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HEMP - BIOCHEMICAL DIVERSITY AND MULTIPLE USES

ELENA TRUȚĂ^{1*}, ȘTEFANIA SURDU¹, CRĂIȚA MARIA ROȘU¹, MARIA ASAFTEI²

Biochemical complexity. *Cannabis sativa* L. is one of the species with the most numerous and diverse uses in economy. It can be grown in most climates, is drought resistant, and requires little fertilizers, minimal pesticides or herbicides. Hemp is a very important plant by economical point of view for fibres, seeds, oils, as well as for the chemical constituents with large medical valences and uses in some diseases therapy or in amelioration of certain health troubles. This plant has an extremely complex and diverse chemical constitution. The hemp plants and the crude drug products thereof (marijuana, hashish, and hash oil) are characterized by a wide variety of chemical compounds. There are almost 500 (483 compounds, according to Grotenhermen and Russo, 2002; 525 compounds, according to Radwan et al., 2009) different identifiable chemical substances. The best-known and most specific class of hemp constituents is represented by the C₂₁ terpenophenolic cannabinoids. Other phenol compounds include flavonoids, spiroindans, dihydrostilbenes, phenanthrenes, and dihydrophenanthrenes. The hemp specific flavour is due to volatile terpenes of essential oils, monoterpenes representing 47.9 to 92.1%, and sesquiterpenes 5.2-48.6% of total terpenes (Mediavilla and Steinemann, 1977). This specific flavour is used in training up of the dogs in view to track down the drug storehouses. The physiological role of volatile oils and resin is less known, probably they contribute to insect attraction or they act as protective mechanisms against animals. Also, the ether oils diminish the plant transpiration at high temperatures.

Hemp seeds contain 25-35% oils, 20-25% protein, 2—30% carbohydrates, and 10-15% insoluble fibres and minerals (Pate, 1999 – cf. Peiretti, 2009).

Compounds like friedelin, epifriedelinol, β -sitosterol, carvone and dihydrocarvone were isolated from roots (Sethi et al., 1978). Seeds contain oils (Petri, 1988), while among plant organs flowers are richer in oils than leaves (Lemberkovics et al., 1979). *The fatty acid composition* of fruits is of great interest, because of their use for nutritive and pharmaceutical purposes. If the complete fruit and seed are similar in this aspect, some differences are in the outer layer (Mölleken and Theimer, 1997) (Table 1).

Table 1. Variation of fatty acids in fruit compartments of Chinese hemp varieties

Fatty acid	Fruit (%)	Seed (%)	Shell (%)
Palmitic acid	6.23	7.60	6.83
Stearic acid	2.65	2.48	2.34
Oleic acid	10.22	10.38	37.74
Vaccenic acid	1.27	1.69	4.85
Linoleic acid	56.42	54.92	34.42
γ -Linolenic acid	2.45	2.72	0.97
α -Linolenic acid	18.60	17.45	11.30
Arachidic acid	0.60	1.07	0.78
Octadecatetraenoic acid	0.54	0.50	Not detected (<0.3%)
Eicosenoic acid	1.02	1.19	0.77

The seeds of *Cannabis sativa* L. represented an important source of nutrients in ancient cultures. Seed extracted oils contain more than 80% unsaturated fatty acids: linoleic acid (18:2 *omega*-6), α -linolenic acid (18:3 *omega*-3), γ -linolenic acid (18:3 *omega*-6), stearidonic acid (18:4 *omega*-3). In oil hemp, the *omega*-6:*omega*-3 ratio is 2:1 and 3:1, these values being

considered optimal for human health (Callaway, 2004). The presence of unsaturated fatty acid and of tocopherols recommends the use of oil hemp in nutritional and pharmaceutical and medical purposes, because it decreases the risk of cardiovascular diseases, of some cancers and of senile macular degeneration. Tocopherols act as antioxidants and prevent against oxidation of unsaturated fatty acids. Fatty acid profile and amount in seed oils vary depending on developmental stage of hemp plants, genotype, age, harvest year, environmental conditions (Peiretti, 2009). Approximately 50% from unsaturated fatty acids is represented by α -linolenic acid. In studied provenances, the α -linolenic acid was detected only in seeds, this compound lacking in the other phenophases. Thus, the seeds are the unique source for γ -linolenic acid in hemp. In the 51 genotypes studied in 2000-2001 period, Kriese et al. (2004) evidenced a variation of oil content ranging from 26.25% to 37.50%. In the flower and the leaf of the same plant, the total amount of essential oil is 0.08-0.15%, the flower being richer than leaf with 0.01% (Lemberkovics et al., 1979).

The most important *proteins* of hemp seeds are edestin, zeatin, and zeatin nucleoside, very digestible and with a significant content in all essential amino acids. Arginine, a semi-essential (conditional) amino acid is found in very high levels in hemp seeds. The only amino acids not reported in hemp are cystine, asparagine, glutamine, hydroxyproline, and hydroxylysine.

Regarding the vitamins and pigments, in hemp have been reported vitamin K and two pigments (carotene and xanthophylls).

The data regarding the synthesis of *flavonoids* in *Cannabis* genus are sometimes contradictory, having a limited systematic value, because of the use of different analytic methods or of different plant organs, plant age, use of various provenances. Flavones Quantitative analysis of flavones, polyphenols and polyphenols in different sexual phenotypes evidenced different levels depending on plant sex and on analyzed organ (Truță et al., 2002).

Now, hemp is cultivated on large surfaces on globe, in agricultural and industrial purposes, for seeds, fibres, oils, but it is also cultivated as medicinal plant and in pharmacological purposes. These last utilizations are the consequence of the presence of psychoactive *terpenophenols*, generically named *cannabinoids*. The most important cannabinoids are Δ^9 -tetrahydrocannabinol (Δ^9 -THC), CBN (cannabinol), and CBD (cannabidiol), which chemically are a benzotetrahydropyran, a dibenzopyran, respectively a diphenol, followed by cannabichromene (CBC) and cannabigerol (CBG), as well as some derivatives (Page and Nagel, 2006) (Fig. 1). These compounds are criteria distinguishing between the hemp chemotypes (especially Δ^9 -THC and CBD, and THC/CBD ratio). Genotype, climate conditions (temperature, light, humidity), developmental stage, plant age, drying modality of biological material are factors controlling and determining the cannabinoid phenotype of hemp plants.

Geographic origin is also an essential factor – the most varieties synthesizing high THC amounts originated from latitudes situated at south of 30°N (Hillig and Mahlberg, 2004). Although in Europe only Indian hemp varieties were considered as producers of hallucinogens, in last years it was evidenced that psychoactive principles are synthesized also in European varieties, especially in drought conditions and at high temperature (Ciulei et al., 1993). To form THC, the hemp plants need daily average temperatures over 32°C, on long periods, conditions rarely encountered in Romania.

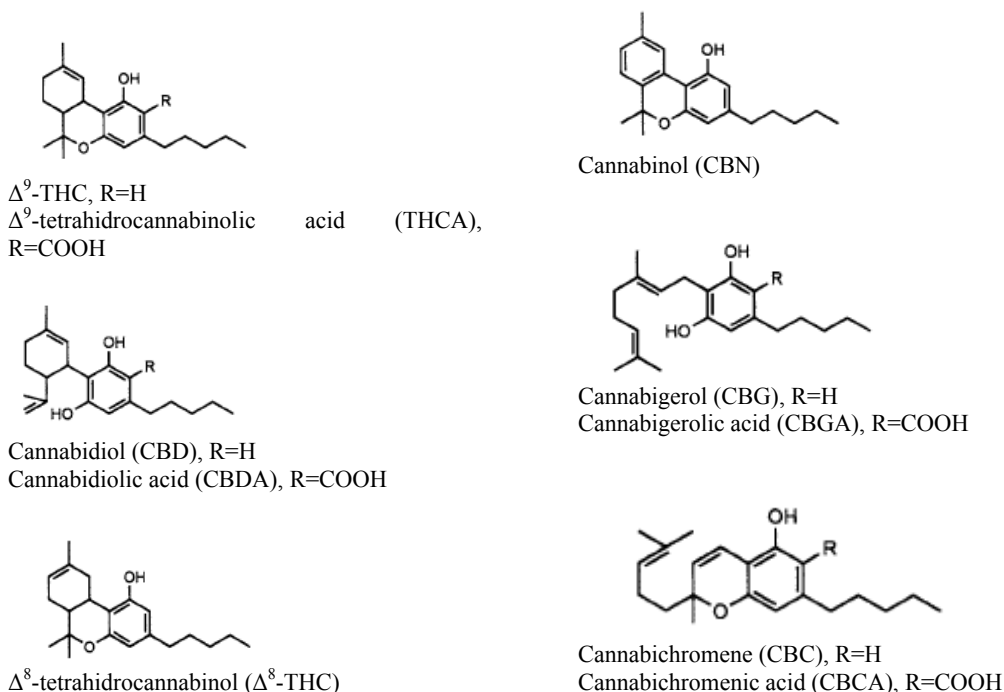


Figure 1. Structure of main cannabinoids (cf. PAGE and NAGEL, 2006)

The highest levels of THC are produced by Indian hemp (*C. indica*) and Chinese hemp (*C. sinensis*). The hemp varieties have similar cannabinoid patterns, but the percentage of each compound varies very much, because of ecological and ontogenetic factors (Turner et al., 1979). The most psychoactively cannabinoid is Δ^9 -THC. CBD is inactive from this point of view, but it is used as marker in identification of hemp chemotypes. In stalks, the cannabinoids are present in smaller quantities than in leaves and inflorescences, and the inflorescences generally contain higher levels than leaves, although the cannabinoid spectrum is the same. Regarding the cannabinoid presence in hemp seeds, the opinions are different. Doorenbos et al. (1971) presented a classification of hemp parts, including the seeds, in the decreasing order of cannabinoid levels: *bract*, *flower*, *leaf*, *small stalk*, *big stem*, *root*, *seeds*, but Mölleken and Husmann (1997) not evidenced the presence of cannabinoids in hemp fruits.

The male phenotypes were considered less active from pharmacological point of view, but at present it is known that the general tendency – the female plant produces a higher quantity of cannabinoids – refers only to the total cannabinoid level (THC + CBN), because in male plant the cannabichromene content is higher (Krejci, 1975; Taylor et al., 1985). The comparative analysis, performed by Ohlsson (1971) on THC levels in male and female plants grown in similar environmental conditions, not evidenced significant differences. More evident was the variation of cannabinoid ratio in relation with ontogenetic stage (Marshman et al., 1976). Numerous data plead for preponderance of genetic factor in determination of fibre and cannabinoid content, but without to cancel some environmental influences. It seems, indeed, that a higher stability of cannabinoid pattern characterizes the descendance of the same variety, even if the environmental

conditions are different. It is, also, possible that the real THC content to be the resultant of action of environmental conditions, but the ratio of main cannabinoids to be under genetic control. However, the role of environment in the phenotypisation of this character must not be neglected.

Depending on THC and CBD content, some authors distinguished three cannabinoid phenotypes, the plants with <0.3% THC being considered as not having psychoactive potencies (Small and Beckstead, 1973) (Table 2).

Table 2. Cannabinoid phenotypes in hemp

Phenotype	THC%	CBD%
I (drug type)	>0.3 (both sexes)	<0.5 (both sexes)
II (intermediary type)	>0.3 (female)	>0.5 (both sexes)
III (fiber type)	<0.3 (female)	>0.5 (both sexes)

Fournier and Paris (1979) accepted 0.5% THC as maximum content tolerable for fiber hemp (Table 3).

Table3. Chemotypes in hemp

Phenotype	THC%	CBD%	THC/CBD
Fiber hemp	<0.5	>0.5	<1.0
Resin hemp	>0.5	<0.5	>1.0

Fournier et al. presented in 1987 another classification of hemp chemotypes (Table 4), in which the drug chemotype has THC >2%.

Table 4. Classification of cannabinoid phenotypes in hemp

Chemotype	Drug type	Intermediary type	Fiber type
THC (% d.w.)	>2	>0.5	<0.3
CBD (% d.w.)	-	>0.5	>0.5
THC/CBD	-	>0.5	<0.1

Bruneton (1995) considers the drug (narcotic or resin) type that CBD non-producing and having THC >1%, while the most variants with THC <0.3%, cultivated in temperate zones, constitute the fiber type. Grotenhermen and Russo (2002) accept the existence of three hemp types and take into consideration the psychoactivity of main chemical compounds of respective chemotypes (Table 5).

Table 5. Chemotypes of *Cannabis sativa* L.

Chemotype	Products	Main cannabinoids	THC content	Psychoactivity
Drug type	marijuana, hashish	Δ^9 -THC	1-20%	Yes
Intermediate type		Δ^9 -THC, CBD	0.3-1.0%	Possible
Fiber type	fiber, edible oil	CBD	<0.3%	no

It was tried to establish some morphological criteria serving to find the psychoactive forms. Small et al. (1976), Petri et al. (1988) consider the density of resin glands to be a morphological criterion to differentiate and select the drug producing hemp, although Turner et al. (1978) results infirmed this fact. Nor other morphological characters (fruit traits, internode length, stem diameter etc.) have not discriminatory value in relation with cannabinoid synthesis.

Pharmacological valences. Therapeutic potential. Risks of some cannabinoid use.

Resin, secreted by hemp specific hairs is differently named, depending on its origin (*charas* - in Asia; *hashish* – in Mediterranean East; *chira* - in North Africa). Resin is harvested by special procedures and then is processed in view of consumption. Marijuana (marihuana) is a colloquial name for dried leaves and flowers of cannabis varieties rich in Δ^9 -THC, while hashish is an Arabic name for cannabis resin or compressed resin glands, containing 5-20% Δ^9 -THC. But there are several regional differences regarding the employment of the terms cannabis, marijuana, hemp, hashish. Leaves and flowered tips of female plants are collected, dried and cut in small

pieces, this mixture being known as *bhāṅg* and *ganja* – in India; *kif* – in Algeria and Morocco; *takroui* – in Tunisia; *habak* – in Turkey; *haschich el keif* – in Syria and Lebanon; *djomba*, *liamba*, *riamba* – in Central Africa and Brasil; *dagga* – in Austral Africa; *marijuana* – in America; *grifa* – in Mexico. Often, the hemp material is mixed with various tobacco sorts.

The first formal report on use as drugs of some hemp constituents dates by 5000 years, these compounds being administrated against malaria, constipation, muscle pain, birth pain; in mixture with wine, they were used as surgical anaesthetic (Robson, 2001). Hemp plants have been utilized to prepare remedy cures in Ayurveda and Chinese medical systems (as analgesic and anaesthetic) (Bruneton, 1995). Assyrian people used hemp products as „incense”, and Scythians „made dizzy” with vapours freed by the passage of hemp plant over hot stones. The doctors of British Army from India or Napoleon expedition in Egypt had the main responsibility in hemp introduction in Europe, in 19th century. Then, due to narcotic properties, hemp preparations became to be ingested in intellectual societies (as a consistent paste – „dawamesk”).

By time, the pharmacological valences of hemp were took into consideration and exploited in medical field, but the inconsistency of therapeutic activity, the bad storage of preparations, the difficulty to establish the optimal doses, as well as the risk showed by synthetic hypnotics and analgesics determined the gradual abandonment of hemp and its disappearance from the most occidental *pharmacopoeias* in the first moiety of 20th century. The hemp remained in British Pharmacopoeia until 1932, and in British Pharmaceutical Codex until 1949. THC was firstly isolated in 1964, and the first synthetic pharmaceutical cannabinoid product (*Marinol®*) was approved in USA in 1985 (Stott and Guy, 2004). Now, the clinical utilization of cannabinoids is restricted to oral administration of Dronabinol and Nabilone. Dronabinol is a synthetically manufactured (-)-trans-isomer of Δ^9 -THC, and *Marinol* is a Dronabinol preparation in sesame oil, available in USA, Canada and some European countries. Nabilone, marketed in UK, Canada, and in some UE countries, is a synthetic derivative of Δ^9 -THC with a slightly modified structure and was marketed starting from 1983. With regard to pharmacological activity, 1 mg nabilone corresponds to 10 mg Dronabinol.

The interest for cannabinoid utilization in medical purposes was relatively recent reactivated and is based on positive effects induced by synthetic cannabinoid administration subsequently to chemotherapy in different cancer forms, in order to diminish the secondary effects (nausea, vomiting), as well as on their utilization in attenuation of anorexia associated weight loss in AIDS. Also, the cannabinoids inhibited the tumour cell proliferation in animal cell cultures and in laboratory animals, accelerating cancer cell apoptosis in some forms of astrocytoma, glioma, neuroblastoma, pheocytochroma etc. (Guzmán, 2003). They are usually well tolerated, and do not produce the generalized toxic effects of conventional chemotherapies. Other beneficial effects on some patients with cancers are pain diminution, muscle relaxation, and sleep amelioration (Walsh et al., 2003).

Δ^9 -THC (Δ^9 -tetrahydrocannabinol) is the most active *Cannabis sativa* compound; cannabinol, which is produced in large amounts is a weak cannabimimetic agent; and cannabidiol, which is abundant but has no cannabimimetic activity. Cannabidiol (CBD) is the nonpsychotropic hemp constituent, but it is important due to its numerous pharmacological activities. Ligresti et al. (2006), studying the anti tumour activities of five natural cannabinoids (CBD, CBG, CBC, CBDA, and THCA), confirmed that CBD is the strongest inhibitor of cancer cell growth and shows the lower cytotoxicity on non-cancer cells. The effect depends on CBD structure, because the addition of COOH group, like in CBDA, dramatically reduced the anti tumour effect. These characteristics plead for CBD utilization in cancer therapy.

The hemp cannabinoids are antiemetic, analgesic (the analgesic effect is comparable to aspirin effect); they reduce the intraocular pressure, diminish the anxiety, induce welfare in some neurological troubles, AIDS, and cancers. The drug therapy of muscle spasticity in multiple sclerosis generally has a low efficiency, but the patients recognized a cramp amelioration and pain relief after administration of some hemp preparations. Dronabinol reduced weight loss and stimulated appetite and weight gain in AIDS and several forms of cancers (Robson, 2001).

Cannabinoids exert protective effects on cardiovascular system, in myocardial ischemia (Grant and Cahn, 2005); also, they determine the improvement of some aspects of cognitive function (Ranganathan and D'Souza, 2006). Canniprene, another compound isolated from hemp, is a dihydrostilbene with inhibitive effect on lipoxygenase and cyclooxygenase, but also showing anti-inflammatory properties (Elsohly et al., 1990).

Other metabolites with significant biological activity have been isolated especially from high-potency hemp (Δ^9 -THC > 10%, w/w). For example, 4-acetoxy-2-geranyl-5-hydroxy-3-*n*-pentylphenol and 8-hydroxycannabinol displayed significant antibacterial and antifungal activities, respectively, while 5-acetyl-4-hydroxycannabigerol displayed strong anti leishmanial activity (Radwan et al., 2009). Other compounds manifested anti malarial or cytotoxic activities.

In time, numerous excesses have been produced in utilization of hemp chemical constituents, as result of lack of standardization procedures and of deficient dosage. Therefore, the treatments can induce grave effects – intoxication, sedation, dizziness, mouth dryness, decrease of blood pressure etc. If the small doses of THC firstly induce euphoria and relaxation, the increase of doses and extension of ingestion or inhalation time will determine grave manifestations: decrease of motor coordination, disturbance of long time memory and of verbal communication, anxiety, tinnitus, hallucinations, and in extreme situation the paranoid psychosis can install.

Verification of teratogenic potential of hemp etheric extracts on rat pregnant females determined the decrease of pregnancies as result of abortion increase, weight diminution of new born rats, decrease of descendance survival rate, fetal malformations (microcephaly, phocomelia). Other authors also reported the presence of fetal malformations in mice, rats, hamsters, and rabbits (Persaud and Ellington, 1968), but in other experiments their presence was not confirmed (Miras, 1965). It seems, however, that the pure THC (responsible for psychotropic effects) not induces teratogenesis (Singh et al., 1981).

The long time administration of hemp extracts determined gonadal lesions at the *Presbytis entellus entellus* males, but also induced liver disorders, with significant glycogen decrease and transaminase increase (Dixit, 1981). In rats was evidenced the blocking of luteinizing hormone and of ovulation (Nir et al., 1973).

In humans, Harmon and Aliapoulis (1972) described three males with gynecomastia associated to the excessive marijuana consumption. Marijuana can too determine the decrease of plasma testosterone level (Kolodny et al., 1974).

Even non-psychoactively compounds like CBD can have some cytotoxicity, dependent on dose and administration duration, reflected in reduction of rat hepatocytes viability in primary cultures, cytoplasmic changes, inhibition of DNA and protein synthesis, modifications of enzyme activities (Cohen and Stillman, 1976; Braut-Boucher et al., 1981).

Other hemp uses. Out of utilization of hemp extracts in pharmaceutical and medical domain, a number of varieties are producers of high quality stem fibres – basic material for textile industry, as well as of vegetal oils (20-40% in seeds). China is currently the prime producer of hemp textile. China had an uninterrupted hemp trade for approximately 6000 years.

Because of the terpene presence, the hemp essential oils are utilized to make soaps, creams, perfumes, or in aromatherapy. The hemp seeds extracted siccative oils serve to the preparation of dyes and soaps.

The seeds constitute food for exotic, domestic, and cage birds (parrots, canaries), while the residues remaining after oil extraction from seeds are administrated as „flat cakes” (30% proteins, 10% fats). These are used either in this form, singles, either they are added in concentrated fodders to feed those animals subjected to fattening (600 g „flat cakes” are equivalent, as nutritional value, to 1000 g cereals grains). In the case of pregnant cows, these cakes must be restrainedly utilized because they can produce abortions. After refination, the oils can be used in preserves industry.

The tow, which represents 40-50% from fibre production, is utilized in tapestry domain and as insulating material. Bags, cords, cables, water hoses, mattress cloth, tent canvas, and knapsacks are made by hemp fibres.

Hemp is a valuable, viable source of woody biomass. One acre (4074 m²) of hemp is approximately 75% cellulose, whereas one acre of trees is only 60%; hemp can give two crops per year, whereas trees give one crop every 20-30 years. The wooden residues („puzderia”) resulting after primary processing of hemp stalks and representing 45-50% by stalk weight constitute an important foundry fuel and is basic material for coal briquettes. Hemp stalk can be converted into 500 gallons (1 UK gallon=4.54 liters) of methanol/acre (<http://www.harbay.net/>).

The wooden residues or whole plant are utilized to obtain paper or special plates used to protect against noises in furniture industry and in constructions, while the P, K, Ca - rich ash remained after wooden residue combustion is applied as fertilizer. Also, husk resulted after seed harvesting represents a valuable fertilizer (10 t hemp husk is equivalent to 40 t stable refuse).

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PURIFICATION OF A NOVEL ALDEHYDE-DEHYDROGENASE WITH WIDE SUBSTRATE SPECIFICITY

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Keywords: computer-modeling, oxidoreductase, binding site, sugars

Abstract: The pAO1 megaplasmid of *Arthrobacter nicotinovorans* encodes two different pathways: one for nicotine metabolism and a putative sugar catabolic pathway. An open reading frame, orf39, from the latter pathway was cloned, purified to homogeneity and partially characterized. It consists of a monomeric NAD/NADP-dehydrogenase acting on various aldehyde as glutaraldehyde or butyraldehyde with an Cys residue in the active site. A possible catalytic mechanism is postulated.

INTRODUCTION

Plasmids are simple genetic elements, independent from the bacterial chromosome, involved both in vertical and horizontal-gene transfer. Most of the time, the plasmids encode different properties (resistance to antibiotics, to highly toxic compounds) which give the host cell an evolutionary advantage. The ability to un-common compounds as carbon and nitrogen sources is such an advantage, allowing the bacteria to be present in many environments as natural autochthonous microflora with a high potential for bioremediation of pollutants. Several plasmid-encoded pathways were described (ex: for metabolism of phthalate (10) or naphthalene (23)) but only few are completely elucidated.

The presence of the 165- kb pAO1 megaplasmid inside the cells of the gram positive soil bacteria *Arthrobacter nicotinovorans* allows this microorganism to use nicotine as sole carbon and nitrogen sources. The complete sequence of this plasmid was determined and two putative pathways could be described (15): on one hand the nicotine-degrading pathway, fully characterized by Brandsch (6) and on the other hand an yet unknown putative sugar-catabolic pathway. The overall GC content of the pAO1 plasmid indicates that nicotine-catabolism gene clusters are a new acquisition, being attached during the evolution to an older plasmid, containing the sugar-catabolic pathway. Recently shown analogies of the pAO1 encoded pathway for nicotine metabolism and the chromosome encoded one from *Nocardioides* sp. strain js614 (12) would suggest an horizontal gene transfer.

The sugar-catabolic pathway is comprised of several genes, among which a putative cellulase, an ABC-transporter system gene cluster and a cluster of several dehydrogenases and oxidoreductases. This last cluster probably encodes the last steps of the pathway, connecting it to the general metabolism of the cell. A part of this cluster is ORF39, a putative succinate-semialdehyde dehydrogenase and ORF40, a putative oxidoreductase. The ORF40 was found to encode a tetrameric sugar-dehydrogenase (20) containing Zn.

Our current study is focused on the ORF39 protein and its possible role in the cell. By cloning the gene in the expression vector pH₆EX₃ (4), we were able to express it as a recombinant His-tagged protein and to easily purify it to homogeneity.

MATERIAL AND METHODS

Isolation and cloning of orf39. The orf39 was isolated by PCR using the primers in table 1 and a suspension of *Arthrobacter nicotinovorans* cells as template. Directional cloning (24) of the fragment containing the *orf39* in the pH₆EX₃ vector was achieved by using *Bam*HI și *Sal*I (NEB, U.K) enzymes and Rapid DNA ligation Kit, Roche). Transformed *E. coli* XL1 Blue competent cells were selected on plates containing ampiciline (50 µg/ml) and the recombinant plasmid was checked for the presence of insert by restriction enzyme digestion.

Table 1. Oligo-nucleotides used for isolation of *orf39*

Primer	Sequence
Orf39forw	5'-CAG CCA TCG TGA TCA GCA ACA AGG-3'
Orf39rev	5'-GTT GAA GGG TCG ACG CTG AGG GTT AG-3'

Protein expression was done using auto-inducible medium as described elsewhere (19). **Protein purification** was achieved using standard IMAC techniques (3) on Fast-Flow Ni-chelating Sepharose (Amersham Biosciences, Sweden). All buffers used in the purification process had 10 mM β-mercaptoethanol final concentration. **Native molecular weight determination** was done using gel permeation chromatography on an HiLoad 16/60 Superdex 200 column connected to an AKTA Basic FPLC system. **Protein concentration** was assayed using the dye-binding method of Bradford (5). **SDS-**

PAGE was performed using the discontinuous system of Laemlli following the procedure described by Sambrook, 1989(24).

Enzyme assay was developed by following the guidelines described by de Caballero et. al. (8), Marchal and Branlant(17), Farrbs et al. (11). The assay mixture was phosphate buffer 100 mM pH 8,4, 10 mM β -mercaptoethanol, 1 mM NAD(P)⁺ and 20 μ g purified enzyme. The reaction was started by adding the substrate at 33 mM final concentration. The formation of NAD(P)H was monitored at 340 nm for 2 min. Enzyme activity was expressed as nanomoles NADH formed per minute per microgram enzyme (molar extinction coefficient for NAD⁺ 6220 M⁻¹*cm⁻¹). Tertiary structure predictions were performed with Jpred (9). For structure based sequence alignment the Seqioa program (7) was used. The pictures were generated from the alignments files using ESPrnt (13).

RESULTS AND DISCUSSIONS:

ORF39 encodes a monomeric protein. The recombinant protein obtained by cloning *orf39* in pH6EX3 has the N-terminal sequence as follows: HHHHHLVPRGSATRSIM, where the methionine in bold is the native START codon. This allowed for an one step purification process of the protein from the *E.coli* cell lysate using mobilized metal affinity chromatography. The purified enzyme had a relative molecular weight of 51, 44 kDa, in good accordance with the theoretical mass. The purity of our preparations was very high (over 95% on SDS-PAGE, fig. 1).

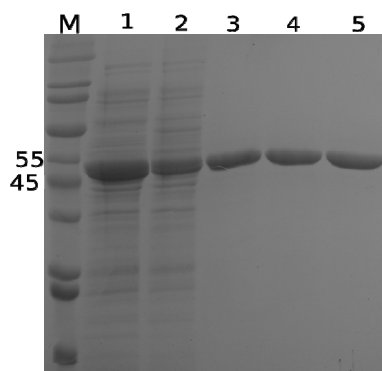


Figure 1. *Orf39* encoded protein was purified to homogeneity.

M –Molecular Weight Marker Sigma Wide Range

1 – Crude extracts (25 μ g)

2 – Cell free extract (25 μ g)

3, 4, 5– Purified protein (10 μ g)

A BLAST search performed at the NCBI servers has shown that the ORF39 is similar at the sequence level with several enzymes belonging to the aldehyd-dehydrogenases family. Class I and II of this family is comprised of dimer and tetramer proteins, while class III enzymes are trimers (2). In order to establish native state of this enzyme in solution, a gel permeation chromatography was performed. Approximately 1.6 mg purified ORF39 were injected on a HiLoad 16/60 Superdex 200 column. The protein eluted as a single peak at with a molecular weight of 64.8 kDa. This would indicate that the enzyme is a monomer. This is the first report of a monomeric aldehyde-dehydrogenase (see below), as all known enzymes with aldehyde-dehydrogenase activity described are dimers, trimers or tetramers.

ORF39 is an aldehyde-dehydrogenase. The purified enzyme was used for functional tests, in order to establish its physiological role. Several substrates were tested and are listed in table 1. The enzyme is able to dehydrogenate several aliphatic and one aromatic aldehyde forming probably the corresponding acids. From the data presented in table 1, it can be seen that the enzyme seems to prefer long-chain substrate.

Table 2. Dehydrogenase activity of ORF39 on various substrates

Substrates	nmoli NADPH/min/mg proteină*
Butiraldehyde	229.1
Glyceraldehyde	93.78
Glutaraldehyde	77.71
Benzaldehyde	36.17
Formaldehyde	26.8
Succinic-semialdehyde	Not Detected

Dehydrogenase activity could be detected with both NADP⁺ and NAD⁺, but the speed of the reaction decreases with the

last co-enzyme (Table 3). Such dehydrogenases acting on aldehydes with both co-enzymes are common, being described also by Veladi et al., 1995 (25) and Ahvazi, 2000 (2). In conclusion, the enzyme encoded by the *orf39* from pAO1 megaplasmid is an NAD(P)- aldehyde-dehydrogenase (ALDH) and we will refer to it with this name from here after (18).

Table 3. Relative activity of ALDH with NAD⁺ and NADP⁺

Substrate (33 mM)	Specific activity with NAD ⁺ (nmoles NADH*min ⁻¹ *μg ⁻¹ enzyme)	Specific activity with NADP ⁺ (nmoles NADPH*min ⁻¹ *μg ⁻¹ enzyme)
Gliceraldehyde	176.85	281.35
Glutaraldehyde	64.31	233.12
Butiraldehyde	24.12	458.2

ALDH has an Cys residue in the active site. A lot of time was lost in developing a purification procedure for this enzyme. Following common IMAC techniques and guidelines, the enzyme was always inactive and migrated on SDS-PAGE as a double band. As it can be seen in figure 2, panel A, adding reducing agents as DTT or β-mercaptoethanol to this batches of purified inactive enzyme did not abolished this behavior.

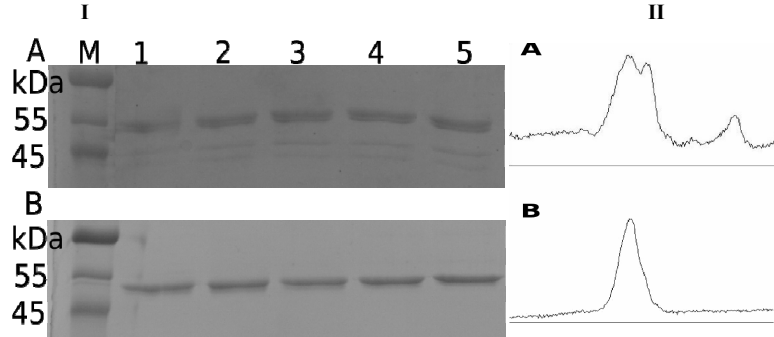


Figure 2. Different SDS-PAGE migration patterns of ALDH purified without (A) or with (B) β-mercaptoethanol. I. SDS-PAGE with 1 microgram ALDH. Loading buffer has 1. o mM 2. 10 mM 3. 50 mM 4. 100 mM 5. 500 mM 2-mercaptoethanol final concentration II. Gel-densitometry of sample 3 (densitometry were realized with ImageJ (1)

This behavior is commonly explained in the literature by a di-sulfur bridge between two Cys residues. This would result in the formation of a loop on the amino acid chain of the protein, loop which is translated in a decrease of the relative molecular weight on SDS-gels with approximately 2 kDa (14).

Using from the starting of the purification process 10 mM β-mercaptoethanol in all the buffers has lead active enzyme. In this case, the protein migrated as a single band on SDS-PAGE (figure 2, B). So, without the reducing agent, the formation of a di-sulfur bridge is possible and leads to enzyme inactivation, either by changing the tertiary structure of the protein, by blocking the active residue or by both mechanisms. A sequence based alignment was performed with the computer generated model of ALDH and the crystal structures of two aldehyde-dehydrogenases, one from sheep (PDB id. 1bxs (21)) (30.4% identity) and one from human (PDB id. 1cw3, (22) (29.7% identity). The alignment (fig. 3) showed that the only highly conserved residue is Cys 266, which is involved in the catalytic mechanism of 1bxs and 1cw3.

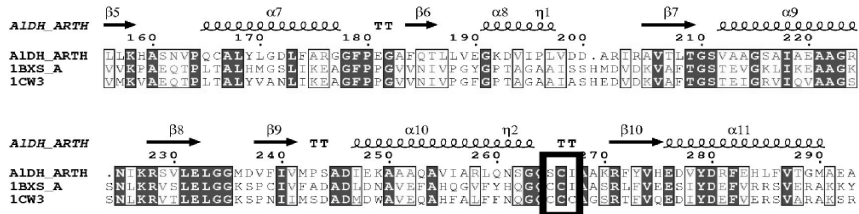


Figure 3. Structure based alignment of ALDH of *Arthrobacter nicotinovorans* and aldehyde-dehydrogenases from sheep – 1bxs and from human - 1cw3 in the catalytic site region. Probably the mechanism by which ALDH from *Arthrobacter dehydrogenates* the substrates is similar to that described for

other aldehyde-dehydrogenases by Marchal and Branlant, 1999 (17) and Ki Ho (2005) (16). The Cys residue from the catalytic site is involved in the formation of a covalent intermediate during the catalytic process. Blocking this residue by a di-sulfur bridge would impair the formation of this intermediate, and those, render the enzyme inactive.

CONCLUSIONS

The ORF39 was cloned, expressed and purified to homogeneity. It consists of an novel aldehyde-dehydrogenase of 52.44 kDa, which unlike the other enzymes of its class is an monomer in solution. Active enzyme preparations required the presence of 10 mM reducing agents in all the buffers. Without the reducing agent, the enzyme runs on SDS-PAGE as a double band typical for di-sulfur bridge formation. Structure based sequence alignments showed that Cys 266 is highly conserved, indicating its implication in the catalytic process and explaining the behavior of this enzyme. In order to fully establish the biotechnological potential of this enzyme, further investigations for characterization of the enzyme (heat stability, pH stability, Km, Kcat,) are underway.

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A COMPARATIVE STUDY ON THE ACTIVITY OF HEPATIC AND MUSCULAR CATALASE IN FRESHWATER FISH SPECIES

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SABINA IOANA COJOCARU ¹

Key words: catalase, muscle, liver, common carp, bighead carp, crucian

Abstract: The paper performs a comparative determination of the hepatic and muscular catalase activity in three 2 summer-old cyprinids species, namely common carp (*Cyprinus carpio*), crucian (*Carassius auratus gibelio*) and bighead carp (*Aristichthys nobilis*), all coming from an intensive growth system. The results obtained evidence higher values (3.58 times higher in common carp, 6.55 higher in bighead carp and 2.07 higher in crucian, respectively) recorded by this marker-enzyme of oxidative stress, for all species under investigation, at the level of liver - known as the main center of substances' metabolism.

INTRODUCTION

As generally known, all aerobic organisms possess catalase - a biocomponent enzyme belonging to the class of oxidoreductases, with hemine or feroporphyrin IX as prosthetic group. The literature of the field suggests that the proteic part is not absolutely necessary for life, once its absence - possibly resulting from a genetic accident, may be compensated, at least partially, through destruction of the hydrogen peroxide catalyzed by peroxidases and - especially in animals - by glutation peroxidase. By the presence of the functional thiolic group of cysteine, the latter one protects the thiolic enzymes against the detrimental action of oxygen while, by its oxidated and reduced forms, it forms an important redox system (BODEA *et al.*, 1964).

According to some authors (RUDNICK, 1967; BRAUNBECK *et al.*, 1987; ORBEA *et al.*, 2000; SÓLE *et al.*, 2004; FERNANDEZ - DIAZ *et al.*, 2006), in the case of fish, catalase is an *adaptation enzyme*. Thus, a study on its activity in the rainbow trout grown in floatable cages showed that the activity of the hepatic and muscular enzyme gets modified as a function of water temperature, density of fish batches, quality of the administered food and age of the individuals (BATTES *et al.*, 1974 - 1975).

As to the percent distribution of catalase in the formation of the hepatic cell, the following values were recorded: 66% in hyaloplasma, 18% in microsomes and 16% in the nucleus (RADHAKRISHNAN and SARMA, 1966).

The literature of the field also shows that, in the case of fish, environmental factors such as: temperature, salinity, season, as well as the feeding habitat induce modifications in the peroxisomal enzymatic activity, seen as also depending on the species (FAHIMI and CAJARAVILLE, 1995; ROCHA *et al.*, 2003), catalase being well-known as an enzymatic peroxizomal marker (AEBI, 1984). It has been also demonstrated that the season, age and sex influence the morphology of peroxizomes from the hepatic tissue in fish. Thus, in the *Mugil cephalus* species, the hepatic peroxizomes tend to increase, both along the summer and in aged individuals (ORBEA *et al.*, 1999). More than that, in *Salmo trutta*, the individual sizes of the peroxizomes and their total volume per hepatocyte, but not their number, get modified during the annual reproduction cycle, in both genera (ROCHA *et al.*, 1999).

Several studies have been devoted to the influence of some chemical substances (aluminium, cadmium, uranium, phenanthrene, endosulfan, phenyl-carboxylic acids, petroleum, etc.) on the catalasic activity in various tissues: sanguine, hepatic, renal and branchial (AINY *et al.*, 1996; OTTO and MOON, 1996; MCFARLAND *et al.*, 1999; VARANKA *et al.*, 1999; SÓLE *et al.*, 2000; IKIĆ *et al.*, 2001; PANDEY *et al.*, 2001; JENA *et al.*, 2002; ACHUBA and OSAKWE, 2003; BUET *et al.*, 2005; GULCIN *et al.*, 2005; LIMA *et al.*, 2006; SUN *et al.*, 2006).

The present paper systematizes the results of the investigations on the activity of catalase, an enzyme actively involved in the oxidative stress, from the hepatic and muscular tissue of some autochthonous and allochthonous cyprinids (common carp, crucian and bighead carp) from the second growth summer, coming from an intensive growing system.

MATERIALS AND METHOD

The experiments were performed on two summer-old representatives of common carp (*Cyprinus carpio*), crucian (*Carassius auratus gibelio*) and bighead carp (*Aristichthys nobilis*) from the Piscicultural Farm of Vlădeni - Iași district. Fresh samples of hepatic and muscular tissue have been taken over, on which the activity of catalase was determined titrimetrically, with potassium permanganate, the results obtained being expressed in mg oxygenated water/ml/30 min. (COJOCARU, 2008).

In the end, the results were statistically interpreted by the Anova test, the unifactorial pattern (FOWLER *et al.*, 2000; ZAMFIRESCU and ZAMFIRESCU, 2008).

RESULTS AND DISCUSSION

Catalase, universally occurring in nature, evidences its activity in all aerobic microorganisms, as well as in the cells of plants and animals. At cellular level, the enzyme occurs almost exclusively in peroxisomes, reducing the level of the hydrogen peroxide, while it is absent in chloroplasts. Hydrogen peroxide is the most stable of all active species of oxygen, being a very strong nucleophilic oxidative agent, responsible for the inhibition of the enzymes from the Calvin cycle. Besides other enzymes, it is one of the most efficient catalysts known up to now, the reactions it catalyzes being essential for life.

The enzyme acts as a regulator of the H_2O_2 level, on also acting as a detoxification agent, as the oxygenated water has a toxic effect upon the tissues, thus protecting the cell through catalysis of the oxygenated water formed in the cells under the action of aerobic dehydrogenases.

A first objective of the present investigation involved determination of the catalase activity in the hepatic and muscular tissue of the two year-old common carp. Thus, as also graphically evidenced (Fig.1), the activity of catalase in the muscle (4.69 mg oxygenated water/ml/30 min.) represents but 27.86% of the one present in the liver (16.83 mg oxygenated water/ml/30 min.), which might be explained through the enzyme's involvement in processes producing considerable amounts of oxygenated water.

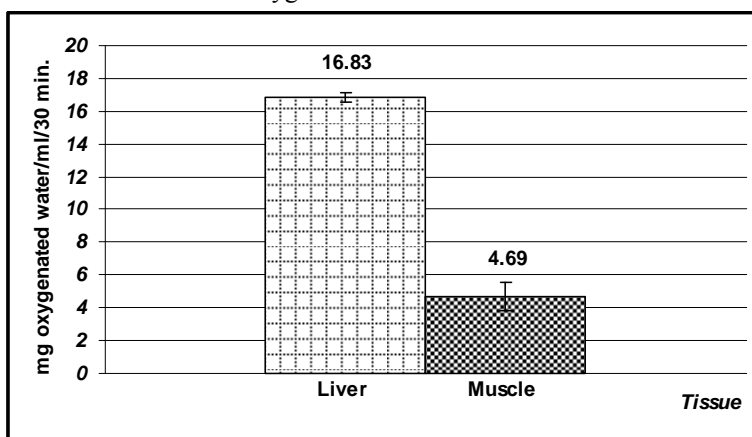


Fig.1. Hepatic and muscular catalase activity in two summer-old *Cyprinus carpio*

In the bighead carp (Fig.2), mention should be made of the fact that, on one side, the catalasic activity records much lower (about 7 times) values in the muscular tissue, comparatively with the hepatic one while, on the other, this is diminished in both tissues, comparatively with the *Cyprinus carpio* representatives, significant differences being evidenced in the muscular tissue, where the catalasic activity is two times higher in common carp.

The distinctly higher values recorded for the hepatic tissue agree with the literature data, which evidence - each time - maximum activities in the hepatic tissue (BATTES *et al.*, 1974 - 1975).

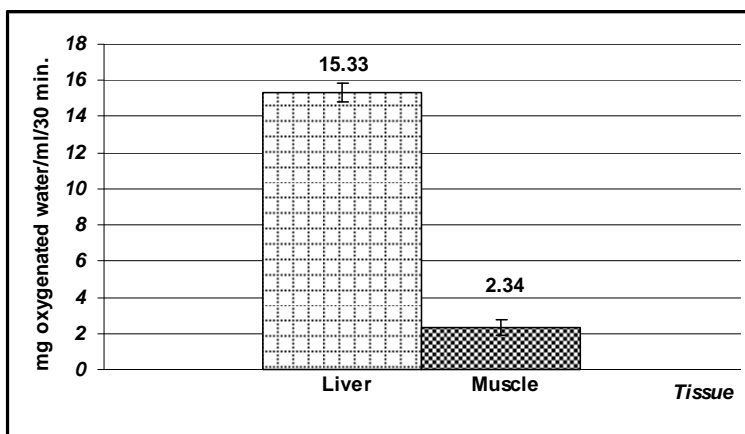


Fig.2. Hepatic and muscular catalase activity in two summer-old *Aristichthys nobilis*

The latest species taken into study was the crucian, a case in which the catalase activity values recorded were comparable with those of the previously-investigated species, mention being made of the fact that maximum values are registered at the level of the muscular tissue (7.85 mg oxygenated water/ml/30 min.). Another observation refers to the moderate difference observed between the muscular and the hepatic activity, comparatively with the previously-analyzed species, the enzymatic activity at muscular level being about 48% lower (Fig.3).

The present results agree with the literature data, according to which, in the same organism, the maximum activity of catalase should be registered in the liver, followed by erythrocytes, brain, spinal marrow, gonads, muscle and liver (PANIKER and IYER, 1972).

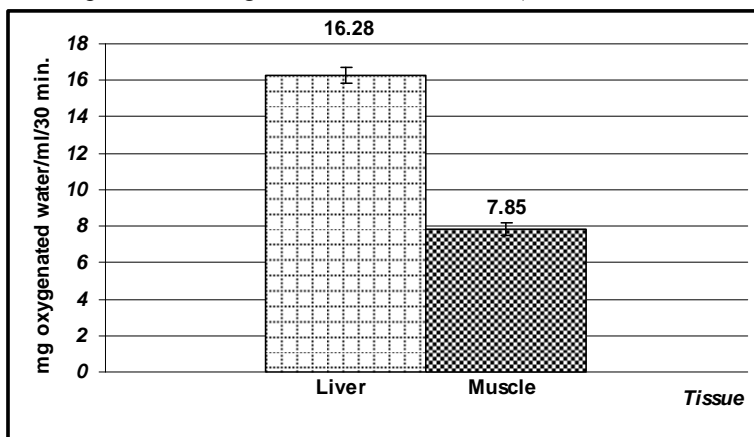


Fig.3. Hepatic and muscular catalase activity in two summer-old *Carassius auratus gibelio*

The last objective of our researches was to test the statistical significance of the obtained data, on the basis of the Anova test, the unifactorial pattern with an equal number of observations

in the cell. Consequently, the null and alternative hypotheses of the test could be established, while a comparison between the critical and the calculated factors (*calculated F* and *critical F*) led to the acceptance of one of them (whether significant differences on the catalasic activity in the cyprinids species under study are observed or not).

As to the hepatic tissue, the results obtained are listed in Tables I - II, *calculated F* (15.3402) being higher than *critical F* (3.8852), which suggests the existence of certain differences from one species to another (Fig.4).

Table I. Summary of the Anova test unifactorial pattern of catalase activity from the hepatic tissue in two summer-old *Cyprinus carpio*, *Aristichthys nobilis* and *Carassius auratus gibelio*

Species	Count	Sum	Mean	Variance
<i>Cyprinus carpio</i>	5	84.15	16.83	0.1011
<i>Aristichthys nobilis</i>	5	76.67	15.33	0.2658
<i>Carassius auratus gibelio</i>	5	81.43	16.28	0.1936

Table II. Calculated and critical values of the factors of catalase activity from the hepatic tissue in two summer-old *Cyprinus carpio*, *Aristichthys nobilis* and *Carassius auratus gibelio*

Source of variation	SS	g. l.	\overline{SS}	Calculated F	P	Critical F
Internal	5.733	2	2.866	15.3402	0.0004	3.8852
External	2.242	12	0.186			
Total	7.976	14				

SS = squares sum, g. l. = degree of freedom, \overline{SS} = mean squares sum
p = probability

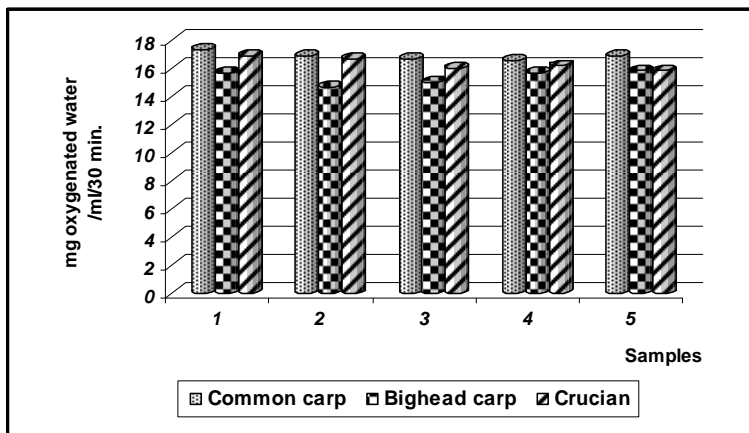


Fig.4. Comparative representation of hepatic catalase activity in two summer-old *Cyprinus carpio*, *Aristichthys nobilis* and *Carassius auratus gibelio*

Equally, the data on the muscular tissue were statistically processed (Tables III - IV), the observation being made that the calculated value of the factor is significantly higher than its critical value (110.6139 *versus* 3.8852), which supports the observation that, in the muscle, catalase records oscillating values from one species to another, representing 59.74% in common carp and 29.8% in bighead carp, respectively, comparatively with the values registered in crucian (Fig.5).

Tabel III. Summary of the Anova test unifactorial pattern of catalase activity from the muscular tissue in two summer-old *Cyprinus carpio*, *Aristichthys nobilis* and *Carassius auratus gibelio*

Species	Count	Sum	Mean	Variance
<i>Cyprinus carpio</i>	5	23.48	4.69	0.7353
<i>Aristichthys nobilis</i>	5	11.73	2.34	0.1936
<i>Carassius auratus gibelio</i>	5	39.27	7.85	0.1069

Tabel IV. Calculated and critical values of the factors of catalase activity from the muscular tissue in two summer-old *Cyprinus carpio*, *Aristichthys nobilis* and *Carassius auratus gibelio*

Source of variation	SS	g. l.	\overline{SS}	Calculated F	P	Critical F
Internal	76.389	2	38.194	110.6139	1.8552	3.8852
External	4.143	12	0.345			
Total	80.532	14				

SS = squares sum, g. l. = degree of freedom, \overline{SS} = mean squares sum
p = probability

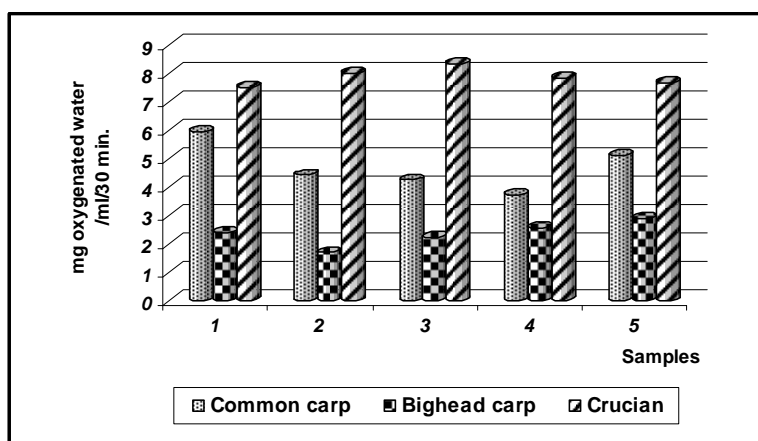


Fig.5. Comparative representation of muscular catalase activity in two summer-old *Cyprinus carpio*, *Aristichthys nobilis* and *Carassius auratus gibelio*

CONCLUSIONS

A comparative analysis on the activity of catalase in the hepatic and muscular tissues of two summer-old *Cyprinus carpio*, *Aristichthys nobilis* and *Carassius auratus gibelio* representatives permits the conclusion that, on one side, significant difference are recorded, in the investigated tissues, as to their enzymatic activity - much higher values being found out, each time, in the liver - while, on the other, as to the different behavior of the enzyme from one species to another, the minimum activity being observed, in both tissues, in bighead carp.

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CORRELATIONS BETWEEN THE PROTEINEMY AND GLYCEMY OF SOME CYPRINIDS AND THE ANTIPARASITARY TREATMENTS APPLIED

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Key words: cyprinids, proteinemy, glycemy, antiparasitary treatments

Abstract: The paper analyzes the modifications produced in some biochemical indices (proteinemy and glycemy), determined in the blood of certain one year-old culture cyprinids, namely: common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Aristichthys nobilis*), subjected to some prophylactic antiparasitary treatments. The experiment was performed between April 2007 and April 2008, in 0.5 ha ponds, each basin being populated with 79% common carp (245 g/piece), 11% silver carp (475 g/piece) and 10% bighead carp (425 g/piece). In the reference pond, no treatments were applied, while the experimental variant was prophylactically treated both in the moment of pond's filling (April 2007) and during the growing period, with **trichlorfon**, applied in preventive doses of 0.1 mg/L, in two steps, and **calcium chloride**, 2 kg/ha, twice a week, respectively. The concentration values of the biochemical indices were determined one year after the experiment (March-April 2008). The results obtained attest that the preventive anti-ectoparasitary treatment applied to the three fish species has positive effects on their physiological condition, generally, on proteinemy and glycemy - especially. In the treated silver carp, spring proteinemy is 23% lower, while glycemy is 35% lower - comparatively with the reference. In the treated common carp, the two biochemical indices show an increasing tendency, with 19% in proteinemy and 27% in glycemy - respectively.

INTRODUCTION

The researches devoted to the evolution of some biochemical parameters, such as blood glucose, proteinemy etc., in culture fish are justified by their significance for estimating the general health condition of the animals, as well as the possible conditions of the food, technological and parasitary stress (Kebus *et al.*, 1992; Barryet *et al.*, 1993; De Dominis *et al.*, 1993; Bau *et al.*, 1994; Rehulka, 1996). Some investigations have evidenced important modifications of such indices under conditions of severe hypothermy and over-density stress, in culture cyprinids (Misăilă *et al.*, 2005). Consequently, the biochemical response of common carp, silver carp and grass carp to such stress conditions is manifested in a 27.7 - 94.8% increase of glycemy (comparatively with the initial values) and in a 2.8 - 27% decrease of proteinemy, respectively.

The preventive application of some antiparasitary treatments in the basins of the pre-development and growth of such fish is expected to bring about a higher functional prosperity of the general physiological condition of the treated fish, comparatively with the non-treated ones, as a result of the running diminution of the parasitary stress.

Generally, the normal concentration values of seric proteins in culture cyprinids from the third growth summer range between 3.1 - 4.7 g/dL, a direct correlation being sometimes established between the proteinemy values and the health condition of adult common carp populations (C₂₊) from various ponds (Patriche, 2007). Consequently, if the values of proteinemy are >3g/dL, the common carp is healthy, while values < 3g/dL show that the fish is potentially ill and, finally, proteinemy values below 1.8 g/dL serum indicate affected organisms. For the moment, the existing data provide no values for younger fish (first and second growth summer).

As to the values of glycemy in adult cyprinids, they oscillate - according to the same authors - between 40 and 90 g/dL serum, certain correlations being again suggested between the glycemy levels and the quality of the fodder, starvation condition of the fish, growing density etc.

The present paper describes the preventive antiparasitary treatments applied as early as the moment of ponds populating, the biochemical response of the fish being compared for the two variants: with and without treatment application, on considering the values of proteinemy and glycemy, dosed both in the end of the growing period (November) and in the end of the cold season (April).

MATERIALS AND METHOD

The investigations - developed between April 2007 and April 2008 at the Research and Development Station for Aquaculture and Aquatic Ecology of Iași - were performed on two parallel variants of an experimental pattern. The

batches were put into two ponds (each with a surface of 0.5 ha), each of them populated with a polyculture formula, including: 79% common carp (245 g/piece), 11% silver carp (475 g/piece) and 10% bighead carp (425 g/piece).

In the reference batch, the experiment involved no antiparasitary treatments, while the experimental variant was prophylactically treated both in the moment of ponds filling (April 2007) and during the whole growth period, with preventive doses of 0.1 mg/L tichlorfon, administered in two steps and calcium chloride (2 kg/ha), twice a week, respectively.

Fish feeding consisted of a granulated concentrated fodder, prepared according to the SAPROFISH 32/SA-1 receipt, the daily administered ratio representing 3-5% of the existing piscicultural biomass. Fodder composition was represented by 32% brute protein, 7% cellulose, 13% humidity and 8% fats. The ratios were periodically updated, on the basis of the control weighing results, performed monthly, by the polling method.

In the end of the experimental period, five fish from each species have been taken over, both from the reference, and the experimental pond, after which blood samples were collected.

Glycemy was determined by the methods with orto-toluidine (Artenie *et al.*, 2008), and proteinemy by the refractometric method, on an ABBE type refractometer (Artenie and Tănase, 1981).

RESULTS AND DISCUSSION

As generally known, proteinemy and glycemy represent especially important parameters, both for evidencing the nutritional condition of fish under certain experimental conditions, and for correctly evaluating the thermal, nutritional and parasitary stress conditions, once known that glycemy is probably the most suggestive index applied in the diagnosis of stress and of its severity, as well.

The results of the investigations, graphically plotted in Figures 1 and 2, may be compared as a function of both the experimental moment (November and April) in which proteins and blood glucose were dosed, and the experimental variant (with or without treatment) considered for analysis.

As to the first situation, one may observe that, in the spring determination, the values of proteinemy (Fig. 1) are higher than the autumn ones, for all species and all variants. Thus, in the case of common carp, the increase of spring proteinemy is 37-57% higher than the autumn values while, in the case of silver carp, the increase is less pronounced (of 3-27%), the bighead carp registering the highest increase (29-88%). A possible explanation might be that, in April 2008, water temperature had already attained values at which fish active feeding was quite intense, comparatively with the values recorded in November, the fish from both variants rapidly restoring their blood protein reserves.

Another observation refers to the fact that, each time, the values of such increase are higher in the reference than in the treated batch. Apparently, such differences are unusual, nevertheless they come from the percent values recorded in spring and autumn, seen as lower - in absolute value - in the untreated than in the treated fish.

A similar analysis on the evolution of glycemy (Fig. 2) shows an important increase in the spring values, comparatively with the autumn ones, both in the common carp and silver carp from the reference batch (60-133%), and in the treated batch (52-63%), comparable values being observed in the case of bighead carp.

As to the second motivation (experimental variant), it is estimated that proteinemy takes higher values in the treated batch comparatively with the reference, in both experimental moments, *i.e.*, 18-19% higher in common carp and 11-62% higher in bighead carp, respectively. In silver carp, the reference values are comparable with those of the experimental variant, even 5-23% higher. A possible explanation for the higher proteinemy in common carp and bighead carp may be related to the additional physiological comfort installed, through reduction of the parasitary stress.

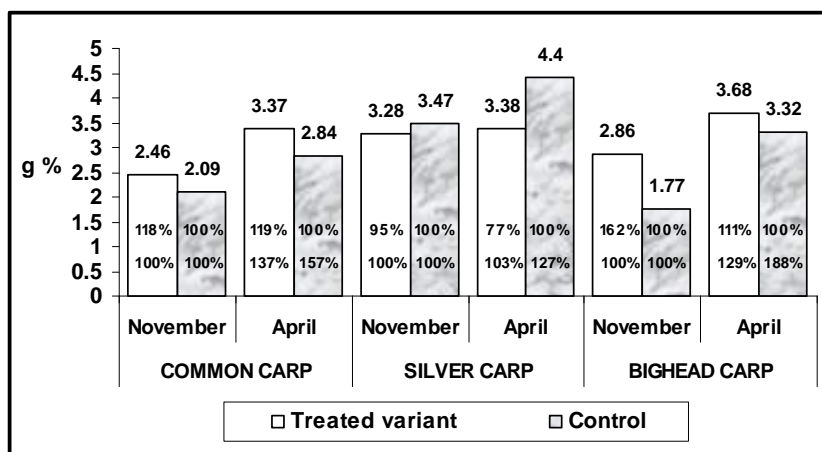


Fig.1. Proteinemia in the fish under experiment

* The top percents represent the comparison as a function of the experimental variant (with or without treatment)

** The bottom percents represent the comparison as a function of the experimental moment (November and April)

Glycemy, instead, records a non-uniform evolution, more exactly, in the case of common carp, the values registered in the treated batch are higher than in the reference, both in autumn (+33%), and in spring (+27%), suggesting the existence of some additional energetic reserves, comparatively with those of the reference. In bighead carp, mention should be also made of a +12% increase in glycemy, in November, in the treated batch, *versus* the reference, while, in spring, the glycemy values in the treated bighead carp decrease with -6%, such a diminution being observed - in the treated silver carp - both in autumn (-7%), and in spring (-35%).

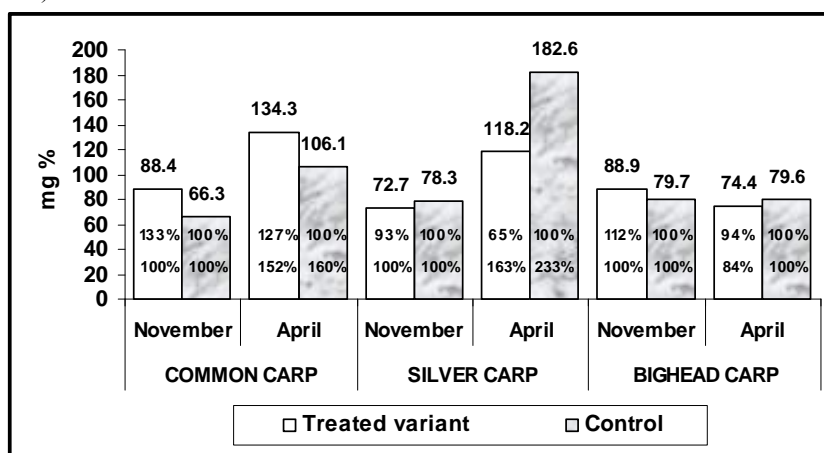


Fig.2. Glycemy in the fish under experiment

* The top percents represent the comparison as a function of the experimental variant (with or without treatment)

** The bottom percents represent the comparison as a function of the experimental moment (November and April)

The higher glycemy values recorded in the two species of Asian cyprinids from the untreated batch suggest a more intense stress condition in the untreated fish and a more difficult adaptation to the wintering conditions of our country.

CONCLUSIONS

1. The levels of proteinemy and glycemy in the three species of cyprinids under investigation range between the normal variation limit cited in the literature of the field for three year old-ages, any increase in glycemy over these values, in fish from the second summer, being also a consequence of the post-wintering stress.

2. In April, proteinemy evidences higher values than in November, for all species and in all experimental variants, the fish rapidly restoring the blood protein reserves, under conditions of an early initiation of active feeding.

3. The evolution of the glycemy values shows an important increase in spring dosage, comparatively with the autumn one, especially in the common carp and silver carp from both experimental variants.

4. Proteinemy attains higher values in the treated batch, comparatively with the reference, in both experimental moments, especially in common carp and bighead carp, which is the result of the additional physiological comfort induced by a more reduced parasitary stress.

5. In the two species of Asian cyprinids from the untreated batch, the values of glycemy are higher than those recorded in the treated variant, which suggests a possible intensification of the stress conditions in untreated fish, as well as a more difficult adaptation to the wintering conditions of our country.

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RHIZOSPHERE BACTERIA HELP PROTEIN ACCUMULATION IN SOYBEAN SEEDS

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Keywords: rhizosphere bacteria, soybean, protein, lipids, soluble carbohydrates

Abstract: The use of rhizobacteria as biofertilizers is one of the most promising biotechnologies to improve primary production with low inputs in fertilizers. In this context, the main goal of this study was to establish if the interaction between different rhizobacteria strains with *Glycine max* L. plants have a positive effect on the total soluble protein, carbohydrates and lipids content of soybeans. Our results revealed that there are no significant differences between the seeds produced by inoculated plants and those produced by the non-inoculated plants regarding soluble reducing carbohydrate content, lipid content and relative humidity. In the case of soluble protein content, rhizobacteria inoculated plants produce beans that contain a greater amount of soluble protein per gram. No qualitative differences could be shown, making our tested rhizobacteria strains an appealing strategy for improving soybean protein production.

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is a subtropical legume; temperatures of 25 to 30° C are optimal for its growth, nodulation and N₂ fixation.

Soybean is one of the most important sources of edible oil and protein in the world (Li et al, 2004). Soy products are the main ingredients in many meat and dairy substitutes. They are also used to make soy sauce, and the oil is used in many industrial applications.

Rhizobacteria are root colonizing microorganisms which are known to be in constant communication with plants roots. Previous studies have shown that some roots-associated bacteria called *plant growth-promoting rhizobacteria* (PGPR) can stimulate growth and development of soybean plants (Glick et al., 1999). The beneficial effects of the PGPR are direct plant growth promotion, mobilization of insoluble nutrients (e.g. phosphate) resulting enhancement of uptake by the plant (Lifshitz et al., 1987), and production of antibiotics toxic to soil-borne pathogens (De-Ming and Alexander, 1988). Co-inoculation studies with PGPR and *B. japonicum* have also demonstrated that increased soybean plant root and shoot weight, grain yield, plant vigour, nodulation and nitrogen fixation can result from the presence of the PGPR (Lie and Alexander, 1988; Verma et al., 1986; Yahalom et al., 1987).

Plant-microorganism interactions are very complex and often have important consequences for the plants. For example, host-pathogen interactions are detrimental to one of the two organisms involved. In compatible interactions, plant disease develops. In incompatible interactions, a resistant host plant establishes a set of defense mechanisms against the pathogen, collectively referred to as Systemic Acquired Resistance (SAR) - Mithöfer, 2002. Some bacteria that live in the rhizosphere are able to modify nodule formation and biological nitrogen fixation (BNF) when they are co-inoculated with rhizobia (De Freitas et al., 1997). However, the mechanisms used by these bacteria to produce the effects mentioned, are not well-understood. The phytohormones are implicated in nodule formation in one way or another (Hirsch, et al., 1997). Common mechanisms used by rhizobacteria to alter nodule formation or biological nitrogen fixation include the release of phytohormones such as auxins, gibberellins, cytokinins and ethylene, or the alteration of endogen levels in the plant (Hirsch, et al., 1997). The effects of some phytohormones are indirect, as they stimulate root growth, providing further sites for infection and nodulation. Systemic induction of secondary metabolites such as flavonoids are implicated in these bacterial effects (Andrade, et al., 1998). A further well-studied mechanism is the ability of some PGPRs to reduce disease caused by foliar pathogens, by triggering a plant-mediated resistance mechanism: Induced Systemic Resistance (ISR) (Van Loon, et al., 1998).

Many studies involving PGPR showed plant growth promotion, but only under gnotobiotic conditions (Glick, et al., 1995) or in potting media (Fuhrmann and Wollum, 1989), where these bacteria do not compete with the normal array of soil microorganisms. PGPR must be rhizosphere competent and able to survive in soil. Traits associated with rhizosphere competence and survival in soil include an ability to tolerate a reasonable range of abiotic factors including temperature, pH and moisture (Sylvia et al., 1998).

The use of PGPR as biofertilizers (preparations of microorganism(s) that may be a partial or complete substitute for chemical fertilization) is one of the most promising biotechnologies to improve primary production with low inputs in fertilizers (Bashan 1998), through any of the many mechanisms possible: biocontrol, nutrient mobilization, phytohormone production and nitrogen fixation (Glick, et al., 1995). Usage of isolated bacteria from crop plant's rhizosphere for productivity increase may be an alternative to organic fertilizers (Compant et al. 2005). The main goal is to reduce the pollution and to preserve the environment in the spirit of an ecological agriculture.

Although this biotechnology has so much to offer, the mechanism of interaction between the plant and the microorganism has yet to be cleared out. It is yet unknown when, during the vegetative cycle of the plant, the interactions with the microorganisms are more effective, when these interactions are desirable and when are not.

In this context, the main goal of this study is to establish if the interaction between different rhizobacteria strains with *Glycine max* L. plants have a positive effect on the total soluble protein, carbohydrates and lipids content of soybeans.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Several bacterial strains were isolated from the roots of *Glycine max* L. on Bunt Rovira nutrient medium as described by (Ștefan et al., 2006). Numerous recent studies show a promising trend in the field of inoculation technology. Mixed inoculants (combinations of microorganisms) that interact synergistically are currently being devised (Bashan 1998). Microbial studies performed without plants indicate that some mixtures allow the bacteria to interact with each other synergistically, providing nutrients, removing inhibitory products, and stimulating each other through physical or biochemical activities that may enhance some beneficial aspects of their physiology, like nitrogen fixation. It still has to be demonstrated that these bacterial synergistic effects also benefit plant growth.

For soybean seed treatment, a preculture was obtained by growing the selected strains in a mixed culture on liquid LB-medium (Ausubel et al., 2002) for 24 h on a orbital shaker at 28^o C. 10 ml of this preculture was used to inoculate 1L of LB medium, the culture being further incubated for 48 h in the same conditions.

Plant cultivation and inoculation in field conditions

The inoculation of soybean seeds was carried out with a mixture of rhizobacteria strains obtained as described above (final culture density - 64 x 10⁶ CFU/ml). The soybeans seeds (Pioneer PR91M10/91M10) inoculated or non-inoculated with rhizobacteria were planted using a small experimental seeding machine.

The experiment was conducted during 2008 within Ezareni Didactic Farm of Iasi, in a cambic cernoziom soil with adobe clay texture and good fertility, moderate humus and highly nitrogen content, moderate mobile phosphorus supply, highly potassium content and a very low acid reaction, almost neutral.

The plants were grown in ecological conditions, without using organic fertilizers and pesticides. After harvesting, the beans were collected and further analysed.

Soluble proteins extract preparation. 1 gram of germinated beans was homogenized using a mortar and pestle for 3 min and resuspended in 10 ml of chilled TrisHCl 0,1 M pH 7.5. After 10 min of extraction at room temperature, the homogenized was separated of insoluble cellular debris by centrifugation for 15 min at 4000 rpm using a Hettich Universal 320 centrifuge. The clear supernatant was used for quantification of protein content and for SDS-PAGE.

Soluble protein content was assayed by the dye-binding Bradford method using the Roti-Quant reagent from Roth (Karlsruhe, Germany).

SDS-PAGE. The protein content analysis was done using SDS-PAGE on 5-20 % gradient gels. The gels were casted according to (Ausubel et al., 2002) using a Sigma gradient maker and an TV400YK (Scie-Plas, UK) electrophoresis module (20 cm in height, 20,5 cm in length and 1 mm thick). Approximately 125 µg proteins were mixed with SDS loading buffer (50 mM DTT, 2% SDS, 0,1% bromophenol blue, 10 % glycerol), boiled for 5 min at 95^o C and then loaded on the gel. The gel was run at 150 V/gel for 30 min and then at 300 V/gel for approx. 3 hours. Protein staining was achieved using the standard Coomassie Brilliant Blue R 250 method (Sambrook et al., 1989).

Soluble carbohydrate content – was assayed using 3,5-dinitrosalicylic acid method as described by (Artenie et al., 2008) and an UV-VIS spectrophotometer (Beckman Coulter DU 720 Life Sciences). The results were expressed in g glucose/100 g analyzed material.

Lipid content – the dried seeds were extracted with a Soxhlet apparatus followed by a gravimetric measurement (Artenie et al., 1981).

Statistical analyses. All assays were done in triplicates. For each sample the mean, standard deviation and standard error was calculated. The statistical significance of the differences between samples was tested using the T-test (Fouler et al., 1998).

RESULTS AND DISCUSSIONS

The economic value of *Glycine max* L. seeds reside in their high protein and lipid content. Together, lipids and protein content account for about 60 % of dry soybeans by weight; protein at 40 % and oil at 20 %. The remainder consists of 35 % carbohydrate.

Soybeans contain significant amounts of all the essential amino acids for humans making it a primary ingredient in many processed foods, including dairy product substitutes. According to the Food and Drug Administration USA, "soy protein products can be good substitutes for animal products because, unlike some other beans, soy offers a "complete" protein profile (Henkel et al., 2000).

In order to improve the production of soybean proteins several strategies were developed: usage of chemical or organic fertilizers, pesticides, developing of transgenic soybean plants (Hinchee et al., 1988). Most of them have a negative impact on the environment. That's why the present study is focused on the development of environmental friendly methods for improving the soybean protein production using rhizobacteria as biofertilizers (Bashan, 1998).

Our previous observations showed that some rhizobacterial strains isolated from soybean roots have a positive effect on plant growth and development processes in field conditions (Stefan et al., 2008). In the present study we aimed to investigate the rhizobacteria effects on the quality of the harvested beans. Several biochemical indices for the nutritional value of beans were taken into account including total soluble protein content, soluble carbohydrates and lipids content. The results of our tests are presented in Table 1.

Table 1 –Biochemical parameters of the experimental variants used in this study

Biochemical parameters	Non-inoculated control (mean \pm standard error)	Inoculated sample (mean \pm standard error)	P(T<=t) two-tail
Soluble protein content (mg/g)	425,52 \pm 6,22	438,13 \pm 3,87	0,002
Soluble reducing carbohydrate (mg glucose/g)	2,44 \pm 0,087	2,53 \pm 0,08	0,47
Lipid content (%)	22,08 \pm 0,39	21,76 \pm 0,28	0,52
Relative humidity (%)	6,02 \pm 0,06	6,13 \pm 0,077	0,31

As it can be seen from the data presented, there are no significant differences between the seeds produced by inoculated plants and those produced by the non-inoculated plants regarding soluble reducing carbohydrate content, lipid content and relative humidity.

In the case of **soluble protein content**, rhizobacteria inoculated plants produce beans that contain a greater amount of soluble protein per gram. Although small, the recorded difference between sample and control is statistically significant as proved by the results of the "t" test ($p < 0.002$) – Fig. 1. Considering an average soybean production of 1.5 T/ha, the above mentioned difference can be translated into an increase of protein production with aprox. 19 kg of soybean protein/ha.

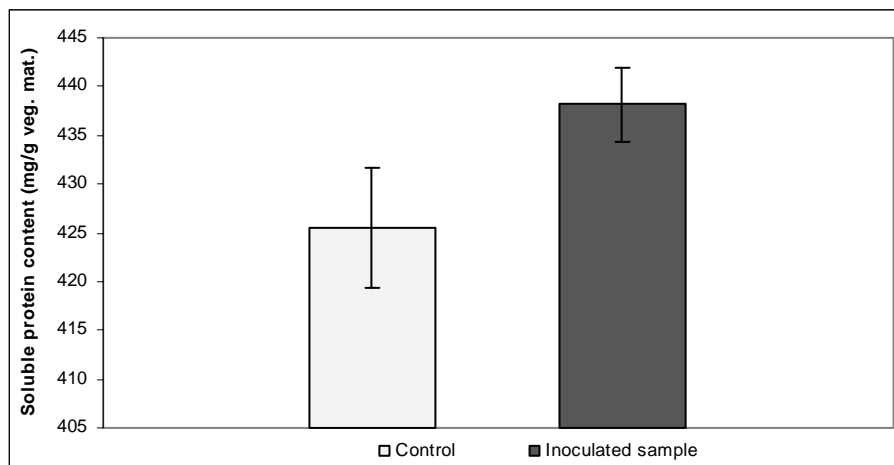


Fig. 1 – Total soluble protein content of beans produce by inoculated and non-inoculated soybean plants

Such improvement in total protein production can be achieved also by amplification of various genes in the genome of transgenic soybean plants (Li et al., 2004) or by usage of important amounts of chemical or organic fertilizers. Both alternatives have their own disadvantages: usage of GMO (genetically modified organisms) is an idea strongly rejected by the public opinion; chemical fertilizers have a well establish negative impact on the environment. A better alternative would be than to use rhizobacteria as biofertilizers. A question arises: does this bacteria influence only the plant growth by mobilizing nutrients from the soil (Sylvia et al., 1999) or they induce some qualitative modification in the protein pattern? In order to asset this problem an SDS-PAGE electrophoresis was performed with the protein extracts (Fig. 2).

As it can be seen from Fig. 2, there are no significant qualitative differences between the protein electrophoresis pattern of seeds produced by non-inoculated and rhizobacteria inoculated soybean plants. In this case the tested rhizobacterial strains help protein accumulation only by a mechanism which involves probably only an increased availability and uptake of nutrients from soil.

CONCLUSIONS

The overall conclusion is that in field conditions rhizosphere bacteria isolated from soybean plants roots can stimulate protein accumulation in soybean seeds. Our test recorded only quantitative differences regarding total soluble protein content between seeds obtain from inoculated and non-inoculated soybean plants. No qualitative differences could be shown, making our tested rhizobacteria strains an appealing strategy for improving soybean protein production.

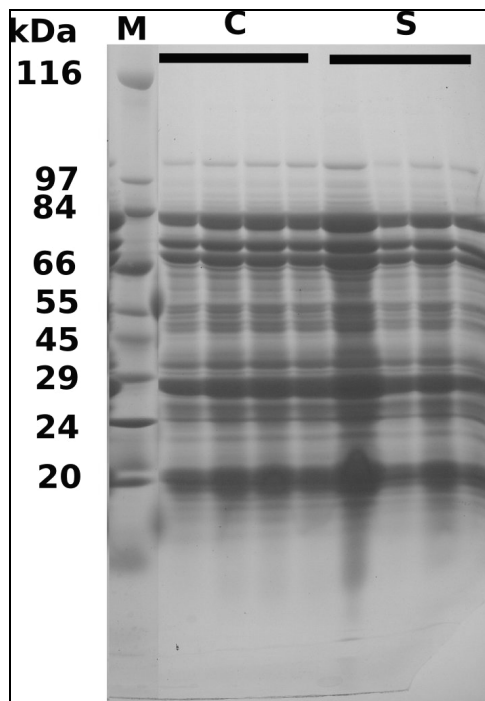


Fig. 2 - SDS-PAGE of total soluble proteins: M-Sigma Wide Range protein marker; C-non-inoculated control; S-rhizobacteria inoculated sample (125 micrograms proteins were loaded on each lane)

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CONSIDERATION ON THE PEROXIDASE ACTIVITY DURING HIPPOPHAE RHAMNOIDES SEEDS GERMINATION EXPOSED TO RADIOFREQUENCY ELECTROMAGNETIC FIELD INFLUENCE

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Keywords: electromagnetic field, oxidative stress, reactive oxygen species, peroxidase.

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

INTRODUCTION

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

MATERIALS AND METHODS

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

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

RESULTS AND DISCUSSION

Peroxidase, unlike the other two, is an enzyme widespread in all cellular compartments (cell wall, chloroplast, cytosol, endoplasmic reticulum, mitochondria, peroxisome, plasma membrane) (Bakalovic et al, 2006; Passardi et al., 2007; Koua et al., 2008), where the function is to neutralize hydrogen peroxide using various electron donors (Heldt, 2005).

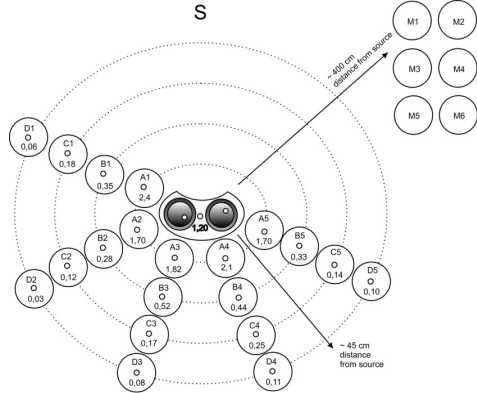


Fig.1. Schematic representation of the experiment. At the center are the two radio stations, around which were arranged the Petri Dishes. In the center of each plate is indicated the magnetic induction in μT .

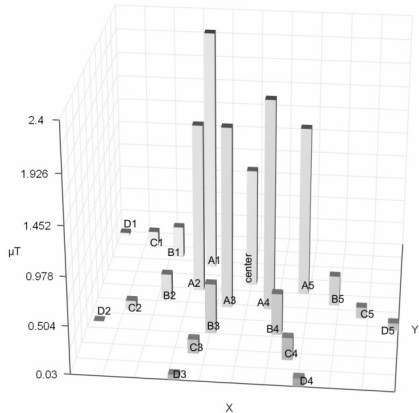


Fig.2. Spatial representation of the intensity of magnetic induction (μT) in relation to the probes arrangement.

Highly reactive oxygen species are generated during aerobic metabolism, and they are normally detoxified by cellular defense mechanisms that involve superoxide dismutase. This enzyme converts superoxide radicals to hydrogen peroxide, which is then converted to water by peroxidase (Taiz and Zeiger, 2002). Peroxidases are heme-containing glycoproteins encoded by a large multigene family in plants. Studies have suggested that peroxidase plays a role in lignification, suberization, auxin catabolism and self-defense against pathogens, salt tolerance and senescence (Atak et al., 2007).

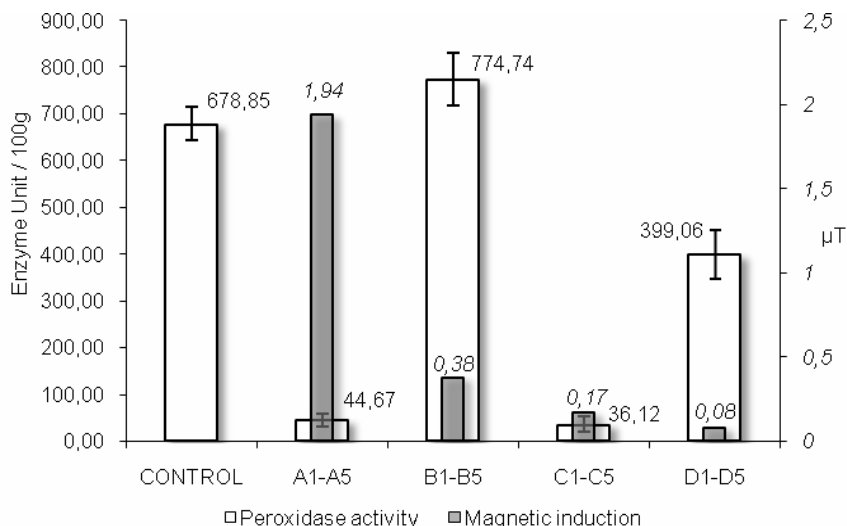


Fig.3. Variation of the peroxidase activity and magnetic induction

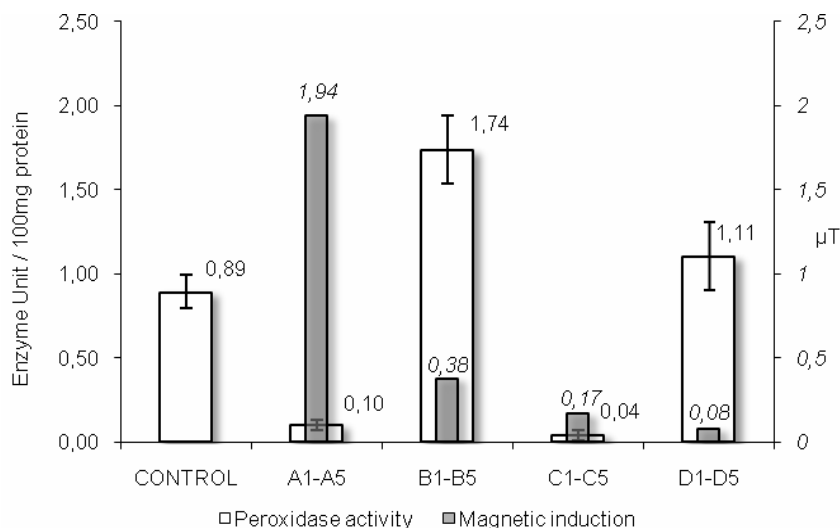


Fig.4. Variation of the peroxidase specific activity and magnetic induction.

The experiment conducted has shown that activity of this enzyme undergoes major fluctuations, as can be seen in fig. 3 and fig. 4. It might say that this pattern of fluctuation could be due to influences exerted by the different intensity of magnetic induction from a cellular compartment to another, which is to have different amounts of enzyme.

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

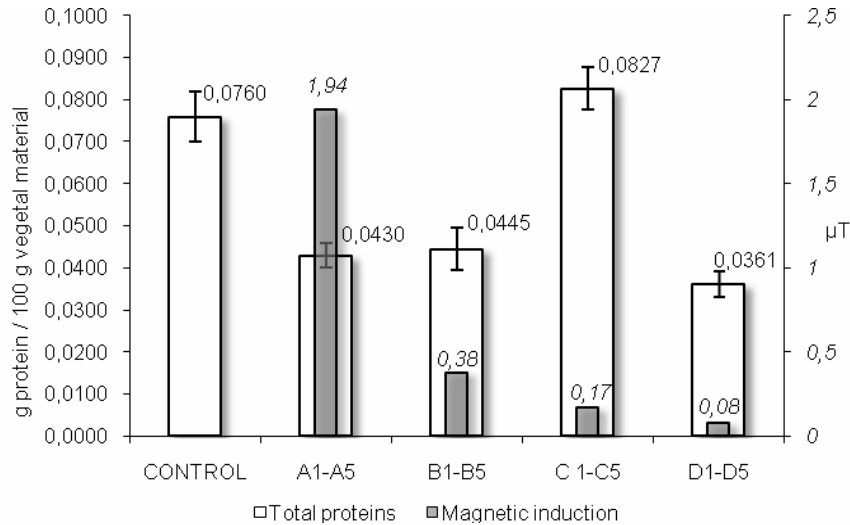


Fig.5. Variation of the total protein quantity and magnetic induction.

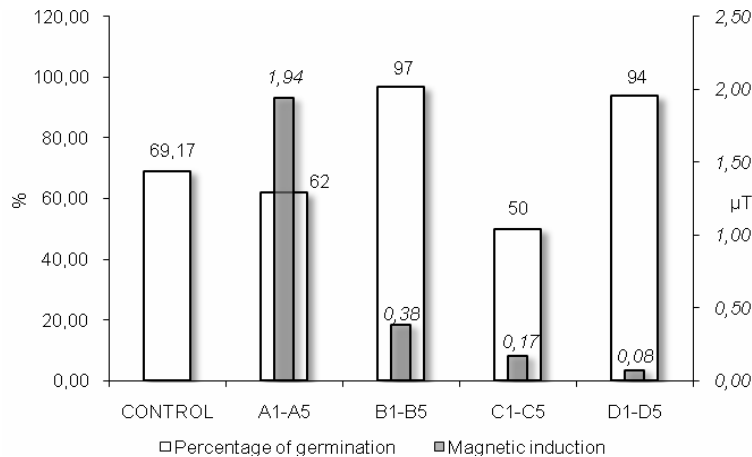


Fig.6. The percentage of seeds germination and the variation of magnetic induction

Percentage of seeds germinated (fig. 6) during the experiment indicates that low intensity magnetic induction can have a stimulating effect. This is observed for samples B1-B5 and D1-D5, where the percentage of germination reached very high values, 97% respectively 94%. In the other two cases, compared with the control, there is a negative trend in germination. In case of A1-A5, high intensity magnetic induction does not seem to have affected germination (percentage difference being only 8% compared to control), where C1-C5 can be considered a decrease of 19%. From germination behavior of toward various intensities of magnetic induction, it may find an indirect correlation between enzyme activities involved in removing the effects of oxidative stress. Since peroxidase takes part to other processes, very high activity in cases B1-B5 and D1-D5 in consistency with high rates of germination may be due to the involvement of this enzyme into other metabolic processes closely related to germination and growth (Atak et al., 2007). From this perspective, the low activities recorded in samples A1-A5 and C1-C5 cannot be attributed to the direct effect of EMF.

CONCLUSIONS

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

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INFLUENCE OF REFRIGERATION LENGTH AND OF SUGAR ADDITION ON ASCORBIC ACID CONTENT IN SOME NATURAL JUICES

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Abstract: Following some studies concerning the main factors influencing the concentration of vitamins within food raw materials, especially ascorbic acid, this work tries to evidence the influence of the refrigeration temperature and of the sucrose addition on content of this vitamin in three types of natural juice. The biological material was represented by orange, strawberry and kiwi natural juices, obtained from these fruits by means a crushing out process. For each type of juice there were made up samples without addition and samples with 5% and 10% sucrose addition. The ascorbic acid determination was carried out from fresh juice as well as from juice kept in refrigerator, at certain time intervals (24, 48, 96 și 168 hours). The storage of these juices under refrigeration conditions has determined percentage reductions of vit. C content of these ones. Thus, after 168 hours of storage, in the three types of analysed juices the highest loss of vitamin have been registrated in samples without sucrose addition, and the least ones in samples with 10% addition. The comparison of vitamin C values in the three analysed juices, subjected 168 hours to refrigeration process, has evidenced that the highest loss of ascorbic acid have been in orange juice, and the least one in kiwi juice.

Keywords: vitamin C, sucrose, orange, strawberry, kiwi

INTRODUCTION

There are some factors which negatively influence the vitamins concentration in food raw materials, such as: high temperatures over certain values, the freezing, the presence of oxygen and of some chemical substances, certain pH values etc.

During food raw materials processing, the level of their vitamins can be negatively affected through some operations such as: manipulation-conservation after harvest of vegetable produce and of meat and aquatic produce, collecting-storage of milk, cutting- chopping end/or scalding, boiling of fruits and legumes, cereals grinding, the adding of chemical substances into legumes, fruits, meat (Adrian and Petit, 1970; Ferrando and Mainguy, 1970; Flanzky, 1970; Scriban, 1970; Ulrich and Delaporte, 1970; Banu et al., 2003).

As to vitamin C, its concentration can be reduced with various percentages, depending on type of processing of raw material containing this vitamin. Thus, three months potatoes storage leads to loss of 50%, the sterilization, but especially vegetables boiling lead to vitamin loss between 47% and 82%. The addition of bicarbonate, used to soften some legumes, contributes to diminish of vitamin C concentration (Banu et al., 2003).

The storage of legumes for an year at temperatures around -10° can lead to vitamin C loss of 80–90% (Selman, 1994).

The addition of anthocians, sugar and even starch seems to have a protecting action on vitamin C (Banu et al., 2003).

Retention of ascorbic acid is better in rapid drying at high temperatures than in slower drying at lower temperatures. Drying methods that expose the food to air result in losses of vitamin C due to oxidation. On the other hand, freeze drying, which is carried out in the absence of oxygen, does not cause loss of vitamin C (Ball, 2006).

According to Selman (1994), the inefficient blanching causes some loss of vitamin C by oxidation, as well as by leaching.

As to microwave heating, the ascorbic acid content is higher in vegetables cooked by microwave heating than by conventional methods (Hill, 1994).

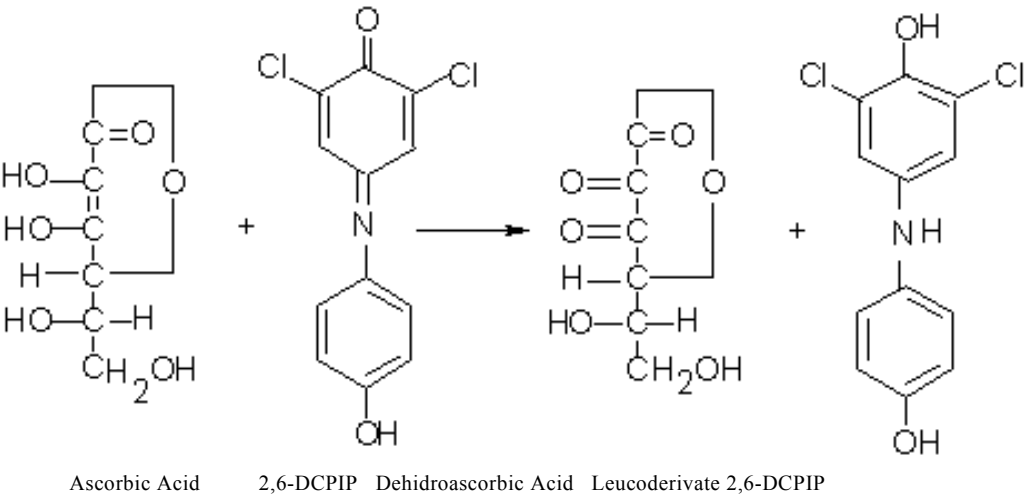
In this work it was studied the variation of vitamin C (ascorbic acid) concentration within three types of natural juices, without and with sucrose addition, stored at +2°C, certain time periods.

MATERIALS AND METHODS

The biological material was represented by natural juices of orange, strawberry and kiwi, obtained from these fruits by means a crushing out process. In order to limit the contact with air and the possibility of vitamin C oxydation, at once after obtaining the juices were rapidly poured into plastic bottles (300 ml capacity) tight closed, and introduced into refrigerator at +2° C.

For each type of juice there were make up samples without addition and samples with 5% and 10% sucrose.

The ascorbic acid determination was carried out from fresh juice as well as from juice kept in refrigerator, at certain time intervals (24, 48, 96 și 168 hours). The method was based on reduction of 2,6-Diclorophenolindophenol (2,6-DCPIP) to the leucoderivate of 2,6- DCPIP, by ascorbic acid (Artenie and Tănase, 1981):



RESULTS AND DISCUSSION

In the table 1 are rendered the values of ascorbic acid content in orange juice.

Table 1 The orange juice ascorbic acid content, with and without sugar addition, stored certain time periods in refrigerator (2° C)

Ascorbic Acid (mg %)			
Juice type Storage length	Sucrose free juice	Juice with 5 % sucrose	Juice with 10 % sucrose
*0 hours	59.752	59.752	59.752
24 hours	58.520	58.784	59.004
48 hours	56.056	56.241	56.454
96 hours	51.744	52.028	52.849
168 hours	37.576	39.295	42.482

* Blank sample

As seen, both in the sucrose free orange juice and in samples with sucrose addition, the vitamin C concentration has gradually decreased once with juice keeping in refrigerator. The highest differences between samples, which has begun to come into view after 96 hours, have appeared after 168 hours of storage. The greatest value was registered by juice with 10% sucrose (28,91% vitamin loss beside blank), and the least was registered by sucrose free juice (37,12% vitamin loss beside blank).

The table 2 reproduces the values of ascorbic acid content in strawberry juice.

Table 2 The strawberry juice ascorbic acid content, with and without sugar addition, stored certain time periods in refrigerator (2° C)

Ascorbic Acid (mg %)			
Juice type Storage length	Sucrose free juice	Juice with 5 % sucrose	Juice with 10 % sucrose
*0 hours	56.056	56.056	56.056
24 hours	52.976	53.167	53.486

Ascorbic Acid (mg %)			
Juice type Storage length	Sucrose free juice	Juice with 5 % sucrose	Juice with 10 % sucrose
48 hours	50.512	50.988	51.339
96 hours	48.048	48.113	48.543
168 hours	41.272	42.105	42.766

* Blank sample

Also in strawberry juice it can see reductions of vitamin C concentration, once with increase of the samples keeping length in refrigerator at +2° C. Less marked then in the case of orange juice, in strawberry juice, after 168 hours of storage, the vitamin C loss has been 26,38%, in sucrose free juice sample, and 23,71% in juice sample with 10% sucrose. Along the whole storage period, but in the last determination (after 168 hours) the vitamin C concentration values of the three analysed samples have been close.

In the table 3 are rendered the values of ascorbic acid content in kiwi juice.

As seen in the table, in kiwi juice, the vitamin C loss have been the least beside orange and strawberry juices. Thus, after 168 hours of storage at +2° C, in sucrose free juice sample the vitamin C concentration has reduced (beside blank) with 20%, and in juice sample with 10% sucrose has reduced with 16,25%.

Table 3 The kiwi juice ascorbic acid content, with and without sugar addition, stored certain time periods in refrigerator (2° C)

Ascorbic Acid (mg %)			
Juice type Storage length	Sucrose free juice	Juice with 5 % sucrose	Juice with 10 % sucrose
*0 hours	70.840	70.840	70.840
24 hours	68.992	68.998	69.166
48 hours	67.760	68.005	68.537
96 hours	65.296	66.108	67.214
168 hours	56.672	58.851	59.332

* Blank sample

CONCLUSIONS

The storage under refrigeration conditions (+2° C) of some natural orange, strawberry and kiwi juices, with and without sucrose addition certain time periods, has determined percentage reductions of vitamin C content in these juices.

After 168 hours of storage, in the three types of analysed juices the highest vitamin loss have registered in sucrose free juice samples, and the least ones in juice samples with 10% sucrose.

The comparison of vitamin C values in the three analysed juices, subjected 168 hours to refrigeration process, has evidenced that the highest loss of ascorbic acid have been in orange juice, and the least one in kiwi juice.

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THE INFLUENCE OF THE ULTRASOUND TREATMENT ON SOME PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS IN *SPINACIA OLERACEA* L. SEEDS

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Keywords: *Spinacia oleracea*, ultrasounds, respiration intensity, catalase

Abstract: The paper presents the results of a study regarding the respiration intensity and the activity of the catalase in the *Spinacia oleracea* L. seeds, subject to the action of ultrasounds with the frequency of 23 and 36 kHz, the electrical power of 7,4; 49,7; and 30 V.A., at the time intervals 20, 30, 40 and 60 seconds. The results obtained emphasize a stimulation effect of the germination of seeds and a slight intensification of the activity of catalase and the respiration intensity.

INTRODUCTION

The ultrasounds are acoustic waves with higher frequencies than the audibility threshold of 16000 Hz (Dimitriu et al., 1990). Because of the high frequencies and the high energy that they release in the propagation media, the ultrasounds produce a series of physical-chemical effects which lay at the basis of their use in different fields: physics, chemistry, biology, medicine, agriculture, industry etc.

The researches carried out by a series of authors (Ausländer et al., 1966; Albu et al.1968; Raianu and Zanvetor, 1969; Dăbală et al., 1970; Albu, 1980) have emphasized the stimulating effect of ultrasounds on the germination, growth and development of plants and also on the vegetal production. In addition, the experiments carried out by us with the seeds of *Pastinaca sativa* have emphasized a stimulating effect of ultrasounds on the intensity of respiration and activity of some oxidoreductases (Stratu et al., 2005). We determined the fact that the sensitivity of seeds to the treatment with ultrasounds varies according to species, used acoustic parameters, time of exposal and morpho-physiological characteristics of the seeds.

Based on these considerations, we proposed to test the effect of the ultrasound treatment on *Spinacia oleracea* seeds – species which germinates with more difficulty and has a lower germination faculty.

MATERIAL AND METHOD

As biological material we used *Spinacia oleracea* seeds from 2007 harvest, acquired from Unisem Iași. The seeds, introduced in the water, were subjected for ultrasound field treatments by two modalities:

- by using an electric high frequency generator with the possibility to modifying in stages the electric power;
- by using an ultrasound bath.

We achieved ten experimental variants (four control variants and six treatment variants) according to the values of the used acoustic parameters and exposal time (Table 1). After carrying out the treatment, the seeds were placed in Petri plates for germination under laboratory conditions.

Table 1. The experimental variants

Crt. no.	Experimental variants	Exposal time [s]	Acoustic parameters	
			Frequency [kHz]	Electric power [W]
1.	M1	20	-	-
2.	M2	30	-	-
3.	M3	40	-	-
4.	M4	60	-	-
5.	V1	20	23	49.7
6.	V2	30	23	49.7
7.	V3	40	23	7.4
8.	V4	60	23	7.4
9.	V5	40	36	30
10.	V6	60	36	30

In these experiments we have studied the seeds respiration intensity and catalase activity.

The seeds catalase activity was determined by iodometric method which consists, in principle, in the evaluation of the peroxide consumption in a determined time interval (Artenie and Tanase,1981).

The seeds respiration intensity was evaluated by Warburg method (Boldor et al.1981)

The investigated parameters were determined at 6, 8, 10, 12 and 14 days since the beginning of the experiment.

RESULTS AND DISCUSSIONS

Spinach (*Spinacia oleracea*) is an herbaceous, annual plant in the family of *Chenopodiaceae*, with a large nutritional (rich source of iron, calcium, vitamin A, vitamin C, vitamin E, vitamin K, vitamin B₉, niacin, opioid peptides, omega 3-fatty acids, magnesium, selenium and several vital antioxidants) and medicinal (remineralized, antianemic, antiscorbutic) values, and melliferous, originating from Central and southwestern Asia, cultivated since the IV century. The fruit is a spherical, edgy or thorny achene, of greenish, grayish or fawn color.

The reserve substances of the spinach seeds are represented by 24% proteins and 7% lipids (Burzo et al., 2000).

The observations made during the deployment of experiments emphasized an effect of stimulating the germination of seeds in the case of variants treated with ultrasounds.

The seeds respiration intensity during the germination process reflects the physiological activity of the embryo and represents an indicator of the metabolic activity and the stress state.

In all the experimental variants, the respiration intensity has, generally, a similar dynamics: it present high values at 6, 8, 14 days and reaches the maximum value in the 12th day since the beginning of the experiment (figures 1, 2 and 3).

Comparatively to the control, in the variants treated with ultrasounds we notice values similar to it, in some cases, slightly higher.

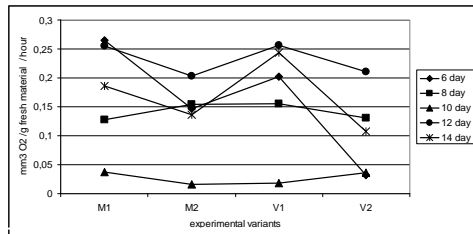


Figure 1. The respiration intensity in *Spinacia oleracea* ultrasounds treated seeds – V1 and V2 variants

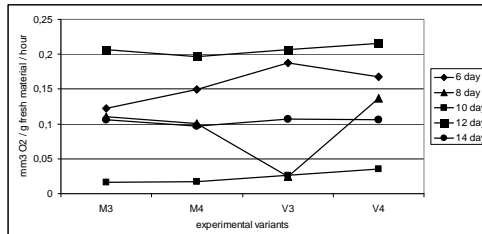


Figure 2. The respiration intensity in *Spinacia oleracea* ultrasounds treated seeds – V3 and V4 variants

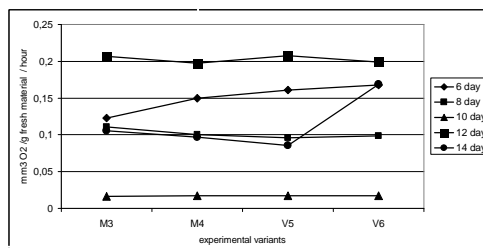


Figure 3. The respiration intensity in *Spinacia oleracea* ultrasounds treated seeds – V5 and V6 variants

The tendency of stimulating the respiration intensity is more evident in the following variants, presented in the decreasing sequence: V4, V3 (the electric power: 49,7 V.A.; the

frequency: 23 kHz; the time of exposal: 40s and 60s ; V6, V5 (the electric power :30 V.A.; the frequency: 36 kHz; the time of exposal: 40s and 60 s.).

The favorable influence on the respiration is due to the mechanical effects that contribute to the tegument corrosion and the increase of the water absorption capacity and chemical effects that accelerate the oxidoreduction processes.

The control respiration dynamics confirms the data from the specialty literature mentioned for the analyzed species and for other species such as: *Lactuca sativa*, *Zea mays*, *Glicine max*, *Lathyrus odoratus*. (Bewley and Black, 1978, cited by Burzo et. al., 1999).

The catalase is an enzyme from the group of oxidoreductases. Through the peroxidase action (supplementary to the catalytic function), the catalase intervenes in the respiration of plants, being indirectly an indicator of the metabolic activity.

In the analyzed interval the catalase activity has a similar activity (with increasing and decreasing stages) in all the experimental variants. The most intense enzymatic activity was determined in the 12th day since the beginning of the experiment (figures 4, 5 and 6).

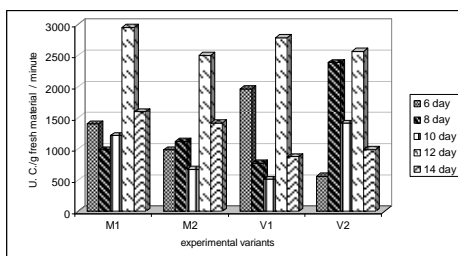


Figure 4. The catalase activity in *Spinacia oleracea* ultrasounds treated seeds - V1 and V2 variants

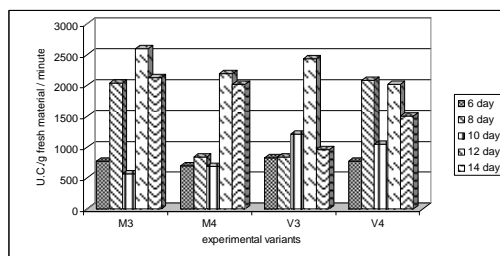


Figure 5. The catalase activity in *Spinacia oleracea* ultrasounds treated seeds - V3 and V4 variants

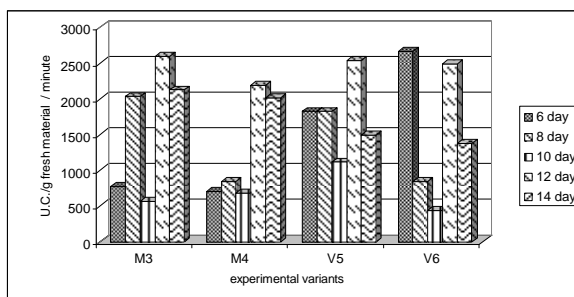


Figure 6. The catalase activity in *Spinacia oleracea* ultrasounds treated seeds – V5 and V6 variants

The oscillations in the catalase activity are in connection with the degradation processes of the endosperm and cotyledons reserve substances and synthesis of some necessary substances for nutrition and embryo growth. On the other hand, they could be determined by the treatment with ultrasounds which intensify the enzymatic activity.

In comparison with the control, we determine a stimulation of the catalase activity in the V4 variants (at 6, 8, 10 days since the experiment beginning); V2 (at 8, 10, 12 days since the

experiment beginning); V5 (at 6 and 10 days since the experiment beginning) and V6 (at 6 and 12 days since the experiment beginning).

The results obtained in the case of the control variants confirm the data from the literature, according to which, the catalase activity increases a lot since the germination beginning and reaches a maximum after a certain number of days, according to the species, after which it begins to decrease (Burzo et. al., 2000).

CONCLUSIONS

Our results emphasize specific value variants of the catalase activity and respiration intensity according to the ultrasound parameters used and the exposure time.

The seeds tested “respond” to the treatment with ultrasounds through a slight intensification of the catalase activity and the respiration process.

The effects of the treatments with ultrasounds noticed in the case of experiments carried out with the test species *Spinacia oleracea* confirm the data from the specialty literature determined for other species with economic value.

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STRUCTURAL CHARACTERISTICS OF *CHRYSANTHEMUM MORIFOLIUM* RAMAT (ROMICA CULTIVAR) REGENERATED *IN VITRO*

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Abstract: The micropropagation of *Chrysanthemum morifolium* Ramat (Romica cultivar), belonging to the collection of “Anastase Fătu” Botanical Garden from Iasi (Romania) was achieved through tissue culture technique and involved callus induction followed by shoot multiplication, rooting and establishment of plantlets in soil. The purpose of this study was to determine the range of variation in certain structural characters of the vegetative organs of *in vitro* regenerated plants at *Chrysanthemum morifolium* Ramat (Romica cultivar). The material subjected to the comparative anatomical analyses was represented by vegetative organs of the parent plant (PP) and regenerated plant (RP), on mature stage. The density of glandular and non-glandular hair (mm^{-2}) on both leaf surfaces was statistical analysed using “t” test at 0,05 confidence level. Despite the great opportunity of genetic variation in callus cultures, the regenerated plants differ not in their structural appearance from the normal plants.

Key words: *Chrysanthemum morifolium*, callus, organogenesis, anatomy, glandular hair, non-glandular hair.

INTRODUCTION

Chrysanthemum morifolium Ramat is well known, not only as an ornamental plant, but also as an important medicinal plant and a major source of natural products (flavonoids, sesquiterpene lactones, essential oils, triterpene diols and triols) used as pharmaceuticals.

It has been found to possess antibacterial, antifungal, antiviral, antispirechetal and anti-inflammatory activities (Wang *et al.*, 1998, Kishimoto *et al.* Ohimiya, 2006, Ukiya *et al.* 2001, Ukiya *et al.* 2002).

Indirect regeneration of *Chrysanthemum morifolium* Ramat (Romica cultivar) through callus cultures represents an unconventional alternative to preserve, to perpetuate and to exploit the source of raw material for natural compounds.

The present study aims at a detailed histo-anatomical analysis of the vegetative organs (adventitious roots, stem and leaf) of *in vitro* and *in vivo* *Chrysanthemum morifolium* Ramat (Romica cultivar) plants in order to determine the range of structural variation in the regenerated plant.

MATERIAL AND METHODS

Plant material

The mature *Chrysanthemum morifolium* Ramat (Romica cultivar) plants, belonging to the collection of “Anastase Fătu” Botanical Garden from Iasi (Romania) were used for initiation of “*in vitro*” cultures (Vântu, 2005, Vântu, 2006)

Explants sterilization

Stem fragments cutting from the mature plants were sterilized with 3% sodium hypochlorite for 10-15 minutes and again washed thoroughly with sterilized distilled water.

Callus culture

The sterilized stem fragments were cut into rectangular pieces of approximately 5 mm in diameter. Stem explants were implanted apical side down to stimulate the natural flow of auxins and carbohydrates.

The callus cultures were established from stem explants on Murashige and Skoog, (1962) medium, supplemented with 2 mg^{-1} 2, 4-dichlorophenoxyacetic acid and $0,2 \text{ mg}^{-1}$ kinetine. Segments of approximately 1 x 1 cm of these calluses were subcultivated on Murashige and Skoog, (1962) medium supplemented with the same combination and concentration of the growth regulators.

Plant regeneration

The callus differentiated into shoots when transferred to MS medium containing 2mg/l kinetine and 0,2 mg/l 2,4 –dichlorophenoxyacetic acid.

The shoots originated from callus tissue were transferred to Murashige and Skoog, (1962) medium without hormones for three months in order to obtain the whole plant (Vântu, 2006)

Histo-anatomical analysis

The material subjected to the comparative anatomical analysis is represented by vegetative organs of the parent plant (PP) and regenerated plant (RP), on mature stage. Adventitious roots, leaves and fragments of stem (cutting from lower, middle and upper level of the plant) were fixed and conserved in 70% ethylic alcohol. Free hand transverse sections were performed using a razor blade. The sections were coloured with ruthenium red and iodine green. The

observations and photomicrograph were done with an Olympus BX51 research microscope equipped with Olympus E-330 photo camera.

Morphometric assessment

For the comparative morphometric assessment between parent plant and regenerated plant (on mature stage), the density of glandular and non-glandular hair (mm^{-2}) on both leaf surface was measured. These characters were analyzed on paradermal stem and leaf sections under 200X magnification using an Olympus BX51 research microscope. For each parameter 10 measurements randomly chosen was made.

Statistical analysis

Data from the morphometric assessment were analyzed by using “t” test (EXCEL) at 0,05 confidence level. Independent samples and unequal variances were assumed.

RESULTS AND DISCUSSIONS

Plant regeneration through callus cultures

The morphogenetical potential has been established to be greater in callus cultures derived from stem explants. The organogenic callus grows rapidly and shows early an extensive organization. The vigorous callus pieces, about 5 mm transferred to fresh medium at intervals of 4 weeks maintain the organogenic callus lines.

The callus differentiated into shoots when transferred to MS medium containing 2mg/l kinetine and 0,2 mg/l 2,4- dichlorophenoxyacetic acid.. After 30 days, green shoots appeared and roots were developed in MS medium with no growth regulators. Root induction on shoot cultures was achieved by subculturing the shoots.

Comparative vegetative anatomy between *in vitro* regenerated and parent plant

In the regenerated plant resulted here by subculturing the shoots, the adventitious root has an endogenous origin and presents primary structure. The stele is of triarch type, showing a multiseriate pericycle and a developed vascular system. The cortex is hypertrophied and do not end with an obviousness endodermis.

According to Toma *et al.* (1985), *in vivo* *Chrysanthemum morifolium* Ramat (Romica cultivar) plant, the adventitious roots formed on the rhizome passes early from primary to secondary structure, resulted from the activity of both lateral meristems (cambium and phellogen). In the primary structure, the stele is of triarch type and the endodermis has Casparian strips.

The excellence of the root system is a key factor for the success of the acclimatization process (Gonçalves *et al.*, 1998). The incomplete vascular connection between shoot and roots of cauliflower rooted *in vitro* resulted in insufficient water translocation to the shoot, endangering the acclimatization of the new plants (Grout & Aston, 1977). The histo-anatomical differences observed here in the adventitious roots formed on the shoots of regenerated plant could change the performance of plant acclimatization.

Despite the stressing conditions of *in vitro* culture media, the stem and leaf of the regenerated *Chrysanthemum morifolium* Ramat (Romica cultivar) plant conserve the structural layout of the parent plant ones.

Along the stem (Fig. 1), from top to base, the passing from primary to secondary structure may be observed. Comparatively with the parent plant, the histological characters of the regenerated plant stem are similar, with small differences: the absence of the cortical hadrocentric vascular bundles, the hypertrophy of the cortical parenchyma, the absence of an obviousness endodermis (see table I and II).

In both parent and regenerated plant, the foliar limb (Fig. 2) has bifacial heterofacial structure, the mesophyll being differentiated in one layer of palisade and 4-5 layers of spongy parenchyma cells. Each lateral vascular bundle is surrounded by a parenchymatous theca.

Both epidermis of *Chrysanthemum morifolium* Ramat (Romica cultivar) leaf in regenerated and parent plant bear apart from stomata, glandular hairs (producing of essential oils) and non-glandular hairs. The foliar limb is amphistomatic, the stomata being of anomocytic type. The glandular hairs have uni- or bicellular gland. The non-glandular hairs are multicellular, with shuttle-like shape on superficial section.

Table I. Comparison between the structure of the upper third of stem in *Chrysanthemum morifolium* Ramat (Romica cultivar) *in vitro* regenerated and parent plant

Stem structural layout (primary structure)	Histological features	
	Regenerated plant (on mature stage)	Parent plant (on mature stage)
epidermis	single-layered with stomata, glandular and non-glandular hairs	single-layered with stomata, glandular and non-glandular hairs
cortex	parenchymatous-cellulosic of meatic type	-parenchymatous-cellulosic of meatic type - cortical vascular bundles of hadrocentric type
stele	- collateral vascular bundles(disposed on a circle) + isolated groups of phloem elements - secretory canals (localized in the medullary rays or at the big vascular bundles periphery)	- collateral vascular bundles(disposed on a circle) + isolated groups of phloem elements - secretory canals (localized in the medullary rays or at the big vascular bundles periphery)
pith	parenchymatous-cellulosic of meatic type	parenchymatous-cellulosic of meatic type

Table II. Comparison between the structure of the lower third of stem in *Chrysanthemum morifolium* Ramat (Romica cultivar) *in vitro* regenerated and parent plant

Stem structural layout (secondary structure)	Histological features	
	Regenerated plant (on mature stage)	Parent plant (on mature stage)
peridermis	the phellogen is differentiated from the hypodermic layer	the phellogen is differentiated from the hypodermic layer
cortical parenchyma	- meatic type - numerous cells with pericline and anticline division walls	meatic type
endodermis	not evident	Casparyan type
stele	- bundles of secondary phloem (at the outer part) - periphloemic cordons of sclerenchymatous fibers - secondary lignified xylem ring - primary vessels surrounded by cellulosic parenchymatous cells	- bundles of secondary phloem (at the outer part) - periphloemic cordons of sclerenchymatous fibers - secondary lignified xylem ring - primary vessels surrounded by cellulosic parenchymatous cells

	(in the inner part)	(in the inner part)
pith	-parenchymatous –lignified (in the outer part) - parenchymatous-cellulosic of meatic type (in the central part)	- parenchymatous –lignified (in the outer part) - parenchymatous-cellulosic of meatic type (in the central part)

According to Hazarika (2006), the special conditions during *in vitro* culture results in the formation of plantlets of abnormal morphology, anatomy and physiology. Tissue culture conditions that promote rapid growth and multiplication of shoots often results in the formation of structurally and physiologically abnormal plants.

The results of our histo-anatomical study revealed that in *Chrysanthemum morifolium* Ramat (Romica cultivar), the stressing conditions of *in vitro* culture media and of the confined environment do not affect the structural layout of the vegetative organs.

Comparative morphometric analysis between *in vitro* regenerated and parent plant

The density of glandular hairs was found to be greater in both foliar surfaces of the regenerated plant than in the parent plant ones (Fig. 3). The “t” test indicated significant differences ($P \leq 0,01$) in glandular hair number between regenerated and parent plant.

With respect to the non-glandular hairs, leaves of regenerated plant were more (on upper surface) or less (on lower surface) hairy than those of parent plant (Fig. 4). The “t” test indicated no differences ($P > 0,05$) in non-glandular hair number between regenerated and parent plant.

The leaf surface represents the interface between the plant and the environment. This lead to study the surface of *in vitro* grown plant leaves in comparison to the *in vivo* mother plants (Bandyopadhyay *et al.*, 2004). According to Brutti *et al.* (2002), the trichomes of *in vitro* leaves are poor developed and scanty distributed. Despite this observation our morphometric results revealed no significant differences in non-glandular hairs number between *Chrysanthemum morifolium* Ramat (Romica cultivar) regenerated *in vitro* and parent plant. The density of glandular hair increases in regenerated plant leaves comparatively with the parent plant ones.

The stress in *in vitro* culture may thus contain both destructive and constructive elements: it is a selection factor as well as a driving force for improved resistance and adaptive evolution (Gaspar *et al.*, 2002). The frequency of somaclonal variation would depend on the culture protocol applied during the *in vitro* process, particularly on the hormone composition of the medium and the number of subcultures (Ducos *et al.*, 2003).

Thus, the organogenic regeneration procedure using in our study would not cause destructive structural variations of vegetative organs in *Chrysanthemum morifolium* Ramat (Romica cultivar).

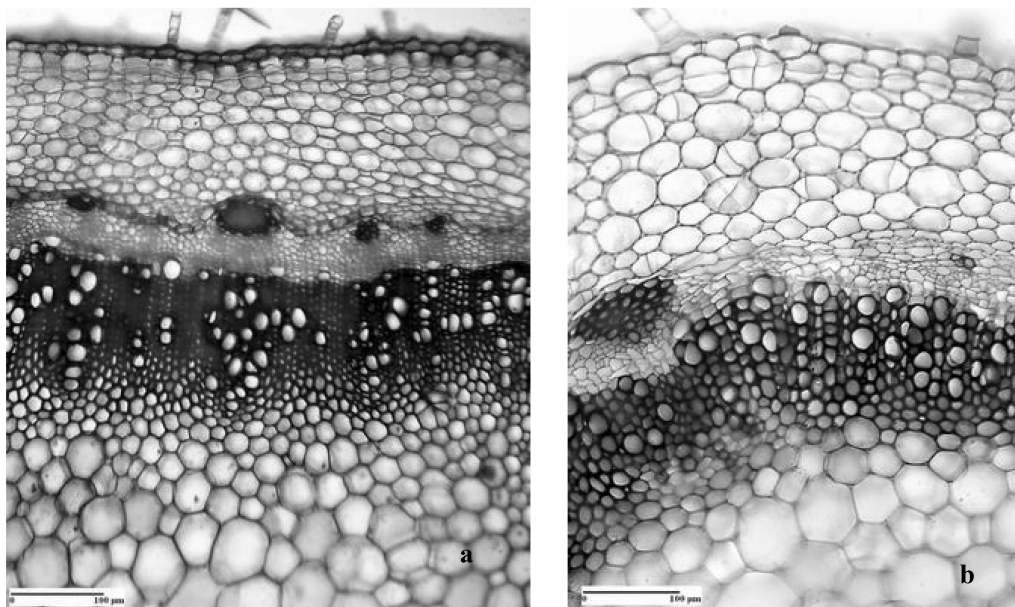


Fig. 1. Cross-section through the lower level of stem in *Chrysanthemum morifolium* Ramat (Romica cultivar) parent plant (a) and *in vitro* regenerated plant (b).

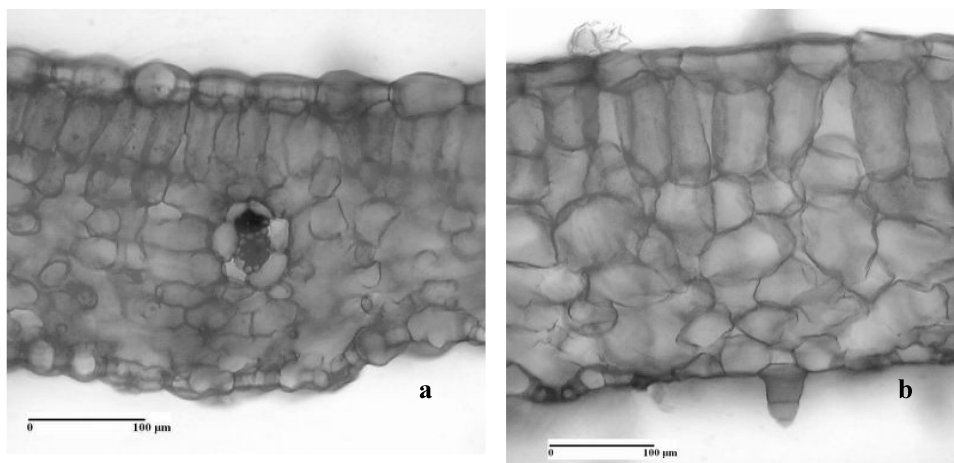


Fig. 2. Cross-section through the mesophyll of leaf in *Chrysanthemum morifolium* Ramat (Romica cultivar) parent plant (a) and *in vitro* regenerated plant (b).

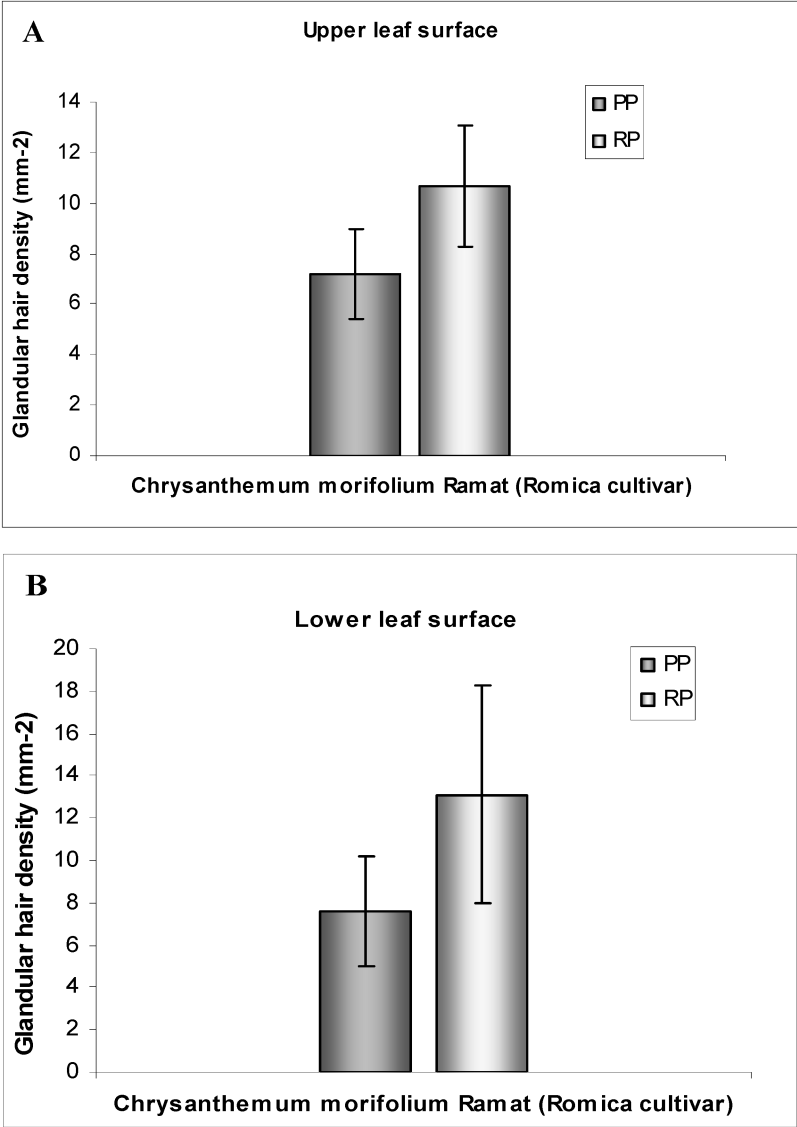


Fig. 3. Comparative glandular hair density on the upper (A) and lower (B) leaf surfaces in *Chrysanthemum morifolium* Ramat (Romica cultivar) parent plant (PP) and *in vitro* regenerated (RP) plant (\pm s.d., n=10).

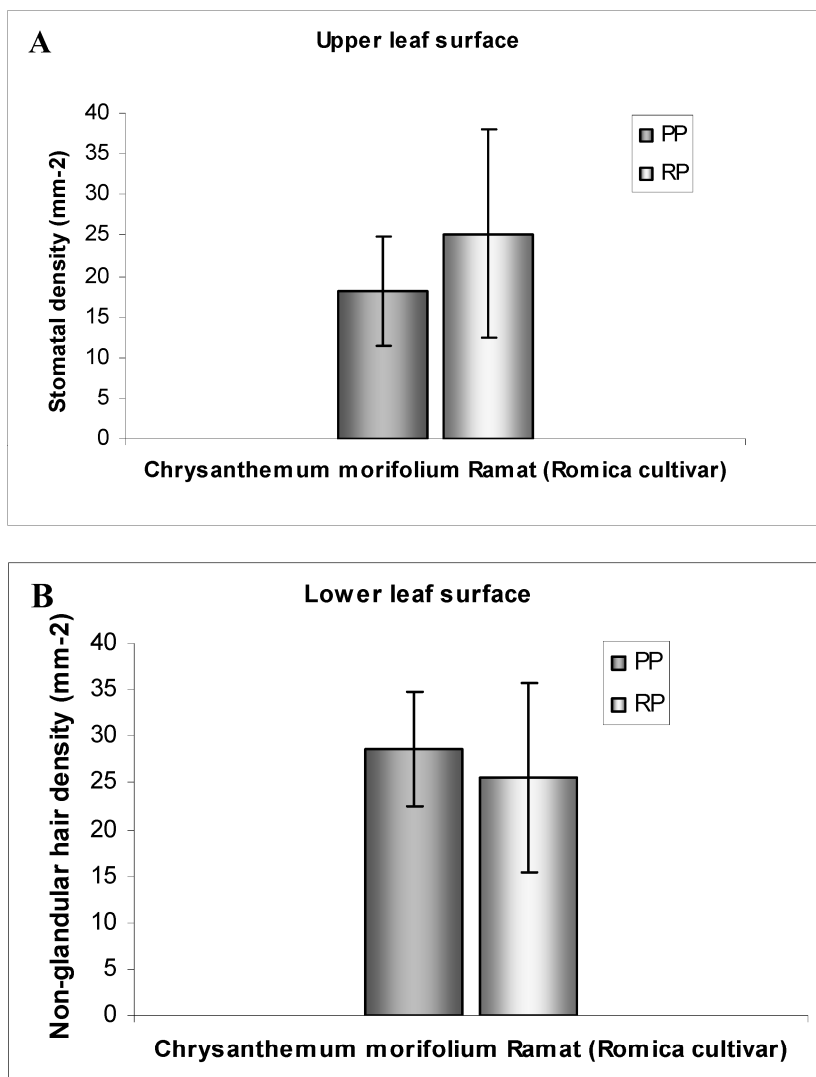


Fig. 4. Comparative non-glandular hair density on the upper (A) and lower (B) leaf surfaces in *Chrysanthemum morifolium* Ramat (Romica cultivar) parent plant (PP) and *in vitro* regenerated plant (RP) (\pm s.d., n=10).

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CYTOGENETIC EFFECTS OF IRRADIATION WITH UV AT 6 ROMANIAN CULTIVARS OF *PHASEOLUS VULGARIS* L.

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Key words: chromosomal aberrations, UV radiations, bean, *Phaseolus vulgaris* L., cultivar.

Abstract: In the actual conditions of increased UV radiation level on Earth surface, the analysis of chromosomal aberrations which may appear during cell division in root apex is important for the estimation of mutagenic effect of UV. The study proves that the cytogenetic effect of UV-B on 6 Romanian bean cultivar (Vera, Ami, Ardeleana, Avans, Star, and Diva), exposed to UV-B/white light for 0,5h; 1,5h; or 3h is very similar, the DNA lesions leading to the same types of chromosomal aberrations, and with aberrations frequency correlated with the mitotic index. For all cultivars, the percent of mutations occurrence are similar to the natural induced ones, proving the low mutagenic effect of UV.

INTRODUCTION

Ozone selectively filters out the shorter UV wavelengths, so the most important consequence of the ozone depletion is an increase in the amount of UV-B (280-315nm) radiation reaching the earth's surface (Björn *et al.*, 1999a). The shift of spectral UV composition towards shorter wavelengths has on higher plants damaging effects, including DNA damage (Buchholz *et al.*, 1995). UV-B is efficiently absorbed by plants and can cause damage to nucleic acids, proteins, lipids, plant pigments, also direct, as well as mediated by reactive – oxygene-species (Bornman *et al.*, 1997).

Chromosomal aberrations occurred during cell division, as response to mutagenic agents, has been investigated in several plant species, but information of behavior shown by different cultivars of the same species can be important for the study of individual variability in a population. In the present study, we examined the chromosomal aberrations induced by UV-B radiation at six romanian cultivars of *Phaseolus vulgaris* L. In *Phaseolus vulgaris* L, UV-B caused reduction in the levels of carotenoid and of chlorophyll *a*, *b* (Strid and Pora, 1992).

The choice of *Phaseolus vulgaris* as biological material for investigations, can be explained by the importance of seeds and legumes in humans nutrition, being considered since centuries the poor peoples “meat” because of the increased level of high quality proteins, and the high energetically level, and due to the importance in soil amelioration, being used as very good previous culture plant for many other species.

MATERIAL AND METHODS

Biological material: *Phaseolus vulgaris* seeds of 6 Romanian cultivars

Mutagenic agent: UV-B radiations. Light sources designated UV/white light, is a mixture of:

1. 2 L 40 W/73 (UV-A), Osram
2. 2 TL 40 W/18 (Blue Light), Philips
3. 1 TL 40 W/12 (UV-B), Philips.

Fluency rate of UV-B lamp is 3 W m^{-2} , total fluency rate is 8 W m^{-2} .

The lamps were positioned in the next order: 1, 2, 3, 2, 1.

The Osram L 40 W/73 (UV-A source) includes sufficient UV-B radiation to activate UV-B photoreceptors (Beggs, Wellmann, 1985).

Relevant WG type cut-off filters (Schott and Gen, Mainz, Germany) were used for the experiment: WG 305 with 11, 9 W m^{-2} fluency rates, WG 320 (11 W m^{-2}) and WG 360 (8, 3 W m^{-2}). The last one was used for UV-B control. The filters cut UV-B radiation with certain wavelength (50% transmission for the given wavelength). The quartz filter (Q) does not cut off any UV radiation, has 100% transmission for the full UV spectrum.

Working steps:

1. Seeds germination: 20 seeds for each experimental variant, from each cultivar were sown in transparent plastic 9/9 cm boxes filled with vermiculite (Deutsche vermiculite Dämmstoff GmbH Sprockhövel), in growth chamber, in dark, at 25° C.

2. UV-B/white light irradiation: for each cultivar, there were 6 experimental variants (5 treatment variants and 1 control). For treatment variants, 72h old seedlings (15 for each variant) were irradiated in boxes covered with cut-off filters (WG 360, WG 320, WG 305, WG 275, Q) for 0,5h; 1,5h; or 3h with the light source described above. For control variant, 15 seedlings (72h old) were kept in dark at 25° C.

3. Cytogenetic studies: after irradiation, roots were colored by Feulgen method and microscope slides were prepared using root tips of 0, 5 – 1 cm, following Squash techniques for cytogenetic studies (Cîmpeanu *et al.* 2002).

RESULTS AND DISCUSSIONS

The frequency of cells with chromosomal aberrations

If we consider the values induced by different wavelength UV (for **0,5h** irradiation time) in ana-telophase (A-T) of the 6 bean cultivars (Vera, Ami, Ardeleana, Avans, Star, Diva), it could be noticed that the frequency of cells with chromosomal aberrations increase with the decrease of radiation wavelength (meaning UV-A, to UV-B and finally UV-C kind of radiation). The maximal number and types of aberrations were found in the case of full spectrum UV (filter Q), especially for Ami and Diva cultivar, following Vera, Star, Ardeleana and Avans (Fig. 8).



Fig. 1. A-T with expelled chromosomes, Ardeleana, Q, 0,5h

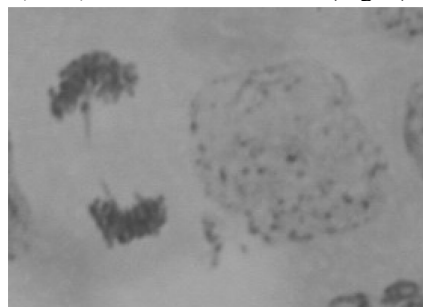


Fig. 2. A-T with broken bridge, Ardeleana, WG275, 0,5h

For **1,5h** irradiation time, results were similar regarding aberrations frequency connected with cultivar type: the frequency of cells with chromosomal aberrations increase with the decrease of radiation wavelength, the maximal number and types of aberrations were found in the case of full spectrum UV (Fig. 9).



Fig. 3. A-T with broken bridges, retardate chromosome, Vera, Q, 1,5h



Fig. 4. A-T with broken bridges, expelled, retardate chromosome, Star, WG32 01,5h

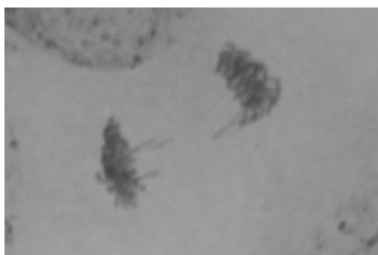


Fig. 5. A-T with broken bridge, retardate chromosome Avans, WG275, 1,5h

For **3h** irradiation time, it could be observed the increase of aberration frequency for all irradiations variants (WG 360, WG 320, WG 305, WG 275, Q), meaning UV-A, UV-B, UV-C and full spectrum of UV, proving the importance of treatment period next to UV harmfulness (increasing with the decrease of wavelength) in disturbing cell division (Fig. 10).

The Ardeleana, Star, Diva, Ami and Vera cultivars reacted very similar to Avans. Aberrations types were in order of their occurring frequency: simple or multiple bridges, retardate chromosomes, expelled chromosomes (Fig. 1), simple or multiple bridges combined with chromosomes or chromosomal fragments (Fig. 2, Fig. 3, Fig. 4) and in a very low percent some other aberrations types as more than one retarded chromosomes, expelled genetic material, and multiple division poles. Ami cultivar presented also binucleated cells.

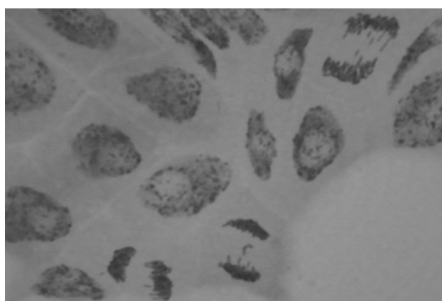


Fig. 5. A-T with multiple broken bridges, retardate chromosome Diva, Q 3h

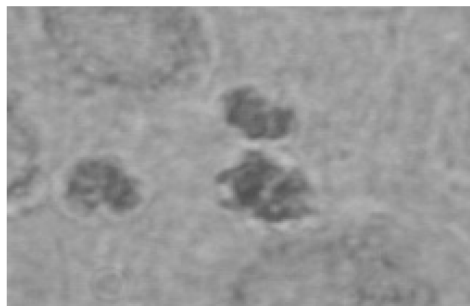
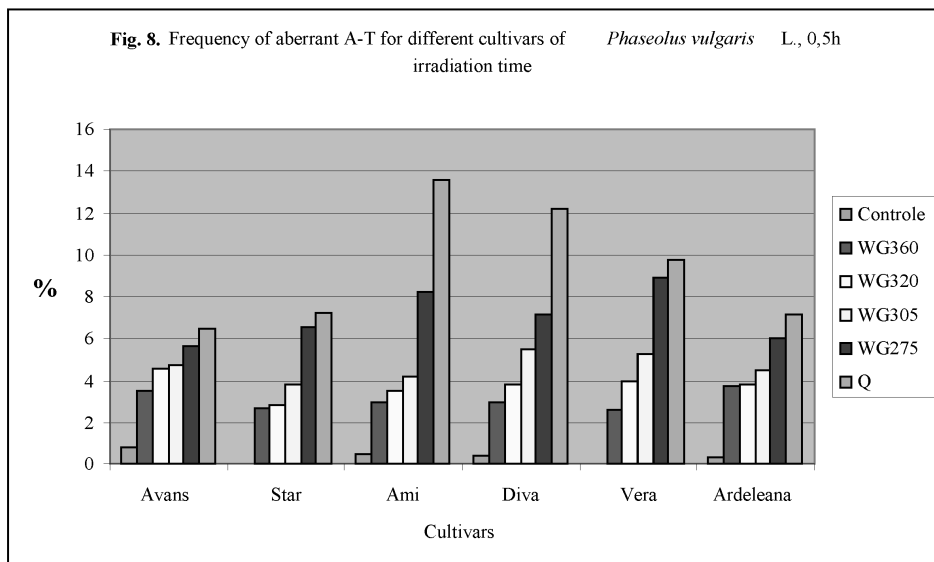


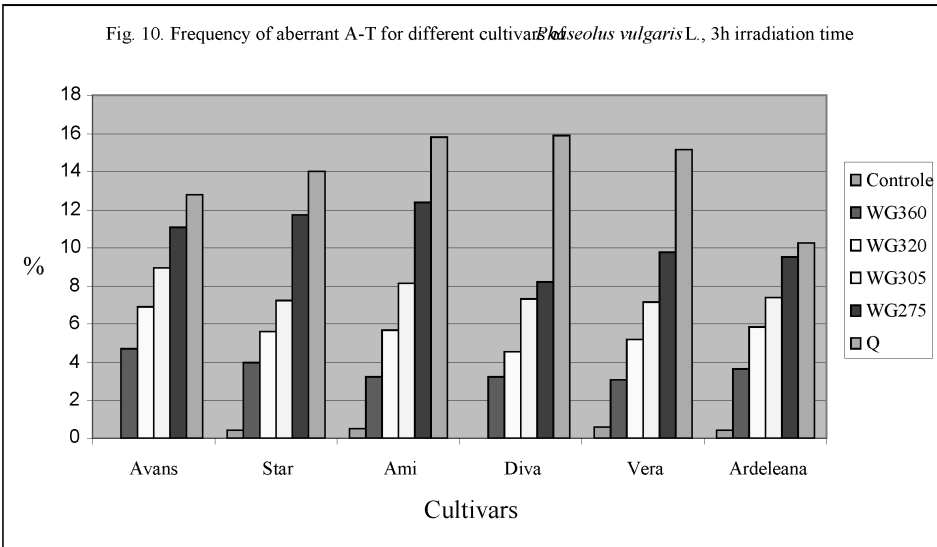
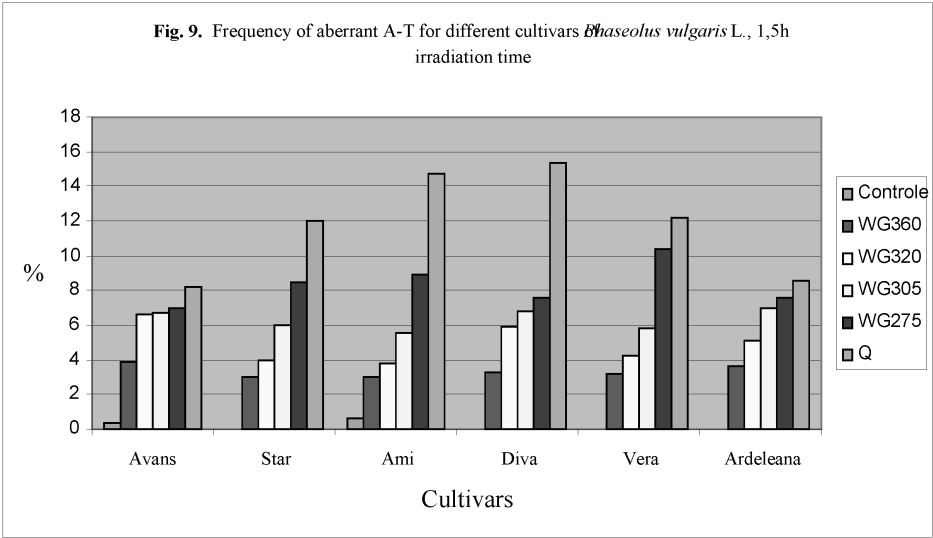
Fig. 6. Tripolare A-T, Avans, Q, 3h

The most frequent aberration types induced by UV, in the case of Avans cultivar, are multiple broken bridges, but could be observed also combinations between bridges and retardate or expelled chromosomes, one or more retardate chromosomes, unequal and also tripolar anaphase (Fig. 5, Fig. 6).



After analyzing aberrations type, it can be noticed that in the case of all investigated cultivars, UV irradiation induced lesions at DNA level but also affected division spindle.

For all the 6 cultivars, the results regarding the aberrations frequency can be correlated with the mitotic index. The aberrations frequency value increases and the mitotic index decrease (as a plant protection mechanism) correlated with the decrease of UV wavelength and increase of irradiation time (Băra, Grama-Țigănaș, 2005). The mitotic index decrease, proves an inhibition of cells division, shown that a supra UV-B dose could cause reduction in plant growth and in biomass production, similar to some other studies (Sullivan and Terramura, 1989, Tosserams *et al.*, 2001).



CONCLUSIONS

For all 6 investigated cultivars, the frequency of aberrations induced by UV, increase with the decrease of wavelength and with the increase of irradiation time, but the percent of mutations occurrence are similar to the natural induced ones, proving the low mutagenic effect of UV.

After analyzing aberrations type, it can be noticed that in the case of all investigated cultivars, UV irradiation induced lesions at DNA level but also affected division spindle, occurring multipolar ana-telophase, retardate chromosomes.

All the 6 cultivars reacted very similar, regarding both aberrations type or aberrations frequency, as response to irradiations with different UV wavelength for different irradiation time, so no cultivar can be considered the most resistant.

The maximal number and types of aberrations were found in the case of full spectrum UV, correlated with the mitotic index decrease.

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

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Keywords: barley, hydroalcoholic rosemary extract, mitotic index, chromosome aberrations

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

INTRODUCTION

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

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

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

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

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

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

MATERIAL AND METHODS

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

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

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

The results obtained are presented in Tables 1-4.

RESULTS AND DISCUSSIONS

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

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

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

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

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

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

1.2. Frequency of the cells with chromosome aberrations

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

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

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

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

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

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

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

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

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

2.2. The frequency of the cells with chromosome aberrations

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

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

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

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

CONCLUSIONS

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

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

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ACUTE LIPOPOLYSACCHARIDE ADMINISTRATION IMPAIRED IMMUNE RESPONSIVENESS IN NORMAL RATS

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Keywords: lipopolysaccharide, corticosterone, leukocyte, immune response

Abstract: Involving of endotoxin lipopolysaccharide (LPS) on immune response modulation was examined in adult male Wistar rats. Acute LPS injection (25μg/kg, i.p, Sigma) increased serum corticosterone, suggesting significant effects on hypothalamic-pituitary-adrenal axis (HPAA) activity. In addition, acute LPS administration significantly decreased the number of leukocyte, the number of lymphocyte, total serum protein, antibody titer, body weight and significantly increased albumin-globulin ratio, suggesting that LPS significantly impaired immune responsiveness. Taken together, our data provide further support for modulation of immune response during acute LPS administration.

INTRODUCTION

The neuroimmune response is characterized by a bi-directional communication between peripheral immune cells and the central nervous system. Of particular importance during times of infection is the activation of the centrally mediated febrile and corticosterone responses, which aid the innate immune system in the effective clearance of the pathogen while limiting the extent of inflammatory damage (Schobitz et al., 1994; Jiang et al., 1999). Inhibition of these innate immune responses has been shown to significantly affect the morbidity and mortality with infection (Kluger et al., 1998; Jiang et al., 2000; Nadeau and Rivest, 2003).

There is growing evidence that the neonatal immune environment can program the subsequent neuroimmune responsiveness of the adult. A single challenge with the bacterial endotoxin lipopolysaccharide (LPS) during the neonatal period can significantly alter the febrile, neuroendocrine, neurochemical and behavioral responses of the adult (Shanks et al., 2000; Boisse et al., 2004; Ellis et al., 2005; Spencer et al., 2005). A heightened neuroendocrine response, resulting in increased levels of corticosterone, has been shown to underlie the attenuated inflammatory responses to an adult LPS challenge (Ellis et al., 2005), highlighting that programming of these centrally mediated responses by the neonatal immune environment can alter other facets of the innate immune response as well. However LPS, derived from gram-negative bacteria, represents only one type of immune stimulus, which is mediated by the toll-like receptor (TLR)-4 pathways (Tapping et al., 2000).

The aim of the present work was to study the effect of acute activation of the immune system induced by LPS administration.

MATERIALS AND METHODS

Animals

30 male Wistar rats weighing 200-250 g at the start of the experiment were used. The animals were housed in a temperature- and light-controlled room (22°C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water ad libitum. Rats were treated in accordance with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and all procedures were in compliance with the European Council Directive of 24 November 1986 (86/609/EEC).

Acute LPS administration

The rats were injected, once, with 25μg/kg LPS (lipopolysaccharide from *Escherichia coli*, serotype 0111:B4; Sigma) in 250 μl saline i.p. The saline vehicle received the same doses of physiological saline (0,9% NaCl solution). 7 days postimmunization, whole heparinized blood was collected. To determine the count of leukocyte (μl), blood sample was taken with a leukocytes pipette and diluted (1/20) with the Türk solution. One drop of hemolyzed blood was transferred onto Neuberg's haemocytometer, on the counting area of the haemocytometer and than coverslipped. The blood sample was therefore monolayered in a space of 0.1 mm height. The total number of leukocyte was in a 5mm² area was counted and expressed as the number of leukocyte from 1 μl whole blood.

The blood samples were examined in a light microscope KRÜSS model with a magnification of 400 x. The leukocyte formula was determined by means of blood smears stained with May Grünwald-Giemsa. To determine total serum protein, antibody titer and albumin/globulin ratio we used the Weichselbaum method (biuret test for proteins and the technique for albumins).

Corticosterone ELISA

Blood samples (300 μl) were collected in heparinized tubes and immediately centrifuged at 4°C and plasma was stored frozen at -80°C until it was assessed. A corticosterone ELISA kit was used to assay the plasma samples for

free corticosterone. The precision and sensitivity of this assay was: interassay variability, 7.8–13.1% CV; intra-assay variability, 6.7–8.0% CV; and sensitivity, 27 pg/ml. Corticosterone concentration is expressed as $\mu\text{g/dl}$.

Statistical analysis

All data are presented as the mean \pm S.E.M. Comparisons between groups were performed using Student's *t*-test. $p < 0.05$ was taken as the criterion for significance.

RESULTS AND DISCUSSIONS

1. Effect of acute LPS administration on immune responsiveness of rats

Experimental data were registered 7 days after acute LPS administration. Acute LPS administration, once, significantly impaired the total number of leukocyte ($p < 0.05$) (Figure 1.), the total number of lymphocyte ($p < 0.05$) (Figure 2.), total serum protein ($p < 0.05$) (Figure 3.), antibody titer ($p < 0.03$) (Figure 4.) and body weight ($p < 0.05$) (Figure 5.) and significantly increased albumin-globulin ratio ($p < 0.02$) (Figure 6.) compared to the saline treated-groups. In addition, LPS increased serum corticosterone concentration ($p < 0.05$) (Figure 7.), suggesting significant effects on HPAA axis.

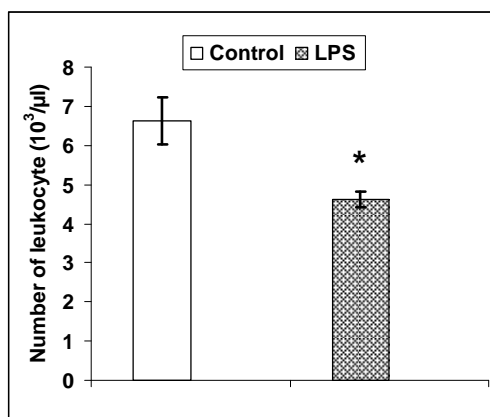


Figure 1. Changes of the total number of leukocyte tested 7 days after acute LPS administration ($25\mu\text{g/kg}$).

Values are means \pm SEM ($n=15$ per group). * $p < 0.05$ vs. Control group.

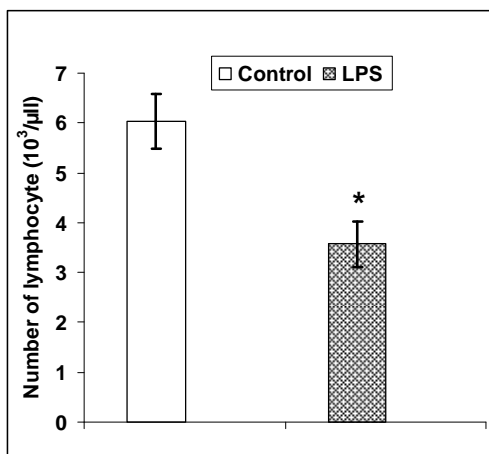


Figure 2. Changes of the total number of lymphocyte tested 7 days after acute LPS administration ($25\mu\text{g/kg}$).

Values are means \pm SEM (n=15 per group). *p<0.05 vs. Control group.

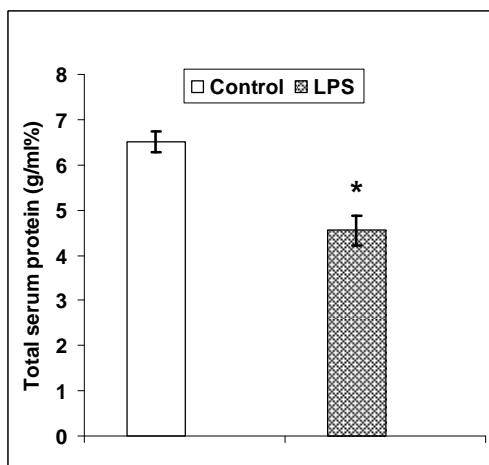


Figure 3. The effect of acute LPS administration ($25\mu\text{g/kg}$) on the total serum protein level tested 7 days after immunization. Values are means \pm SEM (n=15 per group). *p<0.05 vs. Control group.

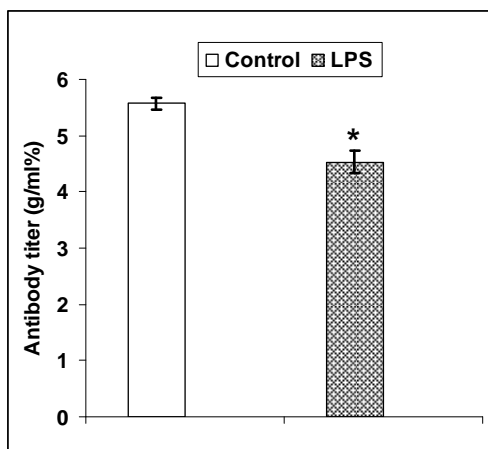


Figure 4. The effect of acute LPS administration (25 μ g/kg) on the antibody titer tested 7 days after immunization. Values are means \pm SEM (n=15 per group). *p<0.03 vs. Control group.

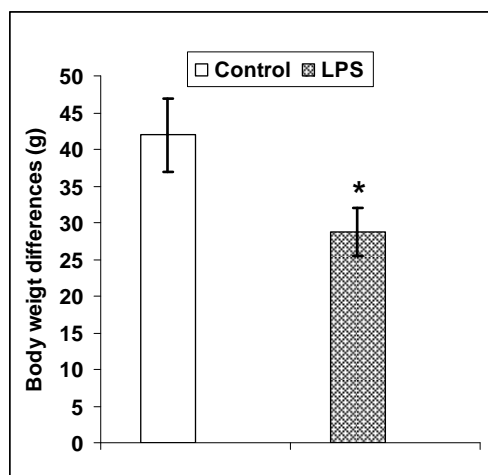


Figure 5. Effect of acute treatment with 25 μ g/kg LPS on body weight. Values are means \pm SEM (n=15 per group). *p<0.05 vs. Control group.

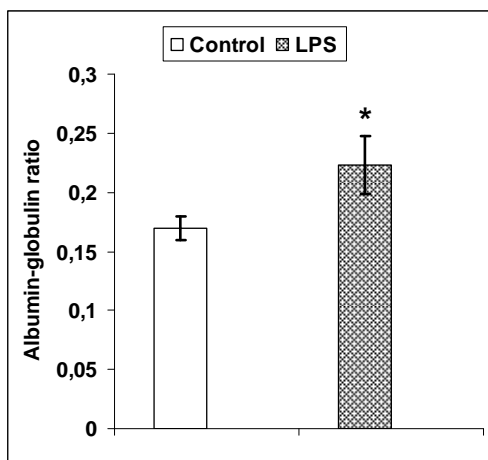


Figure 6. Effect of acute treatment with 25μg/kg LPS on albumin-globulin ratio. Values are means ± SEM (n=15 per group). *p<0.02 vs. Control group.

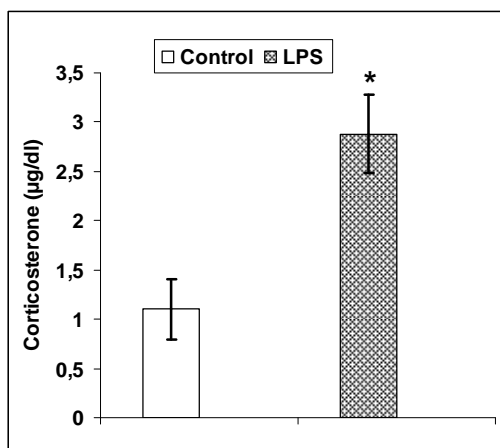


Figure 7. Effect of acute treatment with 25μg/kg LPS on serum concentrations of corticosterone. Values are means ± SEM (n=15 per group). *p<0.05 vs. Control group.

The present study highlights several important findings regarding the effect of acute LPS administration on immune response modulation of male Wistar rats. First, we have shown that a single dose of LPS, acute administrated could impaired the immune responsiveness of rats, in term of decreased the total number of leukocyte and lymphocyte, total serum protein, antibody

titer and body weight. Second, we have shown that acute LPS administration induced stress, in term of increased serum corticosterone level.

Peripheral administration of low doses of LPS is a very useful model for immune activation. It acts by binding to TLR-4 receptors in various organs, including endothelia. This ultimately results in the synthesis and secretion of IL-1. Not surprisingly, LPS elicits very similar neurochemical, endocrine, and behavioral responses to those of influenza virus and IL-1. It elevates plasma concentrations of ACTH and corticosterone, indicating HPA axis activation. It also increases brain 3-methoxy,4-hydroxyphenylethylene glycol (MHPG, the major brain catabolite of norepinephrine), indicating activation of the brain noradrenergic systems, preferentially in the ventral (diencephalic) system, and increases brain 5-hydroxyindoleacetic acid (5-HIAA, the major catabolite of serotonin), indicating activation of brain serotonergic systems, and increasing brain concentrations of tryptophan (Trp) (Dunn, 1992a,b). Each of these responses resembles those to IL-1. The differences are that the increases in plasma ACTH and corticosterone and the neurochemical responses were significantly slower, perhaps reflecting the delay involved in the induction of IL-1 by LPS. The distinction between the MHPG responses in the dorsal and ventral projection systems is less marked with LPS than with IL-1, and LPS also exhibits a significant activation of dopaminergic systems (i.e., increases in 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) not normally observed with IL-1.

Our results provide further support for involving of LPS on immune response modulation of rats.

CONCLUSIONS

On the basis of our results obtained by LPS administration, we can conclude that peripheral administration of acute LPS impaired immune responsiveness in rats and increased stress response.

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OVEREXPRESSION OF P53 IN GASTRIC CARCINOMAS AND IT'S CORRELATION WITH LAUREN AND GOSEKI CLASSIFICATION

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Key words: gastric cancer, P53

Abstract. Despite their importance, prognostic factors in gastric cancer are not yet well known. The study included surgical specimens from 40 patients with gastric carcinoma. All specimens were fixed in 10% buffered neutral pH formaldehyde and paraffin embedded. Histological sections were stained using current techniques: haematoxylin-eosin, trichromic van Gieson, and Alcian blue. We used Laurén histological classification with two main types of gastric carcinoma: intestinal and diffuse and Goseki classification with four main types of gastric carcinoma. We assessed p53 immunoreactivity. (DO 7 Biogenex). Using Lauren classification, immunoreactivity was positive for p53 in 62.5% of the cases: 7 cases of well differentiated intestinal low index gastric carcinoma, 1 case of poorly differentiated intestinal low to moderate index gastric carcinoma, 15 cases of diffuse moderate to high index gastric carcinoma. Using Goseki classification, immunoreactivity was positive for p53 in 62.5% of the cases: 3 cases with low index and 1 case with moderate index in type I, 3 cases with low index in type II, 9 cases with low to high index in type III and 6 cases with low to high index in type IV. The positivity for p53 was expressed in 66% of the gastric carcinomas with a coloring intensity growth pattern from the well differentiated types to the poorly differentiated types. The percentage of tumors positive for p53 did not correlate with their differentiation degree.

INTRODUCTION

Gastric adenocarcinomas make up 90-95% of all malignant stomach tumors (1). Due to endoscopic investigations correlated with histopathological examinations, early adenocarcinoma has been having increasingly good prognosis, contrary to advanced gastric carcinoma. Epidemiological, immunohistochemical (IHC) and progressive data also confirm the two types of most common histological variants, that is intestinal and diffuse gastric carcinomas (2).

Just as the WHO classification, Goseki classification (1992) relies on histological evidence. Tubular differentiation assessment and intracytoplasmic mucus amount determination enable us to distinguish 4 categories (3):

- Type I – well differentiated tubes and scarce mucus.
- Type II – well differentiated tubes and abundant mucus.
- Type III – poorly differentiated tubes and scarce mucus.
- Type IV – poorly differentiated tubes and abundant mucus.

Numerous studies dwelt on IHC used for prognosis determination, together with protein gene products.

Normal P₅₃ protein has an extremely short life and can be found in very low amounts inside the cells. It cannot be detected in normal cells using IHC techniques. P₅₃ protein produced by the abnormal *p53* gene has a much longer life than normal P₅₃, it gathers inside malignant cells and may be IHC detected by means of Ac anti P₅₃. Theoretically, P₅₃ overexpression as a surrogate marker of gene *p53* mutations is an IHC “test” employed to determine neoplasia – dysplasia. In practice however, we cannot say the same since a correlation between stain and gene *p53* abnormalities is not precise.

Therefore, the purpose of this study is to assess p53 immunoreactivity in gastric carcinoma and its correlation with Lauren and Goseki classification.

MATERIAL AND METHODS

The study material consisted of surgical specimens from 40 patients diagnosed with gastric neoplasm. All specimens were fixed in 10% buffered neutral pH formaldehyde and paraffin embedded. Histological sections were stained using current techniques: haematoxylin-eosin, trichromic van Gieson, Alcian blue, and PAS, which enabled us to classify injuries and determine their differentiation. We employed Lauren and Goseki classification to assess the histological types. (4)

Immunohistochemical stain using the Avidin-Biotin Complex method was performed by means of the Optimax autostainer manufactured by Biogenex. Pretreatment consisted of 12-15min. boiling in citrate solution followed by 2 hours incubation with p53 antigen (DO7 Biogenex – Ready to use). DAB viewing followed by a slight haematoxylin stain (1min) revealed p53 under the form of a brown reaction color with nuclear location (5,6,7).

Statistical processing was performed using the Spearman Rank R Correlation Test, Chi-Square Anova tests.

RESULTS AND DISCUSSIONS

P₅₃ expression in the 40 cases under survey was graded according to a scale based on the percentage of tumor cells with positive nucleus (8)

- 0 negative stain;
- + positive reaction in less than 10% of the tumor cells nuclei;
- ++ positive reaction in less than 10% of the tumor cells nuclei, however less than 33%;
- +++ positive reaction in less than 33% of the tumor cells nuclei.

We interpreted a positive p53 reaction, with distinct homogenous or granular nuclear stain. The reaction product was brown (9).

We also considered that tumors overexpress p₅₃ protein only when we obtained an intense nuclear stain in any of the 3 patterns. We considered negative the cases with an equivocal or poor nuclear stain. Likewise for the cases with extremely scarce or isolated positive nuclei.

We found no positive p₅₃ cells on the normal gastric epithelium neighboring the tumor and the negative witness slides. We detected an overexpression of the P₅₃ protein in 25 (63 %) of the 40 gastric tumors under survey, with a stain intensity and stain pattern heterogeneity (**Table 1**).

Table no.1 *p₅₃ overexpression in the 40 cases under survey*

P ₅₃	No. cases	%
0	15	37.5%
+	9	22.5%
++	6	15%
+++	10	25%
Total	40	

By correlating p₅₃ overexpression with Lauren histological classification we identified the following:

- 8 of the **12 intestinal ADK** (66.6%) have p₅₃ overexpression. This overexpression was marked + in 7 of the cases and ++ in 1 of the cases (this case being moderately differentiated, (**Fig. 1, 2**);
- 15 of the **26 diffuse gastric carcinomas** (57.6%) have overexpression p₅₃ overexpression. This overexpression was marked ++ in 5 of the cases and +++ in 10 of the cases (**Fig. 3**)
- the 2 mixed forms both had P53 protein overexpression, one being marked + and the other ++.

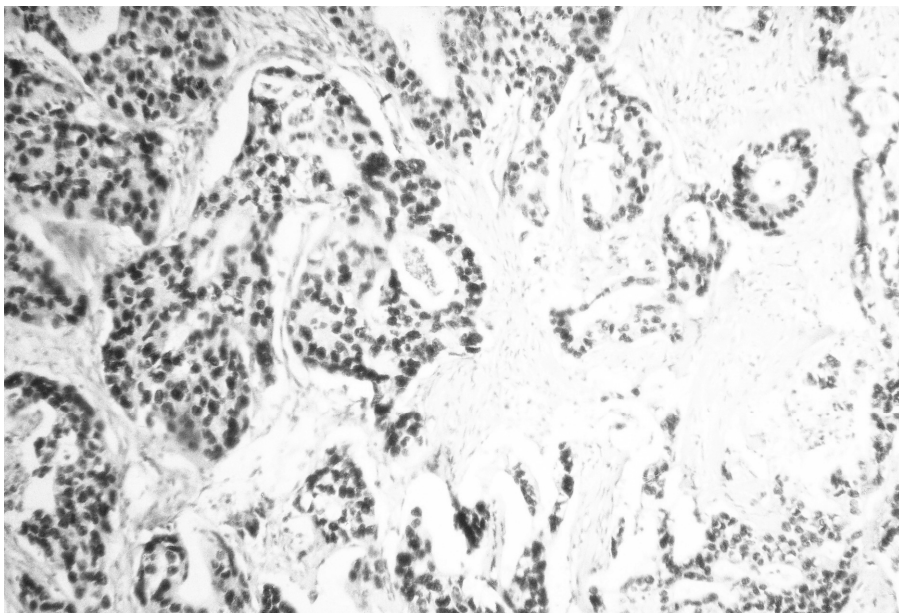


Fig. 1. *Distinct homogeneous or granular nuclear stain revealing p_{53} (++) in a moderately differentiated ADK, IHC x40*

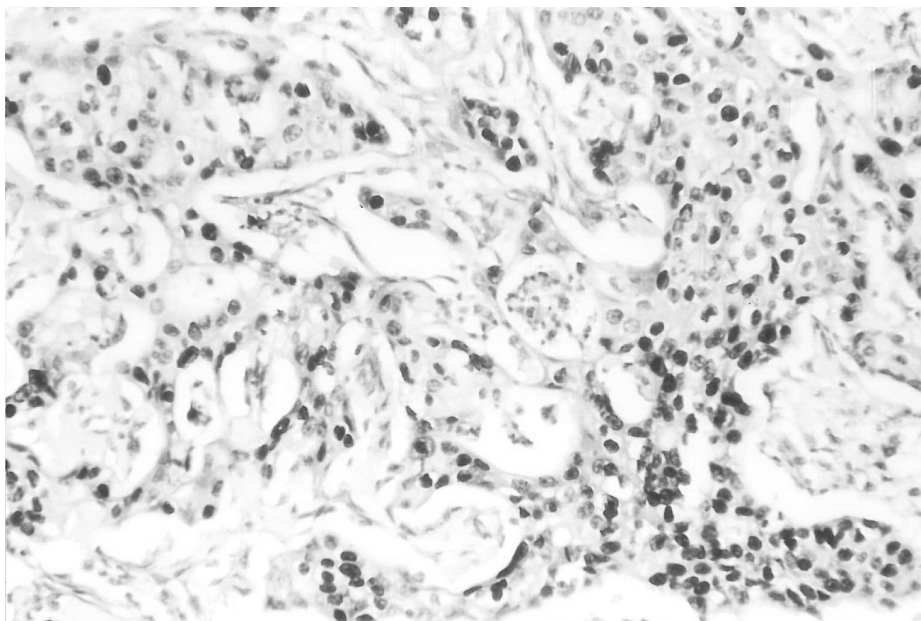


Fig. 2. *Distinct homogeneous or granular nuclear stain revealing p_{53} (++) in a moderately differentiated ADK, IHC x40*

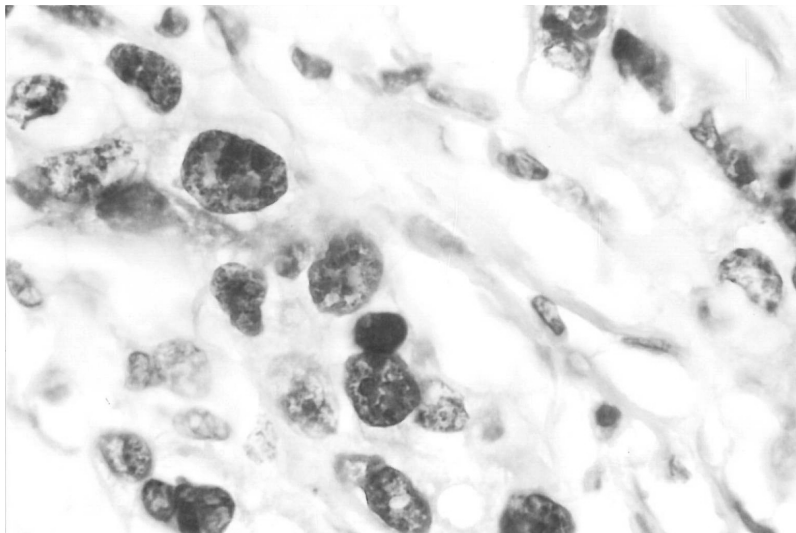


Fig. 3. Distinct homogeneous or granular nuclear stain revealing p₅₃ (+++) in a diffuse gastric carcinoma, IHC x100

Both the parametric Chi-square test and the nonparametric correlation coefficient reveal the existence of a significant correlation between p53 overexpression and Lauren classification ($\chi^2=28.44$, $p=0.00476$, $r=0.6319$, 95%CI) (**Table 2, 3**).

Table no. 2 Parameters considered for the p53 association vs. Lauren classification testing

	Chi-Square χ^2	df	p 95% confidence interval
Chi-Square - χ^2	28.44874	df=12	p=0.00476
Yates Chi-Square	29.71917	df=12	p=0.00308
Correlation coefficient (Spearman Rank R)	0.630572		p=0.01088

By correlating p₅₃ overexpression with Goseki classification we identified the following:

- Type I – 4 (50%) of 8 cases experienced p₅₃ overexpression. This overexpression was marked + in 3 of the cases and ++ in 1 of the cases (whose correspondent was the moderately differentiated form of the Lauren classification) (**Fig. 4**).

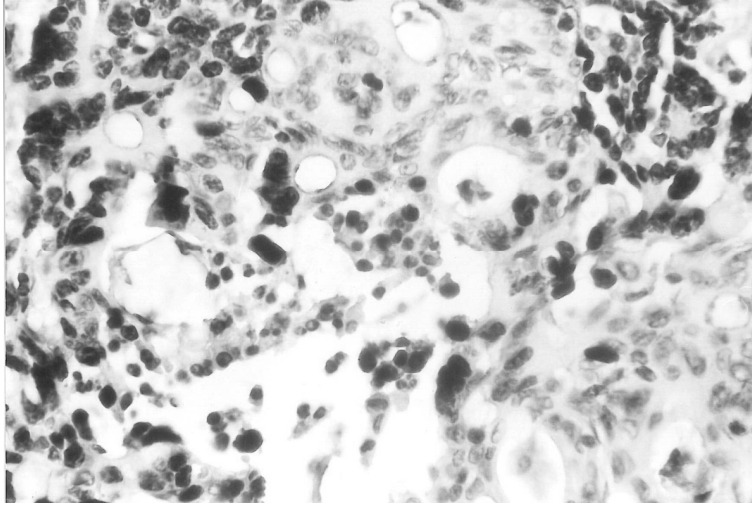


Fig. 4. *Distinct homogeneous or granular nuclear stain revealing p_{53} (++) in a moderately differentiated ADK, IHC x40*

- Type II – 3 (75%) of 4 cases experienced p_{53} overexpression marked +.
- Type III – 11 (68.8%) of 16 cases experienced p_{53} overexpression marked + in 2 cases, ++ in 2 cases and +++ in 7 cases
- Type IV – 7 (58.3%) of 12 cases experienced p_{53} overexpression marked + in 1 case, ++ in 3 cases and +++ in 3 cases (**Fig. 5**)

The 2 mixed cases in the Lauren classification were, in the Goseki classification, a mixture of the I and III forms, both with p_{53} , + overexpression.

The study of the p_{53} association vs. Goseki classification reveals a significant correlation between p_{53} overexpression and Goseki classification ($\chi^2=46.47$, $p=0.009$, 95%CI), accounted for by a high number of cases that have no p_{53} and belong to class I, as well as by a high percentage of cases with p_{53} (++, +++) and belonging to class IV. (**Table 3**)

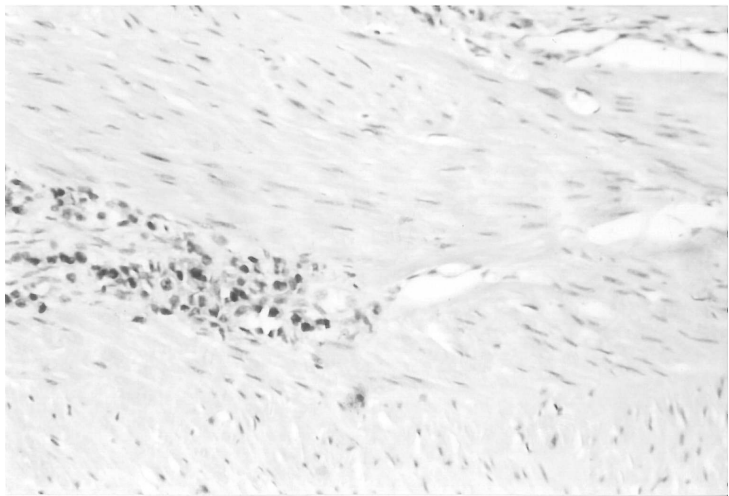


Fig. 5. Distinct homogeneous or granular nuclear stain revealing p_{53} (+++) in a diffuse gastric carcinoma. Muscle invasion, IHC x20 stain

Table no. 3 Parameters considered for the p_{53} association vs. Goseki classification testing

	Chi-square χ^2	df	p 95% confidence interval
Chi-square - χ^2	44.95370	df=9	0.009223
Yates Chi-square	46.47770	df=9	0.005757
Correlation coefficient (Spearman Rank R)	0.5450		0.025434

P_{53} suppressor gene (“wild” type) induces apoptosis, while the mutant protein blocks the function of the “wild” form, inhibiting apoptosis induction. Mutant p_{53} protein accumulation may behave as an immunogenic agent, generating anti- p_{53} antibodies. P_{53} positive immunoreaction is correlated with the amount of mutant p_{53} gene, being able to reveal cell proliferation and apoptosis inhibition; it is mainly observed in the nucleus, as a brown deposit.

In normal circumstances, p_{53} is considered the “genome guardian” (p_{53} protein is a transcription factor monitoring genome stability) or a “dual gene”, since in its normal state it works as antioncogene that blocks cell cycle allowing modified DNA repair, and in its modified state it becomes oncogenic.

DNA alteration triggers p_{53} protein accumulation, which stimulates the *cip1* gene, which in its turn encodes a protein called p_{21} (21 KDa) and inhibits cyclic dependent kinases (p_{21} is also called cip-cdk inhibitor protein). Cell proliferation is thus blocked. When mutation repair is insufficient by cell cycle blocking in G1, then p_{53} induces cell death by apoptosis, designed to prevent daughter cell flaw perpetuation. (10).

Gene p_{53} mutation (tumor suppressor gene) occurs in 50% of the breast cancer patients. P_{53} protein loss and/or alteration further to gene rearrangement may result into cell growth derangement, by replication flaws and genetic accumulations.

Replication is not blocked in cells where p_{53} is inactive, which leads to genetic instability. This instability is expressed during cell division, favoring a quick aneuploid clones or rearranged cells selection.

Systemic gene p_{53} mutation is the most common genetic event occurring in human cancers, as it leads to the inactivation of the specific protein p_{53} -to-DNA bonding function.

Allelic TP_{53} loss occurs in over 60% of the gastric carcinomas, and mutations are found in about 30-50% of the cases. However, these percentages also depend on the mutation screening method and on the size of the specimen. TP_{53} mutations are identified in some cases of intestinal metaplasia, most of the alterations being characteristic of advanced tumors (11).

TP_{53} mutations in gastric injuries are similar to those found in other mainly base transition cancers, especially at CpG nucleotide level.

Systemic mutations determine spatial p_{53} protein configuration by altering the amino acids sequence. Conformation alterations triggered by these mutations (considered critical malignant cell transformation stages) are associated with gene p_{53} product stabilization (12) and mutant protein accumulation in the nuclei of the tumor cells in sufficient quantities to enable their IHC detection. (13).

In order to study the prognostic meaning of protein P_{53} overexpression and its prognostic involvement, we analyzed its expression using the monoclonal anti- p_{53} antibody (Biogenex DO₇-AM).

- Positive p_{53} immunoreaction found in 62.5% of the cases allowed the identification of nuclear protein accumulation tumors, as well as the subclassification of p_{53} positive tumors according to the immunocoloring pattern and the percentage of positive cells in each injury.
- Lauren and Goseki classifications together with p_{53} overexpression enabled us to notice that well differentiated forms (type I in Lauren classification, types I and II in Goseki classification) experienced a predominant stain intensity marked + or ++. The percentage of positive p_{53} tumors belonging to this category varied, ranging from 66% for the type I of the Lauren classification and 50, that is 75%, for the types I and II of the Goseki classification.
- In the diffuse forms (type II in Lauren classification and III and IV, respectively, in Goseki classification), we noticed a stain intensity marked ++ and +++, respectively. The percentage of positive tumors varied from 57.6% (type II in Lauren classification and 64.2, that is 50%, in types III and IV of the Goseki classification).
- If from the viewpoint of the coloring pattern intensity we may state that it grew from well differentiated forms marked + to poorly differentiated forms marked +++ anticipating an aggressive behavior from the standpoint of the percent of positive tumors, we noticed a higher number of p_{53} positive tumors in well differentiated forms (type I in Lauren classification 62.5%, type I – II in Goseki classification, 50-75%) as compared to poorly differentiated forms (type II in Lauren classification 57.6%, type III, IV in Goseki classification 64.2% and 50%, respectively). This enables us to conclude that p_{53} as sole prognostic indicator is not sufficient, which is accounted for by that codon 72 polymorphism, that is by the fact that this is more frequently encoded by a proline then by an arginine. Or, it is well known that the latter is very common in antral gastric cancers.

- Another conclusion drawn from here would be that these facts could explain why a histologically well differentiated tumor, which we could expect to have a good prognosis, may have an aggressive behavior, thus contradicting its histopathological description.

CONCLUSIONS

Positive p₅₃ occurrence in the 40 cases under survey was expressed in 66% of the gastric carcinomas, with an increased coloring pattern from well differentiated forms marked + to poorly differentiated forms marked ++++. This evolution foresees the tumors' aggressive behavior.

As for the percentage of p₅₃ positive tumors, there is no relation between the percent of positive tumors and their differentiation, which makes us conclude that p₅₃ as sole prognostic indicator is not relevant.

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CYTO-HISTOPATHOLOGICAL CORRELATIONS IN UTERINE CERVIX PATHOLOGY

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Keywords: cytodiagnosis, cervical biopsies, cervical intraepithelial lesions, cervical carcinoma, human papilloma virus (HPV), rhabdomyosarcoma

Abstract: Clinical significant cervical lesions are often correlated with epithelial cell abnormalities on cervical smears. The histologic lesions which are found in uterine cervix can not be always established only with conventional cytology. Thus, it is very important that any cytologic abnormality be subsequently correlated with biopsy for certification of a cervical lesion. The results of cervical smears and tissue diagnoses over a five year period were reviewed, being examined the correlations between the cervico-vaginal smears and the surgical specimens. All cervical smears with their subsequent biopsies and hysterectomies, between 2003-2008 were retrospectively evaluated. The patients ages were between 18 and 70 years old. The cervical cytology records were verified and the cytodiagnosis were compared with the correspondent histopathological diagnosis. From the 5700 cervico-vaginal smears, diagnosed in the mentioned period of time, 3835 were „negative for intraepithelial lesion or malignancy”. The others of 1865 presented squamous and glandular lesions, and cervical carcinoma. One case, of a 20 years old girl, had a cytodiagnosis with suspicion of sarcoma, which was confirmed on biopsy and subsequent hysterectomy as a cervical embryonal rhabdomyosarcoma. The exfoliative cytologic cervico-vaginal test is very important in precocious diagnosis of intraepithelial cervical lesions, especially when it is correlated with histopathologic examination. The immunohistochemical and molecular testing of high-risk HPV will contribute to a better management of cervical precancerous lesions.

INTRODUCTION

The primordial function of cervical cytology is in bringing to notice epithelial abnormalities which would otherwise escape detection because of their clinical silence (Kealy, 1986). The cervico-vaginal cytologic test is now widely used both as a screening test in asymptomatic populations and in the follow-up of patients with cervical carcinomas treated by surgery or irradiation. The accuracy of predicting cervical epithelial abnormalities from surface cell samples is now fully accepted. One aspect however in the assessment of performance in cytology is the comparison of the cytodiagnosis with the ultimate tissue appearances (Kealy, 1986). Although it is not always possible to determine the exact histologic change in the cervix on the basis of the cytology smear, when a cervical abnormality is present, this is detected by cytologic examination in the majority of cases (Rosai, 2004). The great success of the cervico-vaginal cytologic test led to the conception among clinicians and large population that it can detect every case of carcinoma. It is important to realise that it is only a screening test and, thus, it will be associated with false-negative results.

MATERIALS AND METHODS

The results of cervical smears and tissue diagnoses over a five year period were reviewed, being examined the correlations between the cervico-vaginal smears and the surgical specimens. All cervical smears with their subsequent biopsies and hysterectomies, between 2003-2008 were retrospectively evaluated. The patients ages were between 18 and 70 years old. The cervical cytology records were verified and the cytodiagnosis were compared with the correspondent histopathological diagnosis. All the cytologic specimens were conventional smears, and these were selected for the present study only among those which were „satisfactory for the result”. The smears were stained with classic Papanicolaou technique and diagnosed using Bethesda 2001 Terminology (Solomon, 2004). The biopsies and hysterectomies were done to the patients with cytologic diagnosis of ASCUS (atypical squamous cells of unknown significance), AGC (atypical glandular cells), HSIL (high grade intraepithelial squamous lesion), and carcinoma (Smith, 2002). The surgical specimens were routinely fixed, paraffin-embedded and stained with Hematoxylin and Eosin. Epithelial abnormalities in tissues were graded as cervical intraepithelial neoplasia I-III (CIN I-III) and squamous intraepithelial lesions (LSIL, HSIL). The cytodiagnoses were correlated with the adequate histopathologic diagnoses.

RESULTS AND DISCUSSIONS

From the 5700 cervico-vaginal smears, diagnosed in the mentioned period of time, 3835 (67,28%) were „NILM (negative for intraepithelial lesion or malignancy)”: 1200 (21,05%) were „within normal limits” and 2635 (46,22%) were „benign cellular changes”. The others of 1865

(32,72%) presented squamous and glandular lesions, and cervical carcinoma, as follows: 1230 (21,57%) were diagnosticated as LSIL (low grade intraepithelial squamous lesion), 408 (7,15%) as HSIL, 120 (2,10%) as ASCUS, 96 (1,68%) as AGC, and 12 (0,21%) as cervical squamous carcinoma. One case, of a 20 years old girl, had a cytodiagnostic with suspicion of sarcoma, which was confirmed on biopsy and subsequent hysterectomy as a cervical embrional rhabdomyosarcoma (figures 1 and 2).

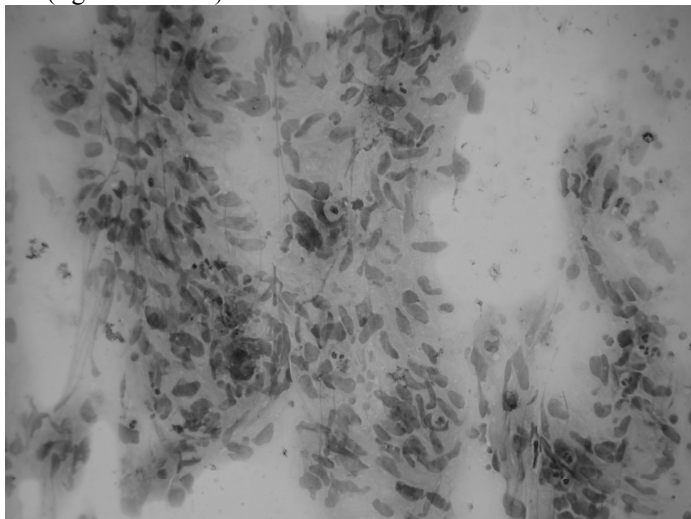


Fig. 1 - Sarcomatous stromal cells, conventional smear, Papanicolau staining x 20

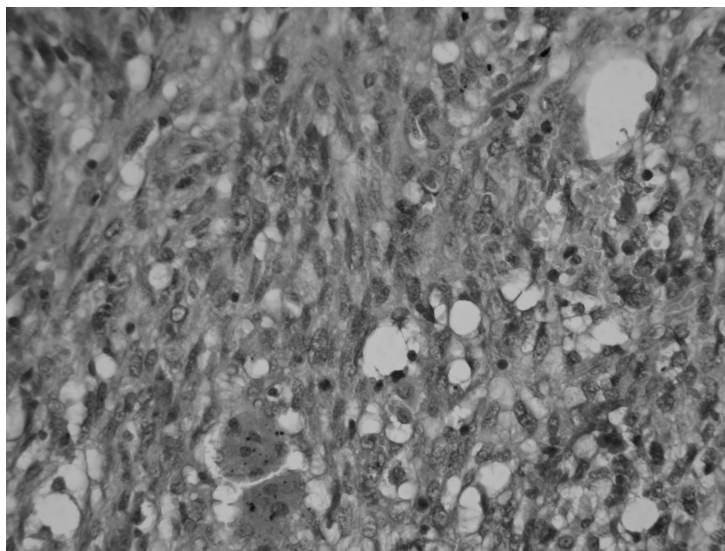


Fig. 2 – Rhabdomyosarcoma, tumoral giant cells, with pleomorphic nuclei, HE x 20

From NILM smears, 53 cases (1,38%) developed cytological cervical lesions, confirmed on subsequent biopsies, at 6 months and one year follow-up. From the 636 abnormal smears with

HSIL, ASCUS, AGC, and cervical cancer there were performed only 380 (59,74%) biopsies. A total of 340 (89,47%) cases presented a total concordance between cytodiagnostic and histopathology, and 40 (10,52%) cases presented minor diagnosis differences. From those 216 cases with ASCUS and AGC, there were performed only 86 (39,81%) biopsies, which confirmed 54 LSIL (CIN I) (figure 3), 22 HSIL (CIN II-III/carcinoma *in situ*) (figure 4), seven endocervical polyps, two glandular-cystic hyperplasia and one in situ endocervical adenocarcinoma (AC). All of the cytologic cervical carcinomas (12 cases) were histologically confirmed. Only 34 patients had total hysterectomy with bilateral adnexectomy.

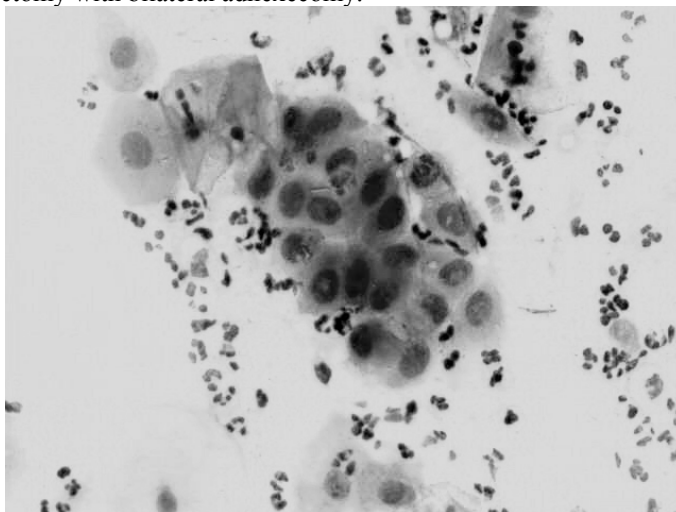


Fig. 3 – LSIL, conventional smear, Papanicolaou staining x 40

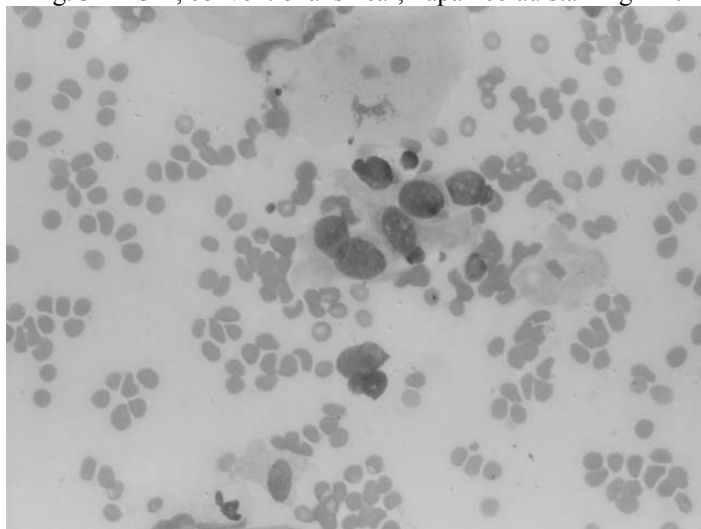


Fig. 4 – HSIL, conventional smear, Papanicolaou staining x 100

The discrepancies which appeared between cytodiagnostic and histopathologic exam were due both to certain fals positive results (postmenopausal pseudokoilcytosis, absence of complete clinical data which could explain the appearance of certain cytologic modifications, like: intrauterine devices, pregnancy, hormonal substitution treatments) and certain prelevation errors of biopsies, which required the repetition of the surgical procedure (Balan, 2007; Tuganova, 2009). The non-concordance between cytodiagnostic and histopathologic exam can be due to a inadequate fixation and staining of the smears. Drying fixation smears are inadequate. Although the squamous cells can be rehydrated, these can not reproduce fine structural details (Kurman, 2002). The glandular cells are more affected by the inadequate fixation. The discrepancy between cytology and biopsy can appear by performing certain superficial histologic sections, which not succeed to enter deep in the lesion. Thus, making deeper sections or turning round the paraffin block for a complete visualization of the epithelium could solve this problem (Kurman, 2002). Most cases which presented on smears cytopatic effect of human papilloma virus (HPV) infection (koilocytes) were confirmed as HPV positive on biopsy (figure 5). The non-concordances were due to pseudokoilcytosis process which appear in atrophic smears. Both the immunocytochemical and immunohistochemical investigation of HPV and molecular testing for high risk-HPV can reduce the diagnostication of advanced forms of precancerous lesions or even invasive carcinoma (Jones, 1996). The correlation between cytology and histology is useful also in verifying the laboratory internal quality and in control of histologic and cytologic processed errors. These problems can be also solved through the microscopic interpretation of smears and surgical specimens by different pathologists (Castle, 2007).

During the investigated period, there was a significant continuous increase in cytological AGC diagnoses. This was probably due to a more intensive education and training of the cytologists and a more defensive cytological practice due to a lack of reliable cytological criteria, aspects which were also described in literature (Scheiden, 2004; Soofer, 2000; Burja, 1999).

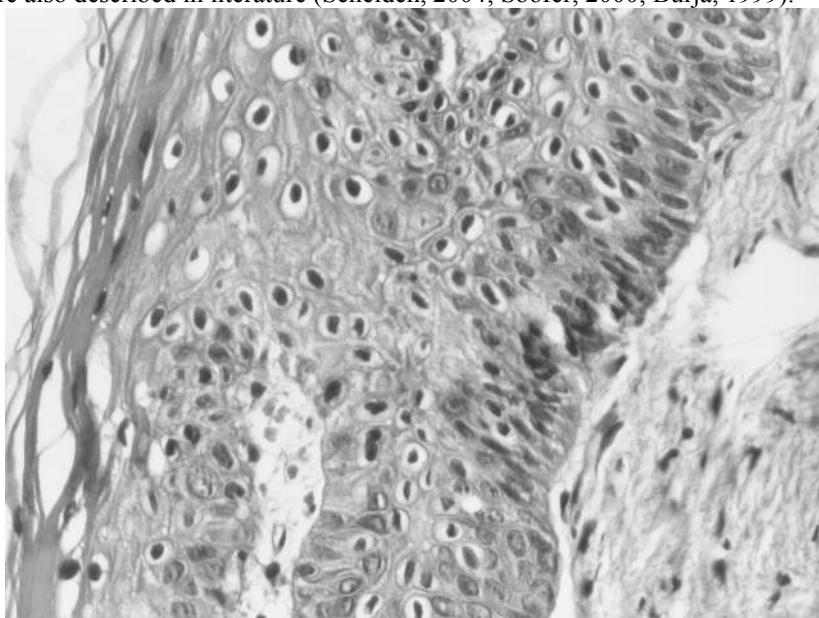


Fig. 5 – LSIL, cytopatic effect HPV, HE x 20

It is widely accepted that the cytological distinction between reactive, inflammatory, and dysplastic or neoplastic altered squamous and/or glandular proliferations is complex and may be controversial for the cytopathologists and surgical pathologists (Solomon, 2004; Scheiden, 2004; Johnson J, 1996).

CONCLUSIONS

The cytotest (Pap test) remains a screening test and it will be inevitably associated with a rate of false negative results. The cervico-vaginal smears are very important in precocious diagnosis of cervical lesions, especially when it is correlated with histopathologic examination. Non-concordance between cytodiagnostic and histopathologic exam is sometimes due to non-observance of some cytologic and histologic processing techniques. The immunohistochemical and molecular testing of high-risk HPV will contribute to a better management of cervical precancerous lesions. Although exfoliative cytology has been proven to be an efficient technique for the detection of precancerous and cancerous squamous lesions of the cervix, it is not a specific test for endocervical, glandular endometrial lesions, and other types of neoplasia.

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EXPERIMENTAL MODELS OF ACUTE PANCREATITIS -CLOSED DUODENAL LOOP MODEL

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Keywords: acute pancreatitis, experimental animal model, closed duodenal loop model, pancreatic ischemia.

Abstract: Randomized controlled studies of severe human acute pancreatitis can be performed only with restriction, especially in children. Some pathophysiological or therapeutic aspects should be first verified through animal experiments. Animal test results can be transferred to clinical practice if the results are based on trials with established models, standardized methods and a study design imitating the clinical situation. This study aims to review the different experimental models proposed for acute pancreatitis, focusing on the experience of the researcher's team with the "closed duodenal loop" model. A closed duodenal loop model was prepared in male Wistar rats. The duodenum was ligated 2cm distal and proximal to the junction of the hepatopancreatic duct. All rats developed severe acute pancreatitis. The histopathological alterations of the pancreas consisted in edema, parenchymal necrosis, thrombosis and hemorrhage. All animals died within 2-5 days from generalized sepsis. Animal experiments are of great value, especially in acute necrotizing pancreatitis because randomized controlled studies are problematic. The most common experimental models of acute pancreatitis are secretagogue hyperstimulation (cerulein pancreatitis), choline-deficient, ethionine-supplemented diet (CDE), duct obstruction/ligation, closed duodenal loop, duct perfusion. Closed duodenal loop model is easy enough from the technical point of view and presents histological characteristics that resemble those of human pancreatitis, but it has the drawback of early mortality.

INTRODUCTION

Acute pancreatitis (AP) varies in etiology, severity, mode of progression and requires a complex treatment. Because changes in evolution of AP are dramatic, its pathophysiologic analysis from clinical cases is difficult. Thus animal models are indispensable for pathophysiological studies, mainly in cell and molecular biology (1). Also, the effectiveness of therapeutic procedures should be examined after testing each substance in the context of legally required study phases, and ideally in the form of randomized controlled clinical studies. In AP this is problematic, due to variations in the causes, degree of severity and disease course, the low incidence of severe forms, the disease onset (usually referred to as the onset of abdominal pain; it cannot be determined exactly). The anatomical site of the pancreas in the retro peritoneum makes this organ rather inaccessible for detailed investigations, so that the treatment of AP is extremely complicated and requires close interdisciplinary work (2).

It has thus become necessary to test new therapeutic procedures and pathophysiological studies in AP first in animal models. The advantages of animal experiments are: standardization, achieved to an extent which cannot be approached clinically; all tests can be carried out in a large number of test animals of the same age and sex, with the same disease severity at the same time after disease onset.

Although animal experiments have contributed to a better understanding of the pathogenesis of AP, using animal models in therapeutic studies is still controversial due to the limited applicability of the results in clinical practice. Examples include cholecystokinin antagonists and protease inhibitors which had a positive effect on the disease course in animal but failed to prove their effectiveness in clinical practice (3).

An ideal experimental model of AP should comply with the following requirements:

1. To show the features of severe necrotizing AP: intra- and extraparenchymal necrosis, signs of systemic inflammation and multiorgan dysfunction syndrome with reproducible mortality;
2. Biphasic disease course: initial systemic inflammatory reaction syndrome (SIRS) with primary multiple organ dysfunction syndrome (MODS), then secondary inflammatory reactions or exacerbation due to infections with secondary MODS; (4-5)
3. Response to therapy;
4. Monitoring: standardized, reproducible and verifiable severity of AP and possibility of continued recording of vital and organ function (2).

It goes without saying that none of the existing models fulfils all these criteria.

Experimental models of acute pancreatitis in rats

There are numerous models of AP and modifications of these, which are differentiated according to the:

1. induction technique (invasive and non invasive models);
2. cause (biliary, obstructive, alcoholic toxic, traumatic, ischemic) or
3. degree of severity (mild/ oedematous or severe/necrotizing) (6-7).

Several laboratory animal species have been used for experimental models of AP.

Although mice have small dimensions and weigh only 20g, there are a lot of papers on murine models, for the invasive diagnostic and therapeutic measures, especially in those with genetic mutation(8). Cats, dogs, sheep or pigs models are expensive and imply ethical objections (9). Rabbits have proved high individual variability of the disease severity even with good standardized induction techniques. Opossum models are good enough but these animals are not easily available (10). For the reasons given, rats are basically ideal test animals for AP.

The common experimental models of AP are:

1. The choline-deficient, ethionine-supplement diet models (CDE) – test animals (mice, rats) develop severe hemorrhagic AP with a big mortality. There are other concomitant causes of death independent of AP, changes in the liver and central nervous system, which contribute to multiple organ failures. This model has a variable disease course, partly due to the animal weight and intake of food (11).
2. Cerulein-pancreatitis – AP is induced by supramaximal stimulation of the pancreas with the cholecystokinin analogue cerulein. Cerulein is injected intraperitoneally (12), subcutaneously or in the femoral vein (13). Cerulein pancreatitis is frequently mild and edematous.
3. Obstruction models – AP is induced by the ligation of the pancreatic duct or bile duct. Reflux of bile and pancreatic secretions alone (without additional exocrine hyper stimulation) leads only to mild pancreatitis without extensive necrosis of infectious complications (14).
4. Duct perfusion models – AP is induced by antegrade and retrograde infusion of bile salts (taurocholate). These models have been used extensively for studying the effect of various therapeutic agents (cholecystokinin antagonists, somatostatin, fibrinolytic agents and cytokine antagonists (15). The induction technique requires a high level of standardization when performing ductal perfusion and the severity of the disease may be too fulminant. This model can be improved by additional noxae not necessarily considered to be involved in causing human AP.
5. Closed duodenal loop (CDL) models – AP is triggered by a reflux of bile and pancreatic secretions following duodenal ligation distal and proximal to the junction of the hepatopancreatic duct (2).
6. Models which expose the animal to the same noxae that trigger AP in man- alcohol, bile duct obstruction, pancreatic ischemia, traumatic damage, toxic substances. This model was not encouraging because the animals showed neither the typical morphological nor the clinical signs characteristic to severe human AP.

Closed duodenal loop models in rats

CDL model of AP is reported to present histological characteristics that resemble those of human pancreatitis (16) and has become one of the most widely used experimental models of AP. The medical literature includes clinical reports of AP associated with duodenal obstruction (17-18).

The factors involved in the development and progression of pancreatitis in the CDL model include: reflux of duodenal fluid, containing activated pancreatic fluid, increased pressure within the pancreatic duct, reflux of bile, infection and impaired pancreatic blood flow. The disease severity depends largely on the perfusion of the closed intestinal loop.(2) The secretory reflux in the closed loop is bacterially contaminated and the intestinal motility and microflora are changed by the lack of bile and pancreatic juice in the intestinal segment distal from the ligated intestinal loop (19). Rats die within 2-3 days from generalized therapy-refractory sepsis.

MATERIALS AND METHODS

Animal preparation: Male Wistar rats, weighing 200-250 g, were used in all experiments. The rats were kept at constant temperature with free access to water and standard rat pellets.

Experimental protocol: The animals received no nourishment other than water ad libitum for a period of at least 18 hours preoperatively.

They were given general anesthesia (induction with ether, then maintenance with atropine 0,1mg/kg, ketamine 1mg/kg and pethidine 1mg/kg, administered intraperitoneally; drug doses were repeated at 30-40 min).

Laparotomy was performed by upper midline incision. To prepare the duodenal loop the duodenum was carefully pierced through and ligated around its entire circumference with 5-0 silk. Locations of ligatures were at 2cm orally and anally of the duodenal entry of the biliopancreatic duct. (Fig.1)

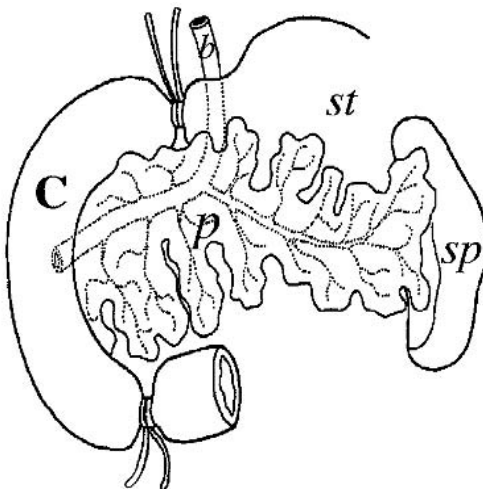


Fig.1 The operative schema: p=pancreas, st=stomach, sp=spleen

In the control group, only laparotomy was performed (Fig.2-3)



Fig.2 Laparotomy in rats



Fig.3 Laparotomy in rats

After surgery, the animals received no nourishment other than water during their survival.

RESULTS

Pancreatic histological changes (compared with a normal pancreas Fig. 4): After the occurrence of death, small samples from the pancreas were fixed by an immersion in Formalin. After paraffin embedding, sectioning and staining with hematoxylin-eosin, the sections were examined light-microscopically. Pancreatic histological changes included interstitial edema, inflammatory cell infiltration (Fig.5), focal necrosis (Fig.6) and hemorrhage (Fig.7).

The average survival period was 24 hours (12- 48hours)

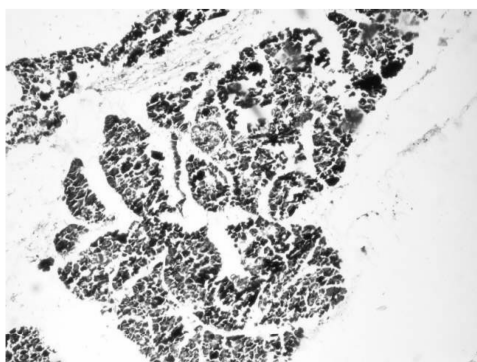


Fig. 4 normal pancreas



Fig. 5 inflammatory cell infiltration

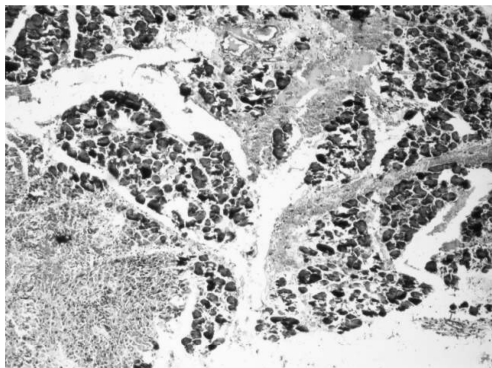


Fig. 6 pancreatic necrosis

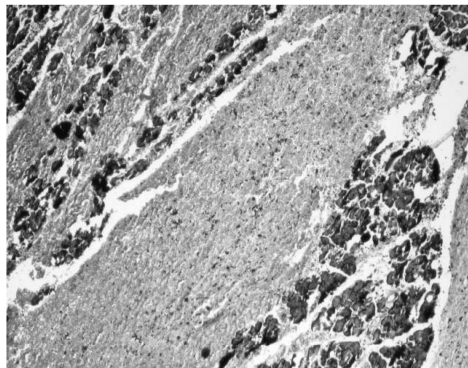


Fig.7 pancreatic hemorrhage

CONCLUSIONS

CDL model in rats leads to the development of AP with marked interstitial edema, focal necrosis and hemorrhage.

The appearance of these lesions and high mortality of the animals are remarkably reproducible.

This model of AP is morphologically and biochemically similar to clinical severe AP.

In this model, the exocrine pancreas was vulnerable to activation of digestive enzymes both inside and outside the acinary cell. The reflux of bile and duodenal contents into the pancreatic duct and subsequent intrapancreatic activation of digestive enzymes have been suggested to play an important role. The high pressure in the duodenum causes a high pressure on the intrapancreatic-duct and the subsequent congestion of pancreatic acinary cells. These pancreatic injuries induce overproduction of many kinds of cytokines and induce systemic inflammatory response with high mortality.

The CDL model has the drawback of early mortality induced by factors such as retention of gastric juice, impaired duodenal blood flow and bacterial infection. To avoid retention of gastric juice, a lot of strategies have been proposed, such as preparation of a gastrojejunal anastomosis, insertion of a tube within the duodenum and temporary release of the obstruction after preparation of CDL, but these surgical invasions have the potential to bias the experimental results.

CDL model is simple to prepare and easy enough from the technical point of view.

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EFFECTS OF ANTI-VEGF AGENTS ON THE OCULAR NEOVASCULAR STRUCTURES IN DIABETIC RETINOPATHIES

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Key words : diabetic rethinopathys, angiogenesis, anti VEGF substance.

Abstract: In diabetic rethinopathys there are lot of anevrisms, neovesels at the peripheric retina and optic nerv disc, and difuz vascular leakage sometimes with severe hemorages. After anti VEGF action the neoocular structures are remised.

INTRODUCTION

Diabetic retinopathy (DR) is a common ocular complication of the diabetes mellitus (DM). DR appears much more frequent to the patients with type I (40%) of diabetes comparative to the type II (20%), and it represents the main cause for blindness in the developing countries. About 90% of the diabetic patients develop DR after 20 years of diabetes (2).

From the pathogenic point of view, DR represents a microangiopathy that primary affects the retinal capillary network (arterioles and venules) and is characterized by loss of the pericytes, thinning of the vascular basement membrane, followed by the proliferation of the endothelial cells (2). At the blood flow level, the modifications consist of deformation of the red cells and increasing of the platelets aggregation leading to the lowering of the sanguine oxygen transport. Consequently, the ischemia develops at the tissues level, while the dilations of the vascular walls (microaneurisms) appear around the affected region. As a consequence of the high levels of the glycemia, different metabolic products appear as a result of aldoreductase pathway (sorbitol, galactitol). These products accumulate at the level of endothelial capillary cells, increasing the cellular volume and the thinning of the basement membrane. Finally, the dilatation of the capillaries, the thinning of the vascular basement membrane and the production of the vasoactive factors due to hypoxia determine the microvascular occlusion, leading finally to the aggravation of the tissue hypoxia and creating of a vicious circle (3). The final consequence of these pathological processes represent the breakdown of the blood-retinal barrier leading to the “leakage” of the plasma components in the retinal tissue, formation of hemorrhages, hard and soft exudates and edema (4).

From the *clinical point of view*, the DR follows the next three stages:

- **nonproliferative** in which the pathological modifications do not pass over the internal limiting membrane,
- **preproliferative** that represents an intermediate stage,
- **proliferative** in which the fibrovascular proliferation pass over the internal limiting membrane.

Recent studies showed the role of the growth and inflammatory factors in the appearance of tissue modifications in DR, this hypothesis being suggested by the high levels of the proinflammatory cytokines in the vitreous according to the severity of the disease: TNF-alfa, ICAM (adhesion molecule), VEGF (vascular endothelial growth factor). Our study has the aim to evaluate the role of the VEGF in the angiogenetic process in DR.

MATERIALS AND METHODS

The examined material was taken from the Ophthalmologic Department of the Railway University Hospital in Iasi and represents a diabetic male, 43 years old patient, having proliferative diabetic retinopathy on both eyes.

The eyes were examined by means of the following techniques:

- **biomicroscopic** examination of the anterior segment of the eye;
- direct and indirect **ophthalmoscopic** examination and **biomicroscopic examination** of the vitreous and the retina;
- **fluorescein angiography** of the ocular fundus realized by using 5 ml fluorescein 10% intravenously injected prior to the digital examination of the fundus. The ocular fundus pictures were taken by using VISUCAM – LITE ZEISS fundus camera in different moments of the evaluation of the patient. The dye contrasts the retinal vasculature revealing also the leakage, the retinal edema, the neovascular network arising on the retina and also into the vitreous, different areas of retinal atrophies, etc.;
- **red-free examination** was also realized by using the same fundus camera and revealed the neovascular fibrous tissue, the hard and soft exudates, the retinal scars and atrophic areas. It can also be used as a way to evaluate in dynamic the patient.

The right eye of the patient was injected with *Bevacizumab (Avastin)*, an anti-VEGF agent used off-label. The dose of 2,5 mg of Avastin was injected monthly and the patient monitorised by using the above mentioned techniques in order to detect possible complications and effects.

RESULTS AND DISCUSSIONS

The first investigation of the patient revealed, during ophthalmoscopy and red free examination, the presence of numerous new vessels covering the whole ocular fundus, including over the optic disc, hard exudates, microaneurisms, superficial retinal hemorrhages and macular edema (fig.1, fig.2 and fig.3).



Fig.1 Right eye, ocular fundus.



Fig. 2 Right eye, fluorescein angiography.

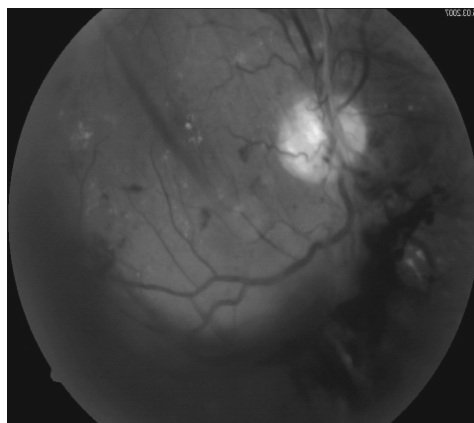


Fig. 3. Left eye, ocular fundus.

One month after Avastin injection, the fluorescein angiography and red free examination revealed in this patient the regression of the neovascular network and reducing in the volume of the fibrous component also. The retinal exudates and edema also decreased, leading to the improvement of the visual acuity of the patient (fig.4, fig. 5, fig.6 and fig. 7).

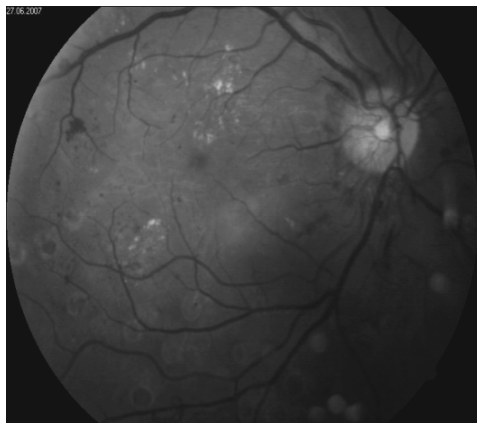


Fig. 4 – Right eye, ocular fundus, bevacizumab, one month examination.

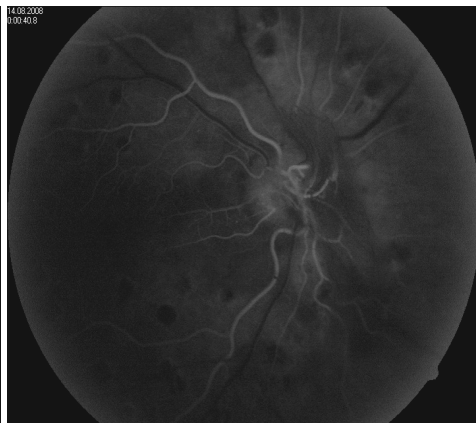


Fig. 5 – Right eye, fluorescein angiography, bevacizumab, one month examination.

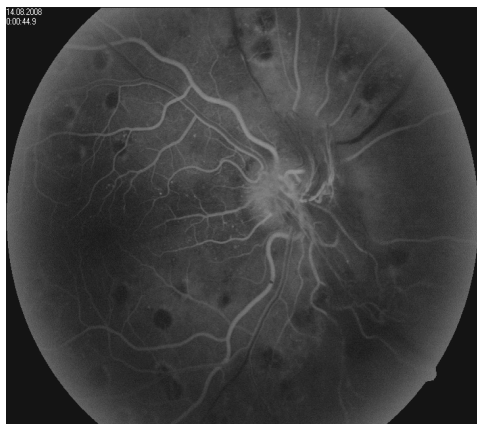


Fig. 6 Right eye, fluorescein angiography, bevacizumab, 10 secondes, one month examination.



Fig. 7 Right eye, fluorescein angiography, bevacizumab, 60 secondes, one month examination.

VEGF that inhibits “in vitro” the retinal endothelial cells’ growth is implicated in the appearance of the modifications in DR. The studies realized on animal models demonstrated a direct relationship between the VEGF concentration and the evolution or regression of the neovascular network. The vitreous concentration of this factor is higher in patients with proliferative DR comparative to the group with nonproliferative DR.

The perturbation of the glucidic metabolism induces troubles at different levels – vascular, neuronal and glial and is accompanied by the high concentrations of VEGF. However, it was also demonstrated that VEGF has an important neuronal benefic role maintaining for a time the survival in hostile conditions (phenomena called “preconditioning”), but it is toxic for the endothelium, aggravating the microangiopathy by increasing the capillary permeability leading to the appearance of edema, exudates and hemorrhages. VEGF-A a member of the VEGF family, has a central role: it is a protein having several isoforms, is present in the majority of the

tissues, free or bound to the extracellular matrix, is synthesized as a response to the hypoxia, estrogens, in hyper- or hypoglycemia, in inflammation, etc. Excessive producing of VEGF appears early in diabetes and represents a factor that starts the retinal proliferation. It is not known the exact mechanism through which the concentration of VEGF increases, but it was demonstrated that, mainly the high concentrations of VEGF determine the alterations of this factor concentrations equilibrium. It was also observed that except glycemia, the advanced glycation products influence the VEGF synthesis.

After the onset of the neovascular proliferation, the progression of angiogenesis is continuing in the less resistant areas. As an example, the absence of the internal limiting membrane at the optic disc level explains the prevalence of the neovascular growth into the optic nerve head. It was also observed that the neovascularization progresses easily when the conjunctive tissue is already developed, and the posterior vitreous detachment represents a convenient region for these new vessels growth (1).

The angiogenesis progresses to the next stage, in which is associated with the formation of the conjunctive tissue. By advancing the proliferative DR, the fibrous component continues to increase with or without developing of the new vessels. The fibrovascular component is oriented to the vitreal cavity or into the retinal surface and covers the optic disc, while the avascular form of the fibrous tissue results usually from the modifications develop at the posterior hyaloid level. The diffuse neovascularization extends until the posterior vitreous detachment appears and is characterized, diabetic patients, much more by the slow decrease of the whole vitreous volume than the formation of certain cavities inside of the vitreous. Thus, an idea was introduced, that the angiogenesis is not producing towards the vitreous cavity, but these vessels are tracked by the vitreous contractions to which they adhere (1). Because of the sudden contractions of the modified vitreous other retinal lesions appear: ruptures of the new vessels and onset of the vitreous hemorrhages, retinal ruptures or retinal detachment.

Another important idea is represented by the effect of anti-VEGF agents on the proliferative DR. Anti-VEGF agents may be used as monotherapy or associated with other therapeutic methods. The studies confirm their major effect in the regression of the retinal lesions that appear during DR and functional recovering of the patients.

CONCLUSIONS

Diabetic proliferative retinopathy is an ocular complication of the diabetes mellitus in which the VEGF seems to play an important role, as the anti-VEGF agents like off-label Bevacizumab has a strong regressive effect on the pathological vitreo-retinal lesions.

The retinas structure modifications in diabetic rethinopathys take a long time, while the administration of anti VEGF produce a more rapid reaction due to a high rate of cells autolysis and heterolysis.

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