *Symphytum officinale* L.", *An. St. Univ. "Al. I. Cuza"*, Iași, XXXII, s. II a Biologie, 51-53.

2. Alexander, M.P., Doijode, S.D., 1995 - "Electromagnetic field, a novel tool to increase germination and seedling vigour of conserved onion (*Allium cepa* L.) and rice (*Oryza sativa* L.) seeds with low viability", *Plant Genetic Resources Newsletter*, 1995, 104, 1-5.
3. Drochioiu, G., Cristea, M., Străjeru Silvia, 1993 - Activitatea catalazică a cariopselor de porumb în procesul îmbătrânirii forțate. *Cercetări Agronomice în Moldova*, XXVI - vol. 1-2 (99)/1993, Iași, 19-25.
4. Dumitru, I.F., 1967 - *Lucrări practice de biochimie*. Edit. Did. și Ped., București.
5. Ellis, R.H., Hong, T.D., Roberts, E.H., (1985) - *Handbook of Seed Technology for Genebanks*, International Board for Plant Genetic Resources, vol. II, Rome, 450-451, 478-480.
6. Khan, A.A., Braun, J.W., Tao, K.L., Miller, W.F., Bensin, R.F., (1976) - "New method for maintaining seed vigour and improving performance", *J. Seed Technol.*, 2, 33-57.
7. Pittman, U.J., Ormrod, D.P., (1970) - "Physiological and chemical features of magnetically treated winter wheat seedlings", *Can. J. Plant Sci.*, 50, 211 - 217.
8. Raicu, P., Anghel, I., Stoian Veronica, Duma Doina, Taisescu Elena, Badea Elena, Gregorian Iuliana, (1983) - *Genetica. Metode de laborator*, Edit. Acad. R.S.R., București, 49-51, 53-54.
9. Zănoagă, C.V., 1995 - Role of the redox state in forming the biological quality of natural waters. *Self-purifications processes in natural waters*, Chișinău, 218-244.

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THE TOTAL DNA QUANTIFICATION FOR THREE TYPES OF TISSUE FROM *CARASSIUS AURATUS GIBELIO* BLOCH

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Keywords: total DNA, *Carassius auratus gibelio* Bloch.

Abstract: We established the total DNA quantity and the variability intervals for three types of tissue (muscle, liver and spleen) from five individuals of *Carassius auratus gibelio* Bloch, to characterize this species from the point of view of this parameter.

MATERIALS AND METHODS

Biological material used for DNA extraction was represented by 5 individuals for each species which come from Larga Jijia - Movileni fishing farm. The establishment of DNA quantity was made after the extraction with phenol: chloroform: isoamylalcohol (25 : 24 : 1) (Ausubel 1995). The technique was used for DNA extraction from fresh tissues. For cellular lyses we used a lyses buffer and K proteinase which were incubated at 37°C for 12 hours. After the incubation period the samples were centrifuged 2 times, each time for separate the first layer from liquid column (figures 1 – 3).

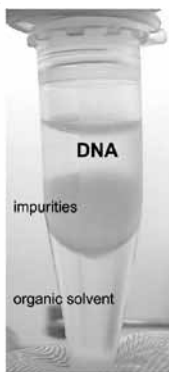


Fig. 1. First separation

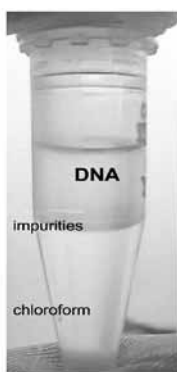


Fig. 2. Second separation



Fig. 3. Purified DNA

After the final separation, the purified DNA is precipitated in absolute ethanol kept at -20°C (figure 4) and centrifuged for pellet obtaining. All pellets were resuspended in TE buffer (pH=8.0) and kept at -20°C.

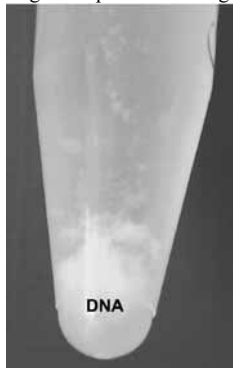


Fig. 4 – DNA precipitation with ethanol at -20°C

RESULTS AND DISSCUSSIONS

Based on absorption values at 270nm and pure DNA extract, we created an etalon curve and a regression equation (figure 5). The regression equation was used to find the real values of DNA concentration from analysed tissues. For a better comparison between all samples, we represented the average values in a graph (figure 6).

For the establishment of total DNA variability intervals were calculated the standard error and the both limits (superior and inferior limits) for each interval. All variability intervals were represented in figure 7.

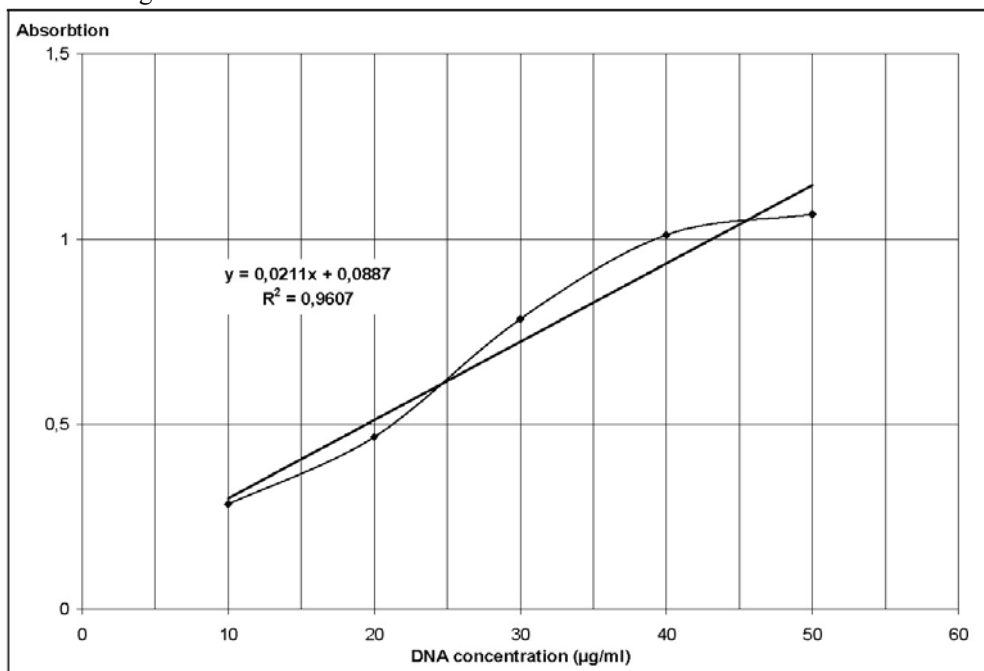


Fig. 5 DNA's etalon curve, corresponding to $\lambda=270\text{nm}$ absorption

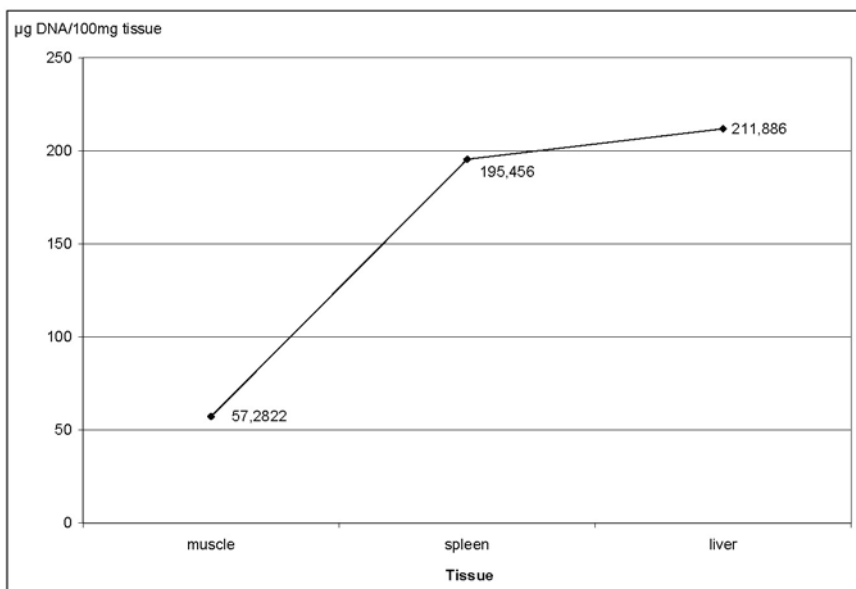


Fig. 6 Graphic presentations for average's values

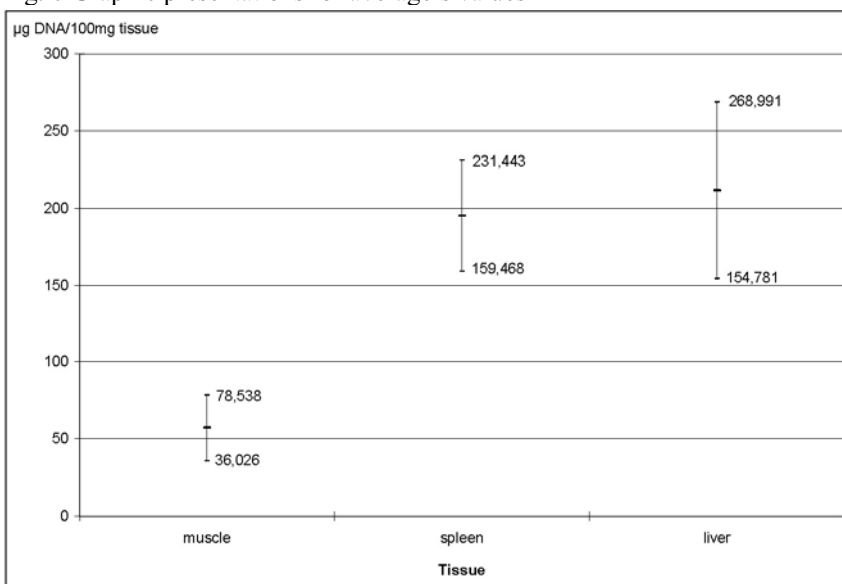


Fig. 7 Variability intervals

From variability intervals graphic representation, we observe that the intervals dimension grows directly proportional with the DNA quantity; for muscle tissue the limits vary between 36,026 μg DNA/100mg tissue (for lower limit) and 78,538 μg DNA/100mg tissue (for higher limit), and for liver, vary between 154,781 μg DNA/100mg tissue (for lower limit) and 268,291 (for higher limit).

CONCLUSIONS

The smallest values are recorded for muscle tissue and the highest for liver tissue.

Referable to variability intervals we can observe that the interval dimensions growth proportional with DNA quantity.

REFERENCES

Ausubel, F. M., Brent, R., Kingston R. E., Moore D. D., Seidman J. G., Smith J. A., Struhl K., 1995– “*Current protocols in molecular biology*”, vol. 1, cap. 2 – Preparation and analysis of DNA. Phenol extraction and ethanol precipitation of DNA, Ed by John Wiley & Sons, Inc. 2.1.1. – 2.1.3.

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DYNAMICS OF LEAF PEROXIDASE ACTIVITY DURING ONTOGENY OF HEMP PLANTS, IN RELATION TO SEXUAL PHENOTYPE

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Keywords: hemp peroxidase, sexual phenotype

Abstract: During vegetation of female and male hemp plants (*Cannabis sativa* L.), five quantitative determinations of peroxidase activities were made (40 days, 55 days, 70 days, 85 days, 105 days). Peroxidase activity presented some differences in hemp plants, between females and males, during their vegetation cycle. In female plants, before anthesis were registered peaks of peroxidase activities. The blossoming of male plants was coincident with the increase of catalytic action of peroxidase. Generally, the male plants displayed greater levels of peroxidase activity.

INTRODUCTION

Plant peroxidases (EC 1.11.1.7) catalyse the oxidation of some organic substrata, in presence of H_2O_2 , and are associated with growth, cell wall formation, fruit ripening, ethylene biosynthesis, resistance to pathogens, reactivity to stress (Frahry and Schopfer, 1998). This enzyme has more isoforms, each of them with an well-defined role in organism. The isoperoxidase pattern is complex, fact evidenced too for hemp (Truță et al., 2002), just this complexity making difficult the understanding of specific functions of enzyme (Yun et al., 1998; Clemente, 1998). Peroxidase is placed in cytosol and cell wall, in several isoforms, genetically different. The cell wall, where there is 6-8% from total tissue peroxidase activity, is considered the site of primary action of plant peroxidases (Fry, 1986). Generally, the idea of intervention of acid peroxidases in lignin biosynthesis is accepted, while basic peroxidases, placed in cytosol, are implied in IAA catabolism, by a decarboxylation step (Lamport et al., 1998; Limam et al., 1998). The chemical composition of hemp is very complex (flavonoids, fatty acids, phenolic spiroindans, dihydrostilbenes, nitrate substances, more than 70 cannabinoids). Although the hemp is a dioecious species, as a consequence of intensive improvement, a lot of sexual phenotypes are cultivated, the most frequent being the monoecious forms. For hemp, it is not known a consistent study on differentiation between sexual phenotypes, regarding morphological, physiological or biochemical traits, in spite of some disparate data. For genus *Cannabis*, variable levels of cellular extract pH are cited, depending on sex. For these reasons, the objective of this study is to identify the existence of some enzymatic differences between the sexual phenotypes of hemp, to complete our previous data obtained from other similar studies.

MATERIALS AND METHODS

We used leaves collected from female and male plants of hemp (*Cannabis sativa* L.), from a population grown in the experimental field of the Botanical Garden of University “Alexandru Ioan Cuza” Iași. The analyses were effectuated in dynamics, starting with plants 40 days old, until 105 days, at intervals of 2 weeks, excepting the last determination, determined by photometric method with o-dianisidine. The principle of this method is to establish the colour intensity of compound obtained by dianisidine oxidation, under the peroxidase activity, in the presence of H_2O_2 . The values of extinctions were determined with a SPEKOL 20 spectrophotometer, at $\lambda=540\text{nm}$. The statistical analysis of the obtained data was performed realized after 3 weeks. Peroxidase activity was using the method described in RAICU et al. (1973). The arithmetical mean (\bar{x}), the standard deviation (SD), the standard error of the mean (SE), the coefficient of variation (CV) and the standard error of the mean, expressed in % (SE %), were calculated.

RESULTS AND DISCUSSIONS

As shown in Fig. 1, the enzymatic activity hasn't a linear evolution, neither in females nor in males. Thus, if 40days old plants have enough close levels, although lightly more increase for males (4.84 ± 0.50 UP/g, comparing with 4.30 ± 0.58 UP/g, in females), until next quantification (55 days) it took place an important progressive increase of peroxidase activity: 6.30 ± 0.45 UP/g, for females, 7.44 ± 0.73 UP/g, for male plants. These values represent an increase with 46.51% for

plants with pistillate flowers and with 53.72% for males, comparatively with the previous determination. In the next two weeks interval, it was observed a diminution of peroxidase activity, more clear in females (5.09 ± 0.55 UP/g), in comparison with plants with staminate flowers (6.83 ± 0.42 UP/g) (70 days). Until 85 days, the values maintained over those registered at 70 days, for both sexual phenotypes: 8.01 ± 0.63 UP/g, in female phenotypes, 8.12 ± 1.31 UP/g, in male plants. Although these values are close, we ascertain, in comparison with former determination, a more marked increase of peroxidase activity in plants with pistillate flowers. The last quantification (105 days) registered a decline of peroxidase average values: 5.18 ± 0.55 UP/g, in female plants, respectively 5.94 ± 0.84 UP/g, in male plants.

A general view on results evidenced a constant maintenance of average peroxidasic activity at a superior level for investigated males, during whole period of vegetation. The activity of this enzyme is correlated with the modifications that accompany the plant development.

In organogenesis, the role of peroxidase is often explained by the double function of this enzyme, involved both in the specific oxidizing of some substrata and in auxin catabolism (Legrand and Bouazza, 1991). By this second function, the peroxidase induce a modulation of morphogenesis, because of the influence on the endogenous hormonal balance.

Because of its intervention in regulation of IAA (indole-3-acetic acid) level, the peroxidase has an indirect role in sex-determining mechanism in hemp, more exactly in stamenogenesis and carpellogenesis. Hemp is one of the species in which a high level of IAA induces the female sex phenotypisation. In hemp, as well as in other monoecious or dioecious plants, the gibberellins, auxins, ethylene and cytokinins have an important contribution to sex expression.

These hormones generally intervene in the derepression of regulator genes which enables the synthesis of specific proteins that control the flower organogenesis. In other species (of the *Mercurialis* genus, for example) the situation is different: in males, auxin is in greater quantities than in females ones, fact in accordance with the peroxidasic profiles, emphasized for females, and attenuated in males.

Excepting the influence on male and female sexual organs, the peroxidase, by diminution or increase of auxin level, are notable effects on plant growth. At small auxinic levels, more or less significant inhibitions of plant growth appear. The idea of a strong peroxidase activity associated with an increased auxin catabolism is generally accepted. In female plants, during vegetation, smaller peroxidasic activities are registered, fact that maintains endogenous auxin at an increasing level and allows the phenotypisation of female sex. In individuals with staminate flowers, the greater peroxidasic activities reflects on diminution of endogenous auxinic phytohormone and in the intervention of some other factors that will induce the stamenogenesis. The results obtained by quantitative determinations of the level of peroxidase activities, in dynamics, in female and male individuals, expressed in greater values for staminate plants, must be analysed and correlated with the results obtained in our previous research (Truță et al., 2002), that evidenced significant differences between the isoperoxidase pattern of the two sexual phenotypes. Thus, the female plants have four bands, and the males presented ten fractions, the genetic determinism of these isoforms being multigenic. For hemp, are still unexplained details about the genes coding these peroxidases.

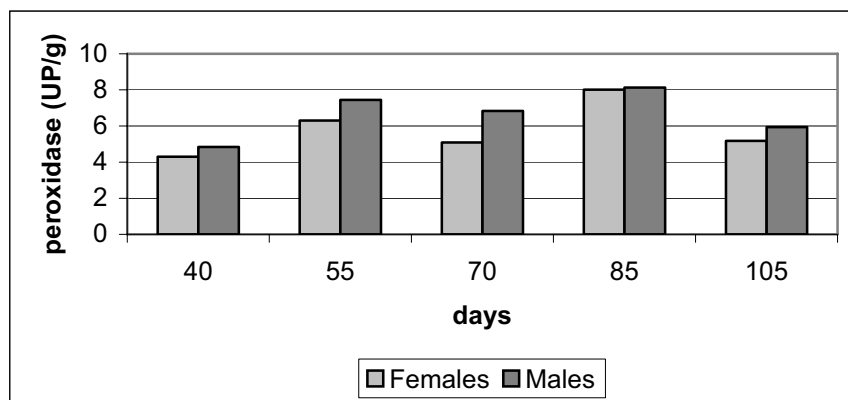


Fig. 1. Diagram of average values of peroxidase activities during ontogenesis of female and male plants of hemp

In investigated female plants we registered peaks of peroxidase activities before anthesis. The carpellogenesis was stimulated by IAA, that increases as result of diminution of peroxidase activity (at 70 days). The blossoming of male plants was early. This moment was coincident with the increase of catalitic action of peroxidase (7.44 UP/g). The other peak of peroxidase activity, at 85 days, could be discussed in relation with the fiber production and the intensification of lignification process. In a previous paper (Truță et al., 2002), significant differences we found, between the isoperoxidase patterns of staminate and pistillate hemp plants, both in the number and stain intensity of bands. The isoenzymatic spectrum was richer for male plants.

CONCLUSIONS

During ontogeny of female and male hemp plants, the peroxidase activity was fluctuant, with periodic increases and decreases.

In female plants, before anthesis were registered peaks of peroxidase activities.

The blossoming of male plants was coincident with the increase of catalitic action of peroxidase in this group of sexual phenotypes.

During all vegetation cycle, the average values of peroxidase activities were greater in male plants than in female plants.

REFERENCES

1. Clemente, E., 1998. *Phytochemistry* 49(1), 29-36.
2. Frahry, G., Schopfer, P., 1998. *Phytochemistry* 48(2), 223-227.
3. Fry, S. C., 1986. *Molecular and physiological aspects of plant peroxidases* (ed. by Greppin, H., Penel, C., Gaspar, Th.), Univ. of Genève, Genève, 169-182.
4. Lamport, D., 1986. *Molecular and physiological aspects of plant peroxidases* (ed. by Greppin, H., Penel, C., Gaspar, Th.), Univ. of Genève, Genève, 199-208.
5. Legrand, B., Bouazza, A., 1991. *J. Plant Physiol.* 138(1), 102-106
6. Limam, F., Chahed, K., Ouelhazi, N., Ghir, R., Ouelhazi, L., 1998. *Phytochemistry* 49(5), 1219-1225.

7. Truță E., Gille E., Tóth E., Maniu M., 2002. *J. Appl. Genet.* 43(4), 451-462.
8. Yun, B. - W., Huh, G. - H., Kwon, S. - Y., Lee, H. - S., Jo, J. - K., Kwak, S. S., 1998. *Phytochemistry* 48(8), 1287-1290.

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CYTOGENETICS EFFECTS INDUCED BY NITRATE OF LEAD ON MITOTIC DIVISION AT *ALLIUM CEPA* L.

SILVICA PĂDUREANU¹

Key words: nitrate of lead, *Allium cepa* L., root meristem, cells in mitotic division, chromosomal aberrations.

Abstract: The paper presents the influence of nitrate of lead upon the mitotic division of *Allium cepa* L. The treatment with nitrate of lead has determined the lessening of the mitotic index and the chromosomal mutations. Also nitrate of lead determined in little proportion cells autopolyploid. The experiment proved that nitrate of lead, known as a polluting agent has a mutagenic potential on the plants.

INTRODUCTION

It is known that the lead is polluting agent very toxic for plants and animals (Ciplea, Ciplea, 1978; Heggstad, 1968; Kihlman, 1966)

At plants, action of the lead demonstrated on various chromosomal aberrations (Pădureanu, 2004; Pădureanu, 2004). Our investigations focused the determination of the mitotic index, the determination of the frequency of the types of chromosomal aberrations from metaphases and aberrant ana-telophases.

MATERIAL AND METHODS

The biological material used in the experiment, was represented by seeds of *Allium cepa* L., harvested from a local population cultivated at the Experimental Didactic Station “V. Adamachi” from the University of Agricultural Sciences and Veterinary Medicine, Iași.

The seeds were put to germination in lab conditions. When the roots reached 15 – 17 mm in length, they were treated with acetate of lead.

Nitrate of lead was used in the form of watery solutions in three concentrations: 5%, 1%, 0.1%.

The time of action of the respective solutions on the radicular meristems was differentiated as follows: 5% solutions acted for 48 hours, 24 hours, 4 hours, 2 hours; 1% and 0.1% solutions acted for 4 hours and 2 hours.

Taking into account the concentration and the time of action of the solutions 8 variants have resulted.

Besides these eight experimental variants, there was also used a control plot and in this case no treatments were applied to the radicular meristems.

For further cytogenetic investigations, the treated and non/treated roots (control) were fixed in Carnoy fixing solution for 24 hours at 4°C then hydrolised with HCl and coloured with the basic colouring matter Carr.

The radicular meristem was displayed using squash technique.

15 preparations and 10 microscopical fields/preparation were examined for all the variants and control.

The microscopical examination was carried out using the optic microscope Nikon Eclipse 600.

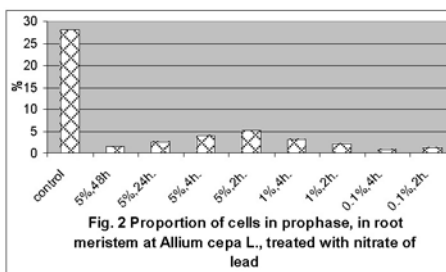
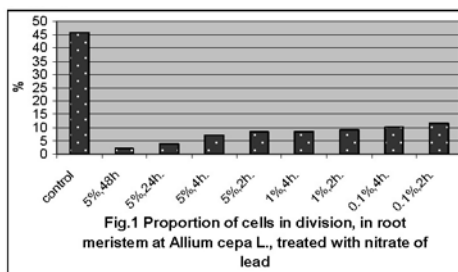
The microphotographies were made with the camera from the endowment of the microscope.

RESULTS AND DISCUSSIONS

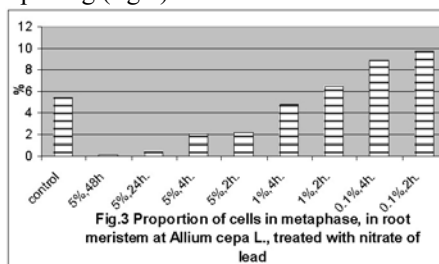
The analysis of the mitotic index

The first aspect investigated, correlated with the mutagenic capacity of the treatments by nitrate of lead on the onion it was represented by the effect of nitrate of lead on mitotic division's stages.

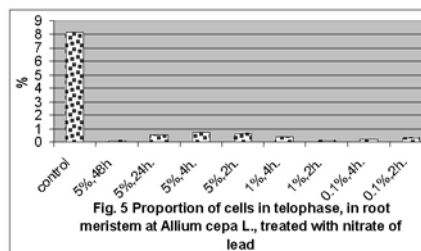
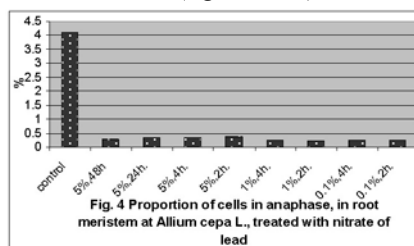
The number of cells in division diminish correlated with the increase the concentrated and time of action of nitrate of lead (fig.1). The situation for each phase of mitotic division is represented in figures 2, 3, 4 and 5. The percentage of the cells in prophase is greater when the concentration is 5%, 2 hours, and other variants is small by comparison with control (fig.2).



The high percentage of cells in metaphase at the variants with 1% concentration (2hours) and 0.1% (4 hours, 2 hours) is surprising (fig.3).

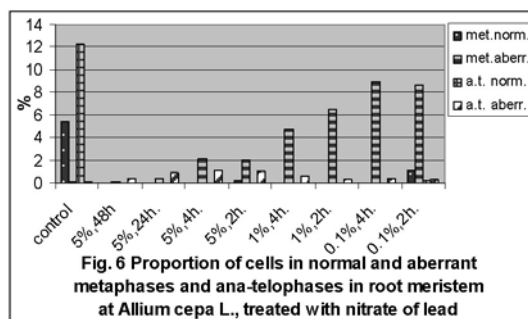


The proportion of cells in anaphase and in telophase remain much low by comparison with control in all cases (fig. 4 and 5).



The analysis of the cells in aberrant metaphase and aberrant ana-telophase

In figure 6 is notice that the highest proportions of aberrant metaphases were induced by 1% and 5% concentrations. Aberrant ana-telophases were produced in high percentages(0.85%, 1.05%, 1.01%) at 5% concentration. On constate that at most cases is not never registered normal metaphases (5%: 48 hours, 24 hours; 1%: 4 hours, 2 hours; 0.1%: 4 hours) and not even normal ana-telophases. This aspect it might due on small number of mitogen cells of respectively variants.

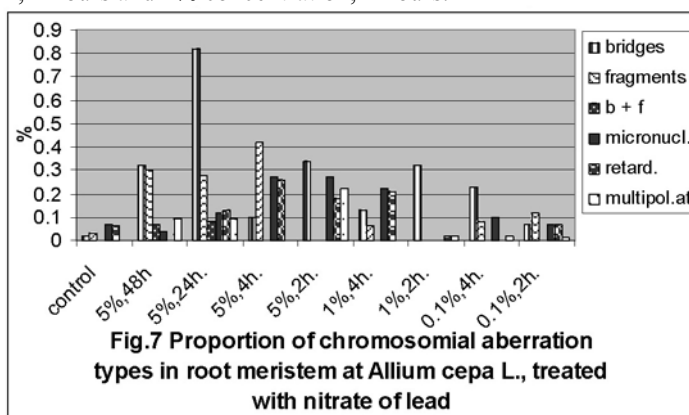


The analysis of the types of chromosomal aberrations

The proportion of the types of chromosomal aberrations induced by nitrate of lead on onion root meristem is graphically represented in figure 7.

This polluting agent induced the thick chromosomal bridges between nuclei by the variant with 5% concentration.

The chromosomal fragments were present at majority variants, excepting the variants with 5% concentration, 2 hours and 1% concentration, 2 hours.



The associations between bridges and fragments appeared only at variants with 5% concentration, 48 and 24 hours.

Micronuclei appeared in interphases, were registered in small percentage at majority variants excepting the variant with 1% concentration, 2 hours.

Retardatory chromosomes appeared at majority variants, excepting the variants with 5% concentration, 48 hours, and 0.1%, 4 hours.

Multipolar ana-telophases were registered at all three concentration of the polluting, but at certain times of action (5%: 4 hours; 1%: 4 hours) were not induced.

A special effect constated at the polluting consist in the presence of many metaphases with picnotic chromosomes sparse in all mixoplasma. Such aberrant metaphase appeared exclusiveness at majority variants, excepting the variants with 5% concentration, 2 hours and 0.1%, 2 hours, when was registered normal and aberrant metaphases.

As a result at this aberrant metaphases appeared c-anaphases characterized by monocromatidic chromosomes pairs wich not separated between that two cellular pols. The

denomination “c-anaphase” is explained by specific behaviour at the chromosome’s cells treated with colchicine (Natarajan, Ahnström, 1969).

Existence the metaphases with picnotic chromosomes and the c-anaphases might be explained owing to the whole inhibition the division shaft by influence the natrium of lead, feeld effect equally under treatments with colchicine.

As a results at this pseudo-anaphases (c-anaphases) appeared much pseudo-telophases characterized by mononucleate cells, wich big nucleus, whose size is double by comparision with diploide normal cells. Such the nuclei are autopoliploides. On surprised diversed stages by formation at this nuclei.

It is fitting much prudence when to affirm the presence of the autopoloides nuclei, but existence of the nuclei with four nucleolus at onion is an strong argument in that sense.

By beside the types of aberrations presenced, on registered other atipic aspects concretized by very prolonged, bacilliform nuclei (21-62 μm), and picnotic, not-functionally nuclei.

It is known that cells at the caliptra has prolonged nuclei, but the nuclei named by us aberrant is not the same with those from caliptra, because they have a specific form, and the nucleolus inspire on aspect at bacillus with spores.

Different aspects of chromosomal aberrations induced by nitrate of lead are presented in figures 8-15.

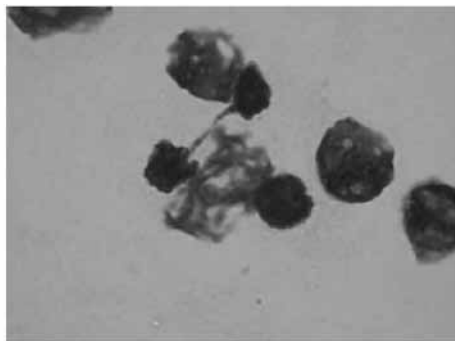


Fig. 8 Telophase with a bridge in root meristem at *Allium cepa* L. treated with nitrate of lead 5%, 24 hours (1000X)

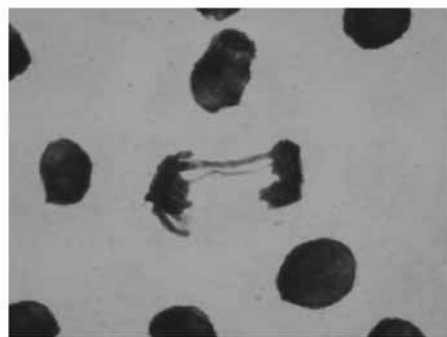


Fig. 9 Ana-telophase with two bridges in root meristem at *Allium cepa* L. treated with nitrate of lead 5%, 24

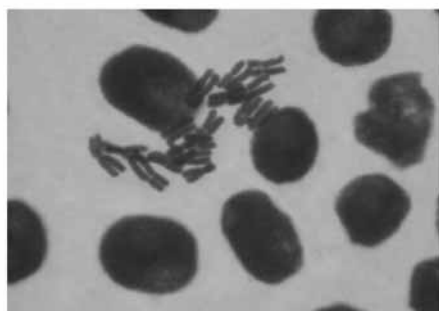


Fig. 10 Metaphase with picnotic chromosomes in root meristem at *Allium cepa* L., treated with nitrate of lead 5%, 4 hours (1000X)



Fig. 11 Anaphase with cleaved chromosomes in root meristem at *Allium cepa* L., treated with nitrate of lead 5%, 2 hours (1000X)

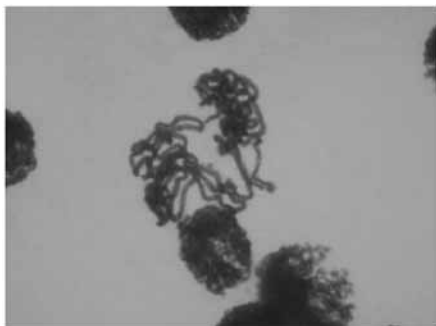


Fig. 12 The forming of a autopoliploide nucleus in root meristem at *Allium cepa* L., treated with nitrate of lead 5%, 4 hours (1000X)

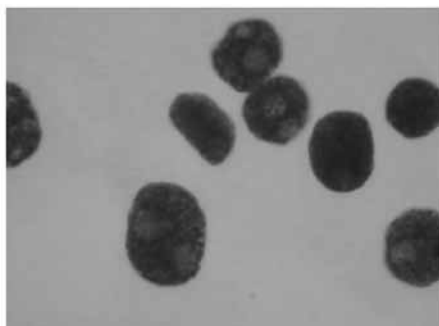


Fig.13 Nucleus with four nucleolus in root meristem at *Allium cepa* L., treated with nitrate of lead 0.1%, 4 hours (1000X)

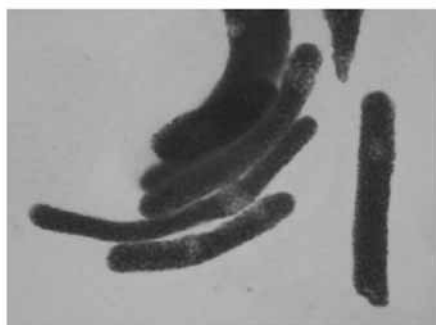


Fig. 14 Prolonged nucleus in root meristem at *Allium cepa* L., treated with nitrate of lead 5%, 4 hours (1000X)



Fig. 15 Prolonged, bacilliform nucleus in root meristem at *Allium cepa* L., treated with nitrate of lead 0.1%, 4 hours (1000)

CONCLUSIONS

Nitrate of lead, known as a polluting agent has a strong inhibitory effect on mitotic division of *Allium cepa* L.

Nitrate of lead has a real mutagenic potential, proof is diverse chromosomal aberrations.

Nitrate of lead seems to be total inhibitory action at mitotic shaft, perturbing the behaviour of the chromosomes in the same manner as the effect of colchicine.

Nuclei with four nucleolus are one argument that nitrate of lead has to be autopolyploid effect.

The side of habitual prolonged nuclei at the caliptra, appeared bacilliform nucleus and picnotic nuclei consequence of natrium of lead.

BIBLIOGRAPHY

1. Ciplea L., Ciplea Al., 1978. *Poluarea mediului ambiant*. Ed. Tehnică, București
2. Heggestad H.E., 1968. Diseases of crops and ornamental plants incited by pollutants. *Phytopatology*, nr. 58, vol.8, 1089-1-98
3. Kihlman B.A., 1966. *Action of chemicals on dividing cells*. Prentice Hall. Englewood Cliffs, New Jersey

4. Natarajan A.T., Ahnström G., 1969. Heterocromatin and chromosome aberrations. *Chromosoma*, nr. 28, 48-61
5. Pădureanu Silvica, 2004. Cytogenetic effects induced by acetate of lead on mitotic division at *Allium cepa* L. *Anale șt. Univ. "Al.I.Cuza"*, Iași, Genetică și Biologie moleculară, tom. V, 282-287
6. Pădureanu Silvica, 2004. Cytogenetics induced by natrium pentacyano-mononitroso-ferrat III on the mitotic division at *Allium cepa* L. *Lucr. șt., Univ. Șt.Agr. Med. Vet. „Ion Ionescu de la Brad”*, Iași, vol.47, seria Agron., 116-121

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THE INFLUENCE OF HEXANITROGEN- COBALTIAT III OF SODIU ON MITOTIC DIVISION AT *ALLIUM CEPA* L.

SILVICA PĂDUREANU^{1*}, MIRELA MIHAELA CÎMPEANU²

Key words: hexanitrogen-cobaltiat III of sodiu, *Allium cepa* L., root meristem,, cells in mitotic division, chromosomal aberrations.

Abstract: The paper presents the influence of hexanitrogen-cobaltiat III of sodiu upon the mitotic division of *Allium cepa* L. The treatment with hexanitrogen-cobaltiat III of sodiu has determined the lessening of the mitotic index and the chromosomal mutations. The experiment proved that hexanitrogen-cobaltiat III of sodiu, known as a polluting agent has a mutagenic potential on the plants.

INTRODUCTION

The cobalt and he's salts are known as pollutants (Ciplea, Ciplea, 1978; Heggstad, 1968; Kihlman, 1966)

At plants, action of the pollutants demonstrated on various chromosomal aberrations (Ahmed, Grant, 1972; Ammore, 1961; Fiskesjö, 1969; Pădureanu, 2004; Pădureanu, 2004). Our investigations focused the determination of the mitotic index, the determination of the frequency of the types of chromosomal aberrations from metaphases and aberrant ana-telophases.

MATERIAL AND METHODS

The biological material used in the experiment, was represented by seeds of *Allium cepa* L., harvested from a local population cultivated at the Experimental Didactic Station "V. Adamachi" from the University of Agricultural Sciences and Veterinary Medicine, Iași.

The seeds were put to germination in lab conditions. When the roots reached 15 – 17 mm in length, they were treated with acetate of lead.

Hexanitrogen-cobaltiat III of sodiu was used in the form of watery solutions in three concentrations: 5%, 1%, 0.1%.

The time of action of the respective solutions on the radicular meristems was differentiated as follows: 5% solutions acted for 48 hours, 24 hours, 4 hours, 2 hours; 1 % and 0.1% solutions acted for 4 hours and 2 hours.

Taking into account the concentration and the time of action of the solutions 8 variants have resulted.

Besides these eight experimental variants, there was also used a control plot and in this case no treatments were applied to the radicular meristems.

For further cytogenetic investigations, the treated and non/treated roots (control) were fixed in Carnoy fixing solution for 24 hours at 4°C then hydrolised with HCl and coloured with the basic colouring matter Carr.

The radicular meristem was displayed using squash technique.

15 preparations and 10 microscopical fields/preparation were examined for all the variants and control.

The microscopical examination was carried out using the optic microscope Nikon Eclipse 600.

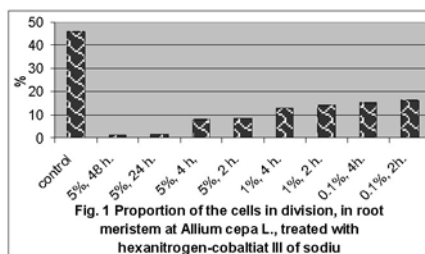
The microphotographies were made with the camera from the endowment of the microscope.

RESULTS AND DISCUSSIONS

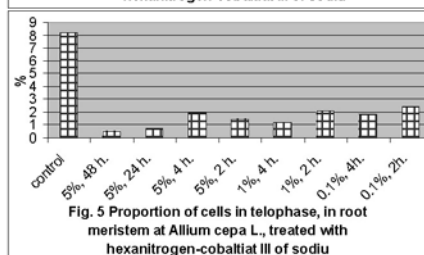
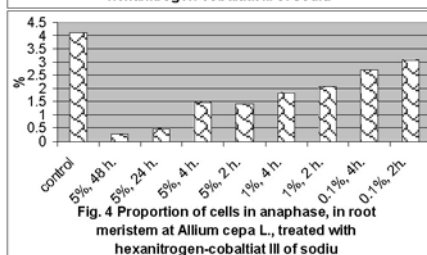
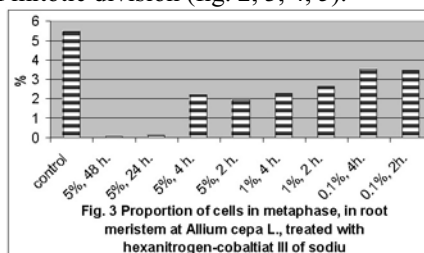
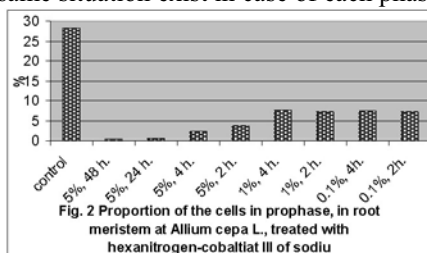
The analysis of the mitotic index

The inhibitory effect of this polluting agent is strong experienced in case of 5% concentration.

At other variants, the percentage of cells in division is a little high, with values correlated in a contrary direction as against the concentration and the time of action of polluting agent (fig. 1).

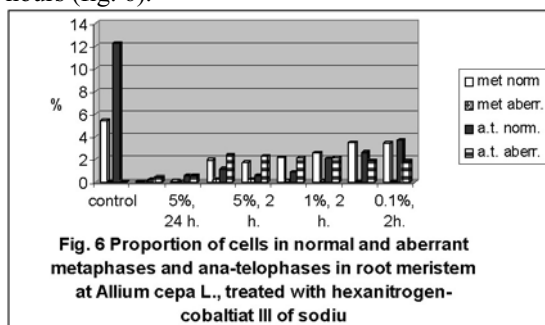


The same situation exist in case of each phase of mitotic division (fig. 2, 3, 4, 5).



The analysis of the cells in aberrant metaphase and aberrant ana-telophase

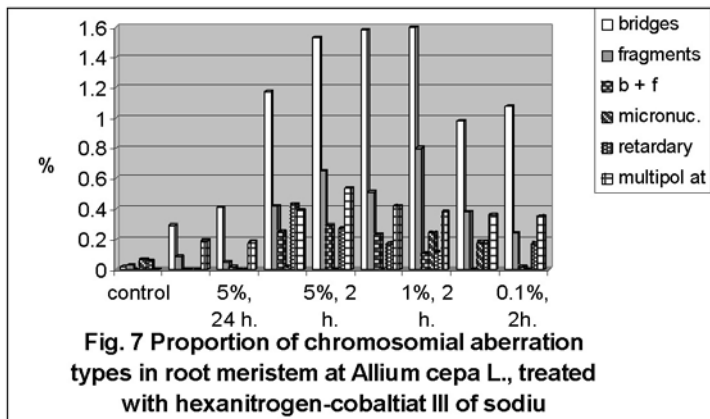
The aberrant metaphases appeared in subunitary percentage to majority variant, excepting of the 5%, 48 hours and 24 hours (fig. 6).



The aberrant ana-telophases appeared at all variants (fig. 6).

The analysis of the types of chromosomal aberrations

The proportions of the types of aberrations induced by complex salt of cobalt to the root meristem of onion are represented in figure 7.



The chromosomal bridges appeared at all variants. At 5% concentrations, 48 hours and 24 hours, the bridges are thick, simple or double, as time at 5% concentration, 4 hours and 2 hours, and 1% and 0.1% concentration, the bridges are thin, multiple.

The chromosomal fragments appeared at all variants in subunitary percentage.

The associations between bridges and fragments absent only at the variants with 5% concentration, 48 hours and 0.1% concentration, 4 hours.

Micronuclei appeared only at three variants: 5% concentration, 4 hours, 1% concentration, 2 hours, 0.1%, 4 hours.

Retardatory chromosomes from metaphase and ana-telophase absent at the variants with 5% concentration, 48 hours and 24 hours.

Multipolar ana-telophases registered in all variants.

Beside the presented, respectively polluting induced the forming of the picnotic nuclei, non-functionally, in subunitary proportion, who is varying directly proportionally with the increase the concentration and the time of action of polluting.

Different aspects of chromosomal aberrations induced by hexanitrogen-cobalt(III) of sodium are presented in figures 8-11.



Fig. 8 Ana-telophase with bridge in root meristem at *Allium cepa* L., treated with $\text{Na}_3[\text{Co}(\text{NO}_2)_6]$, 5%, 24 hours (1000X)



Fig. 9 Telophase with two bridges in root meristem at *Allium cepa* L., treated with $\text{Na}_3[\text{Co}(\text{NO}_2)_6]$, 5%, 24 hours (1000X)



Fig. 10 Multipolar anaphase with multiple bridges in root meristem at *Allium cepa* L., treated with $\text{Na}_3[\text{Co}(\text{NO}_2)_6]$, 1%, 2 hours (1000X)



Fig. 11 Anaphase with ragged bridges in root meristem at *Allium cepa* L., treated with $\text{Na}_3[\text{Co}(\text{NO}_2)_6]$, 0.1%, 2 hours (1000X)

CONCLUSIONS

Hexanitrogen-cobaltat III of sodiu, know as polluting agent has a strong inhibitory effect on mitotic division of *Allium cepa* L.

The cells proportion in division is in opposite direction with the increased of the concentration and the time of action of polluting agent.

Hexanitrogen-cobaltat III of sodiu has a real mutagenic potential, confirmed by diverse types of chromosomal aberrations induced.

Picnotic nuclei represents a characteristic of the hexanitrogen-cobaltat III of sodiu effect.

BIBLIOGRAPHY

1. Ciplea L., Ciplea Al., 1978. *Poluarea mediului ambient*. Ed. Tehnică, București
2. Ahmed M., Grant W.F., 1972. Cytological effects of the pesticides phosdrin and bladex on *Tradescantia* and *Vicia faba*. *Can. J. Genet. Cytol.*, 14, 157-165
3. Ammore J.E., 1961. Arrest of mitosis in roots by Oxygen-lack or cyanide. *Ray. Soc. (London) Proc. B.*, 154, 95-108
4. Fiskesjö G., 1969. Some results from *Allium* tests with organic mercury halogenides. *Hereditas*, 62, 314-322
5. Heggstad H.E., 1968. Diseases of crops and ornamental plants incited by pollutants. *Phytopatology*, 58: 8, 1089-1098
6. Kihlman B.A., 1966. *Actions of chemicals on dividing cells*. Prentice Hall. Englewood Cliffs, New Jersey
7. Pădureanu Silvica, 2004. Cytogenetic effects induced by acetate of lead on mitotic division at *Allium cepa* L. *Anale șt. Univ. "Al.I.Cuza"*, Iași, Genetică și Biologie moleculară, tom. V, 282-287
8. Pădureanu Silvica, 2004. Cytogenetics induced by natrium pentacyano-mononitroso-ferrat III on the mitotic division at *Allium cepa* L. *Lucr. șt., Univ. Șt.Agr. Med. Vet. „Ion Ionescu de la Brad”*, Iași, vol.47, seria Agron., 116-121

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***ECHINACEA* – FROM ETHNOBOTANY TO MODERN PHYTOPHARMACEUTICS**

DANIELA LUMINIȚA ICHIM¹

Medicinal herbs play an important role in health care throughout the world, especially in non-industrialized continents such as South America, Africa and parts of Asia.

In The manufactured drugs are hardly accessible to populations of the non-industrialized countries, the phytotherapy (in modern medicine) of the overpowering industrialized ones will differ quantitatively and qualitatively being determined by the traditions and regionalisms of various cultures. Today, in Japan, 40% of medical practitioners usually prescribe phytotherapeutical preparations, and in Germany, almost 32% of the commercial medicines are the phytotherapeutical ones. (Hansel, 1986, quoted by Ursula Stănescu, 2002).

Some of the most popular european phytopharmaceutical preparations contain an American herb called *Echinacea* and the first commercial European preparation of this plant was made over 50 years ago by Gerhard Madaus, under the name of Echinacin.

Since then this plant has been intensively. Studied, but what is the most important thing is its traditional use given by Native American people, as a remedy for colds, flu and other infections.

The plant s name, *Rudbeckia purpurea* (1753) was given by Linnaeus, the famous Swedish botanist and physician, after Olaf Rudbeckia, a name that one can occasionally find it in botanical and horticultural literature even today.

Echinacea has a few common names in English, but the most widely encountered name is that of Purple Cone – flower, the other ones being: Blak Sampson, Red Sunflower, Comb Flower, Cock Up Hat, Missouri Snakeroot and Indian Head.

As one reviews the articles written between 1850 and 1900 on the medicine of the Native Americans well find out that some authors refer to the use of *Echinacea* by some tribes such as: Cheyenne (sore mouth, gums), Choctans (cough, dyspepsia), Crow (colds, tooth – aches, colic like comb), Daware (gonorrhea), Omaha (septic diseases), Hidatsa (stimulant).

The Eclectics, a group of doctors who depended on botanical medicine in their practice and who were famous from the 1830 s to the 1930 s. They brought *Echinacea* to the fore front of herbal medicine. The Lloyd Brothers made of Specific Medicine *Echinacea* and *Echafolta*, the most famous preparations.

J. King and John Uri Lloyd introduced *Echinacea* to the medical profession.

King provides some botanical information and some uses of the species of *Echinacea purpurea*, writing that the root has a very pungent taste and it has proved to be effective in treating the syphilis.

Echinacea angustifolia has been quoted in John Lloyd s History of *Echinacea angustifolia* and the Lloyd Brothers a Treatise on *Echinacea*, both works being still available.

Around 1870, H.C.F. Meyer began producing a patent medicine which contained *Echinacea angustifolia*, (identified by Lloyd) Hops and Wormwood. Initially, king and Lloyd did not recognize this preparation Meyer mentioned that *Echinacea* proved to be efficient in healing the wounds made by poisonous serpents.

In 1912, in a study made by Lloyd regarding the popularity of vegetable drugs among medical practitioners of the day, *Echinacea* was ranked 11th. Meanwhile, King Began interested in the plant, using it in a preparation (made by Lloyd) for his wife who had cancer at the time and he found out that *Echinacea* retarded the progress of the disease and provided improvement in the pain.

In 1898, King added new materials to his work American Dispensatory and Felter published a monograph about *Echinacea* in the Eclectic Medical Journal under the title The Newer Materia Medica: I. *Echinacea*. Felter referred to the pharmacological action of *Echinacea* calling it antiseptic, alternative and a corrector of the capillary circulation.

While the Eclectics were convinced by the miraculous properties of the plant, the Regulars, or allopaths were critical about it.

In 1915, V. von Unruh, M.D. Wrote an article on the tuberculosis treatment, using a preparation of *Echinacea angustifolia* and *Inula helenium*, seeing the importance of the plant in stimulating the immune system and underlying the fact that his compound did not contain an excess of alcohol, comparatively to Lloyds one.

During a period of three years of study, he has noticed that *Echinacea* stimulates the process of phagocytosis and produces in the blood, effects similar to those produced by the vaccines.

Today, after H. Wagner s study, it is known that *Echinacea* contains water – soluble polysaccharides, that strongly affect the immune system.

The first published report (1897) on the chemical constituents of *Echinacea* was by John Uri Lloyd and it referred to its root which was at first sweet and then acid to taste.

Lloyd's study on *Echinacea angustifolia* was continued by one of his students, S. H. Culter, in his doctoral thesis, published in 1931. Other notable research was carried out by Heyl and Staley, Heyl and Hart who also added the insecticidal activity of the essential oil.

After the prolific amount of material written about *Echinacea* over a 50 years period, it seems outstanding that after 1937 the marketing of *Echinacea* preparations ceased in the U.S. for a long period of time. Meanwhile, the German researchers became interested in testing the plant's properties and Gerhard – Madaus published many studies over the next 50 years.

Echinacea's taxonomy: Division *Spermatophyta*; Subdivision *Angiospermae*; Class *Dicotyledonae*; Subclass *Asteridae*; Classification *Asterales*; Family *Asteraceae* (*Compositae*); Genus *Echinacea*

After some cariological studies it has been established that the vegetal material of cultures, in Europe belongs to the species of *Echinacea angustifolia* and *E. pallida* and not to *Echinacea purpurea* and *E. p.* as it was believed for a long time. (Hodișan V. et Tămaș M., 1984).

The three species differ morphologically, histo-anatomically and biochemically, hence the different quality of the response on the level of the immune system. These are herbaceous plants with verucous leaves; the flowers are a rich purple to pink and the florets are seated round a high cone: seeds, four-sided achenes.

The name of *Echinacea* comes from the Greek hedgehog, i.e. hedgehog, referring to the sharp bracts of the receptacles. The roots, i.e. the rhizomes are shaped as some fragments of different thickness being cylindrical, slightly spiral and having a slight hot – burning taste, at the beginning having and then a sweetish – bitter one. It seems that the hot taste disappears because of the degradation of isobutyl amides, thus, diminishing also – the immune stimulative effect of the medicinal preparations.

The main active principles of *Echinacea* belong to the group of immunostimulators and they are identified to *Echinacea herba* and *E. radix*. To *E. radix*, the active biologic principle having an immunostimulative activity, derived from *E.p.* is the cicaenic acid (it stimulates the phagocytosis).

Echinacea herba, from *Echinacea purpurea* contains much more active principles, which chemically speaking they belong to a different structure: polythalsides, isobutyl amides and polyphenols.

In their studies Moench, Wagner H. and colab. isolated D polythalsidic fractions, on the upper parts of the plant of *Echinacea purpurea*: – 4 – 0 – methylglucoranoarabinoxylan and arabinorhamnogalactan acid which had the capacity to stimulate in vitro the phagocitary activity of the granulocytes (Wagner et. al. 1988).

Among the three species of *Echinacea*, *Echinacea purpurea* is the most important one for therapeutics, being followed by *Echinacea angustifolia* (Stănescu et. al, 2002). Depending on the pharmacological effects induced by the active principles, *Echinacea* has been used for its anti-inflammatory effects: (for polythalsidic fractions), antibacterial and antiviral (for the esters of the caffeic acid), bacteriostatic and fungi static (for polyacetylenic fractions), insecticidal ones (for isobutyl amides).

The use of *Echinacea* preparations in healing the infections is provided simultaneously with the administration of antibiotics and chemotherapy.

Considering the fact that these species have been known all over the world as medicinal herbs but not in Romania, there were introduced in Cluj, in 1982, in cultures 2 species of the same genus, *Echinacea pallida* and *Echinacea purpurea* Moench by the researchers of the Faculty of Agronomy from Cluj Napoca who established the suitable methods for each species. (Oniga Iliora, 1997)

Today, *Echinacea* is a widely sold plant especially on U.S. and Europe's market. The chemical and pharmacological researches demonstrate that the plant is a stimulant of the immune system; therefore the future of *Echinacea* in the international phytopharmaceutics has been assured.

REFERENCES

1. Bauer R., Khan I.A., Wagner H., 1998 – TLC and HPLC Analysis of *Echinacea pallida* and *Echinacea angustifolia* Roots, *Planta Med.*, 54, 426 – 430.
2. Bauer R., Remiger P., 1989 – TLC and HPLC Analysis of Alkanides in *Echinacea* Drugs – *Planta Med.*, 55, 367 – 371.
3. Ioan Ciulei, Emanoil Grigorescu, Ursula Stănescu, 1993, *Plante medicinale. Fitochimie și fitoterapie* – volumul 2, Editura Medicală, București, 690 – 722.
4. Hodișan V., Tămaș M., 1984 – Studiul farmacobotanic comparativ al speciilor *Echinacea angustifolia* Moench și *Echinacea purpurea* (L) Moench – *Farmacia*, XXXII, 4, 203 – 210.
5. Muntean L.S., 1990 – *Plante medicinale și aromatice cultivate în România*, Ed. Dacia, Cluj, 269 – 272.
6. Oniga Iliora – Elena, 1997 – Studiul farmacognostic al speciilor de *Echinacea* (*Asteraceae*) cultivate în România – *Rezumatul tezei de doctorat* – Univ. de Medicină și Farmacie, Cluj – Napoca, 27.

7. Stănescu Ursula, Hâncianu Monica, Miron Anca, Aprotosoia Clara – *Bazele farmaceutice, farmacologice și clinice ale fitoterapiei* – volumul I – Editura Gr.T.Popa, Iași – 2002, 1 – 3; 29.
8. Stănescu Ursula, Hâncianu Monica, Miron Anca, Aprotosoia Clara – *Bazele farmaceutice, farmacologice și clinice ale fitoterapiei* – volumul II, Editura Gr.T.Popa, Iași, 93 – 99; 105 – 144; 216 – 221.
9. Schulthess B.H., Giger E., Baumann W., 1981 – *Echinacea*: Anatomy, Phytochemical Pattern and Germination of the Achene – *Planta Med.*, 57, 384 – 388.
10. Soicke H., Gorler K., Kruger D., 1988 – Glycine – Betaine in *Echinacea* sp. and their Preparations, *Fitoterapia*, 59, 73 – 75.
11. Tamaș M., Hodișan V., 1984 – Actualități în fitoterapie: *Echinacea*, *Practica farmaceutică*, 31 – 40.
12. Tamaș M., Fagarasan E., Hodișan V., Petruța V. 1987 – *Practica farmaceutică*, 158 – 162.
13. Tămaș M., Căpușan I., Constanța L., Orășan R., Filipaș V., 1989 – Tratatamentul local al maladiilor herpetice cu Novastimi – *Clujul Medical*, LXII, 3, 259 – 261.
14. Wagner H., Stuppner H., Puhlmann H., Brekmmer B., Deppe K., Zenk M.A., 1989 – Gewinnung von immunologisch activen Polysacchariden aus *Echinacea* – Drogen und Gewebekulturen *Z. Phytother*, 10, 35 – 38.

1) The Emergency Military Hospital, Iași.

RECENZIE

GHEORGHE HRINCĂ : SISTEMLLE GENETICO-BIOCHIMICE LA OVINE, EDITURA AGATA, BOTOȘANI, 2004, 305+IX PAGINI

Literatura de specialitate din țara noastră s-a îmbogățit, la finele anului 2004, cu o lucrare remarcabilă din domeniul biochimiei și geneticii animalelor domestice. Este vorba despre monografia „ Sistemele genetico-biochimice la ovine ”, publicată în Editura Agata din Botoșani de către doctor în biologie Gheorghe HRINCĂ, cercetător științific principal gradul I la Stațiunea de Cercetare-Dezvoltare pentru Creșterea Ovinelor Popăuți, din Județul Botoșani.

Sintetizând rezultatele cercetărilor proprii și datele existente în literatura de specialitate, conexă cu problematica abordată, autorul - un bine cunoscut specialist în genetica și biochimia ovinelor – reușește să trateze, într-o manieră cuprinzătoare, principalele concepții și direcții de cercetare relative la polimorfismul proteic și mineral la ovine.

Monografia este structurată în patru capitole. În fiecare capitol, pe lângă problemele fundamentale, se discută și metodologia investigării diferitelor sisteme genetico-biochimice la ovine, precum și implicațiile lor practice. Conținutul capitolelor este bogat ilustrat cu tabele, grafice și fotografii color. În bibliografie au fost incluse 319 titluri de cărți și articole, utilizate de autor.

Primul capitol al lucrării, intitulat **Polimorfismul hemoglobinei la ovine**, prezintă structura hemoglobinei, tipurile ontogenice de hemoglobine normale la ovine, metodologia determinării tipurilor de hemoglobină la ovine, determinismul genetic al hemoglobinei la ovine, hemoglobine anormale la ovine, structura genetică a locusului hemoglobinei în populațiile de ovine, în funcție de vârsta și sexul animalelor, de rasă, de condițiile concrete ale mediului de viață și de sistemele de selecție și de creștere folosite. Autorul acordă o atenție deosebită implicațiilor polimorfismului hemoglobinic pentru creșterea ovinelor, încercând să stabilească corelații interesante între genotipurile hemoglobinice și potențialul morfo-productiv la ovine, precum și între acestea și indicii de reproducție, statusul sanatorial, însușirile fiziologice etc.

Capitolul II al lucrării este consacrat **polimorfismului transferinei serice la ovine**. După ce detaliază structura și tipurile genetice ale transferinei la ovine, autorul precizează metodologia determinării tipurilor de transferină la ovine, structura genetică la locusul transferinei în populațiile de ovine, în funcție de vârsta și sexul animalelor, în funcție de rasă, în funcție de condițiile meteo-geo-climatice și sistemele de selecție și de creștere folosite. Aplicațiile polimorfismului transferinic în creșterea ovinelor se concretizează prin corelațiile dintre genotipurile transferinice și potențialul morfo-productiv, indicii de reproducție, însușirile de fiziologie normală și patologică la ovine.

În capitolul III se expun succint aspectele esențiale privind **polimorfismul albuminelor serice la ovine**. Mai întâi se fac referiri la sistemul prealbuminic, apoi la structura albuminei și rolul ei în organismul animal, metodologia determinării tipurilor de albumină la ovine, structura genetică la locus-ul albuminei în cadrul diferitelor populații de ovine în funcție de rasă, aplicații ale polimorfismului albuminic în creșterea ovinelor. Capitolul se încheie cu discutarea rezultatelor unor cercetări care acreditează ideea existenței polimorfismului postalbuminic și la specia ovină nu numai la bovine, porcine și cabaline cum se considera până în prezent.

În capitolul IV sunt inserate informații valoroase privind **polimorfismul potasiului sanguin la ovine**. Astfel, autorul tratează rolul potasiului în organismul animal, discontinuitatea potasemiei, metodologia determinării tipurilor de potasiu sanguin la ovine, tipurile de potasiu sanguin și determinismul lor genetic la ovine, structura genetică la locusul potasiului în funcție de vârsta și sexul animalelor, în funcție de rasă, în funcție de condițiile meteo-geo-climatice și sistemele de selecție și de creștere folosite. Ca și în cazul profilului genetico-biochimic al sistemului hemoglobinic, transferinic și albuminic, se redau unele aplicații ale polimorfismului kalemic în creșterea ovinelor, cum ar fi corelațiile dintre fenotipurile potasice și potențialul morfo-productiv (masa corporală, producția de lână, producția de lapte, producția de piele) la ovine, apoi corelațiile dintre fenotipurile potasice și indicii de reproducție, statutul sanatorial, însușirile fiziologice etc.

Autorul monografiei nu se limitează numai la expunerea datelor experimentale ci interpretează, într-o manieră proprie, diferitele rezultate și degajă direcții de cercetare a aspectelor încă neclarificate.

Cartea doctorului Gheorghe Hrinică, prin conținutul științific de marcă, prin claritatea expunerii problematicei și prin condițiile grafice în care a apărut, se impune lumii geneticienilor, biochimistilor, fiziologilor și, în egală măsură, specialiștilor din zootehnie, medicină veterinară și biotehnologii agricole.

Vlad G. ARTENIE și Ion I. BĂRA

MONSIEUR LE PROFESSEUR DIDIER GUILLOCHON
DOCTEUR HONORIS CAUSA
DE L'UNIVERSITÉ «ALEXANDRU IOAN CUZA» DE IASI

Le 17 février 2005, le Sénat de l'Université « Alexandru Ioan Cuza » de Iași a décerné le titre de Docteur Honoris Causa à Monsieur le Professeur dr. Didier Gérard Daniel GUILLOCHON, Directeur du Laboratoire de Technologie des Substances Naturelles de l'Université de Sciences et Technologies 1 de Lille, France.

*Monsieur le Recteur, Messieurs les Sénateurs, Distingués Invités,
Honorable Assistance,*

Il me revient l'honneur et le très grand plaisir de présenter aujourd'hui, devant vous, les arguments ayant formé le fondement de la décision du Sénat de l'Université « Alexandru Ioan Cuza » de Iași, celle de conférer le titre de Docteur Honoris Causa de l'Université « Alexandru Ioan Cuza » à Monsieur le Professeur dr. Didier Gérard Daniel GUILLOCHON, Directeur du Laboratoire de Technologie des Substances Naturelles de l'Université de Sciences et Technologies 1 de Lille, France.

Cette décision du Sénat de l'Université « Alexandru Ioan Cuza » a été prise sur la base du rapport élaboré par une commission de proposition, ayant comme président Monsieur le Professeur dr. Dumitru I. Oprea, Recteur de l'Université „ Alexandru Ioan Cuza ”, et dont les membres sont : Monsieur le Professeur dr. Ion I. Băra, Doyen de la Faculté de Biologie ; Monsieur le Professeur dr. Vlad G. Artenie, Chef du Département de Biochimie-Génétique-Microbiologie ; Monsieur le Professeur dr. Dumitru C. Cojocaru et Madame le Professeur dr. Erica Maria Nișitan, du Département de Biochimie-Génétique-Microbiologie de la Faculté de Biologie.

Avant de concrétiser les arguments respectifs, permettez-moi de résumer quelques données biographiques et scientifiques concernant Monsieur le Professeur Didier GUILLOCHON, personnalité marquante de la biochimie, de l'enzymologie et de la biotechnologie contemporaines, un des hommes de science qui ont contribué d'une manière substantielle au développement de ces importants domaines des sciences de la vie, aussi bien par des études théoriques fondamentales qu'appliquées, comme résultat d'une activité particulièrement riche dans la recherche scientifique et dans l'enseignement supérieur, activité attestée par une large reconnaissance internationale.

Né le 12 mars 1948 à Suresnes (France), Monsieur le Professeur Didier GUILLOCHON a fait ses études à l'Université « Pierre et Marie Curie – Paris VI », entre 1967 et 1975, ensuite à l'Université de Technologies de Compiègne, entre 1976 et 1986. Dans le cadre de l'Université « Pierre et Marie Curie – Paris VI », Monsieur le Professeur Didier GUILLOCHON a obtenu une Maîtrise de Biochimie, un Diplôme d'Études Approfondies (DEA) de Chimie Organique Structurale et le troisième cycle universitaire en Physique.

Monsieur le Professeur Didier GUILLOCHON obtient le titre de Docteur suite à l'élaboration durant la période 1976-1986 d'une thèse d'état en Sciences Physiques, portant le titre « Contribution à l'étude de la fixation et de l'activation de l'oxygène par l'hémoglobine en technologie enzymatique » et réalisée sous la direction scientifique du Professeur D. Thomas de l'Université de Technologies de Compiègne.

Durant la période 1974-1977, Monsieur le Professeur Didier GUILLOCHON occupe le poste d'attaché assistant en Chimie et Biochimie à la Faculté de Médecine « Bichat Beaujon – Paris VII ». En 1977 il devient assistant des Universités – assistant des hôpitaux de Paris à la Faculté de Médecine « Bichat Beaujon ». En cette qualité, il travaille au même temps au Laboratoire de Biochimie de l'Hôpital « Claude Bernard » jusqu'en 1981, année où il est nominalisé, d'abord sur le poste de Maître assistant, ensuite sur celui de Maître de Conférences en Biochimie à l'Université des Sciences et Technologies 1 de Lille, ville importante du Nord de la France. Ici, Monsieur Didier GUILLOCHON obtient en 1990 le titre de Professeur des Universités de deuxième classe en Biochimie, et à partir de 1995 il honore le titre de Professeur des Universités de première classe en Biochimie à l'Institut Universitaire Technique (IUT) « A » de Lille. À l'Université des Sciences et Technologies 1 de Lille, Monsieur le Professeur Didier GUILLOCHON débute son activité didactique et de recherche scientifique dans le cadre du Département de Biologie Appliquée de l'IUT « A » par l'organisation du Laboratoire de Technologie des Substances Naturelles, installé depuis 2001 au sein de la Polytechnique de Lille appartenant à la même Université. Aujourd'hui, ce laboratoire, dont Monsieur le Professeur Didier GUILLOCHON en est le directeur, dénombre 8 cadres didactiques (5 Maîtres de Conférences et 3 Professeurs), ainsi que de nombreux thésards.

Tout dernièrement, au cours de l'année 2004, Monsieur le Professeur Didier GUILLOCHON a déposé au Ministère Français de la Jeunesse, de l'Éducation et de la Recherche le dossier nécessaire à l'unification du Laboratoire de Technologie des Substances Naturelles avec le Laboratoire de Bioprocédés Microbiens, afin de les organiser en un seul Laboratoire, le Laboratoire de Procédés Biologiques, Génie Enzymatique et Microbien (ProBioGEM) qui permettra la structuration de la recherche scientifique dans le domaine des bioprocédés agro-alimentaires au sein de l'Université des Sciences et Technologies 1 de Lille. Une telle démarche originale, qui deviendra réalité à partir de 2006, possède les motivations suivantes :

- la nécessité de regrouper le potentiel scientifique à l'heure actuelle dispersé au sein de l'Université des Sciences et Technologies 1 de Lille, afin de mutualiser les moyens existants et d'acquérir une meilleure lisibilité universitaire et régionale dans ce domaine ;

- l'opportunité de focaliser les efforts de ce potentiel humain sur une thématique de recherche commune, intitulée « L'optimisation de la productivité et de la sélectivité des bioprocédés enzymatiques et microbiens, ayant comme but la préparation de peptides fonctionnels et à activités biologiques destinés aux industries alimentaire et biotechnologique ».

Dans la recherche fondamentale effectuée au sein du Laboratoire de Technologie des Substances Naturelles, Monsieur le Professeur Didier GUILLOCHON a développé différentes directions dans le domaine des biotechnologies agro-alimentaires et plus spécifiquement de l'application du Génie Enzymatique à la transformation de protéines issues de l'agriculture ou des industries alimentaires, protéines dont on peut mentionner : l'hémoglobine du sang des bovins sacrifiés dans les abattoirs, les immunoglobulines et l' α -lactalbumine du colostrum bovin, protéines foliaires (protéines des feuilles de luzerne) etc. Le thème principal des recherches scientifiques abordées dans le laboratoire sous la direction de Monsieur le Professeur Didier GUILLOCHON vise la protéolyse enzymatique dirigée pour l'obtention et la préparation de peptides possédant des propriétés fonctionnelles et des activités biologiques, par exemple les peptides à action opioïde (c'est-à-dire peptides capables de produire des effets semblables à ceux de la morphine), les peptides à action analgésique, les peptide antimicrobiens, substances pouvant être utilisées en tant que ingrédients alimentaires, aliments-santé où préparés pharmaceutiques. Les objectifs des recherches mentionnées supposent au moins deux approches :

- 1) la modification de la sélectivité de la protéolyse enzymatique par la mise en œuvre des protéases dans des environnements artificiels pour la catalyse hétérogène en systèmes solide/liquide ou systèmes de solvants organiques mono et biphasiques ;

- 2) le couplage de l'hydrolyse protéolytique avec des systèmes d'extraction sélective, ayant comme finalité la conception de réacteurs enzymatiques.

Les résultats de ces amples et diverses recherches ont été concrétisés dans plus de 40 conférences à des congrès internationaux, par 10 brevets d'invention et par 85 articles scientifiques publiés dans de prestigieuses revues internationales de biochimie, enzymologie ou biotechnologie. Fait à retenir, les données présentées dans les travaux de Monsieur le Professeur Didier GUILLOCHON sont citées dans un nombre appréciable de publications scientifiques dans les domaines mentionnés.

Monsieur le Professeur Didier GUILLOCHON accomplit actuellement de nombreuses responsabilités universitaires, dont on peut citer :

- directeur du Laboratoire de Technologie des Substances Naturelles ;

- responsable de la Formation Doctorale « Stratégies d'Exploitation des

Fonctions Biologiques », formation cohabilitée par les Universités de Compiègne, Amiens et Lille ;

membre élu du Conseil Scientifique de l'Université des Sciences et Technologies 1 de Lille ;

- membre élu de la Commission de Spécialistes de la même Université.

Non dernièrement, il est également expert pour le programme « Aliment-Qualité-Sécurité » du Ministère de la recherche, Direction de la Technologie.

Le Laboratoire de Technologie des Substances Naturelles, sous la direction de Monsieur le Professeur Didier GUILLOCHON, promeut de fructueuses collaborations avec d'autres laboratoires de l'Université des Sciences et Technologies 1 de Lille (Laboratoire de Chimie Biologique, Laboratoire de Chimie Organique Physique, Laboratoire de Catalyse, Laboratoire de Génie des Procédés et Technologies Alimentaires), avec de nombreux laboratoires des Universités du reste de la France (Laboratoire de Technologie Enzymatique de l'Université de Compiègne, Laboratoire de Physiologie et Pathologie des Cancers de l'Université de Nantes, Laboratoire de Physiologie du Centre Hospitalier Universitaire (CHU) de Caen, Laboratoire de Génie Protéique de l'Université de La Rochelle, Service de Pédiatrie du Centre Hospitalier Régional (CHR) d'Amiens, Laboratoire de Génie Enzymatique et Cellulaire de l'Université de Technologie de Compiègne), avec différents instituts de recherche (Institut Pasteur de Lille, Institut Pasteur de Paris, Institut Curie de Paris), ainsi qu'avec un grand nombre d'industries agro-alimentaires, sociétés et organismes privés de Normandie, Champagne-Ardenne ou du reste de la France, toutes ces collaborations étant axées sur l'analyse des hydrolysats peptidiques, sur la préparation de produits nouveaux par génie enzymatique et sur la mise au point de procédés enzymatiques.

Dans ce contexte, nous devons mentionner que Monsieur le Professeur Didier GUILLOCHON est responsable du projet régional intitulé «Génie des procédés enzymatiques et protéolyse », dans le cadre du Contrat de Plan Etat - Région Nord - Pas de Calais, 2001-2006, « Qualité des productions agricoles et des procédés de transformation, qualité des aliments et sécurité alimentaire ».

Au niveau national, Monsieur le Professeur Didier GUILLOCHON a été directeur de plus d'une vingtaine de thèses de doctorat et a participé comme membre dans de nombreuses commissions d'attribution du titre de docteur, dans les Universités de Compiègne, Nancy, La Rochelle, Rennes, Caen et Lille. Nous devons également souligner l'implication de Monsieur le Professeur Didier GUILLOCHON dans la préparation de deux Habilitations à Diriger des Recherches à l'Université des Sciences et Technologies 1 de Lille et l'autre à l'Université de N'Gaoundéré, au Cameroun.

Parallèlement aux activités menées en France, Monsieur le Professeur Didier GUILLOCHON a participé à des missions et activités dans d'autres pays, comme s'en suit :

- Dairy Research Center, Université de Laval au Québec, Canada ;
- Département de Biotechnologie Alimentaire de l'Université de Dongseo en Corée du Sud ;
- École Nationale Supérieure des Sciences Agro-Industrielles de l'Université de N'Gaoundéré au Cameroun ;
- Université „ Alexandru Ioan Cuza ” de Iași, et bien d'autres encore.

La première visite en Roumanie de Monsieur le Professeur Didier GUILLOCHON date de mai 1999, lorsqu'il est venu à Iași pour exposer son cours de Génie enzymatique aux étudiants du Master francophone « Enzymologie et Biotechnologie » à la Faculté de Chimie de l'Université « Alexandru Ioan Cuza ». En mai 2000, Monsieur le Professeur Didier GUILLOCHON revient dans l'Université « Alexandru Ioan Cuza » avec la même mission de présenter le cours susmentionné au Master francophone de la Faculté de Chimie. Aussi bien le cours que les travaux pratiques afférents soutenus par Monsieur le Professeur Didier GUILLOCHON se sont déroulés au Laboratoire de Biochimie, au sein de la Faculté de Biologie de notre Université. La visite à Iași de Monsieur le Professeur Didier GUILLOCHON en 1999 a ouvert la série des visites qui ont suivi ultérieurement. En 1999, 2000, 2001 et 2002, Monsieur le Professeur Didier GUILLOCHON a participé en qualité de conférencier invité aux V-ème, VI-ème VII-ème et VIII-ème éditions de l'École d'Été Franco-Roumaine, intitulée «Biologie et Pathologie Moléculaires. Biotechnologies» et organisée entre 1995 et 2004 par l'Université « Alexandru Ioan Cuza » de Iași (co-directeur Professeur dr. Vlad Arteni) en collaboration avec l'Université des Sciences et Technologies 1 de Lille, France (directeur Professeur Émérite Jean Montreuil).

Aux cours de l'École d'Été Franco-Roumaine de Biochimie, Monsieur le Professeur Didier GUILLOCHON a présenté de nombreuses conférences de spécialité devant un large auditoire : étudiants, chercheurs scientifiques et cadres didactiques universitaires, venus aussi bien de Iași que d'autres centres universitaires, tous profondément impressionnés par le contenu scientifique et par la clarté des problèmes exposés. Parmi les 18 conférenciers qui ont honoré le cours des 10 éditions de l'École d'Été Franco-Roumaine de Biochimie, Monsieur le Professeur Didier GUILLOCHON est la seule personne qui a proposé, accepté et développé la collaboration scientifique et didactique du Laboratoire qu'il dirige avec le Laboratoire de Biochimie de la Faculté de Biologie de Iași. Dans ce sens, entre les deux laboratoires a été signé un «Accord de Coopération Scientifique et Technique » sur une période de 5 ans à partir de 1999. Sur la base des discussions portées entre le Bureau de direction du Département de Biochimie-Génétique-Microbiologie, ainsi que la direction de la Faculté de Chimie, avec Monsieur le Professeur Didier GUILLOCHON, il a été convenu premièrement que pour les années universitaires 2000-2001 et 2001-2002 une bourse Socrates soit attribuée à un étudiant biochimiste au Master « Enzymologie et Biotechnologie », afin que ces étudiants puissent suivre le Diplôme d'Études Approfondies (DEA) au Laboratoire de Technologie des Substances Naturelles. Au présent, le premier des deux étudiants est en cours de finalisation de sa thèse de doctorat en co-tutelle, ayant comme directeurs de thèse Monsieur le Professeur Didier GUILLOCHON de la part de l'Université des Sciences et Technologies 1 de Lille, et moi-même de la part de Université „ Alexandru Ioan Cuza ” de Iași.

Entre le 25 et le 29 mars 2003, Monsieur le Professeur Didier GUILLOCHON a participé en tant que membre de la Délégation des Universités de la Région Nord – Pas de Calais au Forum International sur la Coopération Universitaire Francophone, qui a eu lieu d'abord à Bucarest, ensuite dans le Centre Universitaire Iași.

Sur la base du contrat de collaboration entre le Laboratoire de Technologie des Substances Naturelles et le Collectif de Biochimie de l'Université „ Alexandru Ioan Cuza ” de Iași, nous, le Département de Biochimie-Génétique-Microbiologie, nous avons proposé et organisé en 2002, en accord avec Monsieur le Professeur Didier GUILLOCHON, un module Franco-Roumain de Master, intitulé « Bioprocédés dans le domaine agro-alimentaire », module qui a reçu de la part du Ministère de l'Éducation et de la Recherche de Bucarest l'autorisation de fonctionner à la Faculté de Biologie à partir de l'année universitaire 2003-2004. Le 19 février 2005, Monsieur le Professeur Didier GUILLOCHON a présenté une conférence de spécialité devant les étudiants en deuxième année de ce Master.

Dans le cadre du contrat existant entre les deux laboratoires, plusieurs membres du Collectif de Biochimie de la Faculté de Biologie ont bénéficié de la qualité de cadre didactique invité au Laboratoire de Technologie des Substances Naturelles de l'Université des Sciences et Technologies 1 de Lille, Laboratoire sous la direction de Monsieur le Professeur Didier GUILLOCHON. Parmi ces cadres didactiques on peut citer :

- Professeur Ovidiu Toma en 2001 – 2 mois ;

- Professeur Vlad Artenie en 2002 – 1 mois ;
- Chargé de Cours Anca Mihaela Humă – en 2002 – 3 mois, en 2003 – 2 mois, en 2004 – 3 mois ;
- Assistant universitaire Eugen Ungureanu en 2005 – 1 mois.

Monsieur le Professeur Didier GUILLOCHON a également fait donation au Laboratoire de Biochimie du Département de Biochimie-Génétique-Microbiologie d'appareillage, matériels de laboratoire et réactifs chimiques.

À l'occasion de sa visite en 2005 à l'Université „ Alexandru Ioan Cuza ”, Monsieur le Professeur Didier GUILLOCHON nous a honoré par sa participation en tant que référent, le 18 février, à la soutenance publique de la thèse de doctorat en Biochimie intitulée « Immobilisation de certaines protéases sur différents supports avec d'éventuelles applications pratiques », élaborée par Madame Chargé de Cours Anca Mihaela Humă.

À partir de 1999, Monsieur le Professeur Didier GUILLOCHON est référencé aux 5 périodiques internationaux de spécialité : Journal of Membrane Sciences, International Journal of Food Science and Technology, International Journal of Biomolecules, Enzyme and Microbial Technology, Separation Sciences and Technology.

L'activité didactique de Monsieur le Professeur Didier GUILLOCHON est axée sur l'organisation des travaux pratiques et sur la présentation des cours dans des matières comme Chimie Générale, Chimie Organique, Enzymologie, Génie Enzymatique, Chimie et Biochimie, Enzymologie en milieux non conventionnels, et bien d'autres encore. À côté des supports de cours publiés dans ces disciplines, Monsieur le Professeur Didier GUILLOCHON est coauteur du livre intitulé « Biotechnologie, La Science et les Hommes – La Vie », aux Éditions Messidor La Farandole, ainsi que de la monographie « Additifs, réglementation, mode d'utilisation, structures chimiques et fonctionnelles ».

Les mérites scientifiques et didactiques de Monsieur le Professeur Didier GUILLOCHON sont également illustrés par le fait que le Laboratoire qu'il dirige est affilié à la Société Française de Génie des Procédés et le Club Français des Membranes.

Être extrêmement généreux, prêt à n'importe quel moment d'aider, d'accorder son soutien moral ou matériel, homme d'une grande culture, attaché dès le premier instant à l'espace ethnoculturel roumain, qu'il connaît bien et qu'il apprécie profondément, Monsieur le Professeur Didier GUILLOCHON produit une forte impression à tous ceux qu'il rencontre. Le dynamisme et la fermeté qui le caractérisent, accompagnés par l'ouverture et l'approche vis-à-vis les étudiants roumains avec lesquels il travaille, vis-à-vis des jeunes cadres didactiques et des professeurs du Collectif de Biochimie de l'Université „ Alexandru Ioan Cuza ” qui ont été les hôtes du laboratoire dirigé par Monsieur le Professeur Didier GUILLOCHON, toutes ces éléments constituent l'exemple éloquent pour ce que devrait être le véritable magister.

De tous les faits susmentionnés se concrétise l'image d'un grand Professeur – créateur d'école, homme de science complet, avec de grandes qualités morales, de dynamisme social, au même temps admirateur et ami de la ville de Iași, de la Moldavie et de la Roumanie, par l'entière activité que nous avons mentionnée. Pour toutes ces raisons, nous soutenons chaleureusement la proposition de conférer à Monsieur le Professeur Didier GUILLOCHON, par le Sénat de l'Université „ Alexandru Ioan Cuza ” de Iași, le titre de « DOCTEUR HONORIS CAUSA » de la plus ancienne Université de Roumanie.

En fin de ce discours, Monsieur le Professeur Didier Gérard Daniel GUILLOCHON, permettez-moi de vous souhaiter de tout cœur bienvenue parmi ceux qui forment l'Université „ Alexandru Ioan Cuza ” de Iași, de vous souhaiter également de nombreux années en bonne santé et avec la même capacité de travail, dans le but de continuer à militer pour approfondir la collaboration entre le Laboratoire de Technologie des Substances Naturelles et la Laboratoire de Biochimie de la Faculté de Biologie de l'Université „ Alexandru Ioan Cuza”, généralement pour le progrès de l'enseignement supérieur de Biologie et de Biochimie, à partir de cet ancien, riche en traditions et en histoire, Centre Universitaire de Roumanie.

Vlad Artenie