SILVIA NEAMTU

PhD THESIS SUMMARY

Living cells functionality in electric and magnetic fields

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CONTENTS

PART II
EFFECTS OF INTENSE AND SHORT DURATION ELECTRIC FIELDS,
ON LIVING CELLS - BIOMEMBRANE ELECTROPERMEABILIZATION

Keywords

- weak magnetic fields, geomagnetic field, zero magnetic field, geomagnetic activity, Ap index, magnetic resonance mechanisms, electron transfer model, recombination of electron pairs,

- plants, vegetative growth, seedling growth, alfalfa - *Medicago sativum* L., wheat - *Triticum aestivum* L., rye - *Secale cereale* L., tomato -*Lycopresicum esculentum* L., pepper - *Capsicum annuum* L. mexican marigold - *Tagetes erecta* L ., french marigold - *Tagetes patula* L. pot marigold - *Calendula officinalis* L. ultrastructural analysis, fractal analysis,

- bacteria, bacteria growth, *Listeria monocitogenes*, *Salmonella*, *Escherichia coli*, laser light scattering, electrophoresis, SDS polyacrilamide, agarose electrophoresis, cyanobacteria, *Synechocystis*, cell motility, molecular motors, human sperm,

- membrane electropermeabilization, electroporation, critical transmembrane potential, pulsed electric field, molecular transport, DNA transfer, aqueous pore theory, molecular dynamics simulation (MD), electroincorporation, electroinsertion, electrochemoterapy, transdermal electrotransfer,

- electrohemolysis, membrane resealing, electron irradiation, target analysis, red cell cytoskeleton, spectrine, dimensional distribution,

- protein glycosylation, diabetes mellitus type I, diabetes mellitus type II, chronic lymphatic leukemia, Hodgkin lymphoma, bone marrow failure, glycosilated hemoglobin.

1. INTRODUCTION

The purpose of this thesis is to investigate the morphological and functional effects induced by interaction of living systems with electric and magnetic fields.

Nonmagnetic nature of membranes, cells and tissues, allows magnetic fields penetration with negligible attenuation and direct action at various levels of cellular structures and processes. The current problem of the interaction of cellular structure with weak magnetic fields (µT) is given by perception and cellular response threshold.

Geomagnetic field as a major factor of living organisms environment includes a main static component originating in the Earth's core (~ 50 µT) over which there is a natural variable component of interplanetary origin and electromagnetic field of technological sources superimposed. Magnetic fields of ambiental are weak (1-100 µT) low frequency (0-300Hz) fields with a major power-frequency (50 - 60Hz) component generated by transmission and electrical distribution lines. At the cellular level, the energy produced in this range of field parameters are encountered below the cellular perception of thermal noise energy of membrane and does not justify the existence of significant biological response. In the presence of weak magnetic fields the biological response is supported by a broad range of experimental and epidemiological data and is characterized by non-linear dose-response curves with significant efficiencies in time to amplitude and / or frequency, as defined by the "amplitude windows" and by "frequency windows". Biological "windows" for weak magnetic fields are encountered at different levels of structural organization or functional level. A large number of refference publications point to significant correlations between clinical, epidemiological as well as biological processes and natural fluctuations of alternative geomagnetic field component. Geomagnetic disturbances are considered an ecological risk factor that can desynchronize the biological rhythms and other biological processes in the organism or may modify the functional state of the brain. Similarly, reducing the static component of the geomagnetic field may become a risk factor for health, resulting in functional disturbances of various functional systems [Pokhodzey 1998]. At cellular level the shielded magnetic field induces decrease in cell proliferation, changes in cellular ultrastructure accelerates the processes of protein synthesis etc. [Belyavskaia 2004]. Geomagnetic field compensation is perceived as an element of stress in the membrane transport processes [Morariu et al. 1999, 2001 Ciorba and Morariu, Ciortea et al 2001]. Variability of biological response appears to be a general feature of interaction of simple organisms (plants, prokaryotes) with weak magnetic fields.

Systematic study of plants and bacteria growth in the absence of the main component of geomagnetic field (zero magnetic field) as described in the first part of the thesis, fills the overall picture of geomagetic field significance in the development of these organisms and the influence of natural magnetic fluctuations (magnetic storms).

The cell membrane presents on the other hand an important intrinsic electric field maintained by permanent dipole distribution and ion movements between intra and extra-cellular media. This strong intrinsic electric field present at the cell membrane level is an essential barrier for the external electric fields action. An important aspect of biotechnological interest focus the cells interaction with electrical fields is the intrinsic membrane potential modulation through short (µs) an intense (kV/cm) electric field pulses. As the induced transmembrane potential reach a threshold value reorganization of lipid

bilayer occured accompanied by membrane permeability and free intra and extracellular molecular transport. Membrane electropermeabilization is a noninvasive process that can be controlled and modulated by electrical parameters. The successful outcomes that are obtained by the use of this process gave rise to a new biotechnology based on biomembrane electroporation. While experimentally wise the literature is impressive and demonstrates the practical importance of the electroporation technique, the underlying molecular processes of membrane electropermeabilization is still a matter of big focus at present.

The studies that are presented in the second part of the thesis include original contributions regarding both the fundamental issues of membrane electropermeabilization process and also the applications of electroporation as an alternative technique to investigate changes at the cellular level in various pathologies.

PART I

IFLUENCE OF WEAK MAGNETIC FIELDS ON
PLANTS AND BACTERIA GROWTH

The first part of this thesis illustrates the influence of magnetic fields based on the natural processes of cell growth and multiplication of unicellular organisms (such as bacteria and cyanobacteria) and on the vegetative growth of plant. The significance of geomagnetic field in these processes has been proven in condition of static component compensation of geomagnetic field (zero magnetic field). This research is motivated primarily by the scarce studies regarding the response of these organisms in the absence of geomagnetic field. It was intended to achieve full investigation, which in terms of plant development included all stages of plant growth. Experiments were carried out on a significant number of species at different times of the year. Bacterial studies included at least two methods of analysis on different bacterial strains. Preliminary results related to cell motility in zero magnetic fields ends the first part of this thesis.

Experimental data are preceded by a systematization of weak magnetic field bioefects for each biological system investigated.

III. EFFECT OF WEAK MAGNETIC FIELDS ON VEGETATIVE GROWTH OF PLANT

1. Vegetative growth of plants. General considerations

Ontologically speaking, plant development during a life cycle is defined by four stages [Pankaj et al. 2005]: 1. vegetative stage comprising the following phases: i) seed germination, ii) seedlings growth marked by stem elongation and leaf formation, iii) branch; 2. reproductive phase defined by blossom, flowering, fruit formation stage and ripening plant, 3. senescence which comprises the processes that occur until the end of the active life of the plant, 4. latency consisting in the suspension of physiological (however, this process can be reactivated). Unlike the animal kingdom where the base structural pattern of the body and organs is contoured from embryonic stage, plant evolution implies the emergence and development of new tissues and organs.

Seed germination, as well as the transition from dormant state to the active life is a succession of phases initiated by water uptake through cell walls (soak phase) and activation of signaling hormone. Germination is completed once the embryo radicle penetrates the

structures surrounding the embryo (embryogenesis). Balance between expansion capacity of the embryo (growth potential) and the restriction imposed by the surrounding tissue (endosperm) is the main factor of the germination process.

Physiological process of post embryo growth is based on cell division and on the expansion process in the specialized tissues called meristems. These are formed during embryogenesis at opposed poles of seeds in so called apical meristems, and initiate the root and major axis development of seedlings. Plant growth depends on the sustained function of meristems where the balance between cells proliferation and differentiation is strictly controlled.

Initiation of lateral organs of plants such as emergence of leaves, flowers and fruits would imply the increase of the growth rate of cell division in apical meristem on the lateral side of the stem. The cell proliferation located in this area leads to the formation of primordia of lateral organs and to the reinitiation of the first cellular division in the new primordia. Hormonal activity and genetic expression is the main driver of the growth process of young and mature plants.

3. Vegetative growth of plants in zero magnetic fields - Experimental results

The interest for seeds germination and plants growth in the absence of geomagnetic field was obviously related to space research. Older work researches were reviewed by Conley (1969). An important contribution is brought by Soviet and Russian literature [2004]. The main problem of these researches remains the wide diversity of published results.

Zero magnetic field influence on plant growth was mainly studied in shielding conditions. Most of the reported investigation reveals only partial aspects of a plant species evolution in such conditions. The main objective of our research was to extend the analysis to the entire range of vegetative growth. The following points represent the new aspects of our work: a) the experiments were repeated several times with the same material and conditions for a period of a year or more;

b) various geomagnetic activities were simultaneously operative in addition to ZMF and we investigated the possibility that geomagnetic activity had influence on the results;

c) we experimented with some species which have not been previously tested. Plant selection took into account a variety of plant species including both important economically crops (alfalfa - *Medicago sativa* L. var. Euver Lucerne, wheat - *Triticum aestivum*, Gramineae fam, rye - *Secale cereale*, Gramineae fam, tomato - *Lycopresicum esculentum*, Solanaceae fam, pepper - *Capsicum annuum*, Solanaceae fam) and spontaneous plants (mexican marigold - *Tagetes erecta* , - french marigold - *Tagetes patula*, fam Asteraceae, pot marigold - *Calendula officinalis*, Asteraceae fam). With the exception of wheat seeds, selected species were not investigated.

The response of plants in zero magnetic field was assessed in two ways:

1. morphologically in the following development stages: i) seed germination, ii) the seedling growth as defined by the appearance and growth of radicle within 4-5 days, iii) development of young plantlets within a month, iv) mature plant until flowering stage and appearance of the fruit.

2. ultrastructurally through electron miscroscopy analysis of leaves through the last two stages of plant development.

3.1. Zero magnetic field

Static component of geomagnetic field (GMF) was compensated by a Helmholtz coil having a 1.2 m diameter. The longitudinal ax of the device was oriented along the field line at our particular latitude (Fig.3.1).
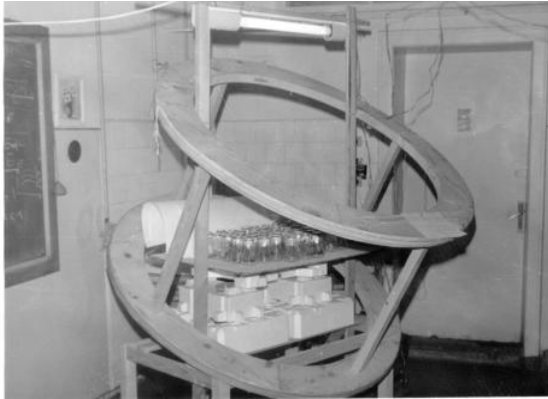
Fig.3.1.
System for static geomagnetic field compensation–Helmholtz coils

This method reduces the intensity of geomagnetic field by a factor of about 100 x by. To monitor the magnetic field variations on the selected area for samples exposure, we used a FLUXMASTER magnetometer with an accuracy of ± 1 nT. In different positions the field varies in the range of 0-500nT. These values include diurnal variations of geomagnetic field. Our device did not compensate the natural geomagnetic field fluctuations occurred during the various periods of the experiment. This allows us to further evaluate the influence of magnetic fluctuations induced during magnetic storms. For each experiment, data was analyzed in relation to daily changes of geomagnetic activity (GMA) represented by Ap index. This is an average index of daily activity recorded by certain magnetic observatories at around Earth and is provided by the National Geographic Data Center USA. As a measure of changes in geomagnetic activity we used the standard deviation values Ap index (SD Ap_{index}) throughout the experiment. The presence of a magnetic storm was marked by the value of Ap and the day it was recorded

3.3. Seed germination in zero magnetic field

The trend of seeds germination follows a sigmoid curve whose slope characterizes the rate of germination. This curve was determined for each species by the percent of seeds germinated at every 3 hours in zero magnetic fields (ZMF -experimental) and in geomagnetic field (GMF) as control. ZMF effect was estimated from the normalized difference of the germination rate in zero magnetic field and geomagnetic field represented by x_o parameter of the Boltzmann fitting curve of the experimental data: $((x_{CMZ}-x_{CGM})/x_{CGM}) * 100$.

As it can be observed from the results presented in Table 1, in the periods with low geomagnetic activity, the speed of seeds germination was not significantly different in CMZ compared with ZMF for all selected plant species [Neamtu and Morariu 2005]. According to these data the lack of static component of the geomagnetic field does not represent a stress for plants in this phase of growth.

Significant differences were observed during geomagnetic perturbed periods in the set of experiments performed on alfalfa seeds [Neamtu and Morariu 2005]. The effect of ZMF on seed germination rate is illustrated in Fig.3.2.a. for nine experiments (a) in parallel with

geomagnetic activity for the period of seeds incubation (b). Significant effects observed in ZMF condition are marked with an asterisk (*) on the plot.

Table 1.
Speed of seeds germination in GMF (C-control) and ZMF (S-sample),
ZMF effect and GMA variation during seeds exposure

Plant species	x_0 (%/hour) C – CGM; S- CMZ	CMZ effect $(x_{CMZ}-x_{CGM})/$ $x_{CGM}*100$ [%]	GMA (sd Ap_{index})
Medicago sativa	M – 30.49 ± 0.87; P – 29.67 ± 0.98	– 2.6	2–10
Tagetes patula	M – 26.5 ± 1.50; P – 26.92 ± 1.09	1.5	2–8
Calendula oficinalis	M – 26.06 ± 0.13; P – 26.03 ± 0.64	0.11	2–8
Triticum aestivum	M – 9.09 ± 0.16; P – 8.79 ± 0.12	– 3.3	2–11
Secale cereale	M – 8.93 ± 0.31; P – 9.11 ± 0.11	2	2–11
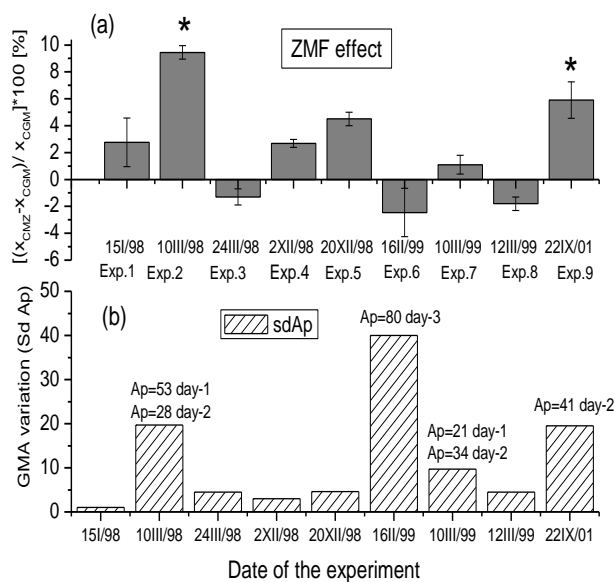
Fig.3.2.a
ZMF effect on alfalfa seed germination (a) and changes in geomagnetic activity associated with incubation periods (b)
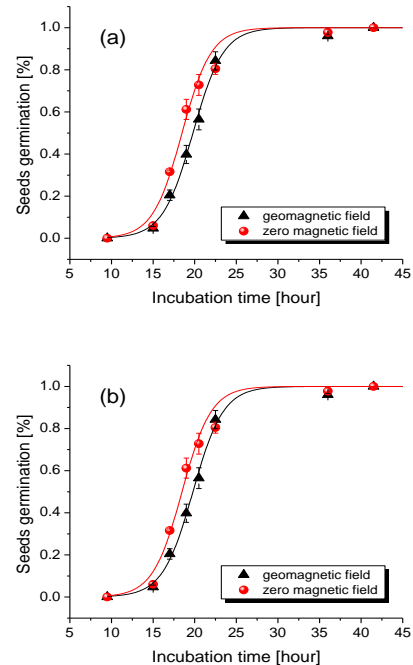
Fig.3.2.b.
Stimulation of alfalfa seed germination in zero magnetic field:(a) Exp 2 (b) Exp 9

In periods of low geomagnetic activity, no statistically effects were recorded. The presence of a major storm (Ap = 53) during the seeds soaking (the first day of exposure) (Exp 2) and a minor storm (Ap = 41) in the second day of incubation (Exp 9) was correlated with significant increases of the germination rate in ZMF (p <0.05). Seed germination period was reduced by 10% in the first case and by 6% in Exp. 9. In Fig. 3.2.b. germination curves in ZMF and GMF are detailed for the two experiments.

The obtained results support the hypothesis of a favorable influence of natural magnetic fluctuations induced by major magnetic storm on the initial phases of germination. They are as well consistent with results of Alexander and Doijde [1995] and Pazur and Scheer [1992] that report significant increases of seeds germination in weak magnetic fields [1.5μT, 5

Hz] and cell division in algae [20-200 μT, 7.5 Hz] in AC magnetic field with frequencies close to Schuman resonant frequencies.

3.4. Zero magnetic field effect on the early seedling growth phase

The early stage of seedling growth in ZMF was quantified after four days of seeds incubation in the dark, by root and stem length. The significance of the experimental data was evaluated on 80-120 seedlings using ANOVA test of the Origin 7.5 at p = 0.5.

The effect of ZMF magnetic field on the growth of seedlings was reported as normalized difference of the growth parameters: $(L_{ZMF}-L_{GMF})/L_{GMF}$ where L refers to the plant, root or shoot length in ZMF or GMF. Each experiment/species was repeated 6-8 times at different times of the year and each experimental period consisted on simultaneous incubation of two or three plant species.

The results indicate a specific response depending on the plant species [Neamtu et al 2009, Neamtu and Morariu 2010]. The most important effect on *Tagetes erecta L.* seedlings appeared on the growth of root which was significantly stimulated during low geomagnetic activities ($p < 0.05$) (Fig. 3.3, Fig. 3.4).
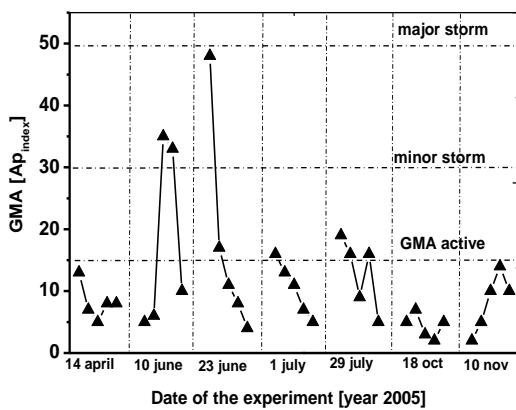
Fig.3.3.
The daily Ap index of geomagnetic activity during the 2005 Values belonging to an experiment are connected by straight lines The date of the first day of each experiment is marked on the abscissa..
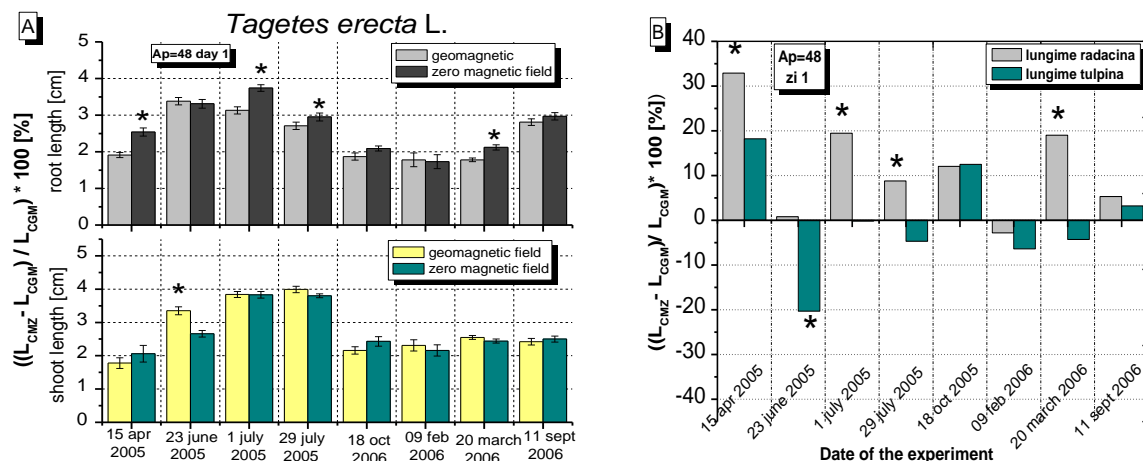
Fig 3.4.
The values of seedlings length growth parameters of Tagetes erecta in ZMF and GMF in different periods of the year 2005 and 2006 (A) and related ZMF effect. Values statistically significant are marked with a star. The presence of magnetic storms is represented by Ap index value and the emergence day.

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In five experiments out of eight, the growth of root was significantly stimulated up to around 30%. Worth noting is the fact that these effects have been highlighted during the spring and summer. A particular situation can be observed for the experiments performed in autumn and winter (October 2005, February and September 2006) when practically no effect of ZMF was observed. This may be associated to the decrease of plant sensitivity out of their natural vegetation period.

The root length remained unaffected, in the presence of a minor magnetic storm (Ap = 48) on the first day of seeds incubation (June 2005) which may be regarded as a masking effect of ZMF root stimulation which occurred during low geomagnetic activity.

Seedling growth of *Triticum aestivum L.* and *Secale cereale L.* in ZMF were dominated by inhibitory effects contrary to the effects observed for so *Tagetes erecta*. The ability of seed to generate roots fell in six of eight experimental periods for wheat (Fig.3.5.).
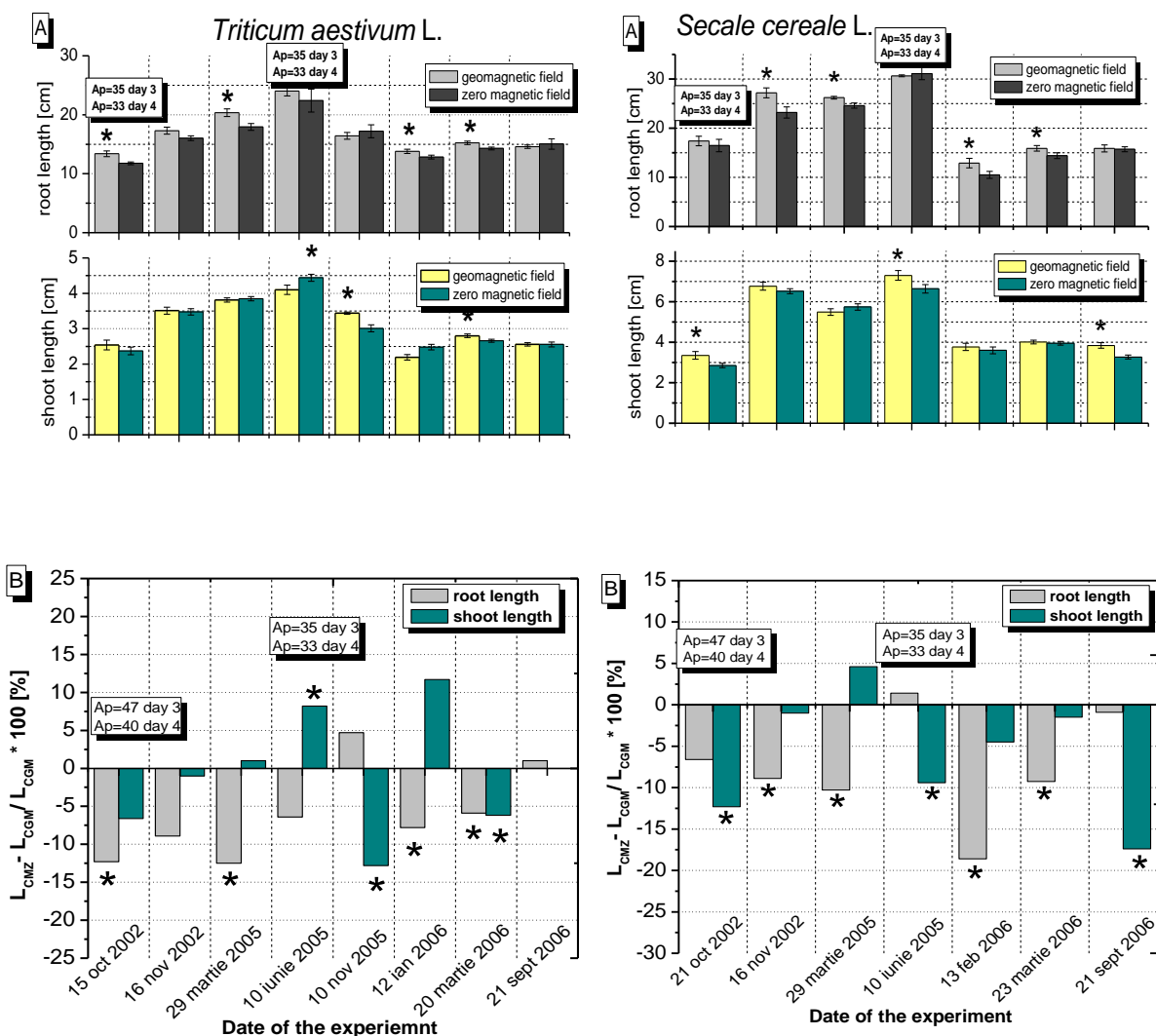
Fig. 3.5.
The values of seedlings length growth parameters of Triticum estivum and Secale cereale in ZMF and GMF in different periods of the years (A) and related ZMF effect.

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We noticed the high sensitivity of the rye in ZMF (Fig.3.5). A significant inhibition of seedling growth was recorded in all the experiments (10% ±0.60, p<0.5) with the maximum in February and September 2006 (~18%).

In different experiments, the seedling growth of *Tagetes patula L.* reveals an inconstant response in ZMF that alternate between significant root stimulation and shoot inhibition. On the other hand, seedling growth of *Calendula officinalis* L. has not been affected, indicating a low sensitivity to changes in magnetic environment MED.

Increased geomagnetic activity such as those equivalent to minor storms do not cause special effects in the growth of seedlings exposed to ZMF.

Stimulation or inhibition of plant growth of some species in the first four days of zero magnetic field exposure could be explained by the enhancement of plants sensitivity during a phase of intense growth (such as the seedling growth), just as the effects of weak AC fields are enhanced by stressors [Ruzic et al. 1998, 2000] or by physiological state of seeds [Aksyonov et al. 2001, Alexander and Doijode 1995]. This point can explain significant response of sp. Tagetes erecta and Tagetes patula during spring and summer periods when plant growth rate is enhanced in front of autumn and winter periods when a slower growth is accompanied by insignificant differences in seedling plants growth in ZMF and GMF

3.5. Effect of ZMF on the growth of aerial part of plants

ZMF influence on the emergence and development of aerial part of plant was followed by growth parameters of young plants (one month old) and mature plants at the end of vegetative stage and by ultrastructural analysis of leaves.

The morphological analysis consisted in monitoring the differences encountered in the phase of plant and leaf emergence and in the statistical evaluation of the differences of stem and leaf length, total length of the plant and its weight, the number of buds, flowers and fruit. Statistical significance of experimental data and assessment of zero magnetic field effect were determined as in the seedling growth case.

ZMF influence emphasized in the early growth stage, is greatly reduced during the development of young and mature plants [Neamtu ei al. 2009, Neamtu and Morariu 2010]. . Statistical analysis shows, for the same species exposed in ZMF, both significant and no growth differences in different periods of the year as as it can be seen in Fig.3.6 for *Calendula officinalis*.

In the absence of geomagnetic field, the development of *T. erecta L., Calendula officinalis L., Triticum aestivum L. and Capsicum L. anuum* was mainly slowed, a trend illustrated by the delay of the emergence and small size of the youngest leaves (Fig. 3.7.). After a month of ZMF exposure plant are less vigorous and the average weight of plants is lower than in the control group [Neamtu and Morariu 2010]. Similar results were reported by Lebedev for barley seedlings exposed three weeks to 10 nT shielded magnetic fields [Lebedev, 1977].

Development of young seedlings belonging to *Tagetes patula* and *Lycopersicum esculentum* sp. is not influenced in ZMF in the first month of life. Prolonged exposure of plants to five month, highlights a negative effect on the development of green mass of mature plant belonging to *Tagetes patula* L. and confirms the negative influence in the development of *Capsicum anuum* L., or insignificant response of sp. *Lycopersicum esculentum* L

In most species the reproductive growth stage was slightly influenced in ZMF. One can speak about an enhancement of growth of young plants (*Tagetes erecta, Tagetes patula,*

Capsicum anuum) and a faster (*Lycopersicum aesculentum*) or slower process (*Tagetes patula*) in the stage of buds and flowers emergence. In this stage of growth, pepper plant development was delayed and disease resistance diminished.
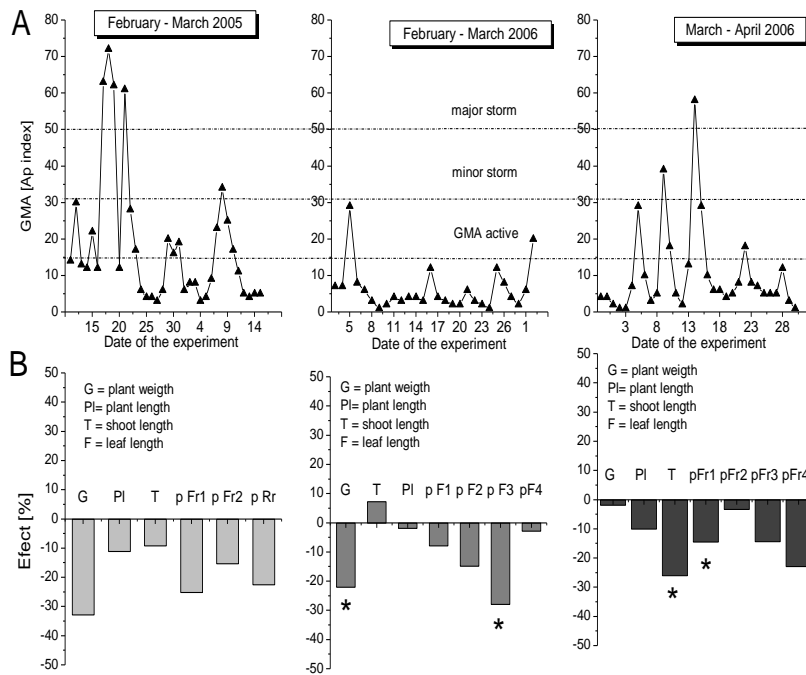
Fig.3.6.
Variation of geomagnetic activity (A) and ZMF effect on Calendula officinalis growth after a month of incubation
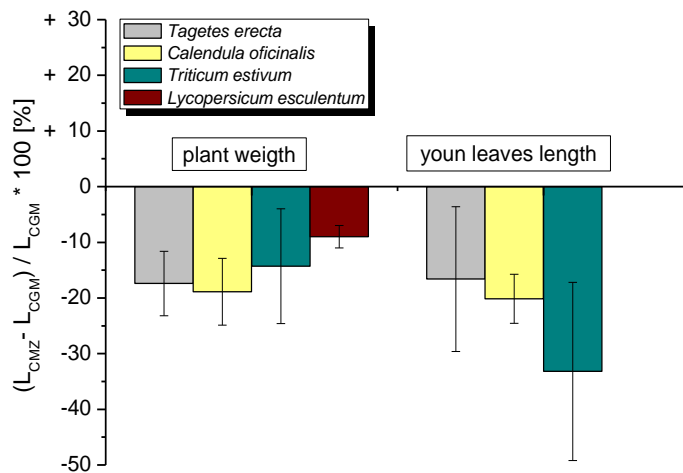
Fig.3.7.
ZMF effect on the growth parameters (weight, leaf length) of Tagetes patula L., Calendula officinalis L., Triticum estivum L., Lycopersicum esculentum L., after a month of incubation. (the average value of several experiments are given in each case)

In most species, the reproductive growth stage was slightly influenced in ZMF. One can speak about an enhancement of growth of young plants (*Tagetes erecta, Tagetes patula, Capsicum anuum*) and a faster (*Lycopersicum aesculentum*) or slower process (*Tagetes patula*) in the stage of buds and flowers emergence. In this stage of growth, pepper plant development was delayed and disease resistance diminished.

ZMF effect well defined in some species in the early stages of growth is significantly reduced by long term exposure of plants, suggesting an increased capacity of plants adaptation in the absence of geomagnetic field.

Analysis of ultrastructural changes at leaf level was performed on images obtained by electron microscopy, following the first chloroplast ultrastructure, which is the main body

of photosynthesis process [Neamtu et al 2009]. Overall picture of this analysis shows that the lack of geomagnetic field induced significant negative changes at this level, primarily on the chloroplast and nuclei of leaf cells of plants studied, resulting in the reduction of their metabolic activities. However, there is some differentiation of each plant response to the lack of magnetic field, due to somatic, phylogenetic and genetic features. Young plants exhibit a greater sensitivity than mature or aging ones. Also, crop plants suffer more in the absence of magnetic field than spontaneous plants.

Quantitative analysis of ultrastructural images of leaves cells, as fractal image analysis, to determine the mass fractal dimension and long-distance correlation analysis properties (FFT and DFA), brings additional information on the ultrastructural changes in leaves of plants exposed in ZMF [Morariu and et al 2006, Buimaga-Iarinca et al 2007, Neamtu et al 2009].

Fractal dimension of an image is perceived as local property. To find the mass fractal dimension of nuclei (including the nucleolus, eucromatin and heterocromatin) and of chloroplasts, we have used their electron microscopy images. A computer program ImageJ with "plugins-site" FracLac was used. The outline of the nuclei and chloroplasts has been selected from the general picture, and then analyzed by Box Counting method.

Long distance correlation is a global property of the system and can bring information about eucromatin structure of nuclei and general structure of the chloroplasts. This property was determined using FFT methods (Fast Fourier Transform) and DFA (Detrended Fluctuation Analysis) for which we used software developed in our research group. The spectral analysis had as an outcome the spectral correlation exponent, β, which is given by the slope of the spectrum in a double-logarithmic representation. Spectral correlation exponent (β coefficient) proved to be an effective parameter to characterize quantitatively the ultrastructural changes in leaves. Significant ultrastructural changes were highlighted in both the structure of chloroplasts and nuclei. In all examined plant species we have found significant differences in the β coefficient determined in plants exposed to ZMF compared to control, in the first month of incubation. These differences become insignificant when the chloroplasts of tomato and pepper plants aged 66 days. For marigold the differences remain even at this age but become insignificant in the reproductive growth stage of plants, after 180 days of incubation.

These results show a higher sensitivity of young plants to the absence of geomagnetic field and also suggest that over time there is a process of adaptation of plants to magnetically change of environmental, to regain normal functionality of plants, related to natural aging process. By analyzing the correlation properties were determined persistent changes in the structure of nuclei in plants exposed to zero magnetic field. In pepper, marigold and *Calendula*, structural changes of the nucleus illustrated by significant differences of spectral correlation exponent, remain both in plants aged 66 days and after 180 days. These observations suggest that the process of plants adaptation is only partial and refers primarily to the chloroplast functionality. The results confirmed most of ultrastructural observations.

IV. INTERACTION OF WEAK MAGNETIC FIELDS WITH BACTERIA AND CYANOBACTERIA

2. Zero magnetic field effects on bacteria growth

Literature provides sporadic data concerning bacterial response given in the absence of geomagnetic field. Shielding the geomagnetic field by a factor of 10, Becker found a 15 times reduction of the size and number of *Staphyloccocus aureus* colonies relative to the control cultures at an exposure time of 72 hours [Becker 1963]. Achkasova also found a depression of vital functions of bacteria in screened geomagnetic field [1973]. Available literature data offers no other information about bacterial multiplication in hipomagnetic field outside the above mentioned references.

The study described in this thesis refers to the analysis of bacteria growth in zero magnetic fields achieved by geomagnetic field compensation and also in 3 Gauss magnetic field. Tested strains were gram-positive bacteria (*Listeria monocytogenes*), gram negative (*Salmonella* and *Escherichia coli*) and nonspecific microorganisms grown on nutrient substrate protein (albumin) in closed cultivation systems ("batch" cultures) [Neamtu et al. 2003]. Cells multiplication was assessed by direct methods in order to determine the number of bacteria/ml cells suspension in parallel with indirect methods of monitoring the decrease of sugar and nutrients reserve in the environment. Additionally it has been investigated the bacteria response in 3 Gauss magnetic field (300μT) was also investigated. The same batch culture system was used to analyze cyanobacteria growth in Zarrouch medium.

2.3. Experimental results

2.3.2. Assessment of bacterial growth and metabolic activity in ZMF

The change of bacteria density/ml suspension was assessed by:

- optical transmission of cells suspension determined spectrophotometrically in the visible spectral range (520 nm - 700 nm) for *Salmonella* (Fig.4.1.), *Escherichia coli* and *Listeria* using the following calibration scale
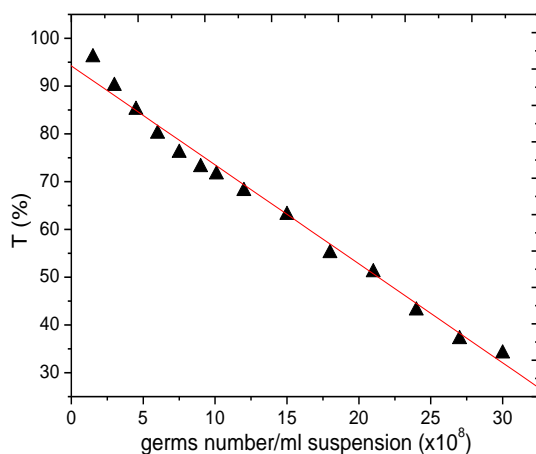
Fig.4.1.
Calibration scale: transmittance - bacteria density / ml suspension of Salmonella. λ = 400nm
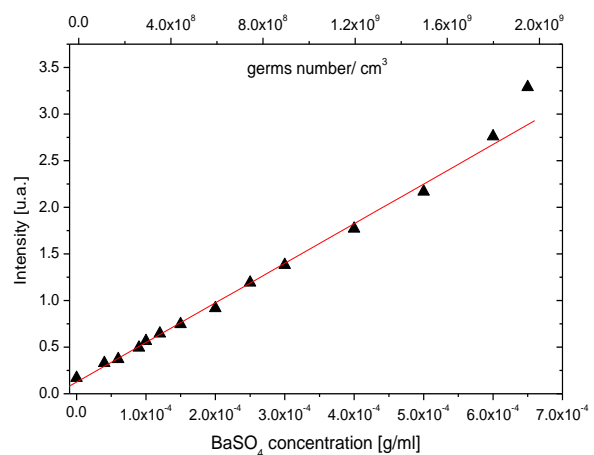
Fig.4.2. Mc Farland scale calibration for 10⁻⁵-10⁻³ range of BaSO4 concentrations determined by light scattering method and the associated bacteria scale density

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- Laser light scattering Mc Farland calibration scale used in order to determine the nonspecific germs contaminaton of 5% albumin solution (Fig.4.2.).

Metabolic activity of bacteria was estimated by sugar and pH medium variation due to specific strains activity (*Salmonella, E. coli, Listeria monocitogenes*) and albumin substrate degradation in the presence of non-specific Gram(-) bacteria.

Degradation of albumin following the development of microbial germs was highlighted by techniques of agarose gel electrophoresis and polyacrylamide gel in the presence of SDS (sodium dodecilsulfat) within 5 days of incubation.

2.3.3. Bacterial growth and metabolic activity in cultures of Listeria monocitogenes, E. coli and Salmonella strain exposed in ZMF

For all bacterial strains tested, the results reveal increased resistance in 3 Gauss magnetic field. In zero magnetic fields, the sensitivity of bacteria seems to depend on bacteria strain. *Salmonella* respond positively in these conditions, the cell density being significantly increased in the exponential phase of growth (Fig4.3). The results indicate favorable conditions for *Salmonella* contamination in the absence of geomagnetic field. The two other strains showed resistance.
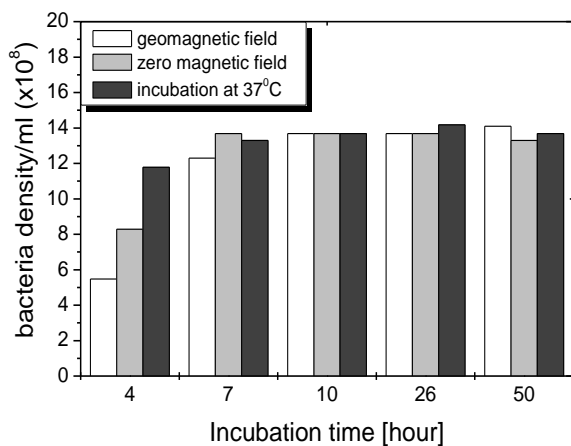
Fig.4.3.
Bacteria growth in Listeria, E. coli and Salmonella cultures exposed in GMF, ZMF, 3Gauss MF at 24ºC and in GMF at 37ºC
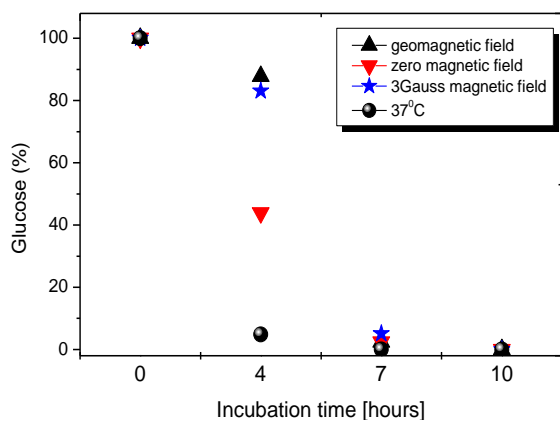
Fig.4.4.a
Glucose degradation in the prezence of Salmonella in CGM and CMZ at 24º C and in CGM at 37ºC
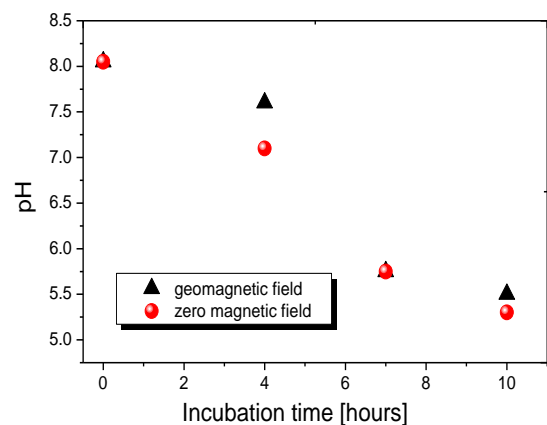
Fig. 4.4.b. pH variation în Salmonella cells suspension exposed in CGM and CMZ at 24º C and in CGM at 37ºC

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In the first 4 hours glucose was significantly reduced in *Salmonella* cell suspension (49.5%) and was entirely metabolized after 7 hours in both in GMF and ZMF (Fig. 4.4.a). In the same time, pH decreases in the cell medium (Fig. 4.4. b). These results show the positive influence of ZMF on *Salmonella* activity and support the data obtained in the bacteria growth tests. A low decrease in metabolic activity of *E. coli* in ZMF is also observed.

2.3.4. Growth and metabolic activity of gram (-) germs on the albumin substrate

In aqueous solution, albumin is a proteic nutrient for bacteria. Our results show significant increases of gram (-) germs contamination of albumin solution in ZMF conditions. After two days of exposure the number of germs/ml suspension has doubled (Fig. 4.5.). The difference of growth was maintained on the third day of incubation.
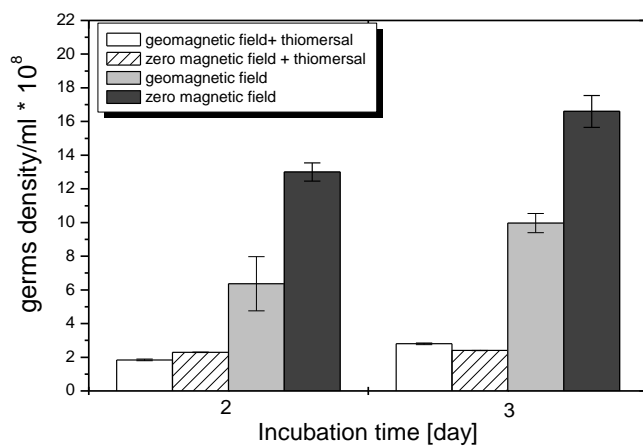
Fig.4.5.
Influence of ZMF on gram (-) germs growth on albumin as nutrient substrate in the presence and the absence of thiomersal as bactericidal agent

Similar results were obtained in both SDS polyacrylamide gel electrophoresis and agarose gel electrophoresis of albumin. Important differences of molecular integrity of albumin were distinguished after three days of exposure. Separation of distinct bands in the original spot of albumin indicates more pronounced degradation in samples exposed in ZMF, that persisted after five days of incubation. (Fig. 4.6.)
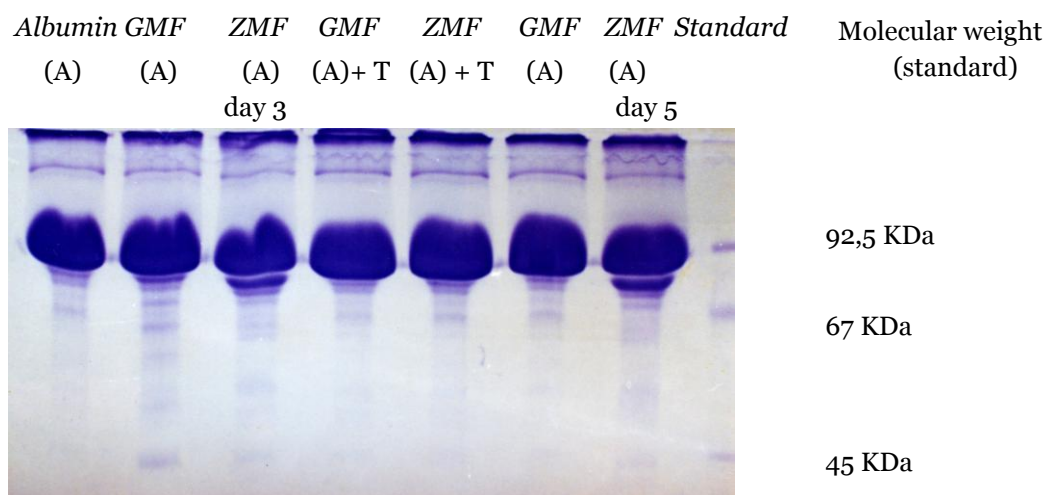
Fig. 4.6.
Polyacrylamide gel electrophoresis of albumin in the presence of SDS after 3 and 5 days of incubation in CGM and CMZ in the presence and absence of bactericidal agent (thiomersal)

In agarose gel electrophoresis, changes became evident after 5 days of exposure and consisted of enlarging the area of migration spot, which results in a decrease of migration speed of protein fractions. These changes reflect a process of degradation more pronounced in ZMF and are in agreement with the results obtained by polyacrylamide electrophoresis.

The results of our investigations show a dependence of bacterial response on species in ZMF and complete the studies of tolerance of bacteria (*Pseudomonas, Enterobacter, E. coli*) to antibiotics [Creanga et al 2004, Poiata et al 2003]. The overall images show that the development of bacterial cultures of *Salmonella* and some strains of *Pseudomonas* virulence is enhanced in zero magnetic fields, while the reactivity of some strains of *Enterobacter* and *E. coli* is depressed or not affected. In other words, the presence of a magnetic field of 25-75 μT, equivalent of the geomagnetic field intensity, is a protective environment against contamination with *Salmonella* and to the virulence of *Pseudomonas* strains. The growth of nonspecific gram (-) germs on albumin substrate also illustrates the protective effect of the geomagnetic field. On the other hand the natural magnetic environment creates favorable conditions for contamination with *Enterobacteriaceae* strains by increasing their resistance to some antibiotics.

3. Cyanobacteria growth in zero magnetic fields. Influence of geomagnetic activity

Cyanobacteria are organisms with growth potential both in the presence of light (by oxygen-based photosynthesis) and in the dark on glycolysis and oxidative phosphorylation. Structural and biochemical characteristics are similar to bacteria, which lack the nucleus and chloroplasts. Cyanobacteria are model organisms to study photosynthesis, nitrogen assimilation and to environmental stress adaptation. Except for a study aimed to examine the possibility of stimulating algal biomass in electromagnetic fields at 50-60Hz frequencies [Serafin et al 1995], we do not know other references on the influence of weak magnetic fields on cyanobacteria growth.

Synechocystis sp are unicellular organisms that are dispersed uniformly in the liquid media, providing a good biological system for cell multiplication studies. Changes of cianobacteria growth in ZMF, and in the presence of natural fluctuations of the magnetic field, was followed on *51 AICB Synechocystis* cell strains, provided by the own collection of algae of Institute of Biological Research, Cluj-Napoca.

3.2. Evaluation of growth rate of cyanobacteria in the CMZ

Synechocystis cell growth was estimated spectrophotometrically from the suspension absorbance at $\lambda = 670$ nm measured daily during a period of 8-12 days. Cell growth curve was characterized by the rate of cells growth quantified by a fit parameter "x_0" obtained by fitting the experimental data with Boltzmann function. ZMF effect was estimated by the normalized difference between the x_0 rate in samples exposed in CMZ and CGM.

3.3. Experimental results

In periods of low geomagnetic activity, the rate of cell proliferation in ZMF ranged below statistical significance ($p > 0.5$). However significant effects were observed during periods with high variation of geomagnetic activity. In a set of nine experiments, the growth rate of cyanobacteria was stimulated under ZMF exposure in all periods where the presence of a storm was encauntered. The obtained results for these cases are presented in detail in Fig.

4.7 a, b. Cell growth curves (right, b-d) are represented in parallel with the daily variation of geomagnetic activity (left, i-iii)).
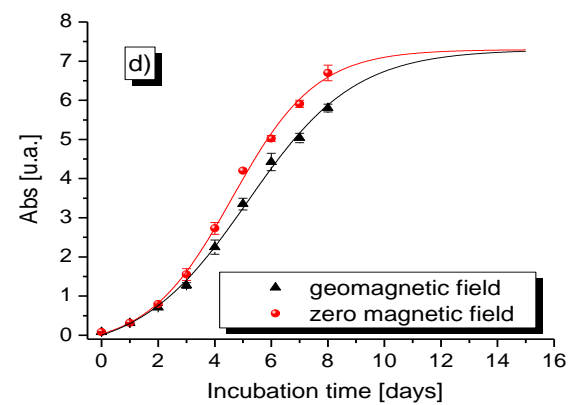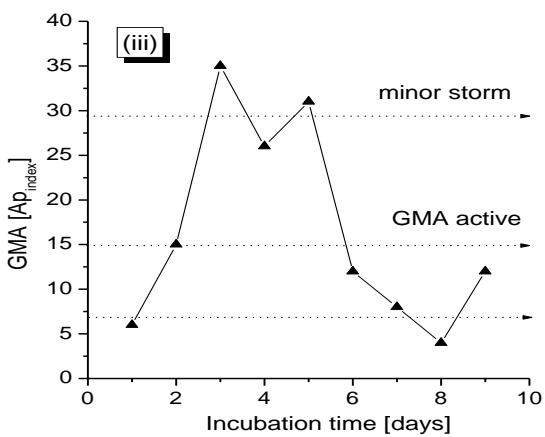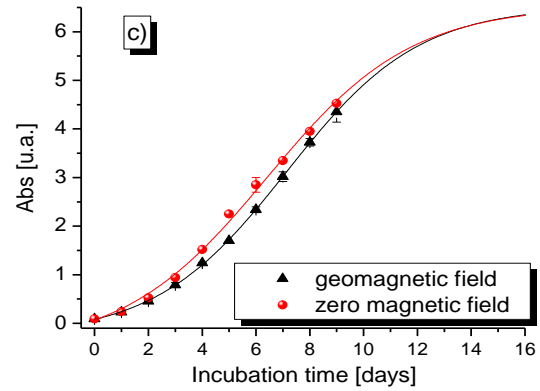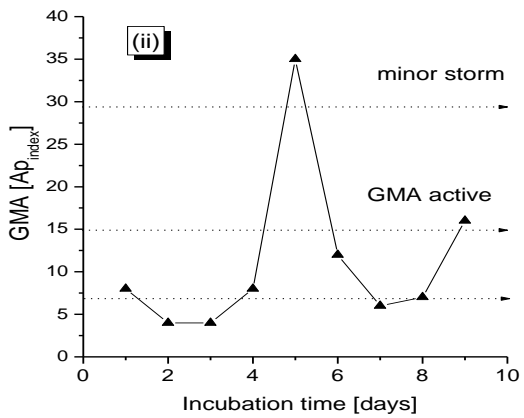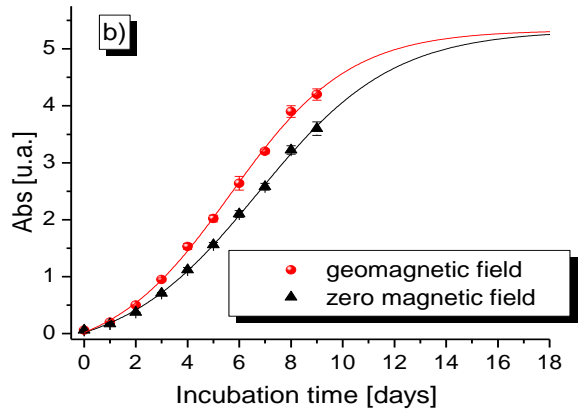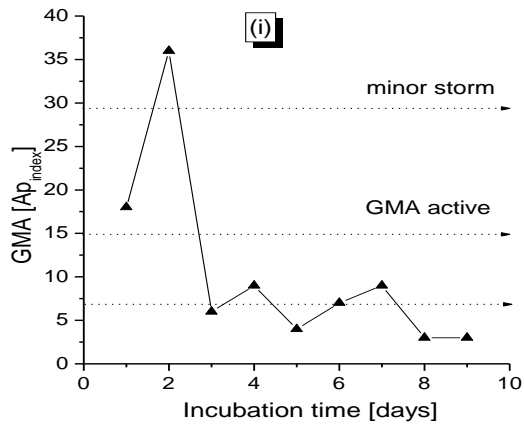

Fig. 4.7a.
Geomagnetic activity variation in the presence of magnetic storms (i,ii,iii)

Fig. 4.7b.
Cyanobacteria growth curves in ZMF and GMF in the presence of magnetic storms

The relation between geomagnetic activity and zero magnetic effect, indicates the following three combinations:

  • the presence of a minor storm (Ap = 35) at the beginning of the incubation period is associated with a significant cell growth rate in zero magnetic field (15%, $p < 0.05$) (Fig.4.7 case i).

  • the presence of a magnetic storms sequence (Ap = 35, 30) in the first period of cells incubation, accompanied by an active period of the GMA (Ap = 26) are associated with significant stimulation of cells growth rate (10%, Fig. 4.7, case iii).

  • the presence of a minor storm (Ap = 35) in middle exponential growth phase is correlated with a diminished response (Fig. 4.7, case ii), the stimulation effect (7%) was similar to the one in periods of moderate GMA variation.

Data presented in this chapter indicates a nonspecific response in the rate of unicellular cyanobacteria growth in the absence of static geomagnetic field component and a sensitive biological response modulated by changing of the variable magnetic component. The results suggest a biological response that is conditioned by the magnetic storm occurrence at the initiation of cell growth, or at the occurrence of magnetic disturbances during the exponential phase of growth.

V. LISTERIA MONOCYTOGENES AND HUMAN SPERM MOTILITY IN ZERO MAGNETIC FIELD- PRELIMINARY DATA

1. Cell motility

Motility is a dynamic process that plays a fundamental role in cellular function as molecular and cellular organelles transport, chromosome segregation in mitosis, or cell shape changes in cell locomotion and simple organisms (prokariote, eukarioate) in response to external stimuli. Cell motility is based on complex processes of self-organization supported by so-called molecular motors [Fletcher and Theriot, 2004]. These consist of a set of proteins and molecular complexes that provide energy for movement, by converting chemical energy released by hydrolysis of the molecule ATP macroergic links or energy stored in transmembrane potential. Known molecular motors can be grouped into five categories of which the best characterized are: (i) the rotating motor, for movement of bacteria, and (ii) linear motor, one of the most common forms of movement found in cell eukariote, in sperm and in some types of protozoa motility. A special category is the motor based on assembly and disassembly of actin, which is based motility of pathogenic bacteria: *Listeria* monocitogens*, Shigella, and Rickett.*

3. Experimental results

Studies presented in this chapter aimed to monitor the motility in ZMF of prokariote organisms (bacteria) and eukariote cells based on different molecular mechanisms. Experiments were performed on: (i) bacterial strains belonging to *Listeria monocytogenes* sp whose displacements is based on actin assembly and disassembly mechanism and (ii) human sperm motility in semen which is driven by linear motors having promoter dineine molecules. [Boldiszar et al. 2001, Neamtu et al. 2005, Truta et al 2005,]

3.1. Exposure of Listeria monocytogenes cultures in ZMF

Listeria monocytogenes cultures were provided by Microbiology Laboratory of USAMV Cluj-Napoca. A particularity of these bacteria is to synthesize cilia at room temperature (21-22OC) which are immobile to 37OC. Motility of bacteria inoculated into on tubes with semisolid agar medium was determined after 48 hours of incubation by moving the ring formed by bacterial culture that developed in culture medium. Parallel tubes were inoculated with bacteria exposed to 3Gauss magnetic field intensity. Cell motility analysis was repeated after one week of removing samples from ZMF and kept at room temperature.

3.3. Motility of Listeriae monocytogenes in ZMF

Differences in bacteria motility were detected after 24 hours of cells incubation in GMF and ZMF [Neamtu et al 2005]. Direct microscopic examination of native preparations showed a low mobility of bacteria incubated in ZMF, observation subsequently confirmed by the migration in soft agar culture (Fig. 5.1).

Fig. 5.1.a
Listeriamonocitogens migration after
48 hours incubation at 21°C.
Control MF 3G, sample MF 3G, control ZMF, sample ZMF
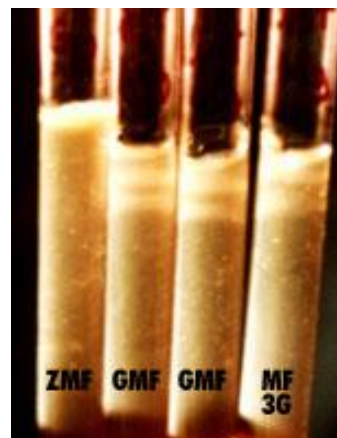
Fig. 5.1.b.
Listeria monocitogens migration after
6 days incubation at 21°C.
Control ZMF, sample ZMF, control MF 3G, sample MF 3G,

The white ring made by the cells growth, moved down the soft agar column similar in GMF and 3G magnetic field but remained at the starting line in the sample exposed to CMZ. These results indicate that in the absence of the geomagnetic field *Listeria* motility is inhibited, but not affected in the intense magnetic fields (3Gauss). The presence of the geomagnetic field seems to be an essential requirement for locomotion of bacteria.

Cell motility was not restored after exposure in ZMF suggesting a lasting effect. Except for samples incubated in ZMF, in all tubes kept and stored at room temperature, within one week it has occurred another opalescent disk into the depths, with a width close to the first ring, as a consequence of continuing the process of migration and multiplication of bacteria (Fig. 5.1.b). The behavior of *Listeria* in ZMF was similar with the response of ependymal cells reported by Sandodze et al [1995]. Ciliary motility apparatus of these cells was practically stopped in compensated geomagnetic field (x 100). These data represent evidence that the movement of cilia and flagellum generated by different molecular mechanisms (actin assembly and disassembly for bacteria and linear motor for ependymal cells) is equally affected in ZMF. The common point in both types of mechanisms is the energy source needed for motility, given by ATP hydrolysis. Effects of inhibition of cellular

processes based on the energy involved in ATP hydrolysis (eg transmembrane ion transport) have also been highlighted in our previous studies on human erythrocytes aging in ZMF [Morariu et al 1999].

3.2. Analysis of spermatozoa motility in human seminal fluids

The velocity of spermatozoa cell is a way of assessing the fertilizing capacity of cells. Fertilizing capability is attributed to the group of cells moving with fast velocity> 16 um/s called rapid spermatozoa. Below this value slow cells fall. Cells motility was estimated microscopically over a period of 30 hours of incubation at different temperatures (17-22ºC) by image analysis method [Neamtu et al 2005] using a CCD camera coupled to a microscope. Trajectories of 15 to 30 cells/2 samples were statistically analyzed every 3 hours, each path consisting of 6-10 lengths/sec. Cell motility in ZMF and GMF was characterized by the ratio of rapid sperm count (viability) and their velocity towards the total number of cells. ZMF effect was determined by normalized difference in the percentage of rapid cells in ZMF and GMF as control.

3.2. Spermatozoa motility in zero magnetic fields

ZMF notice a significant increase in velocity of spermatozoa movement in the first hours of incubation (Fig. 2).

Fig.2.
Speed of the faster spermatozoa at different temperatures

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Stimulation of sperm velocity reaches a maximum that fluctuates in the range of 17-20% at temperatures between 17°C-20°C. Faster cells returns to baseline, while cell motility in GMF drops below this value.

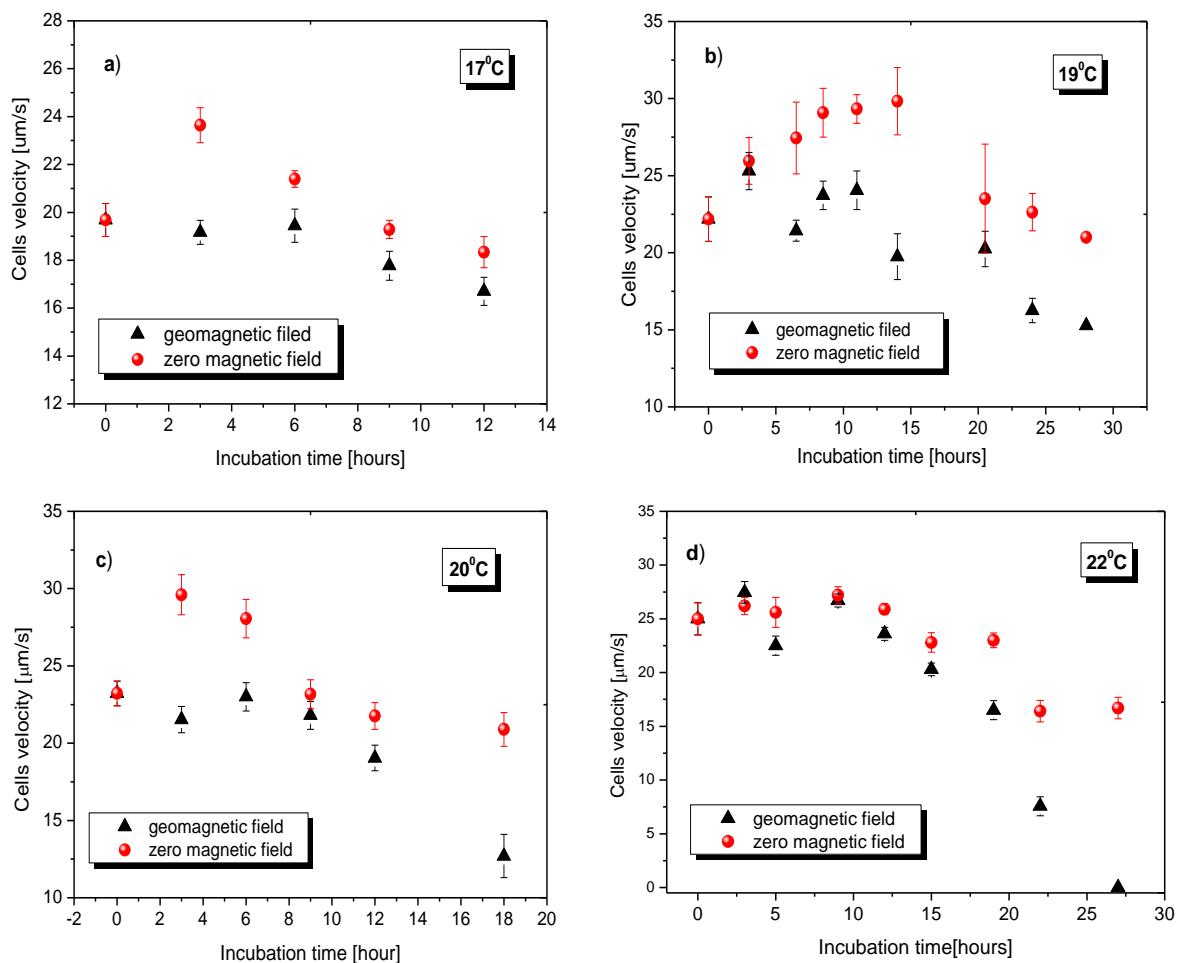The cell velocity is not affected at 22°C in ZMF. Viable spermatozoa move faster with similar velocity both in ZMF and GMF. We consider these conditions as a threshold temperature sensitivity of cells to changes their motility in the presence of magnetic field. Viability curve of rapid spermatozoa is shifted towards significantly higher values in ZMF, but their speed is not stimulated.

The increased of cells velocity in ZMF in the fist hours of incubation at temperatures below 22°C (17-20°C) seem to be a reaction of adaptation to environmental conditions by activating slow spermatozoa motility passing into rapid cells category. In GMF cell velocity varies around the baseline during the first hours of incubation, without being stimulated it then decreases to the minimum value which defines the category of rapid spermatozoa and slow category.

VI. CONCLUSIONS

The first part of the thesis includes analysis of zero magnetic field influence (I) during the vegetative growth stage of some plant species and (II) in the process of bacterial multiplication. The final chapter presents preliminary results on the motility of unicellular organisms (bacteria, sperm) in zero magnetic field.

The original results include the following aspects:

I. THE VEGETATIVE GROWTH STAGE OF PLANT

A systematic study was conducted concerning plants response in the absence of the geomagnetic field throughout the entire vegetative growth period. Eight species of plants were investigated: *Medicago sativum* L. *Tagetes patula*, *Tagetes erecta* L., *Calendula officinalis* L., *Triticum aestivum*, *Secale cereale* L., Lycopersicum esculentum L. and *Capsicum annuum* L. in the following growth phases: i) seeds germination, ii) the early seedling growth defined by the embryo radicle emergence and development across a period of 4-5 days, iii) development of young plants (one month aged) and iv) the mature plant untill the step of flowering and fruit formation.

Original issues are: 1) expand the study of partial aspects of plant evolution in zero magnetic field to analysis of the entire range of vegetative growth stage, 2) monitoring the growth in different periods of the same species and under the same experimental conditions 3) perform comparative tests of plant species that have not been reviewed yet 4) detect the influence of natural magnetic fluctuations under zero magnetic field at different stages of plant development.

1. Seed Germination

The absence of static component of the geomagnetic field does not represent a stress factor during seed germination. The process is positively influenced in the presence of major magnetic storms, if the magnetic disturbance is present in the initial phase of the germination process (water uptake). These results were obtained in seed germination of *Medicago sativa* L. and *Secale cereale* L.

2. Early stage of seedling growth

The response of plants in the early stage of seedling growth is dependent on plant species: 1) *Tagetes erecta L.* and *Medicago sativa* L are dominated by stimulation of growth; while *Triticum aestivum L.* and *Secale cereale L.* are mainly inhibited. *Tagetes patula L* show inconclusive response and *Calendula oficinalis L.* shows a low sensitivity. Significant response can be viewed as an enhanced sensitivity of the plant during some phases in which the growth is intense, such as the post embryo growth.

Increased geomagnetic activity variations equivalent to the occurrence of magnetic storms does not significantly influence this step of plant growth. An increased sensitivity is suggested by *Tagetes erecta L* in the presence of a minor storm in the water uptake step of seeds.

3. Young seedlings growth (one month old)

At different times of the year, statistical evaluation of growth parameters showed for the same species, both significant ZMF effect and on the limit of statistical significance.

The growth of *T. erecta* L., *Calendula officinalis* L., *Triticum aestivum* L. and *Capsicum anuum* L. was mainly slowed, a trend exemplified by the delayed emergence and small size of the youngest leaves. After one month of exposure in the ZMF, plants are less vigorous and the average weight of green mass of plants is lower than control group.

4. Mature plants - five to seven months exposure in ZMF

Reproductive stage of growth is not significantly influenced in ZMF. One can speak about an enhancement of growth in height of young plants (*Tagetes erecta, Tagetes patula, Capsicum anuum*) and a faster process (*Lycopersicum aesculentum*) or a slower one (*Tagetes patula*) at the stage of buds and flowers development.

5. Ultrastructural analysis of leaf cells Ultrastructural analysis of leaves generally emphasize the negative influence of ZMF in the chloroplasts and nuclei from young plants, offset in the natural process of aging in mature plants. There are some differentiations in the response of each plant: young plants show a greater sensitivity than mature or about aging. Also, crop plants (*Lycopersicum aesculentum, Capsicum anuum* L) suffer more in ZMF than plants from spontaneous flora (Tagetes patula L.).

Most of ultrastructural observations were confirmed by fractal image analysis. Some plant species showed persistent changes in the nuclei structure in ZMF conditions suggesting that the process of plant adaptation is only partial and refers primarily to the chloroplast functionality

In conclusion, ZMF effect emphasized in some species in the early stages of growth is significantly reduced by long term exposure of plants, suggesting an increased temporal capacity for adaptation of plants in the absence of geomagnetic field.

II. BACTERIA GROWTH

Our investigations completes the studies concerning the antibiotic tolerance of bacteria [Creanga et al 2004, Poiata et al. 2003] and shows, in ZMF conditions, a response dependent on species. *Salmonella* respond positively while *Listeria monocytogenes* shows a good adaptation: cell multiplication in *E. coli* cultures is not affected or not significantly depressed. The growth of microorganisms on the albumin substrate is significantly enhanced in ZMF. The results indicate that the presence of the geomagnetic field is a protective environment from microorganisms and pathogenic germs contamination such as *Salmonella*

bacteria, without affecting significantly the growth of other bacteria (*Listeria monocytogenes, E. coli*). Bacterial growth is not affected at relatively high values of magnetic field (300µT).

Cyanobacteria growth

In periods of low geomagnetic activity, the rate of multiplication of cyanobacteria is not influenced in zero magnetic fielda. Significant correlation (p <0.05) between the rate of cell population growth and geomagnetic activity variations suggests an increased sensitivity of the cyanobacteria in the presence of minor magnetic storms. Biological response is conditioned by the emergence of magnetic storm at the beginning or during the exponential phase of growth.

III. CELL MOTILITY - preliminary data

Cell motility was studied on non magentotactic unicellular prokaryote (bacterial strains sp. *Listeria monocytogenes*) and eukariote cells (human sperm) whose motion is caused by different molecular motors.

Listeria monocytogenes motility depends on geomagnetic field presence. In zero magnetic field migration of bacteria is inhibited, effect persistent on the subsequent exposure to geomagnetic field, suggesting a lasting effect. In high magnetic fields (300 µT) bacterial motility is not affected.

Different response is given by human sperm motility. In the temperature range 17-22ºC cell velocity is significantly stimulated in the first hours of exposure to ZMF. This response seems to be an adaption response to unfavorable environmental conditions by activating slow sperm motility passing in the category of quick.

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PART II

EFFECTS OF STRONG AND SHORT ELECTRIC FIELDS ON LIVING CELLS - BIOMEMBRANE ELECTROPERMEABILIZATION

1. INTRODUCTION

Electropermeabilization, also known as the electroporation is a membrane phenomenon initiated by applying of strong (kV / cm) and short duration (μs-ms) electric exponential or rectangular shaped field pulses, on cell suspension, cell cultures, or tissue [Rakosy-Tican L. et al. 2000]. It consists on temporarily removed of membrane barrier for intra-and extracellular molecules, once is reached a critical transmembrane potential of about 200-300mV. Above this value membrane permeabilization take place, followed by free transmembane transport of ions and molecules [Neumann and Rosenheck, 1972, Zimmermann et al. 1974; Kinosita and Tsonga, 1977] through the so-called aqueous pores. In a certain range of pulses parameters, this process is reversible.

Membrane electropermeabilization is described as a three step process :

(i) induction (μs) linked to a modulation of transmembrane potential induced by external electic field,

(ii) expansion – a slow process with a lifetime in a time range of ms to minute. The membrane permeated state is controlled by physical and chemical parameters like pulse strength, number and duration, temperature or pulsing buffer composition [Rols and Teissie 1989]

(iii) pores resealing, controlled and modulated by electrical parameters of the applied field. While the induction of pores is assumed to be a lipid domain process, the stability of permeable state after poration suggests that in the pores resealing process other molecular structures are involved [Rols and Teissie 1992, Chang and Reese 1990, Neamtu and colab 1990].

In biotechnology, electroporation of membranes provides the best solutions for solving some applied purposes, being used successfully in all areas involving the manipulation of living cells. Although understanding the molecular mechanisms of the membrane permeability process continues to be an open topic of study, motivated by the limited understanding of the theoretical bases of this phenomenon [Teissie et al. 2005, Weaver 2003, Weaver and Chizmadzev 1996], electroporation technique is now widely used for cellular incorporation of a large diversity of molecules such as pharmacologically active substances, antibiotics, oligonucleotides, RNA, DNA [Favard 2007, Gehl, 2003, Rolls 2006, Golzio et al. 2002] transdermic release of active substances [Prausnitz 1996 Denet et al. 2004 Pavselj et al 2005b], cellular fusion [Zimmermann 1982], membrane electroinsertion of protein [Teissie and Ramon in 1998], sterilization [Rowan et al. 2000 Teissie et al. 2002], and tissue ablation [Davalos et al. 2005, Lavee et al. 2007].

The practical relevance of membrane electropermeabilization comprises the following aspects:
- ➢ is a noninvasive physical process and reversible in known intervals of electrical parameters
- ➢ is influenced by factors that can be controlled and used to modulate the process in the desired way,
- ➢ new cellular systems created are compatible with the organism
- ➢ it provides a direct access to the site of action for active substance and its biodegradation is avoided.

Cellular manipulation by electroportion technique has facilitated the development of new ways of medical therapy.

II. EXPERIMENTAL RESULTS

1. Membrane electroporation technique – Experimental installation

In order to address research topics on membrane electropermeabilization a device was designed and built [Fig.2.1] having a pulse generator designed to generate rectangular or exponential discharge electric pulses with time and amplitude programmable in defined intervals Tab 2.1.

	Rectangular pulses		Exponenţial pulses	
	Pulse length μs-ms	Intensity KV/cm	Pulse length μs-ms	Intensity KV/cm
	20μs -50μs	0-12	10-800μs	0-12
	50μs -100μs	0-8	0,8-8ms	0-4
	100μs -400μs	0-4	8-20ms	0-2
	0.4μs -1.5ms	0-2	20-50ms	0-1
	1.5μs -5ms	0-1		

Table 1.1 Ranges of electric pulse parameters values - amplitude and duration, for rectangular and exponential pulses

The device includes a flow electroporation cell with a processing capacity of 2 ml of biological sample. The cuvette is placed in a thermostat block accurate to 0.5 C in 2-40°C temperature range.

2. CONTRIBUTION OF ELECTRICAL PULSE PARAMETERS IN ERYTHROCYTE MEMBRANE ELECTROPERMEABLIZATION

The values of electrical pulse parameters used for reversible membrane permeabilization range between 1-20 kV/cm amplitude and 10μs - 10 ms pulse duration.

Lower pulse amplitude can generate fields below the critical membrane potential. Instead, excessive field intensity causes irreversible membrane permeabilization, followed by a massive efflux of intracellular constituents.

Our investigations aimed to find the main contribution of the pulse strength and duration on erythrocyte electropermebilizarea [Neamtu et al 2000]. Two different processes were followed: the efflux kinetics of large macromolecules as hemolobin (65,000 Da) (Figure 2.1.) and influx of relative small particles as manganese ions (55 Da) normally impermeable through erythrocye membrane.

Four combinations of electric pulse parameters were tested:

a) E = 4kV/cm, τ = 40 μs;   c) E = 4kV/cm, τ = 100 μs;
b) E = 5 kV/cm, τ = 40 μs;   d) E = 5 kV/cm, τ = 100 μs

The kinetics of hemoglobin efflux reaches a plateau after 30 min due to the molecular balance between the two compartments of the cell (Fig 2.1.). The plateau value gives the permeabilisation efficiency of electric field pulses.

A relevant result obtained from our experiments is related to the fact that different combination of pulse parameters can induce the same permeabilization. A similar hemoglobin and ions permeability is noticed under the action of b) and c) combination (Fig 2.1.).

Fig.2.1

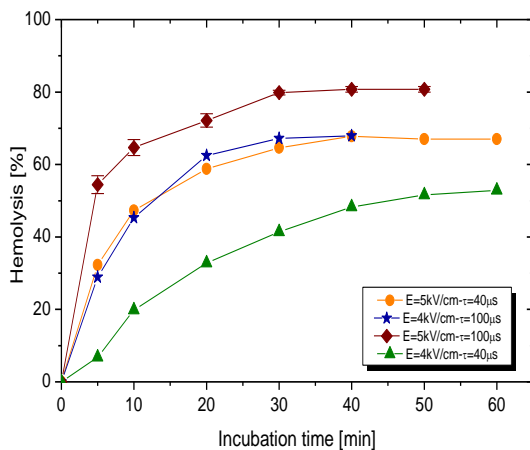*The kinetic of hemoglobin efflux through erythrocyte electroporated membrane at different pulse parameters. Incubation temperature - 4°Cion.*

By exposing cell suspensions to any combination of electric field parameters two distinct cell populations are obtained:

a) erythrocyte ghosts with a low hemoglobin content

b) hemoglobin-containing red cells and partially permeable membrane to manganese ions.

Manganese ions permeability of membranes of the two cell populations differs greatly. Distribution of manganese ions in intracellular compartments is strongly asymmetric, being built almost exclusively in erythrocyte ghosts. The presence of manganese ions both in cell ghost and in erythrocyte sediment revealed the size selectivity of membrane permeabilization process. Different ranges of pulses parameters values induce a selective permeability to different molecular species. The two 'species' tested: hemoglobin and Mn^{2+} ions are considered representative for a wide range of molecular species discriminated by molar mass and the default size.

Restoring membrane integrity and impermeability of obtained cellulat ghosts was analyzed by the membrane permeability to manganese ions of cells electroporated and incubated at 37°C (Fig. 2.2).
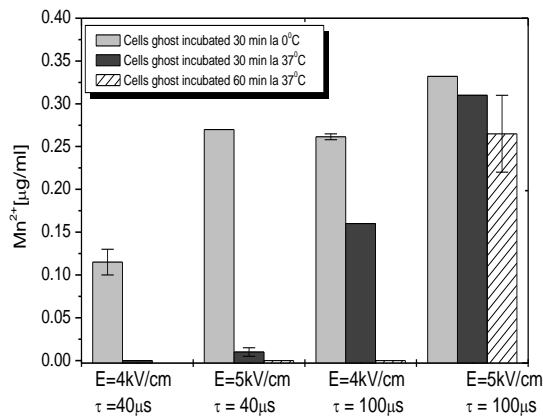

Fig.2.2

Intracellular concentration of manganese ions in cell ghosts obtained by exposing erythrocyte to different parameters. electric pulses

The process of pore resealing was found to be the step that discriminate between the two cases (b) and d) with similar permeabilisation efficiency (Fig 2.2.). Although membrane permeability induced by electric puses E = 4kV/cm, τ = 100 μs emphasized both by molecular hemoglobin efflux and by the influx of manganese ions was similar, restoration of membrane integrity was different and was dependent on length of electric pulse applied. These data confirm that the pulse duration is the main parameter that controls cell membrane resealing step.

3. PORES RESEALING INACTIVATION IN ELECTROPORATED ERYTHROCYTES MEMBRANE IRRADIATED WITH ELECTRONS

Molecular mechanisms of membrane permeabilization continue to be one of the issues much debated in the literature. It is accepted that the induced membrane permeabilization is a lipid domain process, but the stability of permeable state after poration suggests that in the pore resealing process, other molecular structures are involved, mainly the membrane proteins and cytoskeleton [Teissie and Rols 1994, Rols and Teissie 1992, Chang and Reese 1990]. Cell membrane and cytoskeleton protein network are viewed as a complex system, their association is located at the transmembrane protein level through a lipid binding protein, ankirin. Hypothesis of cytoskeleton involvement in membrane electropermeabilization process is supported by several research groups [Rols and Teissie 1992, Chang and Reese 1990].

Our research intends to identify the protein with a dominant contribution that could be involved in the recovery phase of erythrocyte electroporated membrane [Neamtu et al.

1999]. In this connection, we take advantage of the "target analysis" method following the inactivation of protein function caused by irradiation with high energy electron beam. It has been shown that this method provides the opportunity to evaluate the molecular weight of a protein. [Kempner and Macey 1989].

3.1. Target Analysis

The main working hypothesis is that each collision between high-energy electrons and molecular target strongly affects the molecular complex, so that their biological function is completely lost. The fraction of the still active molecule decreases exponentially with the average number of collision:

$$N/N_0 = e-KD \tag{3.1}$$

The average number of "hits" is proportional to the radiation dose D, the constant K containing information directly related to the mass of target molecular system.

The experimentally accessible parameter is the slope of the so called 'inactivation curve' obtained by the logarithmic representation of the fraction of biologically active molecule vs. radiation dose. The molecular weight of the target system is calculated by the relation:

$$Kx = 6.4 \; x109 \; mm \tag{3.2}$$

determined empirically by Kepner and Marcey [1968] and theoretically by Kepner and Haigler [1985].

3.2. Electron irradiation and electroporation

Erythrocyte suspensions were irradiated at liquid nitrogen temperature (270^oK) with an electron beam of 5MeV energy, generated by a linear accelerator. At this temperature the formation of free radicals is ruled out of the water (the dominant side effect which shields the primary effect of radiation).

The membrane electropermeabilization process was quantified by the degree of erythrocytes hemolysis after electroporation. Pulsing was performed at $0\text{-}4^oC$ with square-vawe electric pulses, $40\mu s$ length and the variable amplitude in the range 2-6 kV cm^{-1}, or fixed at 4 kV cm^{-1}. The hemoglobin concentration was determined spectrophotometrically measuring the absorbtion at $\lambda = 410$ nm in the cells medium. The electrohemolysis yield (H/H_0) was given by the ratio between the absorbance of electroporate samples and the control hemolysate.

3.3. Experimental results

The direct effect of electron irradiation on erythrocyte membrane at irradiation doses of 1-6 kGy was followed in both phases of the membrane electropermeabilizare:

1) induction of pereabilisation monitored by efflux of hemoglobin molecules at low temperature (4^oC).

2) restoration of membrane barrier function for these molecules by incubating the electroporated suspension at temperatures between $0\text{-}37^oC$.

While the first phase of the direct action of electron irradiation seems to be irrelevant for the first phase of the electroporation process, thermal recovery process is strongly

affected. The lifetime of permeable state of red blood cell membrane is reduced by increasing the temperature, with simultaneous decrease of the membrane permeability to hemoglobin. This process was investigated for erythrocytes irradiated at four different levels: 1, 2, 4 and 6 kGy, electroporated at E = 4kV cm-1 and incubated for 1 h at increasing temperatures (Fig. 3.3.). The presence of hemoglobin molecules in the extracellular environment was used to quantify the permeability state of cell membrane. An unirradiated sample (as control test was treated in the same way a sample. To estimate the molecular weight of structures affected by radiation by the target analysis method, the results obtained at 37°C were taken into account. In this case, the inactivation curve is given by the logarithm of intracellular hemoglobin variation ln (1-H/H0) versus the dose of irradiation (Figure 3.4). By increasing the irradiation dose, the ability to recover membrane barrier function of hemoglobin molecules is progressively impaired.
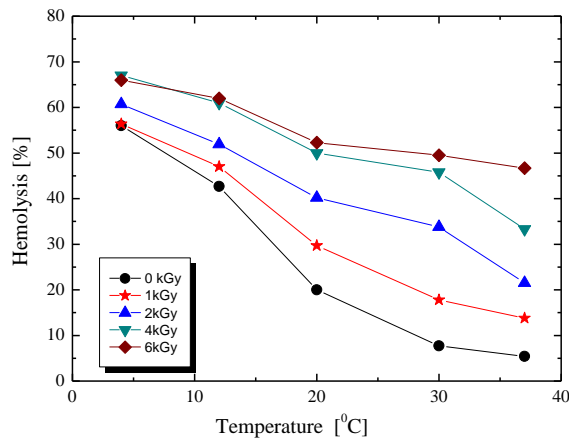

Fig 3.3
The dependence of irradiated erythrocyte electrohemolysis on the incubation temperature. Iradiation with 5 MeV electron beams at doses:1-6 Gy; electroporation E=4kV/cm, τ=40 μs, temperature 4°C

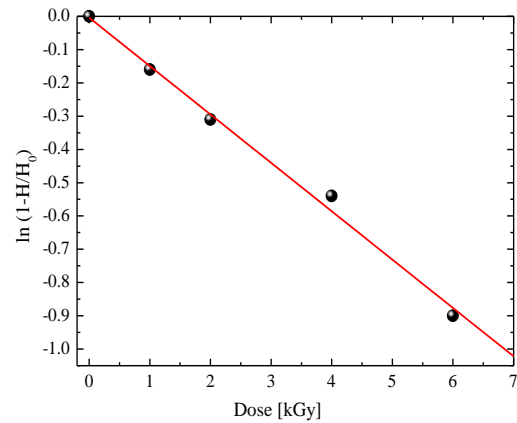*Fig 3.4*
Inactivation of membrane recovery curve (T = 37OC) for various doses of irradiation.

The plot show a slope of K = 0.145 (kGy) -1. Applying this value in the relation (3.2) we obtain a molecular weight M ~ 930kDa, value close to the molecular weight of the spectrin tetramer molecular system. All other membrane protein structures have molecular weight below 400 kDa. Spectrin is the basic component of protein that forms the cytoskeleton network of red blood cells. Our results confirm the involvement of the spectrin network in the recovery of electroporated membrane.

4. DIMENSIONAL DISTRIBUTION OF HUMAN ERYTHROCYTES OBTAINED FROM ELECTROPERMEABILIZATION EXPERIMENTS

For any cell species an effective critical value of transmembrane potential difference can be defined. If the external electric field strength is large enough, all cells whose radius satisfies the limit condition:

$$\psi_c^{ef} = \frac{3}{2} ER \qquad (4.1)$$

will be electropermeabilized.

Electropermeabilization process becomes dependent only on the size of the cell. In a cell population that usually is heterogeneous in size, critical potential induced by the action of electric field is initially achieved by cells with the largest dimension. When increasing the field strength, the percentage of cells that reach critical condition favorable for electropororation also increases. In a certain range of field strength values, all the cells are permeabilizated. Thus, for a normally distributed cells population, electropermeabilization curve shows a sigmoidal dependence (Fig. 2.5).

In the case of erythrocytes electroporation, the curve of electrohemolysis is characterized by two parameters. $E_{1/2}$ parameter is the electric field intensity that induces hemolysis of 50% of the red blood cells population and $R_{1/2}$ assigned to the average cell radius. Based on electropermeabilization process efficiency (η) defined by the ratio between the number of cells permeabilizated and total cell number, we have proposed a relationship that defines the dimensional distribution of cell populations [Turcu and Neamtu 1995]:

$$\eta(y) = 1 - \int_0^{x(y)} f(x^{'})dx^{'} \qquad (4.2)$$

where f(x) is the normalized dimensional distribution function, x and y being dimensionless variables: $x = R/R_{1/2}$ and $y = E/E_{1/2}$. Critical condition given by the equation (4.1) becomes with the new variables:

$$xy = 1 \qquad (4.3)$$

By derivation of equation (4.2) with respect to x, the normalized dimensional distribution function f(x) becomes:

$$f(x) = \frac{1}{x^2}\frac{d\eta}{dy} \qquad (4.4)$$

This formula can be used to characterize the dimensional distribution function given that the efficiency is determined experimentally. The net advantage of this approach is that by using normalized variables have a universal validity, being independent of specific values of membrane conductivity, cell size and electrical properties of internal and external cellular environments.

Size distribution function was verified in experiments carried out in erythrocytes electrohemolysis in two different experimental conditions. Electrohemolysis curves were obtained by unicellular multicellular method.

1. unicellular method - using high dilutions of cell suspension (0.01%) to allow (i) electroporation of a single cell brought between electrodes and (ii) measuring the threshold value of electric field strength that produces cell hemolysis.

2. multicellular method - exposure of cell suspensions with 1% hematocrit. Electroporation involves electric pulses of 60-80% cells. Electrohemolysis efficiency is measured by the number of lysated cell versus total number of cells.

Normalized dependence of the electropermeabilized cells percent on the intensity of applied electric field is represented in Fig.2.5. for two values of pulse duration: 1 ms (A) and 50μs (B).

In Figure 2.6 we present presented histograms of normalized dimensional distribution functions obtained by numerical derivatization of electroporation efficiency dependence.
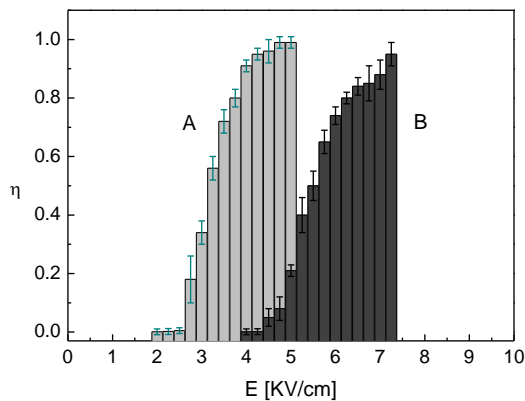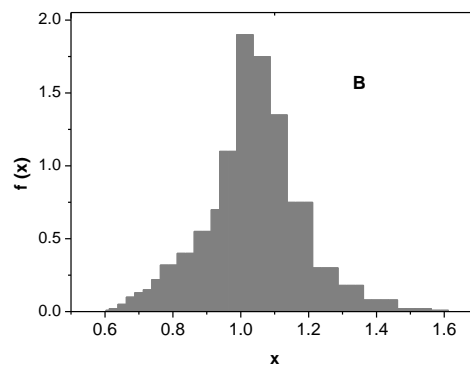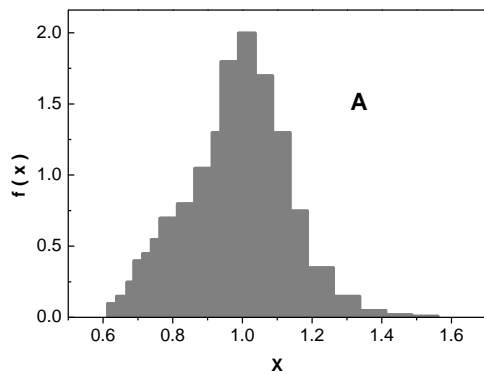
Fig. 2.6.
Normalized dimensional distribution function.
Pulse duration: A –1 ms, B – 50 μs

For experimental conditions that differ in the duration of the applied electric pulse, the calculated dimensional distributions have similar shapes characterized by a relatively narrow peack centered at $R_{1/2}$ and a similar shape. The width of both distributions is ~ 0.3 $R_{1/2}$ which corresponds to about 1μ m

5. MEMBRANE ELECTROPERMEABILIZATION IN DIABETIC AND LEUKEMIA PATHOLOGY

In conditions of permanent (chronic) hyperglycemia, chemical, structural and functional changes of proteins occur in tissues permeable to glucose, leading to known complications of diabetic pathology such as kidney, eye, nerve and vascular diseases. The mechanisms by which glucose in excess can damage protein structures are attributed to increasing (i) enzymatic glycosylation of poteins, (ii) nonenzymatic glycosylation of poteins, (iii) the polyol pathway, (iv) intracellular myoinozitol depletion. Hemoglobin glycosylation leads to abnormal hemoglobins formation: HbA1a, HbA1b, HbA1c with consequences on erythrocyte functionality: low cell flexibility, increase of intrinsic viscosity which is assumed to be a contributing factor to the reduction of the diabetical erythrocytes deformability. Changes in erythrocyte membrane permeability were highlighted also in malignant diseases [Moore et al. 1979]. MRI studies indicated a much slower influx of manganese ions in red blood cells and lymphocytes in cases of chronic leukemia. These results lead to the

supposition that in malignant pathology there is a factor affecting in the same way both directly cell involved in the malignant process (lymphocyte) as well as red blood cells that are not directly affected.

In this context we used electroportion technique to analyze membrane permeability changes of human erythrocyte in the diabetic pathology and leukemia, respectively in chronic lymphocytic leukemia, bone marrow failure and Hodgkin lymphoma (Fig 5.1, Fig. 5.2).
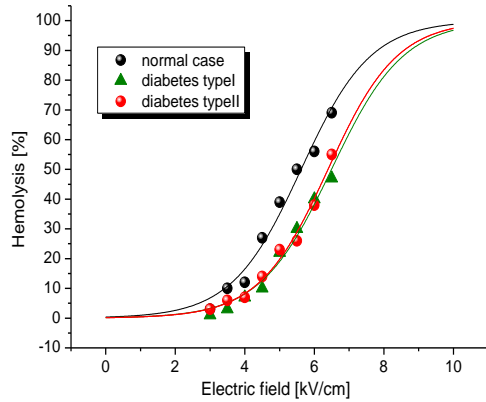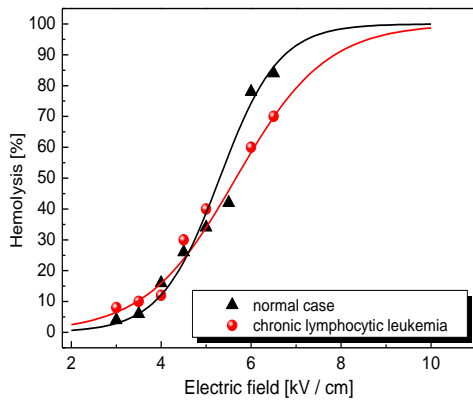

Fig.5.1.
Electrohemolysis in erythrocyte suspensions from healthy subjects and diabetic pathology, diabetes type I and type II

a.
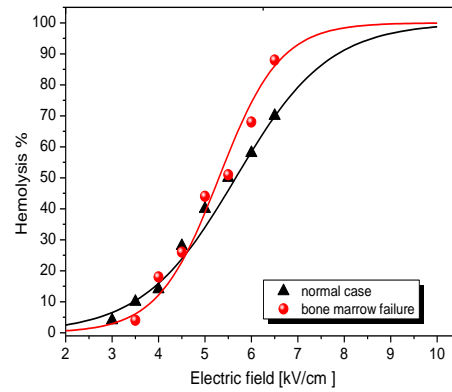
b.
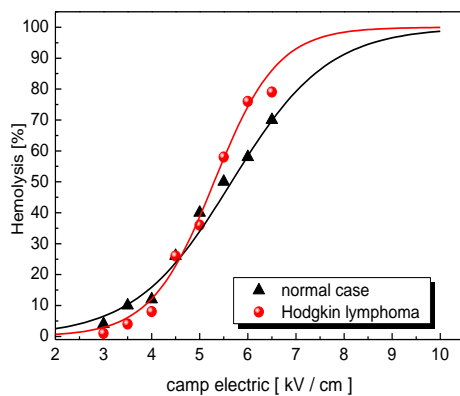
c.

Fig 5.2.
Erythrocyte suspensions electrohemolysis in normal cases and malignant pathology:
a. chronic lymphocytic leukemia
b. bone marrow failure
c. Hodgkin lymphoma

Erythrocyte electropermeabilization was characterized by electrohemolysis of 1% cell suspensions exposed to square-vawe electrical pulses of short duration (100/μs) and amplitude variable in the range 3-7 kV / cm. The characteristic parameter of electrohemolysis curve obtained is $E_{1/2}$ value of electric field strength that causes 50% hemolysis of cells

population. This parameter was used to characterize the permeability changes of the pathological cells compared with health cells.

In diabetes mellitus type I and type II the obtained results show a similar displacement of electrohemolysis curves versus control (Figure 5.1).

E_o relative displacement is 7%, indicating an increased membrane resistance of red cells damaged by excessive glucose in the blood.

In chronic lymphocytic leukemia (Fig.5.2.a), bone marrow failure (Fig.5.2.b) and Hodgkin lymphoma (Fig.5.2.c) membrane electropermeabilisation process is no significantly changed as compared to normal.

The results emphasized the following issues:

- reduction of membrane permeability in diabetic pathology with E_o = 7% is correlated with an increase in hemoglobin glycosylation;
- erythrocyte electrohemolysis is similar in diabetes mellitus type I and type II;
- normal permeability of chronic lymphocytic leukemia, bone marrow failure, lymphoma Hodgkin is correlated with normal levels of glycated hemoglobin.

In diabetes mellitus, the reduced erythrocyte membrane permeability to hemoglobin and the increased membrane resistance to electrohemolysis respectively can be explained by glycosylation of hemoglobin and formation of abnormal hemoglobins. The viscosity of the hemoglobin increases and the volume is enhanced. Following, the diffusion rate in extracellular medium is reduced compared with normal hemoglobin.

III CONCLUSION

The researches presented in this part of the thesis had as an outcome the following original results:

➢ An electroporation device has been designed and developed. It has the capability to generate two types of electrical pulses: rectangular and with an exponential decay. The amplitude and duration of pulses are programmable and they are measured and displayed. The device has the capability of applying 1-10 successive pulses on the same sample during a single pass through the electrodes. Sample cell attached is thermostatically controlled in the range 2-40°C and operates in the flow with a processing capacity of 2 ml.

➢ The main contribution of electric pulses amplitude and length in the membrane permeability process was estimated by electroporation of human erythrocytes using different combinations of pulse parameters. The efflux kinetics of hemoglobin macromolecules (65,000 Da) was tested as well as the influx of manganese ions (55 Da) normally nonpermeable through erythrocyte membrane.

  o It has been proven the ability to control membrane electropermeabilization by adjusting the amplitude and length of applied electric pulse.

  o It has been highlighted the existence of pores of different sizes in the electropermeabilizated membrane of erythrocytes.

  o It has been found that resealing of electroporated membrane is largely controlled by the length of pulse. At the same degree of permeability, restoring the

membrane and recovering its barrier function is achieved in longer periods of time by increasing the pulse duration.

➢ Based on cell size dependence of transmembrane potential, we have proposed a relationship that defines dimensional distribution of erythrocyte population. Size distribution function was verified in two different electroporation conditions.

➢ The protein with major contribution in resealing process of electropermeabilized membrane it was identified. In this connection it was exploited the advantage of the "target analysis" method that offers the possibility to assess the molecular weight of a protein whose function is inactivated by irradiation with high energy electron beam. This method was applied to the results obtained in the recovery phase of the electroporated erythrocyte membranes, initially exposed to different doses of electron irradiation. Inactivation of this phase allows us to identify the protein involved in the recloses of membrane pores as the tetramer form of spectrin. Spectrin is the basic structural unit of the cytoskeleton network. Our results confirm and provide evidence of red cell cytoskeleton involvement in membrane electroperemeabilization.

➢ Membrane electroporation technique was used as an alternative to identify cellular changes in the pathology of diabetic mellitus (type I and type II) and malignant pathology (chronic lymphocytic leukemia, bone marrow failure, Hodgkin lymphoma). Using this technique changes at the cellular level in diabetic pathology have been highlighted. Change of the hemoglobin efflux kinetics through erythrocyte electroporated membrane was correlated with the increase of intracellular glycosylated hemoglobin and was explained by the increase of time constant characterizing the transmembrane diffusion of abnormal hemoglobin molecules with increased viscosity and volume.

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