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SOME CONSIDERATIONS ON THE TRANSITION FROM UNICELLULAR TO MULTICELLULAR LIFE

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Abstract: The transition from unicellular to multicellular life is estimated to have occurred dozens of times in the history of evolution. This paper discusses the results of two recent investigations: Prochnic's investigation (2010) on *Chlamydomonas* and *Volvox* and Anderson's et al. investigation (2016) on the molecular phylogeny of GK_{PID} , which show that the transition from unicellular to multicellular state did not require major genetic restructuring. At times even point mutations resulted in important consequences for the function of some proteins in living organisms. Other molecular mechanisms that contributed to the evolution and the complexity of the living world are also being discussed.

INTRODUCTION

The emergence of eukaryotic multicellular organisms and their diversification was one of the most important events in the history of bioevolution. Eight „major revolutionary transitions” with regard to increases in biological complexity (9, 18) have been identified in the process of evolution of the living world; they involved changes in the replicating units, in the way information is stored and/or transmitted and also in the division of labour. These transitions include the origins of some phenomena and biological events such as: the first cells, the genomes, the genetic code, the eukaryotes, the sex, the differentiated multicellular organisms, the colonial super-organisms and the language. Maynard Smith and Szathmáry, (1995), and Heron (2016) consider that multicellularity was one of the most critical evolutionary innovations.

The diversification of life and the hierarchical organization of the living world are also in Michod's opinion the consequences of a series of transitions: from genes and gene networks to the first cell, from prokaryotic to eukaryotic cells, from unicellular to multicellular organisms, from asexual to sexual populations, and from solitary to social organisms – transitions that required the reorganization of fitness (the transfer of fitness from a lower-level individual to a higher level). In the author's opinion, the evolution of multicellular organisms is an example: from lower-level individuals (cells) into a higher level individuals (multicellular), (20).

It is considered that the transition from unicellular to multicellular organisms has occurred dozens of times within the evolutionary tree during time, despite not having solid proof of it due to extinction or lack of transitional fossils (9). Another interesting aspect is that multicellularity has appeared independently in clades. When seen simply as cellular aggregation into multicellular organisms, the event is estimated to have occurred more than 25 times. Complex eukaryotes are believed to have evolved 8 times: once in the Animalia, three times in the Fungi and six times in three major plant clades (22). Transition in this type of organisms involved the development of some mechanisms of adhesion, recognition, cooperation and communication between cells (with processes like cell signalling, cell proliferation and cell survival also implied) and their association in tissues, organs and precisely coordinated systems. As natural selection acts on phenotypes, it promoted cell aggregations and consequently combinations of traits, being more functional, with better chance at survival and capable of producing more offspring than the unicellular counterparts they derived from, (23, 29, 40). It is obvious that the road from unicellular organisms 3 billion years ago to complex organisms, such as man (with over 200 different types of cells in the body)

was „such a long road", paved most probably with both failure and success, a road we are trying to decipher today using the modern investigation techniques at our disposal.

Hypothesis of the transition from unicellular to multicellular organisms

Three hypotheses have been developed in this respect (17, 28):
a) *Symbiotic hypothesis* - multicellular organisms occurred from symbiosis of different species of single-cell organisms; it is based on symbioses still present today between certain organisms (mutualism: clownfish and sea anemone; protozoans and termites; spider crabs and algae; microbiota in human digestive tract, etc.). The hypothesis has its roots in the *serial endosymbiotic theory* concerning the origin of eukaryotic cell (7), largely accepted by evolutionary biologists, and which by extrapolation was meant to explain the appearance of multicellularity through successive symbioses, a hypothesis that has not been accepted as valid, though;
b) *Syncytial hypothesis (of cellularization)* - multicellular organisms may have evolved from multinucleate unicellular organisms in which an internal membrane may have compartmentalized the cell into several. However, this hypothesis did not gather supporters, either, since it is unlikely for such an organism to have one main nucleus and many micronuclei used for reproduction, (17);
c) *Colonial hypothesis* - multicellular organisms may have occurred from symbiosis between unicellular organisms of the same species, when cells fail to separate following division. This hypothesis was first proposed by Ernst Haeckel in 1874 and it seems to be the most plausible of the three, having the most supporters.

Some characteristics and differences between unicellular and multicellular organisms

In a synthesis article, Niklas and Newman (2013) showed that multicellularity can be either „simple' – when the cells remain in contact with the external environment, or „complex" – when the cells lose this contact, leading to internalization. Differentiation in multicellular organisms is based on some of their characteristics: a) differences in cell specialization, energy consumption/expressed gene, increases in the amount of repetitive DNA (that do not encode proteins); b) the probability for a multicellular organism to return to unicellular state; c) the possibility to estimate when a simple multicellular organism reaches a size or morphology that necessitates specialized tissues for the transport of nutrients (22).

Multicellularity allowed greater size and complexity of organisms, differentiation from numerous cell lineages, coordinated cell division, and sexual reproduction. However, unicellularity was successfully, considering the fact that unicellular organisms are more abundant on Earth than multicellular organisms and have been the only ones populating Terra for ca. 2 billion years (37).

The first unicellular organisms (bacteria) appeared on Earth about 3.5 billion years ago. Multicellularity evolved in myxobacteria ca. 2 billion years ago, the first eukaryotes appeared 1,8-1,4 billion years ago, and animals and plants appeared 600 million-1 billion years ago (Michod, 2007). It took a long time for animals to develop as complex multicellular forms (35). The discovery of the Ediacara fauna (Australia) after the year 1950 certified the presence of metazoans on Earth ca. 650 billion years ago (6). The Cambrian Period was marked by a veritable "explosion" of life on Earth and an obvious diversification of multicellular organisms enhanced by the increased oxygen levels in the waters and in the atmosphere due to photosynthesis (Fig. 1).

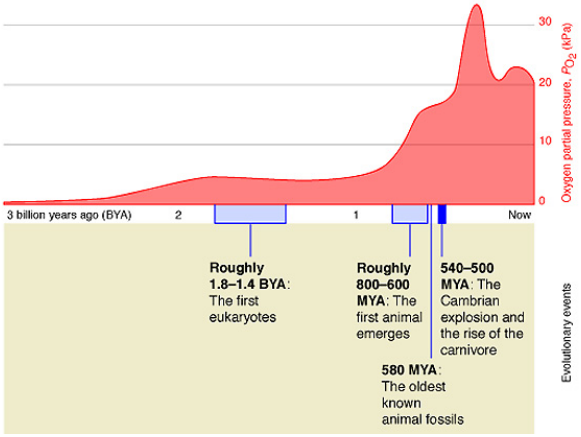

Fig. 1 - Evolution of eukaryotes and oxygen partial pressure on Terra during time (https://www.wired.com/2014/08/where-animals-come-from/)

The presumable transformation series that plants underwent in their transition from unicellular to multicellular state is: unicellular → colonial → filamentous (unbranched→ branched) → pseudoparenchymatous → parenchymatous (we define parenchymatous tissues as those in which cells can divide in all three planes of reference). The transformation series for fungi was shorter, unicellular or siphonaceous (coenocytic) fungi giving rise to multicellular fungi such as asco- and basidomycetes which consist of unbranched filaments that form a pseudoparenchymatous tissue (22). Choanoflagelates and Metazoa shared a unicellular common ancestor. However, some authors (14) consider that this common ancestor might have had an early form of multicellularity that became more robust and was lost in the Choanoflagellate lineage. The mechanism of invention of new genes and their integration to create the network of cell signalling and transcriptional regulation fundamental to metazoans, still remains a mystery.

Niklas and Newman (2013) show that the Holozoa followed the transformational series: unicellular → colonial → parenchymatous. Subsequently, evolution gave rise to functionally differentiated cell types (sponges), multilayered (placozoans), with interior cavities (chtenoforans, cnidarians) and probably those with additional layers and body cavities (mollusks), and segmented (annelids). Animal body plans are various, multi-layered, hollow or with nested cavities, elongated, segmented and appendage-bearing, and with organs with similar morphology. The authors show that even though examples of unicellular organisms descending from multicellular organisms do exist, once an organism reaches complex multicellularity and passes through the export-of-fitness phase, its capacity for evolutionary reversion to a simpler state is reduced, for it has nothing or little to do with selection on fitness grounds.

Since animals have grown in a saturated medium in microorganisms, some authors believe that through their abundance and diversity, microbes have played a major role in the appearance and evolution of animals (35), and moreover, that animals would actually be „host-microbial ecosystems”. McFall-Ngai (2013) considers that bacteria are essential partners in the digestive system of animals (from termites to humans) and that over 1/3 of human genes originated in bacteria (the hologenome theory). Some authors consider that viruses, too, have probably played a

role in the evolution of the living world, in the transition from the ARN to DNA world. Moreover, Koonin and Dolja (2013) show that *"Coevolution of viruses and host defense systems is a key aspect in the evolution of both viruses and cells, and viral genes are often recruited for cellular functions*".

In the table below we present the main differences between unicellular and multicellular organisms (28, 38):

Unicellular organisms	Multicellular organisms
The body of the organism is composed of a single cell. Body organization is simple.	The body of the organism is composed of numerous cells. Body organization is complex.
The activity(functioning) of the organism is carried out/controlled by a single cell.	Different cells in the organism are specialised to perform different functions.
Division of labour is at the organelle level. Low level of operational efficiency.	Division of labour may be at cellular level, tissue level,organ and organ system level. High degree of operational efficiency.
Usually prokaryotic in nature.	They are mostly eukaryotic in nature.
The cell has the same role for itself and for the organism.	The cells have double role: one for themselves and another for the organism.
The cell body is exposed to the environment on all sides.	Only outer cells are exposed to the environment. Inner cells perform other functions.
Any injury of the cell can cause death of the organism.	Injury or death of some cells does not affect the organism, as they can be replaced by new ones.
Small size of the cell body because of the limit imposed by surface area to volume ratio.	The organism can attain large size due to multicellularity.
Life span is usually short due to heavy load of work.	Life span is longer due to limited load of work for each cell type.
Reproduction is by vegetative/asexual methods. They reproduce quickly and in great numbers.	Reproduction is sexual type.
Few introns in genome.	Many introns in genome.
Good capacity of regeneration and power of division.	Capacity of regeneration decreases with increasing specialization. Some specialized cells lose power of division.
Cell differentiation does not exist.	Cell differentiation is evident.
Nutrition by engulfing food.	Nutrition is by specific organs or by food reproduction. They can be autotrophs or heterotrophs.
They are microscopic in nature.	They are macroscopic in nature.

The emergence of multicellularity in green algae in the order Volvocales

Some authors have raised the question of how multicellularity appeared and whether it involved a massive restructuring of the genome. To answer this question, Prochnik et al. (2010)

analysed the genome of two green algae species in Volvocales Order: *Chlamydomonas reinhardtii* - unicellular and *Volvox carteri* - multicellular. Molecular phylogenetic analyses have revealed that the volvocine species evolved from a common ancestor at least 200 million years ago (during the Triassic period) and it took this algae 35 million years to complete the transition (8). Unlike *Chlamydomonas*, *Volvox* are characterized by asymmetric cell division and embryonic morphogenesis, (13). These algae have three independent origins, if we take into account the specialization of their vegetative and reproduction functions. They are flagellated, photosynthetic, facultatively sexual, haploid eukaryotes, with varying degrees of complexity (20). As a matter of fact, the evolution over time of green algae from unicellular to multicellular state followed the steps: - ca. 223 million years ago a species of single-celled green algae began forming aggregates of cells using secreted proteins and sugars; - ca. 200 million years ago cell division in multicellular algae (the number of cells produced) began to be controlled genetically; - then the cells of these algae began to orient their flagella in the same direction so that to control the movement direction of the organism; - ca. 100 million years ago reproductive cells differentiated in some of these volvocine algae and there were developed cells with specialized functions (26).

Chlamydomonas reinhardtii is a haploid unicellular eukaryote species (about 10 μm), with 2 flagella (which enhance motion in water) and can reproduce both sexually and asexually. *Volvox carteri* is a multicellular eukaryote (ca. 350 μm diameter), with relatively low rates of cell differentiation, with only 2 cell types: - ca. 2000 - 4000 small biflagellate somatic cells that form a sphere (a coenobium) with flagella disposed at its surface (which provide coordinated swimming); - ca. 12 - 16 large reproductive cells (gonidia), reproduction is both sexual and asexual (male, female and vegetative colonies), (Fig. 2). The simplicity of their organization has made *Volvox* species a model system useful for exploring multicellularity, as they are organisms made up of a few thousand cells (instead of trillions, like in the case of other organisms) and only two cell types. It is believed that cellular differentiation into sterile somatic cells has evolved at least three times within the clade and further specialization of the reproductive cells occurred in at least two independent lineages, (9).

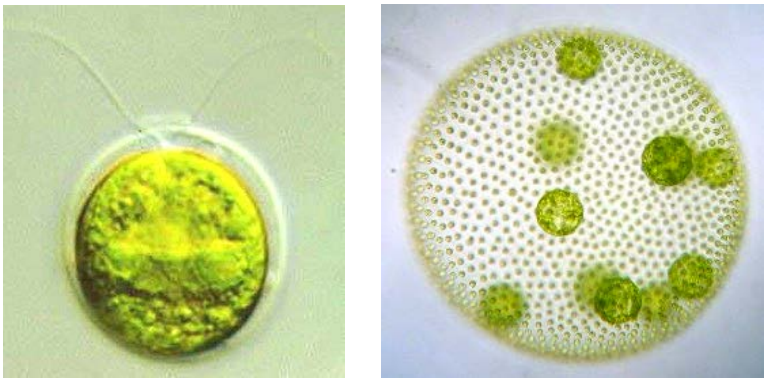

Fig. 2 - *Chlamydomonas reinhardtii* (left) și *Volvox carteri* (right)
(Prochnik et al., 2010)

Prochnik et al. (2010) sequenced the *V. carteri* genome and compared it with that of *C. reinhardtii* (sequenced in 2007) and observed that the two species have a relatively similar number

of protein-encoding genes, 14520 and 14516 gene (only 32 of the genes present at *Volvox* have no relatives in *Chlamydomonas*). The size of the genome at *Volvox* is superior (ca. 138 million base pairs, due to transposons and repetitive DNA) in comparison to *Chlamydomonas* (ca. 120 million pb). Two groups of genes have several clones in *Volvox*: the genes that determine glycoprotein synthesis (which form extracellular matrix) and the genes that encode cyclins (proteins involved in cellular division). In addition, the extracellular matrix in *Volvox* takes up over 99% within the volume of the colony and has, among other things, functions as cell orientation and sex induction (Gille et al., 1983, 1984; Hallman and Kirk 2000 - cited by Herron, 2016).

Following the comparative study on the two species, Prochnik et al. concluded that the transition from unicellular to multicellular life did not require large changes in gene content (number of genes). The transition was also enabled by mechanisms of regulation of gene expression: alternative splicing (ca. 2,9% of genes in *Volvox*), gene duplication, the intervention of cis-regulatory elements of gene expression, and of microRNAs (that post-transcriptionally regulate gene expression), (13, 30).

A mutation with major implications in animal evolution

Early last year, the results of molecular phylogenetic research of a team of American biochemists (published by Anderson et al., 2016), led by Professors K. E. Prehoda, J. W. Thornton and N. King, have aroused a lot of interest and have been widely commented in the scientific world (12, 21, 31, 32, 33, 39), being considered perhaps the most important breakthrough of the year in the field. They found that a seemingly minor genetic event (a mutation/substitution) changed the function of a cellular ancestral protein, which became essential for organizing the multicellular structures of the animal world. What is it about? Here is how Thornton describes the importance of his team's research: *"Our experiments show how biological complexity can evolve though simple, high-probability genetic paths*".... and, "*Before the last common ancestor of all animals, when only single-celled organisms existed on Earth, just one tiny change in DNA sequence caused a protein to switch from its primordial role as an enzyme to a new function that became essential to organize multicellular structures*", (12).

Some authors consider that by phylogenetic studies of relevant living organisms, by comparative investigations on animals and their close unicellular and colonial relatives, one can rebuild cell biology and reconstruct the genome of the last animal's ancestor on Earth - the hypothetical Urmetazoan. Studies of this kind support the idea that Urmetazoan possessed a layer of epithelium-like collar cells, fed on bacteria, reproduced by eggs and spermatozoa, and developed through cell division, cell differentiation and invagination (25).

The Choanoflagellates are considered to be the closest relatives of animals, (3, 5, 14, 16, 25); they are single-celled microeukaryotes or form simple colonies (of equipotent cells) and they live in marine and freshwater environments. There are over 125 known species of choanoflagellates. Cell morphology and feeding behaviour have been conserved and resemble structurally and functionally to a group of specialized cells– *sponge Choanocytes*. Some choanoflagellates may form multicellular colonies through the microvilli collar or through fine intercellular bridges (somewhat similar to the ring canals that link spermatogonia or ovogonia), (3). Fossil sponges have been found in Cambrian (over 500 million years old), organisms with several cell types: *Pinacocytes* (which form the outer layer), *porocytes* (for the pores in the body), *sclerocytes* (producing the spicules of skeletal system), *archeocytes* (with digestion functions, gamete production, tissue repair and regeneration) and *choanocytes* (which cause fluids flow

through the body). Choanocytes strikingly resemble the cells of unicellular choanoflagellates, which has led to the hypothesis that these organisms and animals are sister groups. The next level of complexity reached by animals is represented by hydrozoans, which contain more types of cells than Porifera and are quite complex as true predators (16).

Getting back to *Choanoflagellates*, one of their representatives is *Salpingoeca rosetta*, a uniflagellate organism – which can be unicellular or it can form simple colonies of either chain-like or rosette-like morphologies. Ultrastructural analyses showed that cells in rosette and chain colonies are connected by a combination of intercellular bridges, extracellular matrix, and filopodia (3). The formation of rosettes is regulated by the lipids in the bacteria on which this protozoan feeds. In fact, some authors appreciate that through their abundance and diversity, bacteria "*have exerted important signalling influences on diverse animal and non-animal lineages*" (19, 35). Ample studies in recent years on choanoflagellates, conducted by King, Dayel, Fairclough and others have shown that the rosettes of this protozoan are formed by cell division rather than by cell aggregation, which support the hypothesis that transition from unicellular to multicellular organisms was achieved by repeated cell divisions, (36).

By sequencing the genome of some choanoflagellates there have been identified common genes with multicellular animals (sponges, cnidarians, and ctenophores), such as cell adhesion genes, signalling genes and extracellular matrix genes, which suggests that these genes had developed prior to the transition to multicellular animals. This means that the two groups of organisms share a common ancestor. Genome and transcriptome of *S. rosetta "suggest that the genome of the last common ancestor of choanoflagellate and metazoans contained genes and domains that orchestrate development in modern animals but underwent important changes in gene content and regulation en route to the evolution of the first metazoan."*, (5).

Interestingly, some choanoflagellates which do not form colonies (*Monosiga brevicollis*, for instance) also have in their genome genes that in multicellular animals encode proteins involved in cell adhesion, development and differentiation, which in their case may serve in relation to the environment, (35). The fact that *M. brevicolis* exhibits specific metazoan protein domains involved in signalling and adhesion suggests that they appeared prior to the divergence of choanoflagellates and metazoans. It is presumed that the transition of metazoans from the unicellular ancestor was made through a colonial intermediate– *Urblastea*, composed of choanoflagellate type cells. (5).

Until about 1 billion years ago, the Earth was only populated by unicellular organisms. The emergence and evolution of multicellularity still remains a mystery, although steps have been taken to decipher this phenomenon. In their molecular phylogenetic research, Anderson et al. (2016) show how by duplication and divergence of an ancient guanylate kinase (gk) before the divergence of animals and choanoflagellates from unicellular organisms and the appearance of true multicellularity, a scaffolding protein developed - GK_{PID} (the guanylate kinase protein interaction domain), which function in cell adhesion and mitotic spindle orientation. This protein domain appears to be essential in tissue formation, (2).

The orientation of the mitotic spindle relative to cell axis and neighbouring cells is essential in the formation of the organized tissues. In *Drosophila*, for instance, epithelial cells divide symmetrically - perpendicular to the apical-basal axis, while the neuroblasts divide asymmetrically - parallel to this axis. In both cell types, receptor-independent G-protein signalling involving the GoLoco protein Pins has its definite role in the mitotic spindle orientation. For proper mitotic spindle orientation in neuroblasts and epithelial cells, the Pins protein associates with the Mud protein, both localized on the cellular cortex. For correct orientation of the mitotic spindle in neuroblasts and epithelial cells, the Pins protein associates with the Mud protein, both localized on

the cellular cortex. Moreover, Mud protein localizes to centrosomes during mitosis (independently of Pins) to regulate their organization.

GK_{PID} forms in the animal cell a molecular complex whose functions have been studied in *Drosophila melanogaster* neuroblasts and it plays a similar role in birds and mammals. The complex consists of the GK_{PID} of Dlg protein (Discs-large protein – a membrane associated protein with several molecular domains) which serves as support for mitotic spindle orientation by coupling two molecular partners: an "anchor" protein located inside the cell membrane (Pins - in insects or LGN - in vertebrates) - which indicates the position of the neighbouring cells, and a motor protein belonging to kinesin-3 family (Khc-73) - which binds to the microtubules of the mitotic spindle and pulls the chromosomes (monochromatic) towards the anchor, thus orienting the daughter cells resulted from division relative to their neighbouring cells, (Fig. 3).

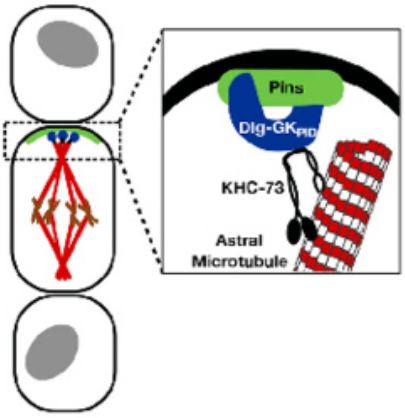

Fig. 3 - The GK_{PID} of the protein Discs-large (Dlg, blue) serves as a scaffold for spindle orientation
by physically linking the localized cortical protein Pins (green)to astral microtubules (red) via the motor protein Khc-73 (black), (Anderson et al., 2016).

Drosophila discs large protein (Dlg) is a member of the membrane-associated guanylate kinase homolog family. It serves in cytoskeleton organization, localization of membrane proteins, and apicobasal polarity of epithelial cells. The *dlg* gene is defined as a tumour suppressor gene, (Woods et al., 1996). Kinesin-3 motor protein family includes: Kinesin-73 (Khc-73) – which plays a role in the mitotic spindle polarity in *Drosophila* neuroblasts, KIF1A in mammals and Unc-104 in *Caenorhabditis elegans* (10).

The GK enzyme and the GK_{PID} protein have similar sequence and structure but they have completely different functions. GK is a univesal enzyme in the living world (it catalyses the transfer of phosphate group from ATP to GMP), whereas the GK_{PID} family proteins are only present in Filozoa (Animals, Choanoflagellates and Filasterea). Observations on the Dlg-GK_{PID} protein and on guanylate kinase suggested that these two proteins may have evolved from an ancient gk enzyme.

One of the members of the research team, Professor Thornton has pioneered technics for reconstructing ancestral genes. In order to verify the above mentioned hypothesis, Anderson et al.

(2016) have evaluated the phylogeny of the protein family- gk enzyme/GK_{PID} protein using maximum likelihood method and the sequences of 224 family members from over 40 animal species (found on various branches of the family tree) they have created their genealogy on computer and by „travelling in the molecular past” of these species, reconstructed the ancient forms of the analyzed proteins, trying to deduce their molecular parent (the sequence of the ancestral protein), (2, 34).

The authors focused on two ancestral nodes, namely Anc-$GK1_{PID}$ protein (from which Filozoa descended) and Anc-gk_{dup} (which existed before the gene duplication) and came up with the hypothesis that transition from the function as a gk enzyme to that of orienting mitotic spindle occurred during the phylogenetic interval between Anc-gk_{dup} and Anc-$GK1_{PID}$.

They synthesized the specific DNA sequences specific and transferred the resulted genes in Drosophila S2 cell cultures, ko for the gk and GK_{PID} genes. They found that Anc-gkdup protein is an active guanylate kinase enzyme and does not have the ability to bind Pins or to orient the mitotic spindle, whereas Anc-$GK1_{PID}$ does not exhibit enzymatic activity but binds Pins with moderate affinity and has high efficiency in the orientation of the mitotic spindle in cell cultures.

Then the authors also reconstructed Anc-$GK2_{PID}$, a more recent progenitor of Dlg proteins in metazoans and found that this protein has high binding affinity for Pins and of orienting the mitotic spindle. On the basis of these results, they concluded that spindle orientation function of $GK1_{PID}$ arose before the divergence of the choanoflagellates and the appearance of metazoans, being an important moment in the evolution of complexity in the animal world (1, 2).

By studying proteic sequences of Khc-73 and Pins in databases, the authors found that Khc-73 orthologues are present in Animals, Choanoflagellates and Filasterea, but not in fungi. Thus, they concluded that Khc-73 gene is as old as the Filozoan ancestor. Then they found Pins orthologues in choanoflagellates (*Salpingoeca rosetta* and *Monosiga brevicollis*) very similar in sequence and domain structure, but not in Filasterea, which indicates that Pins evolved after the emergence of GK_{PID}. They also noticed that *S. rosetta* GK_{PID} can bind the Pins protein of *Drosophila* but not its own Pins, which suggests that this association appeared after animals diverged from choanoflagellates.

Finally, Anderson et al. (2016) sought to identify the genetic mechanism by which the GK_{PID} evolved its capacity to bind Pins and they concluded that the candidate mutations occurred during the phylogenetic interval between Anc-gk_{dup} and Anc-$GK1_{PID}$. They analysed the amino acid changes occurred in two regions of these proteins and noticed only five preserved amino acids in GK_{PID} descendants. Then they tested what amino acid substitutions change its enzyme function for that of binding Pins and found that either of two substitutions (s36P or f33S), in the hinge region of the protein, could produce this change of function.

Besides the worthy contribution to deciphering some mysteries of evolution, the American team’s researches seem to have a great practical impact. One of the team members, Professor Kenneth Prehoda, believes that the information obtained could also be useful in cancer research. It is known that cancer cells cease to function as „team members” (as the author expresses it) and take the individual road of unrestricted division, with a behaviour that resembles unicellular rather than multicellular organisms. If the mitotic spindle of the cells in division is not properly aligned in relation to the surrounding cells, a tumour tissue may form instead of a normal one. Therefore, a thorough knowledge of the molecular events and processes and regulatory mechanisms in unicellular and multicellular organisms could contribute to the development of cancer research, (21).

The work published by the American researchers at the beginning of 2016 stirred up the interest of the scientific world and press, giving rise to many favourable comments but also a number of critics that should be taken into consideration. Professors Prehoda and Thornton themselves, two of the American research team leaders, participated in disseminating information, giving a lot of interviews about the result and the impact of these investigations. Some critics consider, among other things, that working with living cells makes "travelling in the molecular past" difficult, that the experiments on the positioning in Choanoflagellates require a series of clarifications, and that the authors overstate the impact of Khc73-Dlg-Pins complex in mitotic spindle orientation in all animals and the belief that this is the only novelty of animals etc, (33).

Some of these criticisms can be justified, but they should not be exaggerated and the American team's contribution to deciphering an important event in the evolutionary history of the GK_{PID} molecular complex should not be understated. Obviously, it was not this mutation alone that ensured the evolution and complexity of the animal world, as there are multiple factors and mechanisms contributing to this phenomenon: besides gene mutation and acquisition of new genes, there is also gene interaction, gene duplication, gene transposition, regulatory mechanisms of gene expression (alternative splicing, cis-regulatory factors, micro-RNA) etc.

In fact, Anderson et al. (2016) themselves admit that many aspects in this history of animal evolution still remain to be clarified, including how and when the interaction between GK_{PID} and Khc-73 evolved, the mechanisms by which Pins acquired its linker and GoLoco sequences, and the relationship of these components to other pathways and molecular complexes involved in animal spindle orientation.

CONCLUSIONS

The aim of this paper was to show how seemingly minor genetic events occurring in the genome of some organisms can have important consequences for the evolution of the living world.

Two recent studies have been used as arguments: Prochnic's study (2010) – on *Chlamydomonas* and *Volvox* green algae and Anderson's et al. study (2016) - on the molecular phylogeny of GK_{PID}, which show that the transition from unicellular to multicellular organisms did not require major genetic restructuring.

Small differences in gene content can lead to important differences between organisms. At times, point mutations were enough for some proteins in living organisms to acquire new functions.

The transition from unicellular to multicellular organisms was made possible not only the acquisition of new genes, but also by gene interaction, gene duplication (that allowed one of the gene variants to acquire new functions), regulatory mechanisms of gene expression (alternative splicing, cis-regulatory factors, micro-RNA) etc.

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THE EFFECT OF HARVESTING TIME ON ESSENTIAL OILS COMPOSITION OF *THYMUS PANNONICUS* L.

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Abstract: In this paper the authors investigating the possible effect of harvesting time on essential oils composition at *Thymus pannonicus* L., knowing that the harvesting date, time of day and weather conditions is very important for the quality and quantity of essential oils. *Thymus pannonicus* L. is a perennial herbaceous plant, distributed in central and eastern Europe. In Romania these plant is spread all over the country, including two subspecies *pannonicus* and *auctus*. The vegetal material was collected in 2 different phenophases (vegetative and anthesis), during 2 consecutive years. The chemical composition of the essential oil was established by GC-MS analysis with the help of a gas-chromatograph Agilent Technologies coupled to a mass detector. The main chemical components of essential oils are germacrene D (between 8.05% and 17.31%), nerolidol (between 7.74% and 18.49%), farnesol (between 12.95% and 14.77%) and α-terpinyl acetate (between 6.56% and 9.58%). Major differences were registered at the taxa collected in 2013 in anthesis stage, where the main components were carvacrol (42.32%) and thymol (13.98%).

INTRODUCTION

As part of the Lamiaceae family, comprising over 220 genera, *Thymus* is one of the most important genera, due to the number of species (about 350), species that vary greatly from taxonomic and biochemical point of view (Morales, 2002). The species of *Thymus* genus have been used for more than 2000 years as medicinal plants and many of them are still being used today (Rasooli and Mirmostafa, 2002; Tzakou and Constantinidis, 2005; Zarzuelo and Crespo, 2002). Their essential oils are utilized as flavour ingredients in a wide variety of food, beverage and confectionery products, as well as in perfumery for the scenting of soaps and lotions (De Martino et al., 2009). Because of their antiseptic, antispasmodic and antimicrobial properties, the essential oils of *Thymus* species are also used for medicinal purposes (Cosentino et al., 1999; El-Hela, 2007; Jirovetz et al., 2007; Maksimović et al., 2008; Safaei-Ghomi et al., 2009).

Thymus pannonicus L. is a perennial herbaceous plant, distributed in central and eastern Europe. It grows over open dry meadows, grasslands and rocks (Jalas, 1972). In Romania these plant is spread all over the country, including two subspecies *pannonicus* and *auctus* (Oprea, 2005). This species presents vigorous branched stems, covered with hairs with the same length of the axis diameter. The leaves are elliptic or prolonged, 6-12 mm in long and 3-5 mm wide, green in color, both epidermis are covered with hairs, nervures little proeminent. The inflorescence is capitate. The calyx is 3-4 mm long, the corolla is lilac-red, 6-7 mm long (Guşuleac, 1961). *Thymus pannonicus* is commonly used as herbal tea, flavoring agent and medicinal plant due to his biological active substances (such as thymol, carvacrol, geraniol, linalool and other compounds from the essential oil) (Stahl-Biskup and Saez, 2002).

The quality and the quantity of volatile oils from plants can be influenced by a series of environmental factors like the temperature, the radiations and the photoperiod (Yamura et al.,1989). Also, the nutritive material indispensable for plants’ growth, the water, the mineral elements and the nitrogen plays a primordial role on the metabolism products (Rajeswara et al.,1990). On the other hand, the seasonal gradient from the growth period is associated with changes of some environment parameters, like the photoperiod, the air temperature and the available water. The combination of these factors exerts a pressure on the plant that is expressed through influences of the morphology, anatomy, physiology and its productivity. The influence of such factors on the essential oils production was not widely investigated. Thus, the main objective of this paper is to highlight the possible effect of harvesting time on essential oils composition of *Thymus pannonicus*. For this purpose individuals of this species were collected in vegetative and full flowering phases, two consecutive years.

MATERIAL AND METHODS

Vegetal material

The vegetal material was represented by *Thymus pannonicus*, a species that grows wild in the Romanian flora. The species was collected in 2 different phenophases (vegetative and anthesis), during 2013 and 2014, from Fălticeni, Suceava

County, Romania. The identification of taxa was made by Dr. Ioan Sârbu from the Botanical Garden "Anastasie Fătu", Iasi. The collected material was registered and stored in „Alexandru Ioan Cuza" University's Herbarium from Iași.

Isolation and analysis of essential oils

The dried aerial parts of the plant (100g) were subjected to hydro-distillation, for 3 hours, using a NeoClevenger apparatus, according to the method recommended by the European Pharmacopeia (1997). The yield of essentials oils was 0.2% for vegetative phase, 0.8% for anthesis phase and 0.5% for fruiting phase. The obtained essential oils were stored at

+4°C until analysis. The chemical composition of the essential oil was established by GC-MS analysis with the help of a gas-chromatograph Agilent Technologies 6890N coupled to a mass detector (MSD) of the 5975 inert XL Mass Selective Detector type. The conditions for chromatography were: column HP 5MS, mobile phase Helium – discharge: 1 mL/min, injector temperature: 250°C, detector temperature: 250°C, temperature regime from initial 40°C (10 degrees/min.) to 280 degrees, injected volume: 0.1-0.3 µl, splitting ratio-1:100. The DB5 chromatographic column has a length of 30 m an interior diameter of 0.25 mm and a film diameter of 0.25 µm. The separated compounds were identified by means of the NIST spectrum database, and the peak position was confirmed by the Kovats retention index.

RESULTS AND DISCUSSION

Following our analysis of essential oils, a total of 58 compounds were identified, representing between 92.26% and 97.58% of the total number of identified compounds (Tabel 1). The highest number of chemicals (46 compounds) was identified in the volatile oil derived from individuals collected in 2013, in the vegetative stage. The lowest number of compounds (31) was identified in the volatile oil derived from plants collected in 2013 in the anthesis stage. The main chemical components are germacrene D (between 8.05% and 17.31%), nerolidol (between 7.74% and 18.49%), farnesol (between 12.95% and 14.77%) and α-terpinyl acetate (between 6.56% and 9.58%). Major differences were registered at the taxa collected in 2013 in anthesis stage, where the main components were carvacrol (42.32%) and thymol (13.98%).

Carvacrol and thymol are phenolic compounds specific to the *Thymus* genus, known for their wide spectrum of antimicrobial activity (Dorman and Deans, 2000; Lambert et al., 2001; Adam et al., 1998; Manohar et al., 2001). They possess multiple biological properties such as anti-inflammatory, antioxidant, hepatoprotective and anti-tumoral activities (Aeschbach et al., 1994; Alam et al., 1999; Robledo et al., 2005; Skold et al., 1998; Weber and De Bont, 1996; Zeytinoglu et al., 2003).

The geraniol, a monoterpenoid, it was found in higher amounts (13.45%) on the taxa collected in 2013, in anthesis stage. This compound was found in smaller amounts on the other taxa (between 0.48% and 2.21%). The monoterpene geraniol, which is emitted from flowers and herbs (Mockute et Bernotiene, 1999) of many species, has an important role in their overall flavor and aroma. Is also use as repellent (Barnard and Xue, 2004).

α-Terpinyl acetate, nerolidol and farnesol are 3 compounds identified in large quantities in analyzed taxa, except taxa collected in 2013 in anthesis stage. These 3 compounds are important natural flavours and usually are used in perfumes and soaps. Farnesol is also a natural pesticide for mites and is a pheromone for several other insects (Wang et al., 2011).

In generally, a high chemical variability and diversity is observed in the essential oils of *Thymus* species: at least 20 different chemotypes in the genus have been established until now (Tepe et al., 2005). According to Karuza-Stojaković et al., the principal constituents of *Thymus pannonicus* essential oil from southern parts of Vojvodina province were terpinyl acetate, terpinen-4-ol, thymol, carvacrol and geranyl acetate (listed in order of descending quantity). Maksimovic and collaborators in 2008 identified in the volatile oil *Thymus pannonicus*. All., harvested in northern Serbia, a total of 33 constituents, the main being geranial (41.42%) and neral (29.61). Other researchers have identified in the volatile oil of this species large amounts of

thymol (25-41%) and p-cimen (17-38%) (Pluhar et al., 2007).

Table 1. Chemical composition of the essential oil of *Thymus pannonicus*, collected in various phenophases in two consecutive years (2013-2014), from Fălticeni, Suceava County, Romania

Compound	Vegetative stage		Anthesis stage	
	Year		Year	
	2013	2014	2013	2014
α-Pinene	0.21	0.23	0.16	0.91
Camphene	0.21	0.25	0.22	-
Octen-3-ol	0.18	0.26	0.30	0.28
Myrcene	**3.29**	**3.15**	**5.20**	**2.53**
o-Cymene	0.37	0.33	0.25	0.62
Limonene	0.93	0.50	0.09	0.58
Eucalyptol	0.90	0.98	0.27	-
cis-β-ocimene	2.31	1.26	-	2.15
γ-Terpinene	0.33	-	0.68	-
cis- Sabinene hydrate	0.78	0.85	0.39	0.58
Linalool	3.05	-	0.31	2.55
Octen-3-ol- acetate	0.17	0.31	-	0.41
Camphor	0.42	1.09	-	0.54
Borneol	-	0.41	0.45	0.33
Terpinen-4-ol	0.45	0.317	0.13	-
α-Terpineol	0.69	0.261	-	0.62
Nerol	0.46	0.47	0.51	0.26
Linalyl acetate	-	-	-	3.73
Neral	0.46	0.51		0.32
Methyl thymol	-	-	2.50	-
Geraniol	2.21	0.74	**13.45**	0.48
Geranial	0.67	-	0.15	-
Thymol	-	-	**13.98**	-
Carvacrol	-	-	**42.32**	-
α-Terpinyl acetate	**8.52**	**6.56**	-	**9.58**
Neril acetate	0.32	0.65	-	0.33
Linalil acetate	0.45	-	-	-
Geranyl acetate	-	1.37	4.21	--
β-Burbonene	0.79	**4.11**	0.14	5.11
α- Cariophyllene	-	1.50	1.14	2.10
Alloaromadendren	0.44	-	-	-
β- Cariophyllene	1.30	-	-	-
Farnesene	0.18	-	-	-
τ-Murolen	0.30	0.51	-	0.74
Germacrene D	**17.08**	**17.31**	**8.05**	**14.51**
β-Elemene	0.39	-	-	-
γ-Elemene	2.10	1.03	-	1.32
β-Bisabolene	2.56	1.53	0.12	2.72
γ-Cadinol	0.89	-	-	2.32
τ-Cadinol	0.45	-	-	-
τ-Cadinene	0.36	-	0.35	-
γ-Cadinene	1.27	-	0.61	0.54
Elemol	4.30	2.59	-	1.59
Nerolidol	**7.74**	**18.49**	-	**12.51**
Spathulenol	**7.70**	1.18	0.31	-
Caryophyllene oxide	-	2.36	0.73	2.79
Leden	1.36	-	0.13	0.35
Cubenole	0.47	-	-	-
γ-Eudesmol	0.85	-	-	-

Spatulenol	-	-	0.14	1.48
τ-Muurulol	3.68	-	0.15	-
Aromadendrene epoxyde	-	1.31	0.14	1.13
Eudesmol	-	0.55	-	
τ-Murolol	-	1.47	-	1.27
Cis-Trans-Farnesol	-	2.03	-	1.06
Farnesol	**14.18**	**14.77**	-	**12.95**
Farnesal	0.49	0.68	-	1.23
Farnesil acetate	-	0.35	-	0.14
TOTAL %	**96.26**	**92.26**	**97.58**	**92.66**

The yield of plant material, the essential oil content and quantitative composition of plants can be influenced by harvest time, ecological and climatical conditions (Cabo et al., 1982; Putievsky and Basker, 1977; Inan et al., 2011). Regarding the harvesting time, in generally, thyme is most aromatic during the period of blooming (or at the beginning of full bloom); the blooming period being considered the best time for harvesting (Venskutonis, 2002). How-ever, period of vegetation and blooming can be different in various geographical zones depending on their climatic conditions. Also, weather conditions during the day of harvest are very important. In generally, sunny days should be preferred. The plants harvested after rain are difficult to dry and they deteriorate much faster, became inferior from chemical point of view (Kauniene and Kaunas, 1991). Our studies show that the chemical composition is relatively similar for vegetative and anthesis stage (except the taxa collected in anthesis, during 2013); registering differences in the percentages of chemical compounds.

CONCLUSIONS

Our studies have shown that in the case of *Thymus pannonicus* the main chemical components of essential oils are germacrene D, nerolidol, farnesol and α-terpinyl acetate. The changes due to the harvesting period are found only in percentage variations of the compounds, except the plants collected in anthesis in 2014.

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THE ACTION OF T_iO_2, ZnO, Fe_3O_4 NANOPARTICLES ON *SACCHAROMYCES* AND *RHODOTORULA* YEAST STRAINS IN FUNCTION OF THE CONCENTRATION AND DIMENSIONS

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Keywords: nanoparticles, *Saccharomyces*, *Rhodotorula*, proteins, carbohydrates, mannoproteins, carotenoid pigments.
Abstract: The present paper reports on influence of some nanoparticles of metal oxides on *Saccharomyces* and *Rhodotorula* yeast strains. It has been established that the main factors determining cell response to the action of metallic nanoparticles are concentration and dimension, as well as important indices of this adaptive reaction are proteins, carbohydrates, including β-glucans and mannoproteins, and carotenoid pigments content.

INTRODUCTION

Nanotechnologies represents an innovative scientific and economic domain. The unique properties and utility of nanoparticles (NPs) allow application in different fields such as biology, medicine, chemistry, physics etc. (Mrinmoy De et al., 2008, Vaseem et al., 2010, Potara, 2012, Gardikiotis, 2012, Espita et al., 2012, Kiran et al., 2014). It is considered that inorganic nanoparticles due to the new properties, could modify metabolic pathway of living organisms. The application of inorganic nanoparticles at the cultivation of microorganisms represents a recent research domain of nanobiotechnology (Nasr , 2015, Eden Mahendra, 2011). Recent studies have demonstrated that more frequent nanoparticles side effects on cells are: a) nanoparticles uptake by cells followed by the interruption of ATP production and DNA replication; b) the formation of reactive oxygen species (ROS), that leads to cell apoptosis; c) nanoparticles induced cell membrane damages (Sahayaraj, Rajesh, 2011). Insufficient amount of available data on the results of the evaluation of nanoparticles effect on living organisms that tended to be piecemeal has created difficulties of estimating the impact and risks of nanoparticles application in different domains.

Metallic nanoparticles are of great importance. The properties of nanoparticles could be magnetic, bactericidal and catalytic depending on metals. It's important to emphasis the effect of nanoparticles from the perspective of enhancing the technological properties for different opportunities. Biotechnological perspectives on the application of ZnO nanoparticles in different domains are mentioned in some publications (Espita et al, 2012; Vaseem et al., 2010). Comparative with other antimicrobial agents, TiO_2 NPs have attracted a lot of attention due to the high stability, absence of toxicity, low cost, bioactive activity. According to special literature data, minimum inhibitory concentration (MIC) of nanoparticles varies and depends on the microorganism selected for study (Pișkin et al., 2013). The possible mechanisms of TiO_2 action on cellular level have been investigated by other researchers (El-Said et al., 2014; Minju al., 2013) which have characterized some processes of nanoparticles application. The important properties of magnetic iron oxide nanoparticles (NPs) destined for biomedical and biotechnological application have been mentioned in scientific papers of some researchers (Hongtao Cui et al., 2013; Kiran et al., 2014). An important type of Fe_3O_4 nanoparticles is of great interest for biomedicine due to the superparamagnetism and biocompatibility (Guiden et al., 2004).

In the context of developing of technological possibilities of elaboration of new bioproducts for the utilization in different domains of national economy, it is important to study the nanoparticles effect on development and metabolites production at yeasts with biotechnological destination. Thereby, this work aims to determine the mechanism of action of nanoparticles on microorganisms by focusing of the effect of selected nanoparticles on processes affecting biosynthesis of cell components.

The propose for research important scientific problem consists in identification of *Saccharomyces* and *Rhodotorula* yeast response to the nanoparticles action of some metals depending on concentration and dimension for the following investigations regarding processes the biosynthesis modelation of bioactive principles of biotechnological interest.

MATERIALS AND METHODS

Strains, culture medium, cultivation conditions. The following yeast strains: *Saccharomyces cerevisiae* CNMN-Y-18 selected as mannoproteins producer (Usatîi et al., 2013), *Saccharomyces cerevisiae* CNMN-Y-20 as β-

glucans producer (Chiselița et al., 2010), *Rhodotorula gracilis* CNMN-Y-30 as carotenoids producer were used for the study.

TiO_2, ZnO, Fe_3O_4 nanoparticles elaborated by the researchers of the Institute of Electronic Engineering and Nanotechnologies of Academy of Sciences of Moldova (Guțul et al., 2014). Nanoparticles, besides magnetite, were stabilized by polyvinylpyrrolidone (PVP). Suspension of Fe_3O_4 nanoparticles was obtained using the method previously described by (Oterro-Gonzalez et al., 2013). Concentrations of nanoparticles used at the yeast cultivation varied depending on the aim of experiment. The variant without application of nanoparticles served as control sample.

The YPD fermentation medium (Aguilar-Uscanga et al., 2003) specific for selected yeast strains was used for inoculation and submerged cultivation of yeasts. The submerged cultivation was carried out in depth capacity 1 liter Erlenmeyer flask, shaker (200 rpm.) at temperature of 25ºC, aeration rate 80,0...83,0 mg/L, the duration of cultivation 120 hours. Yeast cells, in an amount of 5%, $2x10^6$ cells/ml were inoculated on the liquid medium.

Methods. Protein was determined spectrophotometrically by the Lowry method (Lowry et al., 1951) using bovine serum albumin as standard. The content of total carbohydrates in yeasts biomass was determined using PG T60 VIS spectrophotometer at wavelength of 620 nm with the utilization of anthron reagent and D-glucose as standard (Dey et al., 1993). The β-glucans content in the yeast biomass was determined gravimetrically as described (Thammakiti et al., 2004). Mannoproteins content was determined gravimetrically according to the method (Liu Hong-Zhi et al., 2009, Zhang et al., 1999). Carotenoids pigments were extracted from yeast biomass and measured spectrophotometrically (Frengova et al., 1994, El-Banna Amr et al., 2012, Tămaș et al., 1986). Statistical processing of obtained results was effectuated with set of programs Statistics 7.

RESULTS AND DISCUSSIONS

30 nm TiO_2 and 10 nm Fe_3O_4 nanoparticles were used to establish the role of the nanoparticles concentrations in the cultivation process of yeasts *Rhodotorula* and *Saccharomyces.* The modification of cultivation conditions of yeasts may induce the formation of reactive oxygen species. Carotenoids pigments being the essential components with free radical scavenging property have an important role in regulation of oxidative stress and its negative consequences. The role of carotenoids in adaptive cell reaction present a great interest. The statistical analysis of experimental data on influence of TiO_2 nanoparticles on *Rhodotorula gracilis* CNMN-Y-30 pigmented yeast strain have revealed the significant decrease of carotenoids content at the application of different nanoparticles concentrations. The correlation among pigments content and applied concentration of nanoparticles has demonstrated a strong association R^2 = 0,940 (Figure 1).

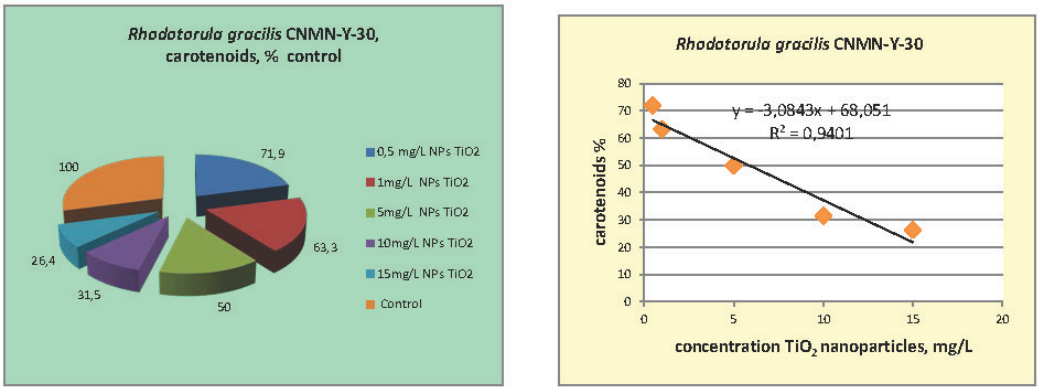

Figure 1. The influence of TiO_2 nanoparticles on carotenoids content in biomass *Rhodotorula gracilis* CNMN-Y-30 depending on concentration.

Under the influence of another type of Fe_3O_4 nanoparticles the β-carotene content, one of the base indices of adaptive response of pigmented yeast strain *Rhodotorula gracilis* CNMN-Y-30 was reduced depending on concentration with 34,5... 85,5%. Low concentrations of Fe_3O_4 nanoparticles are less active than concentrations that surpass the values of 10 mg/l. The correlation between the nanoparticles concentration and β-carotene content was evident and was expressed by coefficient of correlation $R^2 = 0,902$ (Figure 2).

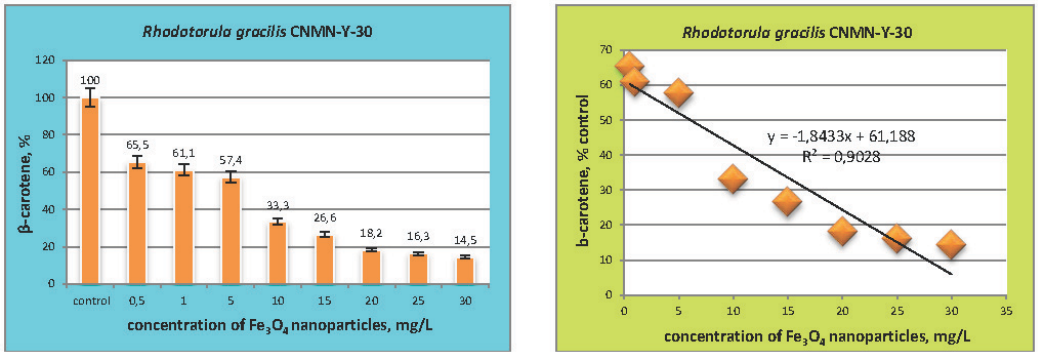

Figure 2. The influence of Fe_3O_4 nanoparticles on β-carotene content in biomass of *Rhodotorula gracilis* CNMN-Y-30 depending on concentration

The effect of TiO_2 nanoparticles on mannoproteins biosynthesis at *Saccharomyces cerevisiae* CNMN-Y-18 strain depending on concentration was significant, also. The obtained results have demonstrated that mannoproteins content increased with 11,9-22,6 % simultaneously with the nanoparticles concentration (Figure 3). The highest values of mannoproteins content were obtained at concentration limits 10-15 mg/L. This effect was caused by the property of yeast cells to uptake nanoparticles inducing redistribution of nutritive medium components and activation of carbohydrate metabolism. Thus, such an analysis of interrelation between the mannoproteins content and nanoparticles concentration can serve as a confirmation that has established a moderate ascending correlation ($R^2 = 0,556$), that argue the hypothesis of the existence of some interrelation in the base of which, according to the regression equation, could be prognosticated the selected indices.

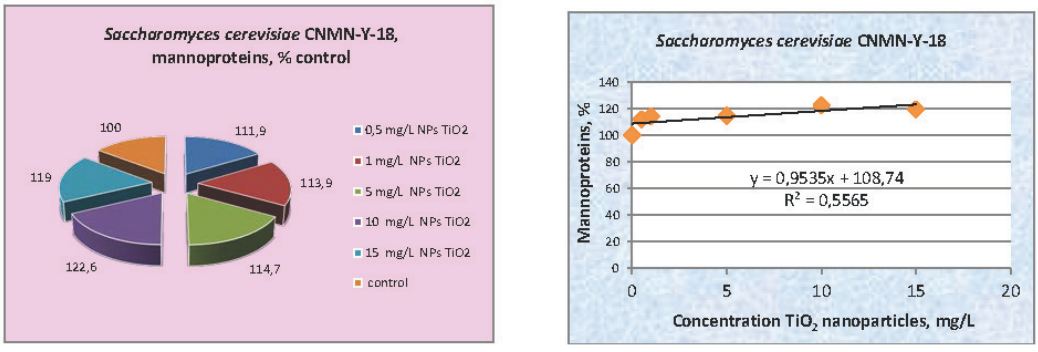

Figure 3. The effect of TiO_2 nanoparticles on mannoproteins accumulation at *Saccharomyces cerevisiae* CNMN-Y-18 strain depending on concentration.

Thereby, it can be affirmed that the study of influence of TiO_2 şi Fe_3O_4 nanoparticles on *Rhodotorula gracilis* CNMN-Y-30 and *Saccharomyces cerevisiae* CNMN-Y-18 strains has revealed that the nanoparticles effect was dependent on concentration that provided alterations of processes of bioactive principles biosynthesis depending on selected biotechnological object. TiO_2 and Fe_3O_4 nanoparticles have a high capacity to inhibit biosynthesis of carotenoid pigments and to induce the mannoproteins biosynthesis.

Another actual scientific problem was the study of peculiarities of nanoparticles action depending on dimension. The investigations devoted to the elucidation of the impact of ZnO nanoparticles on yeasts have demonstrated that the 30 nm dimensions have the capacity to stimulate the biosynthesis of proteins, carbohydrates, β-glucans at *Saccharomyces cerevisiae* CNMN-Y-20 yeast strain**,** qualities that did not manifest other nanoparticles with 10 nm dimensions (Figure 4).

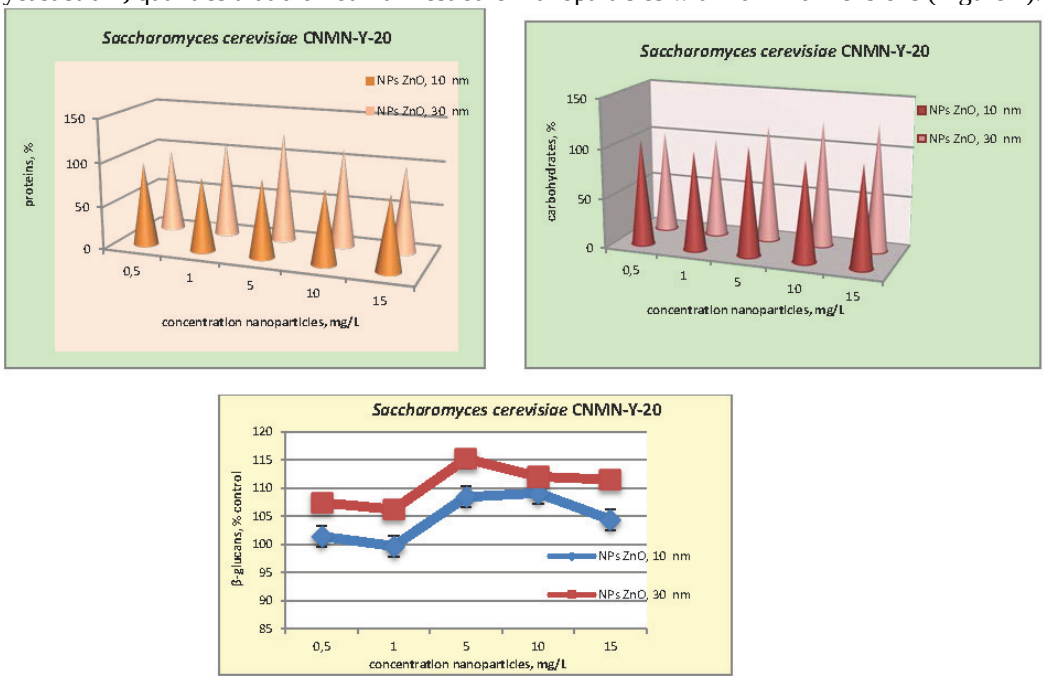

Figure 4. Content of proteins, carbohydrates and β-glucans in *Saccharomyces cerevisiae* CNMN-Y-20 biomass at the cultivation in presence of ZnO nanoparticles with different dimensions.

The similar effects were obtained in the case of study of influence of Fe_3O_4 nanoparticles on biosynthesis pigmented yeast strain *Rhodotorula gracilis* CNMN-Y-30. The research revealed that nanoparticles with 30 nm dimension possessed benefic effects on biosynthesis of cellular components, compared to other nanoparticles of 10 nm that inhibited the biosythesis of proteins, carbohydrates, carotenoids, the last being a part of category of micromolecular antioxidants that protect cells against free radical damage (Figure 5).

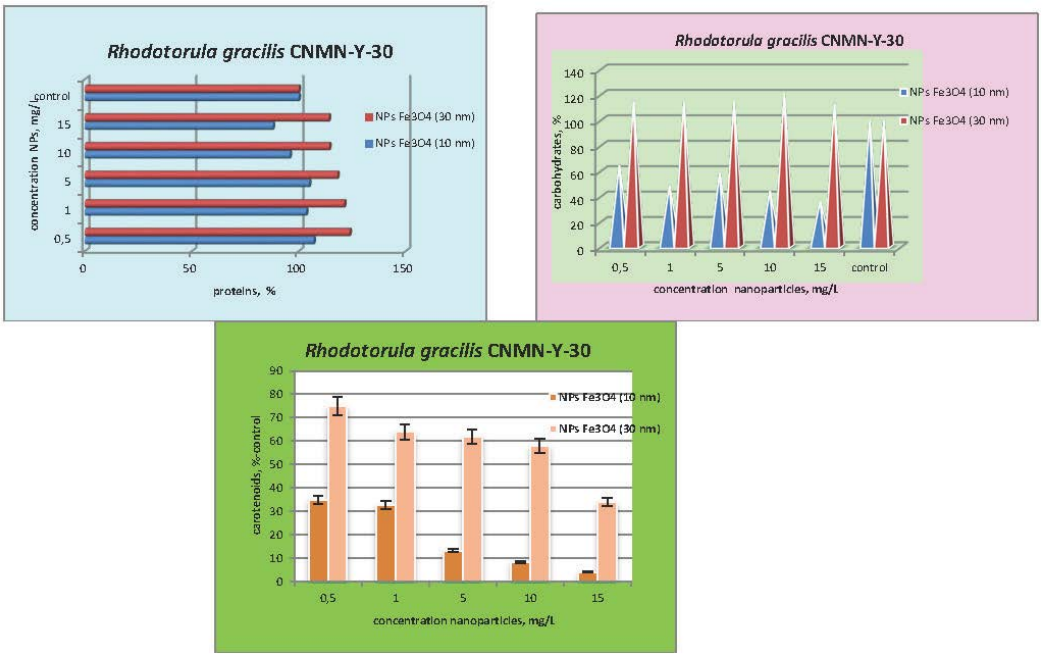

Figure 5. Proteins, carbohydrates, carotenoids pigments content in the *Rhodotorula gracilis* CNMN-Y-30 biomass at the cultivation in presence of Fe_3O_4 nanoparticles with different dimensions.

Thus, the determination of bioactive principles content modifications at *Saccharomyces* and *Rhodotorula* yeasts under the influence of ZnO and Fe_3O_4 nanoparticles has revealed various cell response induced by nanoparticles action depending on dimension. Nanoparticles with the dimensions under 30 nm used at the yeasts cultivation were more toxic as compared with those with larger dimensions. These effects were established by the determination of proteins, carbohydrates, carotenoids pigments content.

CONCLUSIONS

Thus, summarizing obtained results, it can be affirmed that new information regarding the peculiarities of action of TiO_2, ZnO, Fe_3O_4 nanoparticles on *Saccharomyces* and *Rhodotorula* yeast strains. TiO_2, ZnO, Fe_3O_4 nanoparticles induced alteration in synthesis of cellular components of studied yeast strains. Major factors determining cell response to the action of metallic nanoparticles are concentration and dimension. The important indices of this adaptive reaction are proteins, carbohydrates, including β-glucans and mannoproteins, and carotenoids pigments content.

The results obtained at the establishment of the characteristics of influence of TiO_2, ZnO, Fe_3O_4 nanoparticles on biosynthetic processes at *Saccharomyces cerevisiae* CNMN-Y-18, *Saccharomyces cerevisiae* CNMN-Y-20 and *Rhodotorula gracilis* CNMN-Y-30 can serve as a platform for following investigations to modulate biosynthesis of bioactive substances of biotechnological interest.

The research has important implications both from theoretical aspect for the explanation or confirmation of some fundamental hypotheses of the influence of nanoparticles on yeasts, and from practical aspect for the elaboration of some procedures for obtaining of bioactive principles of great interest.

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BIOCHEMICAL INDICATORS IN SOME MICROBIOTA SOILS CULTIVATED WITH CUCUMBERS

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Keywords: unfertilized and fertilized soils, current and potential dehydrogenases, calcined residue, pH, fertilization

Abstract. The study discusses some biochemical investigations (dry matter and water contents, calcined residue, pH, the current and potential dehydrogenase) devoted to a soil (uncultivated and cultivated, unfertilized and fertilized with natural fertilizer at different stages of culture development) harvested from a greenhouse intended for the production of cucumbers. The analysis of the obtained results highlights the complexity of the processes taking place in the soil. These processes depend on the growth and development of plant organisms. Involved in biological oxidation processes, soil dehydrogenase activity provides correlated information related to its health.

INTRODUCTION

Soil is a dynamic, living system whose function is dependent on its condition. The biological component of soil health depends on the number, diversity and health of the present macro-, meso- and microflora. Lately, there is a particular interest in the idea of "functional redundancy" of the microorganisms existing in the soil (Shepherd *et al.*, 2000). The hypothesis is that the number of species of organisms required for the proper functioning of soil processes is inferior to what occurs naturally in most soils (Wardle and Giller, 1996).

From a biological point of view, the soil is an enzymatic system in which the enzymes accumulated together with the enzymes released by the soil microorganisms participate in the biological cycles of the elements thus contributing to soil fertility and implicitly to the creation of conditions for plant nutrition. Enzymes may be indicators of soil quality because they can indicate the microbial activity and implicitly the degree of degradation. Plants provide organic substrates (carbohydrates, amino acids etc.) to the microorganisms involved in the microbiological degradation and they, in turn, produce and excrete their own oxidoreductive enzymes that play an active role in biodegradation (Cunningham and Ow, 1996; Radwan *et al*., 2000).

Enzymes in the soil are subject to complex biochemical processes involving integrated and ecologically connected syntheses that determine soil metabolism. The level of enzymatic activity in the soil system varies due to the fact that in each soil type the amount of organic matter, the composition and activity of living organisms and the intensity of biological processes differ.

Dehydrogenases play an important role in the biological oxidation of organic matter in the soil by the transfer of protons and electrons between substrate and acceptors as well as in the microbial activity measurement (Garcia et al., 1997; Garcia et al., 1998; Garcia et al., 2000). Dehydrogenase activity also provides correlative information on microbial populations in the soil. These enzymes are among the most important endocellular enzymes involved in ATP-producing metabolic reactions and are thought to not accumulate extracellularly in the soil, but exist in intact cells (Denton, 2009).

The aim of the paper is to evaluate the activity of dehydrogenases, in correlation with other biochemical indicators, from soils fertilized with organic fertilizers and non - fertilized, taken from a greenhouse for the cultivation of cucumbers. The dehydrogenase assessment provides information on the biological state of the soil. Assessment of dehydrogenases provides information on the biological state of the soil because, while using oxygen and other electron acceptors, soil dehydrogenase activity is intensified under anaerobic conditions.

MATERIAL AND METHODS

The biological material to be analysed is represented by the soil harvested from a personal property garden in the commune of Matca, located in the central area of Galați County, greenhouse intended for the production of cucumbers. Soil harvesting is a particularly important step in the analysis process because the harvested samples must be representative and at the same time should not introduce changes in the soil composition and qualities due to a faulty technique or improper material preparation conditions.

Considering the fact that the area from which soil samples were collected for biochemical determinations is between 2000 – 5000 m^2, the number of samples required for the average was 2-3 sub-samples. Soil samples were harvested

when they had a low degree of moisture. To this end, we removed the surface layer of soil to a depth of 15 - 20 cm and we harvested 500 g of soil sample from three different points located in relation to the other in the form of chess. Then we removed the vegetal remains (roots, herbs, leaves) and the impurities (splinters, splinters) and we mixed the samples for homogenization. Of the total amount, we have retained about 500 g of soil representing the average sample.

Samples were harvested from unfertilized soils and fertilized with natural fertilizer at different stages of culture development:

1. uncultivated, unfertilized soil;
2. uncultivated soil, fertilized with natural fertilizer;
3. cultivated soil, unfertilized, plants in the vegetative stage;
4. cultivated soil, fertilized with natural fertilizer, plants in the vegetative stage;
5. cultivated, unfertilized soil, plants in the flowering stage;
6. cultivated soil, fertilized with natural fertilizer, plants in the flowering stage;
7. cultivated, unfertilized soil, plants in fruiting stage;
8. cultivated soil, fertilized with natural fertilizer, plants in the fruiting stage.

After sampling, the soil was transported to the laboratory. After the determination of dry matter and water content, it was distributed in Petri dishes where it was maintained to dry under physiological conditions. After drying, the soil was shredded and sifted.

The *dry matter* and *water contents* are determined by the gravimetric method. This basically consists of evaluating the indicator by keeping the biological material at a temperature of 105°C to constant weight. The results are expressed in g of dry matter per 100 g of freshly analysed material. By difference, the amount of water contained in the biological material to be analysed is evaluated.

The method for determining the *calcined residue at 550°C* consists, in principle, in maintaining the sample to be analysed, a determined time at 525 ± 25°C (Mănescu et al., 1978). Keeping the sample to be analysed at the calcination temperature leads to the loss of organic substances and some of the volatile mineral substances.

The results, representing the average of three consecutive determinations, are expressed in g of calcined residue/100 g of soil to be analysed.

To determine the *pH*, we used the electrometric method, basically based on measuring the potential difference between a glass electrode and a reference electrode. The recorded difference varies linearly with the sample pH. The determinations were performed at 20 ± 0.5°C resulting in the average of three repetitions for each experimental variant.

In principle, the method of determining *the current and potential dehydrogenase* activity is based on the ability of these enzymes to transfer hydrogen from different substrates (carboxylic acids, alcohols, carbohydrates) to 2,3,5-triphenyltetrazolium chloride (TTC) which is reduced and switches to red coloured triphenylformazan. Triphenylformazan is extracted with acetone and the colour intensity of the obtained solution, proportional to the dehydrogenase activity, is determined spectrophotometrically (Kiss and Boaru, 1965). To determine the activity of actual dehydrogenases, pre-existing organic substances in the soil will serve as hydrogen donors. To determine the potential dehydrogenase activity, glucose is added to the soil samples, which together with the pre-existing organic substances in the soil will serve as hydrogen donor. Calculation of the current and potential dehydrogenase activity is performed in relation to a calibration curve constructed with TTC reduced to formazan with Zn powder.

RESULTS AND DISCUSSION

In our experimental model, we considered the 1* and 2* samples as control samples for unfertilized and fertilized soils. The analysis of the experimental results indicates the decrease of the dry matter (DM) content of the cultivated soils in both variants, unfertilized and fertilized with organic fertilizer (fig. 1).

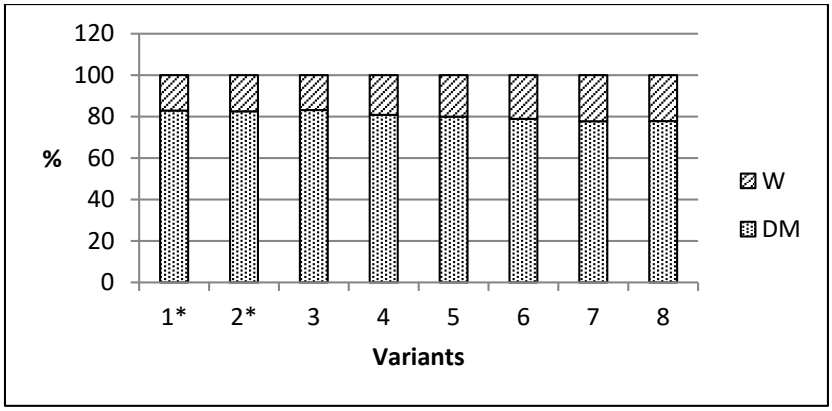

Fig. 1. Dynamics of dry matter (DM) and water content (W) in the soil samples, unfertilized and fertilized with natural fertilizer, harvested at different stages of development of a cucumber culture

In the dynamics of the investigated phenophases, we find the diminishing of the value of the indicator to the fruiting phenophase both in the unfertilized soil version and in the fertilized soil. Considering each phenophase in part, we find the decrease of the dry matter content in the fertilized soils; the diminution becomes almost insignificant in the fruiting phenophase.

The water content of the soils taken to evaluate this biochemical marker has inverse amplitudes to those discussed in the analysis of the dry substance content results.

The results from the determination of the amount of mineral substances are approximate because at the temperature of 525 ± 25°C not only organic substances, but also some of the inorganic substances such as carbonates, nitrates, chlorides or ammonium salts are decomposed. The analysis of the results on unfertilized soils highlights amplitudes describing the trend of diminishing the values of the indicator to fructification phenophase compared to control. The minimum value recorded in fruiting phenophase is 94.09 g% (fig. 2).

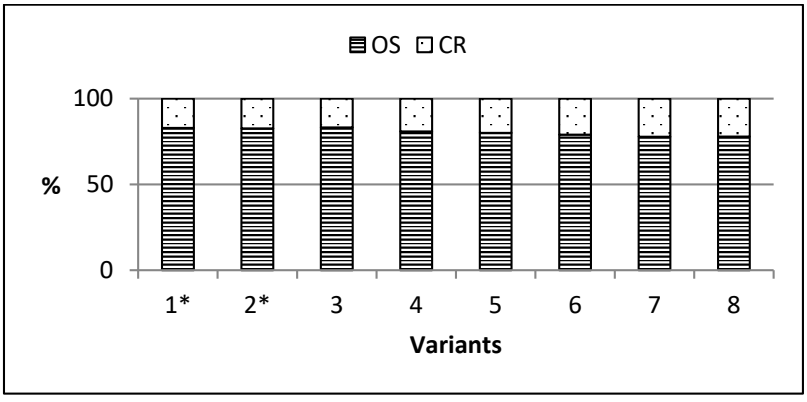

Fig. 2. Dynamics of the calcined residue (CR) and organic substances (OS) in the soil samples, unfertilized and fertilized with natural fertilizer, harvested at different stages of development of a cucumber culture

Compared to uncultivated soil, in the fertilized soils, the values of the amount of mineral substances show a tendency to increase. This is a normal behaviour, considering the higher consumption of organic substances by the plants during their growth and development.

The content of organic substances presents dynamically an inverse aspect to that discussed in relation to the variation in the content of inorganic substances (fig. 2).

The pH, measure of acidity or basicity, is a particularly important feature because it controls many chemical processes that take place in the soil. The value specifically affects the availability of nutrients by controlling their chemical forms. The pH variation curve in unfertilized soil samples records a maximum value in the vegetative stage and a minimum in the flowering phenophase (fig. 3). By comparison with the control, the value of the investigated indicator is superior to the soil taken in the vegetative phenophase of the cucumber culture and diminishes in the case of the soils taken in the flowering and fruiting phenophases.

Fertilized soils have a similar behaviour to non-fertilized soils regarding pH dynamics (fig. 3). The difference is that, although the control samples have identical pH, the fertilized soil samples show in all phenophases higher values of the studied indicator. This behaviour is due to the use of a fertilizer of natural provenance.

The literature mentions that cucumber culture soil should have a slightly acidic to neutral reaction, which requires a pH of 6.5-7.5 (Ghehsareh and Samadi, 2012). The pH value influences the plant's ability to feed the nutrients needed for growth and development.

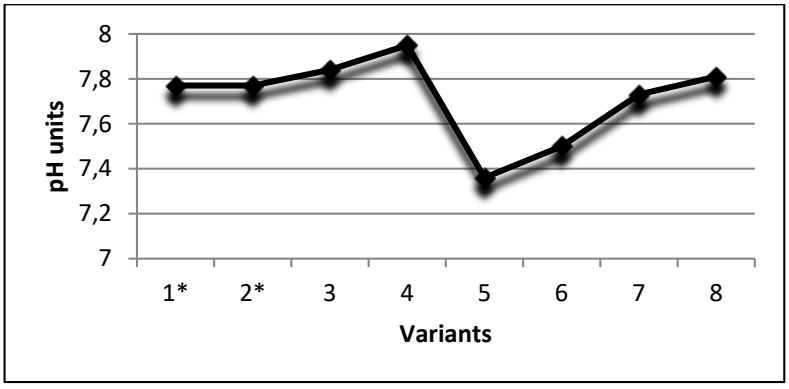

Fig. 3. PH dynamics in the soil samples, unfertilized and fertilized with natural fertilizer, harvested at different stages of development of a cucumber culture

Determination of dehydrogenase activity is a way to immediately assess the metabolic activities of soil microorganisms. Several environmental factors, including soil moisture, oxygen availability, oxidation-reduction potential, pH, organic matter content, depth of the soil profile, temperature, season of the year, heavy metal contamination and soil fertilization or pesticide use can significantly affect the dehydrogenase activity in the soil environment (Wolińska and Stępniewska, 2012; Pandely and Singh, 2006; Brzezinska *et al.*, 1998)

The analysis of current dehydrogenase activity evaluated in unfertilized and fertilized soils, not cultivated and cultivated with cucumbers, generally reveals higher amplitudes of enzyme activity in fertilized soils (fig. 4). The explanation for this behaviour is that the enzyme activity is potentiated by the natural fertilizer that has been applied to the analysed soil. Pesticide studies have

highlighted that, in most cases, they exhibit a temporary inhibitory effect on soil enzymes (Pandely and Singh, 2006). For this reason, we find, in the case of unfertilized soils, a trend of increasing the activity of the investigated enzymes to the fructification stage of the cucumber culture. The maximum enzyme activity is recorded in the final stage of the experiment when the current dehydrogenase activity is 101.81 μg formazan/g analysed soils.

Regarding the fertilized soil, we find that in the vegetative phenophase the activity of the investigated enzyme increases by about 10%, because in the flowering and fructification phenophases the activities recorded for the present dehydrogenases have comparable values of 138.3μg formazan/g and 137.52μg formazan/g, respectively, which mean a decrease of about 34%.

Soil physical conditions have a strong indirect influence on dehydrogenase activity by the changes they make to the soil aeration state. We make these claims because in literature it is known that the dehydrogenase activity is in reverse relation with the air penetration into the pores, the oxygen diffusion rate and the redox potential. As a result, the activity of the enzyme increases with the increase of aerobic activity, which can be translated by the fact that anaerobic or optionally anaerobic members of the microbial association become more important in the total respiratory process of the soil. An important aspect that must be taken into account is that the dehydrogenase activity is in a positive relationship with the soil moisture.

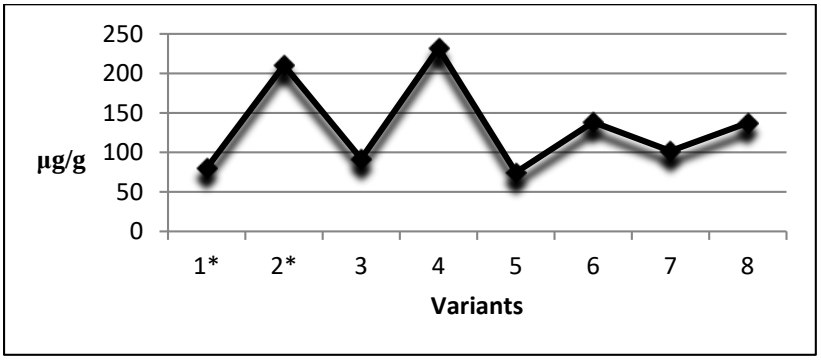

Fig. 4. Current dehydrogenase dynamics in soil samples, unfertilized and fertilized with natural fertilizer, harvested at different stages of development of a cucumber culture

Potential dehydrogenase activity, both in the case of control samples and experimental variants of cultivated soil, shows higher values in cases where the soils are untreated with natural fertilizers (fig. 5). We explain this behaviour by the fact that the fertilization is a factor in disrupting the oxido-reduction reactions involved in the metabolism of microorganism populations as long as the dehydrogenase activity is inhibited by the presence of the products with which the treatment is performed.

In both cases - unfertilized soils and fertilized soils – the samples of cultivated soils have higher values of potential dehydrogenases. The variation curves show increasing trends from vegetative phenophase to fructification, with a slight decrease in flowering phenophase in unfertilized soils, as well as in fruiting phenophase in fertilized soils.

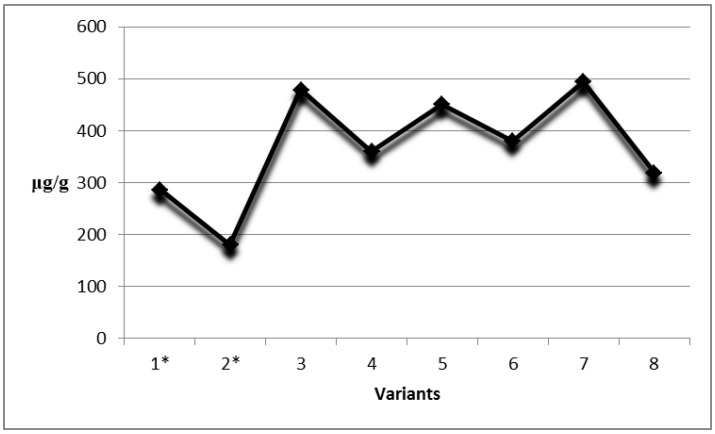

Fig. 5. Potential dehydrogenase dynamics in soil samples, unfertilized and fertilized with natural fertilizer, harvested at different stages of development of a cucumber culture

CONCLUSIONS

The comparative analysis of the results recorded for dry matter quantity in fertilized and non-fertilized soils harvested in different phenophases of growth and development of a cucumber culture, indicates the decrease of the values compared to the control represented by the uncultivated soil. In vegetative and flowering phases, the amount of dry substance diminishes in relation to the control soil.

The amount of mineral substances in non-fertilized soils tends to decrease to fruiting phenophase compared to the control sample. In the case of naturally fertilized soils the tendency is to increase the values of the investigated indicator.

The pH variation curve in unfertilized soil samples records a maximum in vegetative stage and a minimum in flowering phenophase. The pH dynamics in fertilized soils is comparable to that resulting from indicator analysis in unfertilized soils. Recorded amplitudes are superior in fertilized soil samples. According to data from the literature we find optimal levels of pH throughout the experimental model.

The obtained results allow us to appreciate that the current and potential dehydrogenase activities are good indicators of soil biological activity. Enzymes are active in all investigated experimental variants, the particular manifestations being dependent on the fact that the analysed soils are cultivated or not, are treated with natural fertilizer or are taken in a certain phenophase of the cucumber culture. Organic fertilizer, the oldest tool used to improve soil fertility, has to be seen from the point of view of its action due to nutrients as well as soil improvers because it enhances the physical, chemical and biological soil features and thus stimulates many factors of production.

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THE INFLUENCE OF THERMAL PROCESSING ON TOTAL PHENOLIC CONTENT IN POPCORN SEEDS

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Keywords: thermic processing**,** phenolic content, seeds, popcorn

Abstract. The aim of this paper was to search how much the thermal processing can influence the total phenolic content in popcorn seeds. The biological material, used in this work, was represented by popcorn seeds (Zea mays L. var. everta) belonging to eight Romanian local populations. The experiment consisted in three different thermal processings (boiling and steaming for 30 minutes, and roasting for 20 minutes at 150-190°C), followed by determination of total phenolic content in processed grains and in used waters. The estimation of total phenolic contents in seeds extract was carried out through a colorimetric assay, by measuring its reducing capacity with Folin-Ciocalteu reagent. For this purpose, a standard curve was generated, representing the absorbance values of gallic acid standard solutions in relation to their concentrations. As compared to control samples (raw material), the thermal processing of popcorn seeds, by boiling and steaming, resulted in significant losses of phenolic compounds, higher through boiling. The presence of phenols was also found within cooking waters, more in boiling, and less in steaming ones. Compared to the control samples, in the roasted popcorn seeds the phenolic compounds have registered significant increases, probably due to the release of bound phenols by the action of high temperatures and exposure time.

INTRODUCTION

Consumption of fruits, vegetables, and un-polished grains is strongly associated with the reduced risk of developing chronic diseases such as cancer and cardiovascular disease (*Isabelle et al., 2010; Liu, 2004,* cited by *Harakotr et al., 2014b*).

Plant phenolics have potential health benefits mainly due to reactive oxygen species scavenging and inhibition, electrophile scavenging, and metal chelation (*Huang et al., 1992*).

Corn (*Zea mays* L.) is a major cereal used to produce grain and fodder that are the basis for a number of foods, feed, pharmaceutical and industrial products. Due to its adaptability and productivity, it is the third most cultivated field crop after wheat and rice (*Randhir and Shetty, 2005*).

In our diets, corn is a source of macro- and micronutrients (*Gonzalez et al., 2005; Chander et al., 2008*), a rich source of many phytochemicals, including carotenoids (*Chander et al., 2008; De La Parra et al., 2007; Kopselld et al., 2009; Kean et al., 2008, Li et al., 2007a; Lopez-Martinez et al., 2009; Montilla et al., 2011*), phenolic compounds (*Chander et al., 2008; De La Parra et al., 2007; Santiago et al., 2007; Pedreschi and Cisneros-Zevallos, 2007; Lopez-Martinez et al., 2009; Montilla et al., 2011*), anthocyanins (*De La Parra et al., 2007; Pedreschi et Cisneros-Zevallos, 2007; Li et al., 2008*), tocopherols (*Chander et al., 2008, Ibrahim and Juvik, 2009*), and phytic acid, which have multiple functional roles, for example, as antioxidants (*De La Parra et al., 2007; Pedreschi and Luis, 2006; Li et al., 2007b; Graf and Eaton, 1990*), as antimutagens (*Pedreschi and Luis, 2006*), and as inhibitors of colorectal carcinogenesis (*Pedreschi and Luis, 2006; Zhao et al., 2005; Shamsuddin and Ullah, 1989*).

Due to their high antioxidant properties, anthocyanin pigments in corn have anti-inflammatory effects (*He and Giusti, 2010*), can prevent diabetes and obesity (*Tsuda et al., 2003*), heart ischemia–reperfusion injury and hyperlipidemia (*Toufektsian et al., 2008*), and potentially reduce the risk of colon cancer (*Hagiwara et al., 2001*).

Phenolic compounds exhibit pharmacological properties such as: antitumor, antiviral, antimicrobial, antiinflammatory, hypotensive and antioxidant activity (*Cowan, 1999; Shetty, 1997*), being a relationship between the consumption of phenolic-rich food products and a low incidence of coronary heart disease, atherosclerosis, certain forms of cancer and stroke (*Hertog et al., 1993; Diaz et al., 1997; Ito and Hirose, 1989; Ness and Powles, 1997*).

Like other secondary metabolites (anthocyanins and carotenoids), phenolics are influenced by plant species and varieties (*Abdel-Aal and Hucl, 1999; Zhao et al., 2005; Pedreschi and Luis, 2006; Li et al., 2007b; Lopez-Martinez et al., 2009*), and are primarily synthesized through the pentose phosphate pathway (PPP), shikimate and phenylpropanoid pathways (*Randhir and Shetty, 2005*).

By *Turkmen et al.* (*2005*), cooking induces changes in physiological and chemical composition, influencing the concentration and bioavailability of bioactive compounds in food, the thermal treatments decreasing the total phenolics in squash, peas and leek. In sweet corn cooking led to an increase in the level of phenolic compounds (*Dewanto et al., 2002*).

In this paper it has studied if and to what extent the thermal processing can modify the total phenolic content in popcorn seeds, belonging to eight Romanian local populations.

MATERIALS AND METHODS

Research materials. The biological material was represented by popcorn seeds (*Zea mays* L. var. *everta*) belonging to eight Romanian local populations (LP). Dried seeds (moisture content = 8-10 %), coming from crops of the last two years, were used to prepare working and control samples from each LP.

Procedure and research methods. The experiment consisted in three thermal processings, as follows. *For boiling*, they have taken 100 corn seeds of each sample, which have been placed in a stainless steel vessel of three liters capacity. The boiling has done in one liter of tap water in the pot covered, for 30 minutes (timed from the moment when the water began to boil).

For steaming, in each pot, a dense mesh fixed to the walls was mounted, to two-thirds of the vessel bottom. After pouring of one liter of tap water into the bowl, on the sieve were placed 100 seeds that were steamed, within covered pot, for 30 minutes (timed from the moment when the water began to boil).

For roasting, 100 seeds from each sample were roasted for 20 minutes at 150-190°C, in a covered stainless steel vessel.

In order to determine Total Phenolic Content (TPC), first an extract for each seeds sample was obtained, weighing 1 g of grains, which were finely ground and subjected to extraction with a mixture methanol and water (80/20), by stirring, centrifuging and recovering the supernatant (*Adom and Liu, 2002*)

The estimation of Total Phenolic Contents in seeds extract was carried out through a colorimetric assay, by measuring its reducing capacity with Folin-Ciocalteu reagent. For this purpose, a standard curve was generated, representing the absorbance values of gallic acid standard solutions in relation to their concentrations (*Moore and Yu, 2008*). TPC was expressed as mg Gallic Acid Equivalent/g dry seeds i.e. dry weight (mg GAE/g DW).

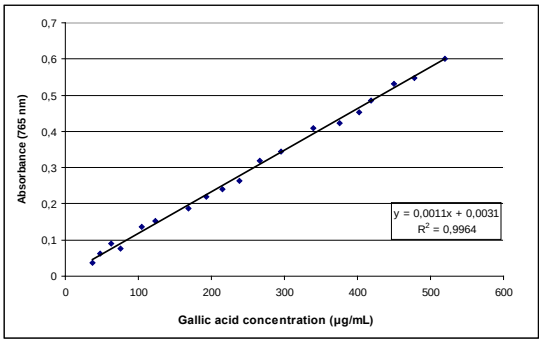

Fig. 1. Standard curve for TPC, using Gallic Acid

Statistical analysis. The data of experiments, coming from four replicates for each determination, were statistically processed using SAS Version 8.02 (*SAS Institute, 2005*)**.** To analyze the significance of differences among samples, generalized linear model analysis was carried out, and for multiple comparisons was used Duncan's multiple range test ($P<0.05$).

RESULTS AND DISCUSSIONS

In the Table 1 are rendered the values of the total phenolic content (TPC) in the eight popcorn seed samples, subjected to thermal processing.

Table 1. Comparative values of TPC in popcorn seeds thermal processed

Popcorn local populations (LP)	TPC (mg GAE/g DW)					
	Raw material*	Boiled		Steamed		Roasted Seeds
		Seeds	Water	Seeds	Water	
LP1 (red)***	8.2±0.7BC**	4.2±0.3DE**	0.8±0.04G	5.8±0.6D	0.4±0.05G	9.9±0.6B
LP2 (purple)	7.8±0.4BC	4.5±0.3DE	0.9±0.07FG	6.8±0.5CD	0.2±0.04G	8.6±0.3BC
LP3 (red)	9.7±0.5B	4.7±0.5DE	1.1±0.05FG	7.9±0.4BC	0.3±0.05G	10.7±0.9A
LP4 (red)	8.5±0.4BC	4.7±0.5DE	0.9±0.06FG	6.8±0.5CD	0.2±0.07G	8.7±0.5BC
LP5 (orange)	6.9±0.5CD	3.8±0.7EF	1.2±0.07FG	5.9±0.3D	0.1±0.03G	8.6±0.7BC
LP6 (white)	5.7±0.5D	4.2±0.5E	0.5±0.08G	4.8±0.6DE	0.2±0.03G	6.9±0.3CD
LP7 (yellow)	6.5±0.3C	3.9±0.8EF	0.8±0.05G	5.8±0.6D	0.1±0.04G	7.8±0.7BC
LP8 (white)	6.2±0.7CD	2.6±0.5F	0.9±0.04FG	4.6±0.3DE	0.1±0.07G	6.5±0.4C

*Unprocessed seeds; **Means with different letters are statistically diferent (P<0.05); ( )***kernel colour

As seen from Tab. 1, the total phenolic content (TPC) in seeds of the eight maize local populations (LP) ranged between 5.7±0.5 and 9.7±0.5 mg GAE/g DW, the highest values being found in red and purple grains.

According to some authors (*Lopez-Martinez et al., 2009; Montilla et al., 2011; Žilić et al., 2012, cited by Harakotr et al., 2014b*), the pigmented corn contains (more) anthocyanins, carotenoids, phenolic compounds, and antioxidant activity than non-pigmented corn.

TPC of seeds was modified after thermal processing (boiling, steaming or roasting), as compared to the control samples (raw seeds). ***Thus, boiling for 30 minutes*** led to a significant decrease ($P<0.05$) of the total content of phenolic compounds in all popcorn seeds, with percentages between 26.3% (LP6) and 51.5% (LP3).

Analyzing the boiling water of seeds, TPC (reported to raw material) was between 8.7% (LP6) and 17.4% (LP5). Significant differences ($P<0.05$) were between LP1, LP6 and LP7 (with close values), on one hand, and LP2, LP3, LP4, and LP5 (with close values), on the other hand. A part of the phenolic compounds was destroyed in each seeds sample by boiling. This fact one can see gathering (mathematically) the content of phenolic compounds from each boiled sample and from its boiling water and comparing it with the control (unprocessed seeds). These losses of total content of phenolic compounds in the analyzed samples ranged between 17.5% (LP6) and 43.5% (LP8), i.e. between 2 and 2.5 times greater than phenols released in the boiling water. These results are consistent with the data reported by some researchers.

Thus, *Harakotr et al. (2014a)*, studying antioxidant components, antioxidant activity, and their changes during traditional cooking of fresh purple waxy corn, found a higher content of phenolics in the boiling water, than in steaming one, because of phenolics losses in the cooking water. Since the sum of phenolics in cooked samples and cooking water consistently differed from their content in raw samples, differences in phenolic content could be, by *Harakotr et al. (2014a)*, due to breakdown of phenolics, which was greater than losses due to leaching into cooking water.

As to seeds steaming, the Table 1 shows that 30 minutes of this thermal processing caused a significant reducing of TPC in all seed samples ($P<0.05$), with percentages between 10.7% (LP7) and 29.2% (LP1).

The analysis of water from seeds steaming, showed the presence of TPC, whose percentages (reported to raw material) ranged from 1.5% (LP5 and LP7) to 4.8% (LP1). As can be

seen in the Table 1, there are no significant differences between TPC values of water from seed steaming.

Gathering (mathematically) the content of phenolic compounds from each steamed seeds sample and from its steaming water, and comparing it with the control (raw seeds), one can see significant differences ($P<0.05$), caused by the destruction of phenolic compounds through steaming. The phenolic compounds losses, through this type of thermal processing, ranged between 9.2% (LP7) and 27% (LP1), i.e. about 6 times greater than phenols released into steaming waters.

Comparing the two types of thermal processing, it can see that steaming has caused losses of phenolic compounds significantly lower than boiling ($P<0.05$).

According to *Harakotr et al. (2014a)*, corn seeds boiling had a more detrimental effect on phenolic acids than steaming. The boiling treatment causes disruption of cellular components with the consequent release of these molecules into the cooking water (*Miglio et al., 2008,* cited by *Harakotr et al., 2014a*). By *Huang et al. (2006)*, steaming treatment causes matrix softening and increases extractability of antioxidant components from the raw materials. The thermal treatment could cause changes in phenolic substances, in the corn, by liberating the bound phenolic compounds into free form (*Xu and Chang, 2009*).

Comparing with control samples (raw seeds), the total content of phenolic compounds in ***roasted corn seeds*** has registered significant increases ($P<0.05$) in all samples (except LP2 and LP4), with percentages between 2.3% (LP4) and 24.6% (LP5). This results are consistent with observations and data reported in some scientific works.

Han and Koh (2011), researching antioxidant activity of hard wheat flour, dough and bread prepared using addition of different phenolic acids, found that the antioxidant activity and residual free phenolic acid content of flour were reduced by mixing, but increased by fermentation and baking.

During thermal treatment**,** Maillard reaction products may protect phytochemicals from oxidation (*Lin et al., 2008*), and according to *Jeong et al (2004)*, heat treatment at 150°C for 40 min. liberated bound phenolics in citrus peels having as result a significant increasing of TPC after treatment.

CONCLUSIONS

The thermal processing of corn seeds, belonging to eight local populations of popcorn (*Zea mays* L. var. *everta*) has significantly influenced the total phenolic content of seeds.

As compared to control samples (raw material), the thermal processing of popcorn seeds by boiling and steaming for 30 minutes, resulted in significant losses of phenolic compounds, higher through boiling. The presence of these compounds was found within cooking waters, more in boiling, and less in steaming ones.

Compared to the control samples, in the roasted popcorn seeds (20 min. at 150-190°C), the phenolic compounds have registered significant increases, apparently due to Maillard reaction products, which have protected phenols against oxidation, on the one hand, and due to the release of bound phenols, by the action of high temperatures and exposure time, on the other hand.

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THE EVALUATION OF UTERINE ELECTRICAL ACTIVITY BY ANTEPARTUM ELECTROMYOGRAPHY

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Abstract. It is known that the cervical length is a predictive factor for pregnancy evolution to term, but there have not been conducted any trials yet to correlate it with myometrial electrical activity on long term. Nowadays, we don't dispose of a simple, widely available technique to objectively monitor uterine contractility during pregnancy, in order to assess the risk of preterm birth. Our aim was to evaluate the relation between clinical elements, the cervical length and the uterine contractility as recorded by antepartum electromyography. In this study were included 113 women with singleton pregnancies, with gestational age 20-29 weeks divided in two groups: 44 women with short cervical length and 69 with cervical length >25mm. The cervical length is indirectly correlated to the mean contraction frequency, and the contraction percentage was significantly lower in the normal cervix group when compared to the short cervix group. The mean number of contractions was slight increase in the short cervix group (11.27±9.59 *vs* 9.80±7.05 contractions/hour). 58% of the short cervix patients present a significantly high number of contractions/hour, of low amplitude, and more than 55% present great amplitude and duration of contractions. About 67% of the short cervix patients present high contraction areas. Maternal activity between 10 am-11pm was more pronounced for both study groups. Electromiography has proven its efficacy and utility in obstetrical practice for identify a short cervix, which is a risk factor for premature birth, and recommend the proper treatment.

INTRODUCTION

It is widely accepted that cervical length is correlated to premature birth risk. Many studies have reported its use in the second trimester (Iams, J.D., et al., 1996; Hassan, S.S., et al., 2000) and the inverse correlation between premature birth risk and cervical length, for the symptomatic patient as well as for the general population (Grimes-Dennis, J. & Berghella, V., 2007). The evolution of a short cervix in asymptomatic patients is unclear (McIntosh, J., et al., 2016).

The most frequent diagnosis that leads to hospitalisation during pregnancy is threatened preterm birth because the patient's individual perception of uterine contractions varies too much. That's why, about half of patients admitted for threatened preterm labour are not in true labour and will eventually deliver at term (McPheeters, M.L., et al., 2005). Almost 20% of symptomatic patients who are diagnosed as not being risky for preterm labour, however, will deliver prematurely (Iams, J.D., et al., 2001). These symptoms are not helpful for predicting the risk of prematurity and lead to unnecessary treatments, on the one hand, or missed opportunities to improve neonatal outcome, on the other hand (Luckovnik, M., et al., 2011).

Whether the pregnancy is low or high risk, one of the risk factors strongly associated with spontaneous preterm birth before 35 weeks is cervical length less than 25 mm, measured by transvaginal ultrasound in the mild trimester, (Iams, J.D., et al., 1996).

The pathophysiology of cervical shortening in asymptomatic women is unclear. Is the shortened cervix the first event, meaning a weak or insufficient cervix which will then lead to premature birth in the absence of uterine contractions, or do these patients actually have asymptomatic contractions that shorten the cervix (Lewis, D., et al., 2005).

Our difficult task is to differentiate uterine contractions that will lead to cervical change, from physiological uterine activity that will not lead to preterm birth (Iams, J.D., 2003; McNamara, H.M., 2003).

Some authors suggested a positive correlation between the increased electrical activity of the uterus and the shortening of the cervical canal (Grgic, O. & Matijevic, R., 2008). It is well known that contractile activity is the result of electrical activity propagation through the myocites and electromyography is a technique that monitors quantitatively this uterine activity by measuring the variation of the membrane potential in myometrial cells (Vinken, M.P.G.C., et al., 2009).

PURPOSE AND OBJECTIVES

The aim was to find a pattern of contractility belong normal pregnancy, without risk, comparing with high risk preterm birth depending of cervical length. Measuring uterine activity in the mild trimester could become an additional tool to estimate the incidence of prematurity in general population.

MATERIAL AND METHODS

This was a prospective observational study conducted at the „Cuza-Vodă" Obstetrics and Gynecology Clinical Hospital in Iași, România. We included 113 women with singleton pregnancies, with gestational age between 20 and 29 weeks. After measuring the cervical length while respecting all the requirements of the Society for Maternal-Fetal Medicine (SMFM), two groups were created: the short cervix group - 44 women with cervical length ≤ 25 mm and the control group - 69 women with cervical length >25mm.
Steps for proper cervical length measurement (SMFM, 2016):
(1)Ensure patient has emptied her bladder.
(2)Prepare the cleaned probe using a probe cover.
(3)Gently insert the probe into the patient's vagina.
(4)Guide the probe into the anterior fornix.
(5)Obtain a sagittal, long-axis image of the entire cervix.
(6)Remove the probe until the image blurs and then reinsert gently until the image clears (this ensures you are not using excessive pressure).
(7)Enlarge the image so that the cervix occupies two thirds of the screen.
(8)Ensure both the internal and external os are seen clearly.
(9)Measure the cervical length along the endocervical canal between the internal and external os.
(10)Repeat this process twice to obtain 3 sets of images/measurements.
(11)Use the shortest best measurement

All patients gave written informed consent to participate and the measurement of uterine activity was performed using non-invasive electromyography (EMG), recorded at the maternal abdominal surface. At the beginning of the study we performed short EMG recordings, up to 2 hours long. Afterwards, the EMG recordings were conducted for as long as the battery charge would last, that is up to 20-23 hours. Because of that, we analyzed the uterine contractility for each hour of the day and for the entire recording.
Uterine activity was monitored using a portable EMG recorder Monica AN24 (Monica Healthcare Ltd, Nottingham, United Kingdom), which has 5 disposable electrodes that must be positioned on the maternal abdomen. Prior to electrode placement, it is necessary prepare the skin, in order to ensure that skin impedance was <5 kΩ in all recordings. The electrodes were positioned as follows: 2 electrodes vertically along the midline, one below the umbilicus (at the uterine fundus) and the second above the pubic symphysis; 2 electrodes horizontally, approximately symmetrical, 3-5 cm from midline, at the edge of the uterus; a ground electrode on the right flank (fig. 1).

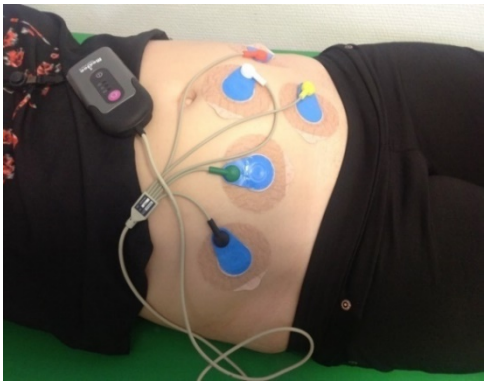

Fig. 1. Placing electrodes on the abdomen (personal collection)

The recordings show five elements: maternal heart rate, fetal heart rate, uterine electrical activity, maternal movements, fetal movements.
The monitored parameters were: maximal amplitude, duration and integral time-amplitude (area of contraction) for each contraction, on one hand, and the characterization of all contractions for each recording, on the other hand: the total number of contractions, the ratio between the number of contractions and the recording length, the maximum, mean and median amplitude, duration, and integral time-amplitude of all contractions, the rate of contractions from recording length, the

index area - defined as the ratio between the sum of all areas of contractions (integral time-amplitude) and the duration of the respective recording.
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RESULTS AND DISCUSSION

We recorded 668 hours in the short cervix group, with a mean of 15.2 hours/woman, and 962 in the control group, with a mean of 13.9 hours/woman (p=0.575). The total number of contractions were significantly increased in the short cervix group (p=0.05): 44 short cervix subjects with 5164 contractions (mean 117.4 contractions/woman) *versus* 69 normal cervix subjects with 5287 (mean76.6 contractions/woman).
Demographic characteristics of these two groups are presented in table I.

Table I. Demographic characteristics in study groups

Parameter	Short cervix group (n=44)	Control group (n=69)	p
Mother age, mean±SD y	31.43±5.11 (19-40)	29.57±4.64 (18-44)	0.047
≥ 3 pregnancy, n(%)	20 (45.5%)	11 (15.9%)	0.001
Nulliparous, n(%)	28 (63.6%)	45 (65.2%)	0.586
Gestational age records±SD, weeks	23.05±2.00 (20-28)	23.90±2.05 (20-29)	0.050
Cervix lenght±SD, mm	17.66±5.83 (5-25)	37.35±5.62 (28-57)	0.001

So, we resume for short cervix group: gestational age in recording is significantly reduced (23.09 *versus* 23.89 weeks; p=0.043), the number of previous pregnancy is increased (45% *versus* 16.5%; p=0.001), and, of course, the cervical length up to 25 mm (17.66 *versus* 37.35 mm; p=0.001).

The highest amplitude is recorded between 11 am and 9 pm (fig. 2).

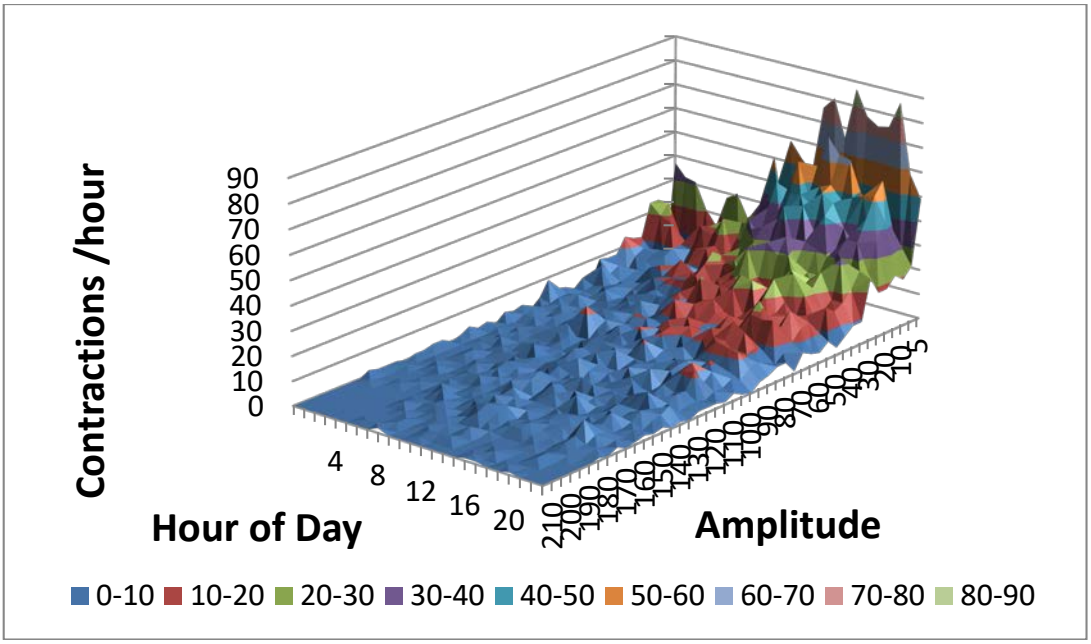

Fig. 2. The diurnal distribution of contractions depending on their amplitude in the control group

We analyzed the signals by each hour of the day, trying to find correlations between maternal movements and uterine contractility in both groups.

We found variability in median maternal movements, probably the circardian physiological rhythms, and we decided to divide the day in two intervals, between 10 am -11 pm=activity period and between 12 pm - 9 am=resting period (fig. 3).

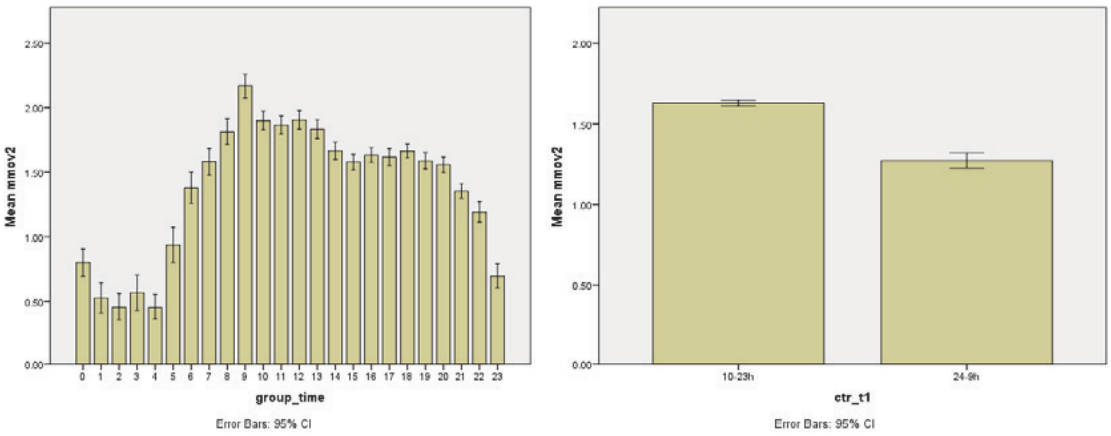

Fig. 3. Mean maternal movements

We tried to characterize all particularities of the recorded uterine activity according to those two different time periods.

There are significant statistical differences of mean contraction amplitude; the patients from the short cervix group had significantly increased contractions during the active period (83.02 *vs* 47.58; $p<0.05$), as well as during the resting period (82.68 *vs* 47.52; $p<0.05$) (fig. 4).

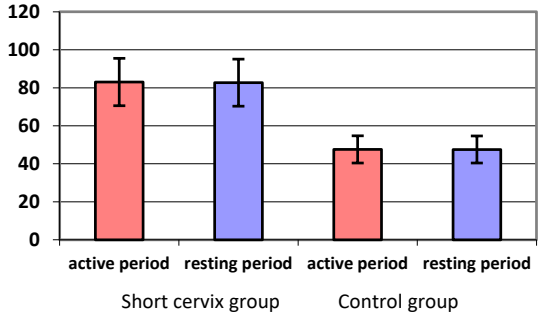

Fig. 4. Mean values of contraction amplitude according to active *versus* resting period in study groups

The mean duration of contraction was a little bit increased in the short cervix group, during both periods, activity (31.77 *vs* 25.48; $p>0.05$), versus resting (31.44 *vs* 24.35; $p>0.05$) (fig. 5).

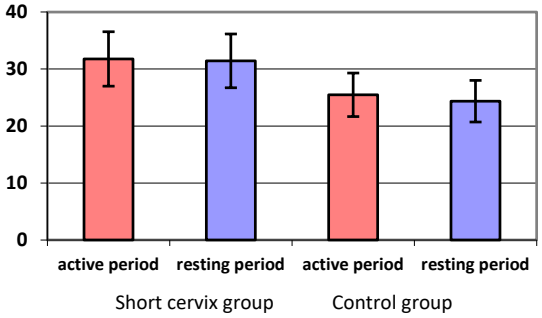

Fig. 5. Mean values of duration of contraction according to active *versus* resting period in study groups

The short cervix group had mean of contraction area significantly increased, during active status (6400 *vs* 3752; $p<0.05$) and during resting status (6238 *vs* 3515; $p<0.05$) (fig. 6).

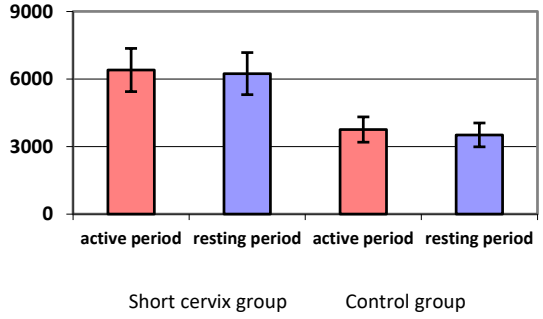

Fig. 6. Mean values of contraction area according to active *versus* resting period in study groups

Maternal activity associated to uterine contractions is significantly increased between 10 am-11 pm, compared to the 12 pm-9 am period, both in the short cervix group (1.66 *vs* 1.25; p=0.005) as well as in the control group (1.67 *vs* 1.25; p=0,005) (fig. 7).

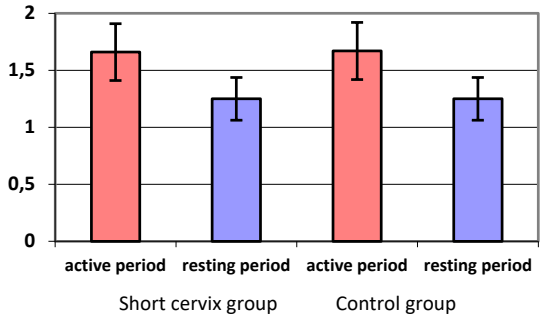

Fig. 7. Mean values of maternal activity according to active *versus* resting period in study groups

The mean number of contractions per hour was slightly increased in the short cervix group, for both active (24.62*vs* 23.50; p>0.05) and resting time (9.09 *vs* 7.38; p>0.05) (fig. 8).

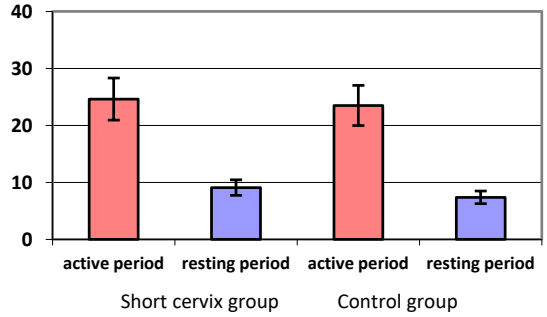

Fig. 8. Mean values of number of contractions per hour according to active *versus* resting period in study groups

Correlation of the parameters of uterine activity to time periods, for both groups (tab. II).

Table II. Statistic differences between utero activity according to active *versus* resting period in study groups

Parameter	Short cervix group (n=44)			Control group (n=69)		
	activity	resting	p	activity	resting	p
contraction amplitude	83.02± 38.60	82.68± 31.54	0.976	47.58[a)] ± 9.04	47.52 [a)] ± 11.57	0.998
duration of contraction	31.77± 4.56	31.44± 4.57	0.882	25.48 ± 2.21	24.35 ± 2.81	0.852
contraction area	6400± 3567	6238± 3235	0.820	3752 [a)] ± 1224	3515 [a)] ± 1425	0.751
maternal activity	1.66± 0.42	1.25± 0.44	0.005	1.67 ± 0.31	1.25 ± 0.49	0.005
contractions per hour	24.62 ± 11.94	9.09 ± 4.6	0.001	23.50± 10.29	7.38± 6.91	0.001
% duration of contraction from record	17.81 ± 10.0	6.18 ± 3.44	0.001	15.77± 11.12	5.75± 3.33	0.001
area index	29.11± 22.30	10.10± 9.55	0.001	28.24 ± 22.98	9.36 ± 8.87	0.001

a) $p<0.05$ short cervix group *vs* control groups

It seems the uterus responds to maternal activity. This is a newly demonstrated phenomenon and it must be confirmed by further studies, because the electrical activity of the abdominal muscles could be mistaken for uterine contractility. As for the timeline of the contractions, we found that between 10 am-11 pm there is more intense uterine activity for both control and short cervix group (tab. II) and this seems to be a physiological event.

There were previous studies to attempt to understand the uterine activity and to quantify the contraction frequency in asymptomatic women. Moore et al in 1994 provided contraction data in low risk pregnancies after 20 weeks of gestation and showed that there is a diurnal variation in uterine contractions but the majority of them occurred during the night (Moore, T.R., et al., 1994). We observed the opposite situations. This could be due to the fact that in our study women were monitored by electrohisterography and, in Moore's study, the contractility was recorded by tocography (the tocodynamometer is a strain gauge positioned over the uterine fundus, witch responds to changes in uterine tension transmitted to the abdominal wall; that's way, the device identifies the frequency of contractions, but not their intensity. It suffers misalignment following maternal movements).

The antepartum uterine activity in normal pregnancy is not known to date. Our study tried to identify, characterize and compare the electromyographic parameters in normal pregnancies and in short cervix pregnancies, with high risk of preterm birth. As far as we know, it is the first long term study (as the duration of each recording) using electromyography. 48.7% of the recordings lasted more than 20 hours. This way, by examining all the recordings, we can evaluate the electrical activity and the maternal activity for the entire 24 hours/day.

The uterine electrical activity was intense between 10 am – 11 pm and correlated with maternal activity; it was manifested through increased frequency and duration percentage of contractions reported to the total recording length, but without modifications in amplitude, duration and area of contractions.

During the active period (10 am- 11 pm), we can see that the uterus contracts more intensely than during the resting period (12 pm – 9 am), in all study groups.

In the short cervix group, we can see that the total number of contractions, frequency, contraction percentage as reported to the total recording length are much more important.

The cervical length is indirectly correlated to the mean contraction frequency, and the contraction percentage was significantly lower in the normal cervix group when compared to the short cervix group.

The mean number of contractions was 7, but we can observe a slight increase in the short cervix group (11.27±9.59 *vs* 9.80±7.05 contractions/hour).

Even though in normal pregnancies there are 2 peaks of contractions frequency, these are at about half of the maximum frequency of the short cervix patients.

The cervical length indirectly correlated with the mean frequency of contractions in the short cervix group.

The mean duration of contractions is around 31.5 for both groups. There are no significant differences between the studied groups, as well as for the two time periods.

The contraction percentage was significantly lower in the normal cervix group than in the short cervix group.

The amplitude of contractions is quite similar between the 2 groups, but more than 58% of the short cervix patients present a significantly high number of contractions/hour, of low amplitude, and more than 55% present great amplitude and duration of contractions.

In both groups, the correlation between the duration and mean contraction area was direct, of medium intensity, statistically significant. About 67% of the short cervix patients present high contraction areas.

Comparing the 2 time periods (active versus resting state), we can see there are significant differences in amplitude and contraction area; these are particularly high in the short cervix group, in the resting phase included.

Maternal activity between 10 am-11pm was more pronounced for both study groups, but without significant differences.
The number of contractions/hour, the contraction duration reported to the total recording length and the area index are significantly higher in both groups in active state when compared to resting state.
Electromiography has proven its efficacy and utility in obstetrical practice. But there are no standardized criteria which can be quantified and reproduced in the general population (Maner, W.L. & Garfield, R.E. 2007, Lucovnik, M., et al., 2012).

CONCLUSIONS

It seems the uterus is responsive to maternal activity. This is a newly highlighted phenomenon and it must be confirmed by other means, because it could be wrongly mistaken for the electrical activity of the abdominal muscles. We must not overlook the influence of the abdominal muscles, especially related to maternal physical activity, not the existence of a permanent uterine tonus, relatively regular, which could be physiological.
Therefore, it is very important to measure the cervical length during second trimester fetal morphology evaluation. We can identify a short cervix, which is a risk factor for premature birth, and recommend the proper treatment. Most of subjects were recruited during fetal second trimester screening.
The Monica Holter Fetal AN24 device has several advantages; it allows long recordings and storing data for further analysis, the signal quality is not influenced by the maternal BMI, fetal position or movements. It is well tolerated because it does not limit the patients daily activities, it does not need strapping for fixation, nor bed rest.
Premature birth screening remains an open discussion, in order to better identify risk factors (uterine contractility, cervical modification, infection), through clinical, biochemical and imagistic methods. Further studies should focus on implementing already known methods, like electrohysterography, and on understanding and interpreting recorded data so that these can be used better and more frequently in our daily practice.

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