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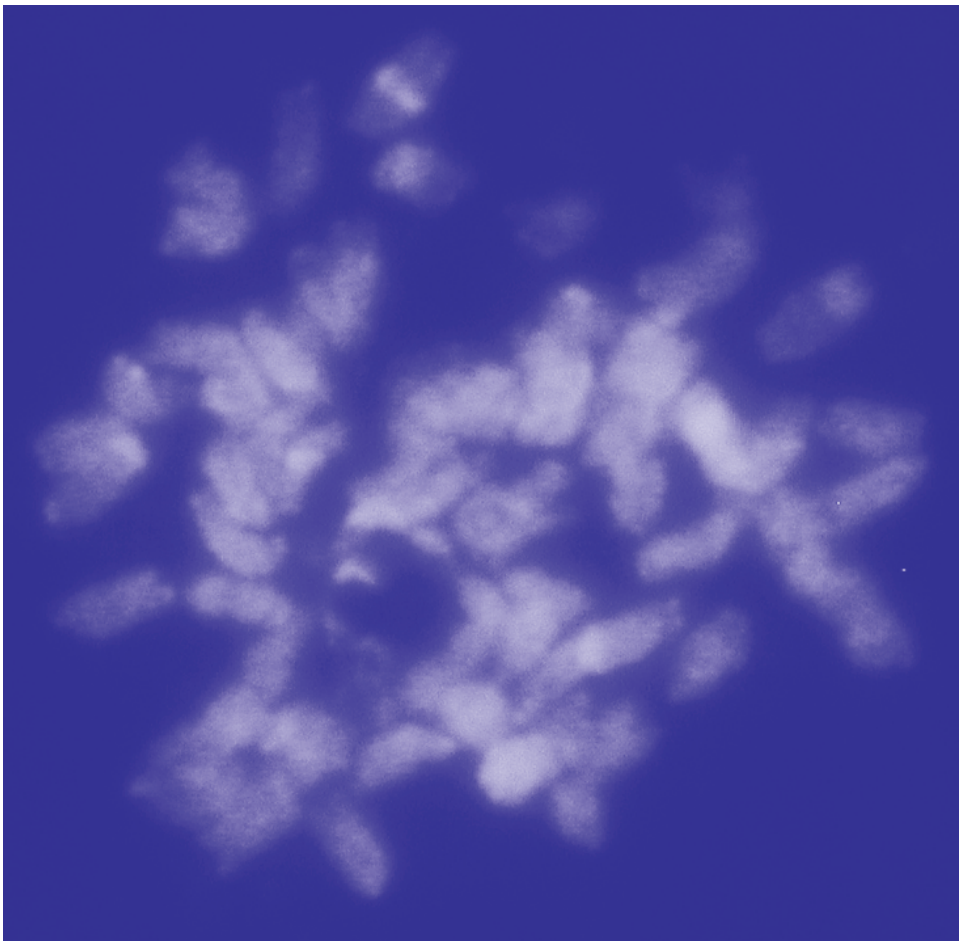

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All authors are responsible for submitting manuscripts in comprehensible US or UK English and ensuring scientific accuracy.

Original pictures on front cover: DAPI staining of the chromosomes of a somatic hybrid between potato and the wild species *Solanum chacoense* (x 2000)

THIS ISSUE IS DEDICATED TO THE CONFERENCE:

"Jubilean and celebratory session –
40 years of plant cells and tissue cultures in Romania
and 40 years of research activity and management
of the Romanian Association of Plant Cells and Tissues
of Professor Dorina Cachiță-Cosma, Ph.D."

19th of March 2016, Cluj-Napoca, Romania

ORGANIZED BY:

The "Babeș-Bolyai" University (U.B.B.),
Faculty of Biology and Geology, Plant Genetic Engineering Group

The Romanian Association of Plant Cells and Tissues (ARCTV)

Institute of Biological Research, Cluj-Napoca (I.C.B.)

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PROGRAM OF THE CONFERENCE:

"Jubilean and celebratory session – 40 years of plant cells and tissue cultures in Romania and 40 years of research activity and management of the Romanian Association of Plant Cells and Tissues of Professor Dorina Cachiță-Cosma, Ph.D."

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Message from the UBB management team – prorector Bálint Markó
Message from the organizers

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 11-12)

=== ORAL PRESENTATION ABSTRACTS ===

Forty Years from the Beginning of the Research in the Field of Plant Tissue and Cell Culture in Romania

Dorina Cachiță-Cosma[1,✉], Aurel Ardelean[1]
and Constantin Crăciun[2]

At the beginning of twenties century, based on the discoveries in cell biology in the previous decades, the German botanist Haberlandt (1902) has founded the concept of cell totipotentiality. The progress registered in biology between the two World Wars has led to the development of a new research field in plant physiology, based on nutrition, biochemistry and cytology that is the culture of plant tissue and cells in vitro. After the Second World War the practical applications of those new discoveries has allowed the development, after 1954, of a series of bio-industries at large scale, as clonal micropropagation of ornamentals and economically important crops, as well as the use of biomass in pharmaceutical industry as a source of secondary metabolites.

This new field has raised the interest and enthusiasm of the researchers in Romania as well. In 1975 three researchers from Romania being specialized abroad, i.e. Cachiţă D., Coman T and Brezeanu A., have learned the in vitro culture technology. Coming back to Romania those researchers have each developed in their home institutes new laboratories for plant in vitro culture. As a result of their research abroad, Cachiţă and Brezeanu did published four papers in European journals (France, Belgium and Germany) as well as one publication in USA.

Starting from 1989 annual symposia was organized in our country, the first being organized by the Biological Research Center and Institute of Agronomy, both from Cluj-Napoca. All presentations were published in the first volume of this symposium. 19th such symposia were organized during the following years but

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only 12 volumes have been successfully published. Prof. Ardelean A., the previous rector of „V. Goldiş" West University Arad, has greatly contributed to the publication of 9 out of 12 volumes, those 9 representing the papers presented at 10 meetings at national level, some of them with international contributors.

We would like also to mention the contribution of Prof. Crăciun Constantin from Babeş-Bolyai University Cluj-Napoca, as an editor of two of the issues of the conferences, as well as its collaboration to ultrastructural studies of in vitrophytoinocula. It is also to be mentioned that in 1990 The Romanian Association of Plant Tissue and Cell Culture has been grounded, organization which has been involved in the organization of all national meetings in plant biotechnology as mentioned before. From 1975 our country was affiliated at The International Association for Plant Tissue Culture (IAPTC), many Romanian researchers participating to the international conferences and congresses taking place around the globe.

=== ORAL PRESENTATION ABSTRACTS ===

Plant Stem Cells – Theoretical and Practical Studies

Dorina Cachiță-Cosma [1,✉] and Constantin Crăciun [2]

In modern biology the theoretical studies on plant cell totipotency has led first to the birth of plant biotechnology and second, more recently to meristemotherapy or gemmotherapy, which interconnects with animal or human cell stem therapies, a very recent field in modern medicine.

In 1994 the Italian author Fernando Piterá, has published „Compendio di gemmoterapiaclinica (Meristemoterapia)", where it is first mentioned the term of meristemotherapy as the use of germs, buds and very young shoots as a treatment because they contain meristems the cells maintained by the plants for their continuous growth.

At global level, but in our country as well, meristemotherapy based on plant stem cells or meristematic cells made up the medical treatments or modern cosmetics, treatments that evolve fast as it does the therapeutic use of animal stem cells.

In the literature there are no data on the use of in vitro plant cells or meristems as resources for meristemotherapy, although the plant biomass which can be obtain in bioreactors in vitro might be a very good source for obtaining fitotherapeutic products. Moreover, if such techniques will be applied the efficiency and profitability might be greatly improved, the production could be extended all year long, the technological flux becoming independent of the season in the temperate zones.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 15-17)

=== ORAL PRESENTATION ABSTRACTS ===

Theoretical and Biotechnological Approaches in the Institute of Biology Bucharest between 1975 - 2015 Based on Plant Cell and Tissue Culture Technology

Aurelia Brezeanu [1,✉] and Gina Cogălniceanu [1]

The year 1975 marked the beginning of research on plant biotechnology at the Institute of Biology Bucharest by the study of in vitro culture of plant cells. The team of researchers was coordinated by Dr. Aurelia BREZEANU within the Laboratory of Plant Morphogenesis and Genetic Engineering.

During the first stage (1975-1983) several basic research topics were studied, that contributed to a better understanding of the biology of the plant development:

- Plant in vitro cytodifferentiation and morphogenesis;
- Apoptosis and senescence in plants, using crown gall tissues infected by *Agrobacterium tumefaciens*;
- The role of endo- and exogenous factors in expressing the androgenetic and/or gynogenetic potential thus regenerating haploid plants used in programs of genetic improvement;
- Protoplasts technology, a pioneer activity in Romania. The studies were focused on microbial protoplasts (yeasts, bacteria) and plant protoplasts and included isolation of protoplasts, their chemical or electric fusion, plant cell regeneration, used in experiments of parasexual interspecific plant hybridization.
- Important results were obtained in the field of microbial genetic engineering and plant transgenesis.

The 2nd stage (1983-1989) was more focused on biotechnological applications using plant cell and tissue cultures. A complex network of scientific cooperation was established with research institutes, experimental stations and universities. Important results were obtained regarding: clonal multiplication, production of virus-free plants, production of haploid plants, somaclonal variability in in vitro cultures, and in vitro stress selection.

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The 3rd stage (1990-1998) was mostly devoted to fundamental research on topics including: gene transfer in plant cells mediated by bacterial plasmid vectors using direct (electroporation and electrotransfection) or indirect methods (co-cultivation), electrostimulation in in vitro systems of cytodifferentiation and morphogenesis by using weak electric fields, the effect of hipersalinity and high aluminum concentration on plant genome, study of cell and molecular processes in plant development using in vitro systems, the study of chimaera gene expression involved in pollinic and somatic embryogenesis in in vitro plant cultures.

The 4th stage (1998-2005) continued fundamental research topics, in the international context focused on the phytohormonal control of plant development. Our laboratory initiated studies on several factors that have a potential role as signals (aliphatic polyamines, jasmonates, external electric current) and on biochemical markers involved in the morphogenetic processes in experimental in vitro systems, both in test and recalcitrant plant species. The mechanisms that induce stress tolerance were studied by simulating in in vitro conditions using stress inductors (PEG) and specific mediators (ABA, AS), thus isolating cell line and/or tolerant individuals. Another major research direction was the potential utility of in vitro cultures to proliferate and biosynthesis of secondary metabolites of biotechnological interest. Thus, the study of Vitis vinifera callus resulted in the isolation of a long-term cell line highly proliferative and with a high production of compounds valued by the pharmaceutical industry (anthocyanins, pycnogenol and resveratrol).

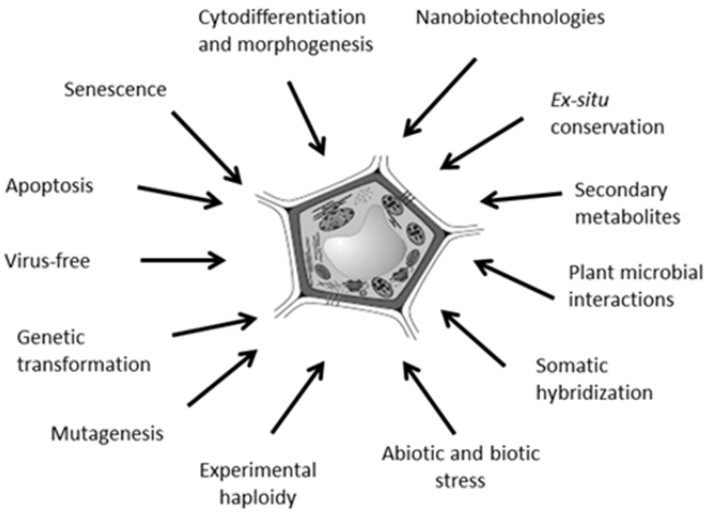

Figure 1. Schematic diagram of the main theoretical and biotechnological research topics at the Institute of Biology Bucharest, during the period 1975-2015 based on plant tissue and cell culture technologies.

The present stage includes several directions of research. One is focused on conservation, by characterizing the in vitro development of protected plant species of Romania for their ex situ conservation. Our studies have established a cell and tissue bank, including a large variety of taxa like bryophyte, lichens, ferns and a large number of vascular plants, from many families.

Plant-microbe interactions, plant in vitro systems for secondary metabolites biosynthesis and plant in vitro systems involved in bio-nano-technologies are our ongoing research topics.

Our laboratory has published over time over 15 books and book chapter and about 1000 scientific papers, both at national and international level. Under the scientific supervision of Dr. Aurelia BREZEANU over 40 doctoral theses in the field of cell biology and plant biotechnologies were finalized. The scientific recognition of the results is also visible in the medals and prizes received.

The results briefly presented in this paper highlight only some of the aspects of the research topics in the Institute of Biology Bucharest in the fascinating field of plant cell and tissue culture over the 40 years of existence.

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 19-20)

=== ORAL PRESENTATION ABSTRACTS ===

The Use of Plant *In Vitro* Systems for the Synthesis of Noble Metal Nanoparticles

Gina Cogălniceanu [1,✉], Monica Mitoi[1], Alexandru Brinzan[1], Gabriela Hristea[2] and Magdalena Lungu[2]

Nanomaterial synthesis using biological organisms is a new research area, defined as nanobiotechnology. In order to prospect and develop green synthesis methods for noble metal nanoparticles (NM-NPs) extracellular and intracellular synthesis using bacteria, fungi, yeast or plants are extensively explored. Noble metal (Au, Ag, Pt, Rh, Pd) nanoparticles are the subject of intensive research, involving synthesis, characterization and applications. Due to their chemical, physical and optical properties, metal nanoparticles are very atractive for a wide range of biomedical applications, such as molecular detection and diagnostics, antibacterial activity, transport of drugs, cancer therapy. Biosynthetic methods using plant extracts proved to be simple and viable alternatives to conventional methods and suitable for developing large scale production. Biotechnological methods of NM-NPs synthesis using plant extracts are advantageous compared to the physico-chemical procedures: i) the synthesis does not require toxic solvents or additives; ii) does not result in toxic wastes for human and the environment; iii) the reactions are not energy dispersive, are relatively fast and in a single phase.

Plant in vitro systems have unexploited advantages for NPs production due to the fact that they are pathogen-free, are independent of seasonal and meteorological variations, are able to produce high amount of cellular mass all year long, offer the possibility to modulate the content of metabolites involved in NPs synthesis using elicitors and/or precursors.

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The aim of this research was to develop a fast, easy, efficient and environmentally friendly methodology of NM-NPs biosynthesis with potential applications in medical, cosmetic, industrial, agriculture field. We used as reducing and stabilizing agent in NM-NPs biosynthesis crude extract of strawberry callus cultures. The NM-NPs obtained were characterized by UV-Vis spectroscopy, TEM, SEM, Zeta Potential Analizer. The NM-NPs size, morphology and stability recommend them for targeted further applications.

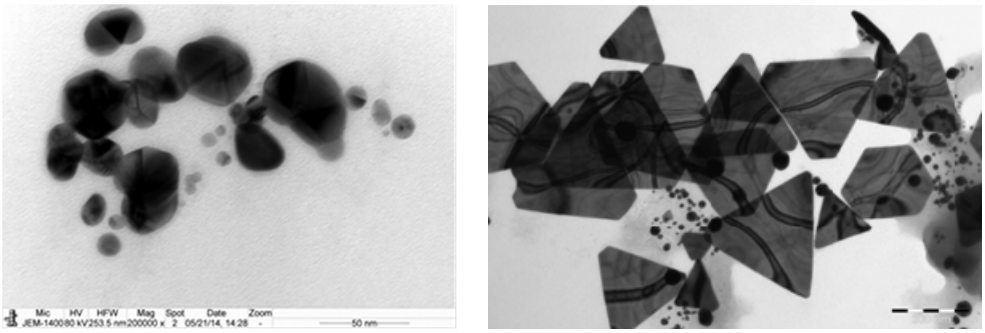

Figure 1. Nano-silver (left) and nano-gold (right) synthesized by crude extract of strawberry callus cultures.

=== ORAL PRESENTATION ABSTRACTS ===

The Application of Combinatorial Biotechnology in Improving Potato Resistance to Biotic and Abiotic Stress

Elena Rakosy-Tican [1,✉], Ramona Thieme[2], Adriana Aurori[1], Imola Erdelyi-Molnár[1], Enikő Besenyei[1], Raluca Alina Mustață[1], Antonia-Maria Mărgineanu[1] and Daniel Cruceriu[1]

The concept of combinatorial biotechnology was introduced in previous presentations to international conferences in order to emphasize the importance to combine many tools of biotechnology, including phenomics, genomics and metabolomics, for the final goal to improve plant resistance to biotic and abiotic stress. This concept is exemplified here by a few examples in improving potato crop, one of the most important security crops worldwide and the third important crop as productivity at global scale. This crop is amenable for such improvement for some reasons: it responds well to tissue culture, somatic hybridization and transformation *in vitro*, its genome was sequenced and has got a rich resource of wild resistant relatives in the center of origin of potato crop. Moreover, potato is one of the crops facing great loses because of many diseases and pests, some of them causing total loss of production. The case studies presented in our work involve the use of sexually incompatible *Solanum bulbocastanum* and sexually compatible *S. chacoense* species as resources of multiple resistance genes, such as resistance to late blight caused by blight potato famine agent *Phytophthora infestans*, Colorado potato beetle and the abiotic stress caused by drought. Another example is genetic transformation with a marker free hair pin construct for PVY resistance combine with stress selection *in vitro* for tolerance to draught.

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=== ORAL PRESENTATION ABSTRACTS ===

Cryopreservation of Ornamental Plants

Adela Halmagyi [1,✉]

The commercial production of ornamental species and varieties is constantly growing and becoming an important part of the economy. New cultivars with horticultural and economic value are produced every year replacing the existing assortments. Over the years several cryopreservation methods have been performed as the only viable procedures for the long-term conservation of vegetatively propagated plant genetic resources. Despite of the growing number of ornamental species cryopreserved in various countries, a large-scale routine application of cryopreservation for long-term germplasm storage in genebanks is still limited. Different cryopreservation methods have been implemented for some vegetatively propagated ornamental species like chrysanthemum (*Chrysanthemum morifolium* L.), carnation (*Dianthus caryophyllus* L.) and roses (*Rosa* x *hybrida*). Chrysanthemum shoot tips were cryopreserved by controlled-rate freezing (highest regrowth after preculture in 0.5 M sucrose and 0.25°C/min cooling rate), encapsulation-dehydration (regeneration after preculture in 0.5 M or 0.75 M sucrose and 4-5 h dehydration in laminar flow cabinet), DMSO-droplet freezing (maximum shoot regrowth after 2 h incubation in 7% DMSO) and droplet-vitrification (highest regrowth after 5 min dehydration in PVS2 100% or 15 min dehydration in PVS2 60%). Droplet-vitrification of carnation and rose cultivars lead to high regeneration after 20-25 min PVS2 dehydration (according to cultivar) while cryopreservation by encapsulation-vitrification of carnation shoot tips after 24 h sucrose (0.5 M) preculture and dehydration at 0°C was the most effective in achieving high rates of regeneration. Although, many cryopreservation studies have been developed to minimize freezing damage and to enhance regrowth, the genetic stability of conserved material still remains a major concern.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 25-26)

=== ORAL PRESENTATION ABSTRACTS ===

Analysis of Genetic Stability of *in Vitro* Plants by Molecular Markers

Anca Butiuc-Keul [1,✉] and Victoria Cristea [2]

Conservation of endangered, rare, vulnerable or endemic plants became of great interest in the last years in order to preserve the natural resources. The most used protocols for plant conservation is *ex situ* conservation in botanical garden collections, or *in vitro* conservation by tissue culture. Both protocols require the previous analysis of the genetic variability in the populations because it is well known that endangered or endemic plants exhibit low level of genetic variation in their population (Hamrick *et al*., 1991). There are several examples of endangered, rare or endemic species with large populations, but individuals are genetically similar or identical. The low genetic diversity variation is due to clonal multiplication or bottlenecks. Thus, knowing the genetic structure and variability is useful before the development of proper conservation strategies. After conservation, the genetic stability of plants should be also investigated, because the success of any conservation method is to preserve populations but the genetic structure and variability as well. Molecular markers as SSR and ISSR are valuable tools for estimation the genetic variability in populations and genetic stability after conservation as well (Varshney *et al.,* 2005).

Several endangered species as *Dianthus giganteus* D'Urv. subsp. *banaticus* (Heuff.) Tutin, endemic for the South-West Carpathians and vulnerable in Romania and *D. spiculifolius* Schur, endemic for Eastern Carpathians and vulnerable in Romania were proposed for *in vitro* conservation. Genetic structure and variability of natural populations was investigated by molecular markers as SSR and ISSR

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previous to *in vitro* culture. After different periods of *in vitro* culture the genetic stability of plants was studied using the same molecular markers and the same PCR condition and programs.

Molecular analysis by SSR and ISSR markers of individuals belonging to natural habitats and *in vitro* plants showed that the genetic differences between somaclones derived from the same individual are low in both species. These differences could be explained by the specific culture conditions. The genetic variability of plants from natural habitats was conserved by *in vitro* culture. Thus, *in vitro* plants could be used for outdoor collection, and for replanting in natural habitats, if necessary.

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R E F E R E N C E S

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 27-28)

=== ORAL PRESENTATION ABSTRACTS ===

Significant Stages in the Evolution of Plant Biotechnology Research in Romania

Camelia Sand Sava [1,✉] and Maria-Mihaela Antofie[1]

Biotechnology is a horizontal domain spread today in almost all others sciences. Even from the beginning of our civilization, humankind accessed biotechnology based on traditional knowledge bringing up today old wine beer or yogurt technologies the major steps in the evolution of this domain took place in the last century. Accessing life either as cells, organisms or part of them or even products of living organisms was part of the classical biotechnology. Today, accessing modelling in living forms and transferring them in new products and services may create nano-technologies. Changing the DNA of the cells will ground modern biotechnology with all implications in our daily life. Romania, is positioned in Central - East Europe and due to political climate it was not an important part in the green revolution that starts late in 1960 in Pakistan. Still, we mention that Andronescu was the first agronomist working in the USA and developing in 1928 the first collection of maize germ plasm following the model already started all over the world after the publication in 1916 of Nikolai Vavilov regarding the theory of centres of origin of species. Over 3000 accession have been developed in the Institute for Agronomy Bucharest being the very first catalyst for maize breeding for our country. Still, due to the Second World War it was almost everything lost and starting later a new strategy for research in the domain. However, the new scientific community lost their connection at the global level and creates delays in further developing these domains. Thus, if during these very years tremendous work have been done in the plant cell culture, only after other 30 years starts (during 1980) starts in Romania the study of plant biotechnology, and reconnecting the scientific world to the global

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level. A tremendous contribution to this recovery process is due to prof. Dorina Cachiță Cosma from University of Cluj and other collaborators from Bucharest. Only in 20 years the evolution of plant biotechnology was really important in our country, scientists reconnecting themselvs to the world. Protoplast cultivation, plant cells cultivation from many species, micropropagation, developing germ plasm collection of th country, the breeding of new hybrids or cultivars in all species of food security interes was only part of this process. We ay mention here succesful results in plant cell physiology, electron-microscopy, electroporation and other new technology all together connected for grounding the esessence of biotechnology in Romania. Also, during 1970 it was registered the first transformations in bacteria and later on during 1990 the first genetically modified plants based on in vitro culture and using bacterial vectors. Since 2000 in Romania nano-technologies are occupying an impressive range in research projects with different implications. We are optimistic that the scientific research in Romania will continue to evolve and to become part of the world culture heritage.

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 29-30)

=== ORAL PRESENTATION ABSTRACTS ===

Physiological Effects of the *in Vitro* Culture on Explants Belonging to *Solanum* Genus

Daniel Cruceriu [1], Imola Erdely-Molnar[1], Zorita Diaconeasa[2], Adriana Aurori[1], Carmen Socaciu[2] and Elena Rakosy-Tican[1, ✉]

A large variety of secondary metabolites are responsible for specific defense mechanisms against stress factors in plant tissues. Among them, phenols and thus flavonoids play an important role, due to their strong antioxidant activity and the fact that their quantity is rapidly increasing if a specific stressful factor appears in the environment. In this context, we evaluated the physiological effects of the *in vitro* culture on explants, by quantifying the total polyphenolic content (TPC), the total flavonoid content (TFC) and the antioxidant activity (AA), both under *in vitro* and *ex vitro* conditions. Our experiments were performed on 2 closely related species of potato: the wild species *S. bulbocatanum* and the potato commercial cultivar *S. tuberosum* 'Rasant' and on two somatic hybrids between them, obtained through protoplast electrofusion. The Folin-Ciocalteu method, the aluminum chloride colorimetric method and the ABTS assay were used to determine the TPC, TFC and AA respectively. Our results prove that both TPC and AA are increasing under optimized *in vitro* conditions, but TFC is lower under the same set of conditions. Therefore, we can state that the *in vitro* conditions represent a stressful factor for the plantlets based on the TPC and AA quantification, but also that flavonoids are a special part of the phenolic compounds, taking into consideration

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their opposite behavior. This divergence might be explained by corroborating 2 different facts: 1) flavonoids are primarily involved in photo-protection against UV solar radiation and 2) UV-B radiation, the major trigger in flavonoid biosynthesis was absent in the growing room, but present under *ex vitro* normal day light.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 31-32)

=== ORAL PRESENTATION ABSTRACTS ===

In Vitro Multiplication of Romanian Endemic and Rare Species *Lychnis Nivalis* Kit

Victoria Cristea [1,✉], Ana Coste[2], Adela Halmagyi[2], Irina Holobiuc[3], Anca Butiuc-Keul[4], Oana Roșca-Casian[1] and Liliana Jarda[5]

Currently, the biodiversity conservation and sustainable development are a major challenge of human society, as it is stipulated in numerous documents: The European Union's Habitat Directive, 1992, CBD Rio de Janeiro, 1992, Global Strategy for Plant Conservation (GSPC) - Nagoya Protocol, 2010, etc. As specified in the GSPC document, the long term conservation, management and re-establishment of plant diversity together with their habitats and ecosystems must be done both *in situ* – through the establishment and/or conservation of species in their natural ecosystems, with minimal management, as well as *ex situ.*

Our studies in the field of *ex situ* conservation through *in vitro* cultures of endemic/endangered plant taxa are focused on species mentioned by Sârbu *et al*. (2007) and in Red Book of Vascular Plants of Romania (Dihoru and Negrean, 2009). *In vitro* conservation of these taxa is based on *in vitro* cultures initiation, micropropagation, medium term preservation and cryopreservation, as well as the study of somaclonal variability, as a consequence of preservation by *in vitro* collections. The already studied and in vitro conserved taxa are: *Dianthus callizonus* Schott & Kotschy, *D. giganteus* D'Urv. ssp. *banaticus* (Heuff.) Tutin, *D. glacialis*

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Haenke ssp. *gelidus* (Schott, Nyman & Kotschy) Tutin*, D. henteri* Heuff. ex Griseb. & Schenk, *D. pratensis* M. Bieb. ssp. *racovitzae* (Prodan) Tutin, *D. trifasciculatus* Kit. in Schultes ssp. *parviflorus* Stoj. et Acht., *D. spiculifolius* Schur, D. *tenuifolius* Schur, and *D. nardiformis* Janka (Cristea *et al*., 2014, Holobiuc *et al.,* 2009 etc.).

The following species *Lychnis nivalis* Kit., *Moehringia jankae* Griseb. ex Janka and *Silene dinarica* Spreng. are *in vitro* currently studied. *Lychnis nivalis* Kit. is endemic for Romania in Rodnei Mountains, considered rare or vulnerable in Romanian Red Lists. In case of *L. nivalis*, *in vitro* culture was initiated from seeds coming from 2 different locations, near Iezerul Lake and near Stiol Lake. $HgCl_2$, H_2O_2 and NaOCl were used as disinfectant agents. The seeds sterilization rate was over 99% and the germination rate, after 83 days, was 60 %. There was studied the influence of 5 cytokinins on the *in vitro* multiplication rate: 6-benzyladenine (BAP), zeatin (Z), 2-isopentenyladenine (2iP), thidiazuron (TDZ) and meta-topolin (mT), each in 2 concentrations – 0.5 and 1 mg/l. The highest multiplication rate was obtained in case of BAP and TDZ, 30 and respectively 28 generated neoplants/inoculum, after 62 days from inoculation.

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=== ORAL PRESENTATION ABSTRACTS ===

In Vitro Multiplication and Conservation of *Euphorbia Canariensis* L.

Adriana Petruș-Vancea[1,✉] and Andrei Fordon[1]

The effect of deuterium depleted water (DDW) (25 ppm D) was studied on the second culture of *Euphorbia canariensis* L., to transfer *ex vitro*. At 18 months of *in vitro* culture, on medium which the distilled water (155 ppm D) was replaced with deuterium depleted water (25 ppm D) are found to the root system, plants are capable to transfer into to the soil, compared to control lot, rootedness, with acclimatization unfit plants. The chlorophyll and carotenoids pigments were increased in presence of DDW. At the end of the acclimatization, *ex vitro* survival rate was raised from 92% to plantlets provided from medium prepared with DDW.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 35-36)

=== ORAL PRESENTATION ABSTRACTS ===

In vitro Culture for *ex situ* Conservation of the Vulnerable Species *Moehringia Janke* Griseb ex Janka

Irina Holobiuc[1,✉], Rodica Cătană[1], Carmen Maximilian[1], Paulina Anastasiu[2] and Victoria Cristea [3]

Moehringia jankae **Griseb.** ***Ex*** **Janka** is an European endemite in the West area of Black Sea Region, being a Dobrogean element which can be found in the East side of Romania (in Hârşova, Moşu Hill, Macin National Park, between Greci and Macin, Consul Hill Reservation, Canaralele Hârşova, Topolog, Tuşan-Măgurele Hill, Cheia Jurassic Recife, Călugareni, Colţanii Mari Rocks) and also in Bulgaria.

The purpose of our study was the evaluation of the natural populations, the identification of new populations in the distribution area and the elaboration of the *ex situ* preservation methodology based on biotechnological approach. Despite plant preservation is made usually in situ in the natural habitats, additionally *ex situ* measures can be useful in different situations, especially for vulnerable taxa, with reduced populations, with limited areas or problems with reproduction or seeds germination. *In vitro* collection establishment as *ex situ* preservation method is based on the elaboration of short and medium term cultures protocols, further long-term procedures can be also applied.

Basic requirements for an *in vitro* storage system are: to maintain genetic stability, to ensure long term storage without loss of viability and to save money, labor and energy. The plants resulted from different period of *ex vitro* maintenance can be used for repopulation programs of the natural habitats or for culture in botanical gardens.

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In the case of *M. jankae*, the initiation of aseptic *in vitro* cultures was started from seeds collected from the plants from Cheia Jurassic Recife. For the endangered plants species, the seeds germination represents an important biological aspect which ensures *in situ* survival of the species and *ex situ* approaches.

Because in this taxon the seeds had a high contamination rate, different methods of sterilization was tested using single or combinations of treatments. The best results were registered in the case of mercuric derivate, despites the sterilization rate was quite low (max. 22 %); meanwhile the germination rate of sterilized seeds was reduced at 14% registered after their vernalization 60 days at 4^{o}C (in the case of the use of thimerosal as sterilising agent).

The plant regeneration from the culture initiated from explants originated from seedlings was evaluated after 4 and 8 weeks taking into account: the % of viable explants; the mean number of nodes/regenerants, the mean length of the tallest regenerated shoot, the rhyzogenesis.

The *in vitro* response of *M. jankae* was good, morphogenesis occurred at good rates on all media tested (on MS supplemented with BAP 1 mg/l+ 0,1 mg/l AIB, MS + zeatine + 0,1mg/l AIB, or adenine sulphate 50 mg/l and 0,5 mg/l AIB and thidiazuron 0,05 mg/l+ IBA 0,01 mg/l).

The acclimatization of regenerated plants was tested on solid, liquid and semisolid substrates. The short term multiplication technology further ensures material for medium and long term preservation, meanwhile producing plants for outdoor collections.

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=== ORAL PRESENTATION ABSTRACTS ===

Buds

Scientific Documentary Film, 1985

Buds - is a documentary film produced by Sahia Film Studios in 1985, with the director Mircea POPESCU and scientific consultant Dr. Aurelia BREZEANU. The film took part a year later in the Scientific International Film Festival in Argentina, winning the highest award, respective the prize *Fritzphil*.

The film is a captivating documentary about the beginnings, the performances and prospects of a new field of scientific research in Romania: Plant Biotechnology. Focusing on the research team coordinated by Dr. Aurelia BREZEANU, whose activity was held in ICEBIOL (currently the Institute of Biology Bucharest of the Romanian Academy), the film presents theoretical concepts in an accessible manner, illustrated with vibrant, sensitive and memorable images. The totipotency of the plant cell, plant genome plasticity, the regeneration of the individuals from tiny and specific tissue fragments of somatic origins, somatic hybridization and embryogenesis are some scientific concepts accurately described in the documentary film that underlie the development of many biotechnological applications. The film was praised in numerous occasions, a detailed review about it being made by Cici Iordache - Abraham in the number 321/1986 in the *Tribuna Romaniei* journal. The author appreciated the scientific performances of the team of researchers coordinated by Dr. Aurelia BREZEANU, their work being developed at the border between fundamental and applied research. Among the results highlighted by the journalist are included: vines free of viruses, highly productive garlic varieties that are disease and weatherproof resistant, seedless watermelon, two new varieties of tobacco, improved varieties of *Datura innoxia* and *Digitalis lanata*. Several aspects were also highlighted as very important: studying the mechanisms of cell control in *in vitro* cultures through macro elements (calcium, magnesium, sodium, potassium), or genetic determinism of plant regeneration from microspores or from somatic cells.

Even after so many years, the film impresses with its freshness and accuracy, describing the first steps of the Romanian research in a field of avant-garde of the time, with results and performances appreciated by the entire scientific community.

=== POSTER ABSTRACTS ===

In Vitro Organogenesis of *Amaranthus Retroflexus* L. with Biotechnological Applications in Glycerol Degradation

Cristian Banciu [1,✉], Anca Manole[1] and Gabriel-Mihai Maria[1]

Glycerol is a secondary product from biodiesel production that is transformed in other useful compounds for polymer industry. The two enzymes that are involved in this process are lipase and decarboxylase.

Our experiments used a very robust plant species (*Amaranthus retroflexus*), that grows on different types of substrate and in a variety of stress conditions (drought, frost, flooding, etc.) to test its survival on culture media with carbon source replaced by glycerol. After aseptic seed germination, plantlets were grown on a calus induction culture medium Gamborg (MS basal medium enriched with NAA and Kinetin). The non-morphogenetic calus obtained was cultivated on four variants with ascending the quantity of glycerol replacing the sucrose as a carbon source.

The survival and the growing rate of the caluses are factors that induce the conclusion of succesful degradation of glycerol by *A. retroflexus*. Further biochemical analyses will reveal the biodegradation pathways and the secondary compounds production.

This could be a viable soution for the polymer industry that uses polyglycerol (one of the sub-products). It offers a solution for consuming the big quatitites of produced glycerol that afects the waste waters and the natural habitats. It could be a challenge for plant biotechnologies to help the industry having a positive impact to the environment.

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=== POSTER ABSTRACTS ===

Morphological, Physiological and Biochemical Investigations of the *in Vitro* Plants of Sedum *Telephium* Ssp.*Maximum* L.

Mirela Ardelean[1,✉]

As the „*in vitro*" cultivation of species of *Sedum* is concerned, the specialty literature does not have scientific papers regarding the gathering of this type of material that could be exploited as a source of active principles. We highlighted the presence of these anthocyanins only at the level of plant inoculum of *Sedum telephium* ssp. *maximum* L., as this interesting phenomenon was not observed in the case of the organs belonging to plants cultivated in natural conditions. The presence of anthocyanins in the root apexes is a novelty in the specialty literature. While analyzing the results regarding the reaction of plant inoculum of *Sedum telephium* ssp. *maximum* L. produced by the propagules cultivated in the medium of *Murashige - Skoog* (1962) (MB - MS) with an input of different growth regulators, which stood at the basis of the experiments carried out within the monitorisation of the reactions of the explants of *Sedum telephium* ssp. *maximum* L., at the presence in the cultivation sub-layer that we modified, we have noticed -at the root apexes of the plants cultivated in MB – MS mediums with input of different growth regulators, using 1.5 mg/l from each of them-, a raspberry-redcoloration of the apexes of the small roots regenerated at the level of different types of explants, either propagules, or callus regenerated from the propagules. These particular reactions observed at the level of plant inoculum of *Sedum telephium* ssp. *maximum* L. determined us to lead our investigations towards the examination of these cells with an optical microscope. After the examination with an optical microscope of the root apexes of the „*in vitro*" plants of *Sedum telephium* ssp. *maximum* L. or of the small roots regenerated at the

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level of callus, we inferred the fact that, both the calyptra cells and the meristem (situated under the apex) have been coloured in red, especially in the case of the plant inoculum cultivated in the MB – MS medium, with a supplement of the mixture KIN and ANA (1.5mg/l from each of them).

Sedum telephium ssp. *maximum* L. calluses grown on *Murashige-Skoog* (1962) growth media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BAP) showed a red coloration of the vacuolar content of some of their cells. It was determined that this phenomenon is due to anthocyanins accumulation under the influence of the growth regulators. Using HPLC analysis, it was found that the highest overall anthocyanins concentration was in calluses grown on medium supplemented with 1.5 mg/l 2,4-D + 2.5 mg/l BAP. Furthermore, the type of the growth regulators (cytokinines or auxines) added individually or as a mixture (in different ratios) in the growth medium, can influence the callus growth rate and what type of anthocyanins is produced preferentially. Also, to the best of our knowledge, this is the first time that the presence of cyanidin 3-glucoside in *Sedum telephium* ssp. *maximum* L. calluses, was reported.

=== POSTER ABSTRACTS ===

Micromorphological Characteristics of Stenoendemic Plant Species *Andryala Levitomentosa* (E.I.Nyarady) P.D. Sell.

Gabriel Mihai Maria [1], Anca Manole[1] and Cristian Banciu[1,✉]

Andryala levitomentosa is a stenoendemic plant species, with a very restricted distribution in an area of about 150 square meters on rocky slopes of the Pietrosul Bistriţei Mountain (Eastern Carpathians, Romania). Due to the narrow distribution and small population size with such a low number of individuals, this species is included in the list of strictly protected plant species (Appendix I) of the Bern Convention, in the Carpathian List of Endangered Species, in the list of the top 50 Threatened Species of the European Flora in need of urgent conservation measures, and in European Red List of Vascular Plants. It has also been included in all the national red lists and books of endangered plant species issued after the species was discovered. This species is still subject of controversies regarding the taxonomic position, despite its discovery more than fifty years ago. Taking into account that micromorphological characteristics are of taxonomic importance this study describes some peculiarities of epidermis of aerial organs including, hairs, stomata, epidermal cells and cuticle.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 45-46)

=== POSTER ABSTRACTS ===

Antibiosis and Antixenosis Properties of the Somatic Hybrids and Backcross Progenies *S. Chacoense* + *S. Tuberosum* Against Colorado Potato Beetle

Imola Erdelyi-Molnár [1], Enikő Besenyei[1] and Elena Rakosy-Tican[1, ✉]

Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is a leading pest of solanaceous plants, which include crop plants like potato, tomato and eggplant. Nowadays CPB spreads throughout Europe, North America, Asia, some parts of North Africa, and the temperate Southern Hemisphere. This pest develops from the egg stage to adult beetles on the host plant, and during this time they can severely damage, even completely defoliate the plant.

CPB is resistant to more than 50 insecticides worldwide and also to Bt pathotoxins.

An alternative method to control CPB could be the use of resistant wild *Solanum* species in potato breeding programs. One of the most effective sources of host-resistance mechanisms to CBP is the natural resistance of the wild species, *Solanum chacoense*. Resistance to CBP is associated with the expression of rare glycoalkaloids, the leptines, which are only produced in aerial tissues.

Somatic hybridization *via* protoplast electrofusion was used to obtain hybrid plants between *S. tuberosum* cv. Delikat and Désirée and *S. chacoense* HL (PI 458310 from the NPGS Sturgeon Bay, USA), respectively *S. chacoense* 1 G (Gross Lusewitz Genebank, Germania). Somatic hybrids with MMR deficiency were also obtained by protoplast electrofusion of cultivated potato and mutant *S. chacoense*, which was transformed with two types of *Atmsh*2 gene: with antisense or complementary negative orientation.

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In this study the deterrent effect of the somatic hybrids with or without MMR deficiency and backcross progenies were determined and its relation with the analyzed plants toxicity against CPB were established

For somatic hybrids and BCs antixenosis effect determination, against adult CPB, a food preference analysis (choice test) was performed. Colorado potato beetle had to choose between one type of somatic hybrid and one type of the parents (*S. tuberosum* or *S. chacoense*). To analyze the antibiosis effect of somatic hybrids against CPB, CPB larvae were fed on SHs and BCs leaves and their development and survival were monitored. The resistance of some hybrid plants was equal, or it approached the resistance degree of *S. chacoense*.

The antixenosis effect of SHs and BCs were correlated with the analyzed plants toxicity and a strong positive correlation between this two properties were observed. Some SHs possessed strong antibiosis and antixrenosis effect against CPB, therefore these plants represent an important step forward in producing pre-breeding lines resistant to CBPs.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 47-48)

=== POSTER ABSTRACTS ===

The Micropropagation Potential and Regenerative Ability from Somatic Embryos of *Vitis Vinifera* Ssp. *Sylvestris* (Gmel.)

Carmen Florentina Popescu [1,✉]

During the last hundred years, the habitat of *V. sylvestris,* as wild grapevines, has been reduced due to pathogens or intensive rivers and forests management. So, in our days, it is present only in certain ecosystems in Romania. The recent papers underlined that *Vitis vinifera* subsp. *sylvestris* is one of the critically endangered subspecies and its conservation in germplasm collection could be an important source of adaptive traits for cultivated grape vines. In a previous paper was presented the morphological characterization based on OIV descriptors of the individual plants collected from some wild grapevine populations growing along the Danube River, providing valuable information. The aim was to establish an *ex situ* collection with plant material tested and confirmed as virus-free and to use it as possible starting plant material for further breeding of grapevine cultivars and rootstocks.

In the present work are presented the results obtained with wild *Vitis vinifera* ssp. *sylvestris* accessions under *in vitro* culture conditions. For long-term conservation or for virus-free plant recovery (if viruses were detected) were applied micropropagation methods starting from meristematic tissues (apex and axillary buds). The *in vitro* development revealed particular aspects and significant differences among wild populations (accessions belonging to 7 different populations) regarding their competence for differentiation, moment of differentiation in inoculated explants, the aspect of proliferative structures, the rates of multiplication and rooting.

The same accessions were tested for their competence in plant regeneration by organogenesis / or embryogenesis starting from somatic tissues. If in the case of petiole explants, for whole plant regeneration, a crucial role had the medium

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composition, in the case of anthers culture, the somatic embryogenesis process and the morphological aspects of the new dedifferentiated structures, proved to be a process strongly dependent by genotype.

Stress selection under controlled *in vitro* condition was applied to investigate the salinity (NaCl) and lime ($CaCO_3$) tolerances of the wild grapevine accessions and to select resistance or tolerant genotypes to these abiotic stresses (salty and calcareous soils). The salt and carbonate chloride stresses induced reduction of differentiation ability of tissues structures and prolonged the period of cultures, which is necessary to overcome the toxic accumulation of certain ions in tissue structures and to restore the growth process.

The results suggested the possible use of selected wild plants either as rootstocks with increased and stable tolerance to these types of environmental stresses (3 accessions of *V. sylvestris*), or to use them for future breeding applications (other 4 different accessions of *V. sylvestris*).

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 49-50)

=== POSTER ABSTRACTS ===

The State of the Art in Inducing PVY Resistance into Potato Cultivars by Biotechnological Methods

Adriana Aurori [1], Carmen Bădărău[2] and Elena Rakosy-Tican[1, ✉]

Inducing resistance to pathogens in different crops was a continuous concern of the researchers and several biotechnological methods have been developed. Due to its high economic importance, potato raised a huge interest in developing such researches and having a significant contribution in refining of several methods. One of the most threatening pathogen of potato is represented by potato virus Y (PVY), this being able to jeopardize up to 80% of the crop yield. The high variability of PVY, as a result of naturally raised mutations or recombination was a continuous challenge, very often leading to overcoming the resistance of different potato cultivars. Today potato virus Y has a high genetic variability with a significant number of strains: PVY^0, PVY^C, PVY^Z, PVY^N, PVY^{NTN}, and PVY^{N-W}. The last two strains: PVY^{NTN} (N-tuber-necrosis) and PVY^{N-W} (N-Wilga) have a rapid spread and produce Potato Tuber Necrotic Ringspot Disease (PTNRD), affecting both leaves and tubers, and hence producing very height yield loses.

Two major approaches for inducing resistance to PVY in potato were performed, one of it is based on pathogen derived resistance (genetic transformation) and the other one relies on the host derived-resistance (somatic hybridization, cis-genesis or intragenic improvement). The application of both strategies is facilitated by the good response of potato to *in vitro* culture, several successes being

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encountered. The aim of the present study was to review the progresses that have been made in the field and to highlight the new direction in obtaining resistance to PVY by "genetically modified" elite potato.

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=== POSTER ABSTRACTS ===

Photosynthetic Response Under Long-Term Drought Stress in Somatic Hybrids Between Potato and *Solanum Bulbocastanum*

Tünde-Éva Dénes [1,✉], Imola Erdely-Molnar[1], Raluca-Alina Mustaţă[1], István Vass[2], András Cseri[2], Imre Vass[2] and Elena Rakosy-Tican[1]

Potato (*Solanum tuberosum*) is a sensitive plant to biotic and abiotic stress, some of the main stress factors affecting potato yield being the drought stress and the late blight disease caused by *Phytophthora infestans*. In order to obtain resistant cultivars to late blight somatic hybrids between cultivated potato and *Solanum bulbocastanum,* carrying two broad spectrum resistance genes against late blight (*Rpi-blb1* and *Rpi-blb*3), were produced previously by protoplast electrofusion (Rákosy-Tican *et al.,* 2015). Somatic hybrids which proved to be resistant to late blight were screened also for drought stress tolerance and the long-term drought stress effect on the plant photosynthesis was verified.

Photosynthesis is a very sensitive process, the presence of an abiotic or biotic stressor leading to several deviations from its normal progress. Therefore, it can be used as an indicator of overall plant fitness. In the first stage of experiment somatic hybrids were pre-selected for drought tolerant genotypes *in vitro*. Taking into account the obtained results genotypes which proved to be drought tolerant and resistant to late blight were selected. These genotypes were introduced on a plant phenotyping platform at Biological Research Centre Szeged, Hungary, where the biomass accumulation under long-term drought was monitored. In the beginning and in the end of the experiment long-term drought stress effect on plant photosynthesis was quantified. Somatic hybrids and the *S. bulbocastanum* parent proved to be tolerant to the applied moderate drought. The integrity of the system

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which directs the light energy from the antenna to PSII was not affected, moreover due to the drought stress the photosynthetic pigments reorganized and their density increased around the active reaction centre in the second measurement. The calculated effective quantum yield revealed that the carbon fixation is negatively affected only by the end of the experiment, these findings sustaining the results obtained from biomass accumulation measurements. In the drought stressed group the quantity of excess energy which was dissipated by non-photochemical process (Non-Photochemical Quenching) was decreased from the first measurement to the second measurement event, that suggests the plants used more efficiently the energy by photochemical process in the end of experiment.

Based on the measurements the effect of drought stress on photosynthesis is more accentuated in the beginning of the experiment, the tolerant clones having the capacity to adapt and by the end the photosynthesis parameters were stabilized. All measured parameters proved that in the somatic hybrids and *S. bulbocastanum* the photosynthesis was enhanced at the beginning of moderate drought stress.

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=== POSTER ABSTRACTS ===

In Vitro Analysis for Drought Tolerance of Different Transgenic Potato Lines Resistant to PVY

Raluca-Alina Mustaţă [1], Imola Erdely-Molnar[1], Tunde-Eva Denes[1], Adriana Aurori[1] and Elena Rakosy-Tican[1, ✉]

Potato is an excellent staple food, but it is very sensitive to diseases, pests and different abiotic factors. Potato viruses are between the diseases causing great losses in potato crop production. In our previous research two different potato cultivars ('Baltica' and 'Désirée') were transformed using *A. tumefaciens* C58C1 pGV2260 with the hairpin construct pRGGYCPiPCY to obtain different potato varieties PVY^{NTN} resistant (Rakosy-Tican *et al.,* 2010).

On the other hand, abiotic stress, such as drought, is another cause which determine the decrease of productivity in potato. One of the main methods for producing drought-tolerant plants is *in vitro* selection under water stress.

The amino acid proline is an organic osmolyte that accumulates in a variety of plant species in response to abiotic stress such as drought (Szabados and Savoure, 2010). Proline accumulation may influence the stress tolerance of the plant in different ways. The stabilization of proteins and protein complexes from chloroplasts and cytosol, protection of photosynthetic apparatus and enzymes involved in ROS detoxification are also some of the important functions, determined by the accumulation of proline (Chaves, 2009).

The aim of this study was to combine gene transfer and *in vitro* stress selection in order to obtain potato varieties 'Baltica' and 'Désirée' that are resistant to PVY^{NTN} and tolerant to drought. The drought stress was simulated in two stages. First, the callus cultures regenerated from internodes were maintained for three weeks on MS-T medium with 5% polyethylene glycol (PEG) 6000. In the second stage, the

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regenerated plants were selected on RMB5 medium with 5%, 10% and 15% PEG. At each stage, the plants were analyzed for viability, root development, the regeneration of axillary buds (Mustață *et al*., 2014) and also at biochemical level, for proline and hydrogen peroxide biosynthesis. The results obtained reveal that three clones of cv. 'Baltica' positive for the hairpin construct of CP (coat protein) gene of PVY^{NTN} and tolerant to drought could be selected also based on enhanced proline biosynthesis, which was significantly higher than in sensitive or control plants. Hydrogen peroxide (H_2O_2) as an indicator of reactive oxygen species synthesis (ROS), evaluated at the end of selections on PEG 6000, reveals a reduced accumulation in stressed tissues as well as a significantly lower concentration in the tolerant potato cv. 'Baltica' transgenic lines. These results suggested that increase of proline contributes to ROS homeostasis in potato clones tolerant to drought and putatively resistant to PVY.

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Mustață, R.A., Molnar, I., Denes, T.E., Rakosy-Tican, E. (2014) *In vitro* stress selection of marker free transgenic potato lines in order to produce potato resistant to both PVY and drought stress, *Sudia UBB Biologia*, **2**, 35-46

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=== POSTER ABSTRACTS ===

In Vitro Micropropagation and Acclimation of *Gypsophila Paniculata* L.

Enikö Selek [1,✉], Lia Mladin[1] and Oana Sicora[1]

Economically important cut flower plant, *Gypsophila paniculata,* from Caryophyllaceae family, was regenerated from shoot tips explants. Murashige and Skoog medium with different concentrations of α-naphthaleneacetic acid and thidiazuron were used for shoot proliferation. The degree of vitrification was reduced by addition of 10 g dm^{-3} agar. An optimum root initiation and development of plants was obtained by using 3 mg dm^{-3} indole-butyric acid. For plants acclimation different periods of time were used. From 4 batches of plants prepared for acclimation in 4 different periods of time those from the and of February have the highest percent of rooting.

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=== POSTER ABSTRACTS ===

Indirect Organogenesis of *Symphytum Officinale* L.

Smaranda Vântu [1,✉]

Symphytum officinale L. is a perennial plant belonging to the Boraginaceae family. The presence of polyphenols, triterpenoids and tannins in this species represents a promising source of natural compounds with high antioxidant activity (Dreger *et al*., 2009). ”In vitro” cultivation of *Symphytum officinale* L was initiated in order to evaluate the cell dedifferentiation and redifferentiation, as an unconvential alternative for plant biomass multiplication (Haaß *et al.*, 1991; Huizing *et al*., 1983).

The initiation of *Symphytum officinale* L. cultures aimed not only to assess the dedifferentiation capacity depending on explant origin and growth regulators, but also to develop a multiplication protocol based on indirect regeneration through shoots, followed by roots development induction. The proliferative capacity was tested on leaf and shoots explants, cultivated on Murashige-Skoog basal medium, testing two auxins: naphtalenacetic acid (NAA) and indolylacetic acid (IAA) and two cytokinines: kinetine (K) and benzylaminopurine (BAP).

The MS medium with 1.0 mg/l IAA and 0,1 mg/l BAP proved to be the best for callus induction from leaf explants. Shoot regeneration was achieved after subculturing the calli on MS medium, supplemented with 1.0 mg/l BAP and 0,1 mg/l IAA. It was found to be the best for multiple shoot regeneration from callus through organogenesis. Root system development was achieved on MS medium without growth regulators. Rooted shoots (plantlets) were gradually acclimatized.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 59-68)

40 Years since the Introduction of Plant Tissue and Cell Culture Techniques in Romania

Dorina Cachiţă-Cosma [1,✉], Constantin Crăciun[2] and Aurel Ardelean[1]

SUMMARY. At the beginning of twenties century, based on the discoveries in cell biology in the previous decades, the German botanist Haberlandt (1902) has founded the concept of cell totipotency. The progress registered in biology between the two World Wars has led to the development of a new research field in plant physiology, based on nutrition, biochemistry and cytology that is the culture of plant tissue and cells *in vitro*. After the Second World War the practical applications of those new discoveries has allowed the development, after 1954, of a series of bio-industries at large scale, as clonal micropropagation of ornamentals and economically important crops, as well as the use of biomass in pharmaceutical industry as a source of secondary metabolites.

This new field has raised the interest and enthusiasm of the researchers in Romania as well. In 1975 three researchers from Romania being specialized abroad, i.e. Cachiţă D., Coman T and Brezeanu A., have learned the *in vitro* culture technology. Coming back to Romania those researchers have each developed in their home institutes new laboratories for plant *in vitro* culture. As a result of their research abroad, Cachiţă and Brezeanu did published four papers in European journals (France, Belgium and Germany) as well as one publication in USA.

Starting from 1989 annual symposia were organized in our country, the first being organized by the Biological Research Center and Institute of Agronomy, both from Cluj-Napoca. All presentations were published in the first volume of this symposium. 19th such symposia were organized during the following years but only 12 volumes have been successfully published.

It is also to be mentioned that in 1990 The Romanian Association of Plant Tissue and Cell Culture has been grounded, organization which has been involved in the organization of all national meetings in plant

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biotechnology. From 1975 our country was affiliated at The International Association for Plant Tissue Culture (IAPTC), many Romanian researchers participating to the international conferences and congresses taking place around the globe.

Keywords: plant tissue culture, research, Romania

Investigations into the *regenerative capacity* of plant tissues and cells have relied on the theory advanced by *Schleiden* and *Schwann,* in 1838-1839, referring to the structural cellular unity of life forms and to the totipotency of cells. It was also in the 19th century that *Vöchting* and *Rechinger* conducted research on the survival and regenerative capacity of plant fragments (cuttings), whose size was progressively reduced. It may be considered that the period of theoretical speculation came to an end at the time when *Haberlandt* (1902) formulated his hypothesis about the totipotency of plant cells, which contain in their genome the entire hereditary information required for the vegetative "reconstitution" of the organism they originated from.

In the period 1902-1922, a consistent experimental research campaign was targeted – in Germany, France and the United States – at proving the regenerative capacity of organ, tissular or cellular plant explants. However, the first practical attempts in this sense were unsuccessful.

As it was proved later, the simplest way to accomplish a plant vitro-culture consists in using explants that contain *meristematic cells* in their morphostructure. In the case of adult cells, the success of such attempts resides in the experimenter's ability to restore the morpho-physiologically differentiated cell to the status of a *meristematic* cell through a *dedifferentiation* process, based on the manipulation of environmental factors. On the other hand, during the "pioneering" period of such researches, *phytohormones* had not yet been discovered. Moreover, the nutritional requirements of phyto-inoculi were not known, in terms of the necessary substances that are indispensable for plant inoculi and that must be present in the culture substrate. At the same time, it is important to be aware of their nature and optimal concentrations in the cultivation medium, for rekindling and supporting the processes of cell multiplication, regeneration and morphogenesis, both in the *primary culture* and, especially, in the *subculture*.

During the period 1922-1939, the first successes were registered in the field of plant vitro-cultures, more specifically in cultures of *zygotic embryos* (*Hanning*), in cultures of *roots* (*White*), in the genesis of *callus*, as well as in its growth and subcultivation (*Gautheret, Nobécourt* and *White*). This is regarded as a *pioneering* stage in the evolution of plant biotechnology (Cachiţă, 2003, see Note 1 at the end of this article).

The progress registered by the techniques of *in vitro* plant cultivation, especially after World War, facilitated the birth of plant biotechnology, which led to what is generically known as the "*green revolution*". This phenomenon was born also thanks to the discoveries of *Morel* and *Martin* (1952), which enabled the creation – in dahlias and, thereafter, in potatoes (1955) – of plants that were impervious to *virus infections*, through the *in vitro* cultivation of meristematic, cauline, apical inoculi, taken from mother plants that had been exposed to multiple viral infections (see *Cachiţă*, 2003; Note 1). This revolution also targeted the multiple implications of plant cell and tissue vitro-cultures not only for the rapid *cloning* of plants through micropropagation techniques, but also for the valorisation of the biomass resulting from the cultures of cells, calluses, roots, shoots or somatic embryos, in phytoreactors, as raw material for the phyto-pharmaceutical and the food industries (dyes, flavours, purées etc.). Depending on how the vitro-culture is organized and conducted, cell cultures may be used for the *bioconversion* or *biotransformation* of certain organic compounds, precursors, in the preparation or transformation of certain *secondary metabolism products*. Cell cultures, on the other hand, are also useful – through somatic embryos – for the production of *artificial seeds*, or for the transmission and expression of genetic information acquired from outside the plants' own genome, through genetic engineering techniques.

In Romania, this field of activity was launched in 1975, when two researchers, namely *Dorina Cachiţă-Cosma*, holding a PhD in Biology, from the Biological Research Centre in Cluj-Napoca (the present-day Biological Research Institute) and *Tatiana Coman*, a horticultural engineer from the Research Institute for Fruit Growing in Mărăcineni, Piteşti, went to Belgium for a specialization in the field of Plant Biotechnology. After their return to the country in the spring of 1976, they founded plant biotechnology laboratories at the institutes they worked for. The findings Dr. *Cachiţă* obtained from the research she had carried out in Belgium were presented, during that period, at several European scientific events and were published in specialized journals from the organizing countries (in Note 2 – at the end of this article – we list the titles of these scientific papers).

It was also during that period that Dr. *Aurelia Brezeanu* (a researcher at the Institute of Biology in Bucharest) visited the United Stated for a specialization in electron microscopy, where she used wheat cell suspensions as an experimental model. The findings of the research Dr. *Brezeanu* carried out on that occasion were published abroad in 1976 (see Note 3).

In the same period, at the Department of Genetics from the University of Bucharest, at the Faculty of Biology, a plant tissue and cell culture laboratory was set up, headed by Prof. Dr. Eng. *Petre Raicu.* The academics and researchers led by Professor *Raicu*, who was the head of this discipline, started publishing, in 1979, the first findings of the studies conducted by this scientific team.

The laboratory of plant vitro-cultures that was opened at the Biological Research Centre in Cluj-Napoca in the summer of 1976 underwent permanent transformations and modernizations and, in time, it trained a large number of specialists in the field of Plant Biotechnology, with research stages in Romania and abroad. Prior to the revolution of December 1989, the team of researchers from this laboratory – led by Dr. *Cachiţă* – included 10 specialists: *Deliu Constantin, Henegariu Octavian, Osvath Tiberiu*, *Zăpârţan Maria, Rakosy-Tican Elena, Cristea Victoria, Dobrotă Cristina, Halmagy Adela, Cătană Corina*, and *Vicol Armeria*. After the revolution, some of these researchers moved to various other universities in Cluj-Napoca or in the country and founded plant vitro-culture laboratories at their new work places, such as the Agronomy Institute and the Pomological Research Centre in Cluj-Napoca, at Babeş-Bolyai University, the Faculty of Biology-Geography (Department of Genetic Engineering) as well as: the University of Timisoara, the Botanical Garden in Jibou, the Potato Institute, the Sugar Beet Institute, the Pastures and Meadows and the Forestry Institutes in Braşov, the Greenhouse Company in Codlea, Braşov County, the Gene Bank in Suceava, the University of Craiova, etc. Since 1990 many of the researchers trained in the field of plant biotechnology have completed their doctoral theses under the supervision of Prof. Dr. *Cachiţă.* Moreover, a series of foreign BSc or PhD students have specialized themselves in the field of plant biotechnology at the Biological Research Centre in Cluj-Napoca.

In our country, *the first scientific manifestation* dedicated to plant tissue and cell cultures was organized as a symposium in Cluj-Napoca, in 1981, by a team led by the research staff of the Biological Research Centre in Cluj-Napoca, by the Director of this institution, Academician Prof. Dr. *Preda Victor,* by Scientific Researcher Dr. *Cachiţă C. D.,* and by Prof. Dr. *Puia Ioan*, who was, at that time, the Rector of the Agronomy Institute in Cluj-Napoca. The symposium rallied the participation of numerous researchers from across the country, and the papers they presented in plenary sessions were included in a volume published by the Agronomy Institute in Cluj-Napoca. Both the symposium and the volume of proceedings were entitled: "Tissue Cultures – Research Instruments in Theoretical and Applied Plant Biology".

Over the years, namely from 1981 to 2007, the symposia on Plant Tissue and Cell Cultures organized in our country, as shown by the bibliography, were held in different localities and, according to the possibilities, the papers delivered in plenary sessions or those presented as posters were published in volumes of proceedings. 19 such symposia have been organized, but only 16 benefited from the publication of their proceedings, since symposia no. 17, 18 and 19 were part of the manifestations held under the umbrella of the Academic Days of the "V. Goldiş" West University in Arad. The papers communicated on those occasions were published in the review "Studia" of the University in Arad. Moreover, in most cases the publishing of the volumes of proceedings from the symposia on plant vitro-cultures was supported by the Rector of the "V. Goldiş" West University in Arad, Professor Dr. *Aurel Ardelean,* who is currently President of this University.

In many cases, the national symposia on plant tissue and cell cultures were organized in Romania within the framework of the events hosted by the National Society for Cell Biology (SNBC). In what follows, we shall present the list of the National Symposia on Plant Tissue and Cell Cultures organized in Romania in the period 1981-2007. Their proceedings were published in the same year or one or two years after the papers were presented at these symposia. In some cases, the papers from two symposia were collated into a single volume:

1. 15-16 December 1981, C1uj-Napoca: The First National Symposium on Plant Tissue and Cell Cultures, entitled: "Tissue Cultures - Research Instruments in Theoretical and Applied Plant Biology"; organizers of the symposium and editors of the volume of proceedings: Preda, V., Puia, I, Cachiţă, C.D., 1981, Tipo Agronomia Cluj-Napoca, 453 p.
2. 15-16 December 1983, Piteşti: "The Proceedings of the Second National Symposium on *In Vitro* Plant Tissue Cultures", Vol. I and II; the organizer of the symposium and editor of the volumes: Ceauşescu I., Academy of Agricultural and Forestry Sciences; the Pomological Research and Production Institute Piteşti – Mărăcineni; the "Nicolae Bălcescu" Institute in Bucharest.
3. 19-21 December 1985, Bucharest: "The Works of the Third National Symposium on *In Vitro* Plant Cell and Tissue Cultures"; organizers of the symposium: the Society for Biological Sciences; the Institute of Biological Sciences, Bucharest; the University of Bucharest; the Research and Development Institute for the Industrialization and Marketing of Horticultural Products; editors: Ceauşescu I. and Anghel I., University of Bucharest Press, 495 p.
4. 7-9 December 1989, Cluj-Napoca: The Fourth National Symposium on Plant Tissue and Cell Cultures, entitled: "*In vitro*" *Explant Cultures - Present and Perspective*; symposium organizer and editor of the volume: Cachiţă, C.D., Tipocart-Braşov S.A. Ed. I.C.B. Cluj- Napoca, 1991, 159 p.
5. June 1993, Bucharest: "The Works of the Fifth National Symposium on Plant Tissue and Cell Cultures"; symposium organizers and editors of the volume: Anghel, L., Brezeanu, A., Cachiţă, C.D., University of Bucharest Press, 1993, 319 p.
6. 10-11 June 1996, Băile Felix – Oradea: The Sixth National Symposium on Plant Tissue and Cell Cultures, entitled: *Actualities and Perspectives in Plant Biotechnology*; symposium organizers and editors of the volume: Cachiţă, C.D., Ardelean, A. and Crăciun, C., Ed. "V. Goldiş" Arad, 1997, 250 p.
7. June 1997, Arad: The Seventh National Symposium on Plant Tissue and Cell Cultures; symposium organizers: Cachiţă C.D. and Ardelean A.
8. June 1998, Buziaş: The Eighth National Symposium on Plant Tissue and Cell Cultures, organizers: Cachiţă, C.D., Ardelean, A. The papers of the symposia held in Arad and Buziaş were published together in one volume, entitled: *"In Vitro" Cultures in Cormophytes*, editors: Cachiţă, C.D., Ardelean, A. and Crăciun, C., Ed. Risoprint Cluj-Napoca, 1999, 407 p.

9. 11-12 June 1999, Constanţa: The Ninth National Symposium on Plant Tissue and Cell Cultures, entitled: *Actualities and Perspectives in Plant Biology*; organizers and volume editors: Cachiţă, C.D., Bavaru, A. anf Brezeanu, A., Ed. "Ovidius" University Press Constanţa, 2000, 186 p.

10. 10-11 November 2000, Cluj-Napoca: The Tenth National Symposium on Plant Tissue and Cell Cultures, entitled: *Jubilee Anniversary: 25 years of Plant Tissue Cultures in Romania*; symposium organizers and editors of the volume: Cachiţă, C.D., Rakosy-Tican, L. and Ardelean, A., Ed. Risoprint Cluj-Napoca, 2002, 436 p.

11. 6 June 2002, Satu Mare: The Eleventh National Symposium on Plant Tissue and Cell Cultures, entitled *Festschrift for G. Haberlandt (100 years since the launching of the theory on plant cell totipotence) and for Morel and Martin (50 years since the creation of the first virus free vitroculture)*; symposium organizers and editors of the volume: Cachiţă, C.D. and Ardelean, A., Ed. Daya, Satu Mare, 2003, 243 p.

12. 5 June 2003, Jibou-Sălaj: The Twelfth National Symposium on Plant Tissue and Cell Cultures, entitled: *Plant Pathophysiology Studied under a Vitroculture Regime*; symposium organizers and editors of the volume: Cachiţă, C.D., Ardelean, A. and Fati, V., Ed. Daya, Satu Mare, 288 p.

13. 9 June 2004, Sighişoara: The Thirteenth National Symposium on Plant Tissue and Cell Cultures, entitled: *Vitrocultures in Cormophytes*, *Experimental Models in Biological Research*; symposium organizers and editors of the volume: Cachiţă, C.D. and Ardelean, A. Ed. Bion, Satu Mare, 311 p.

14. 9 June 2005, Sibiu: The Fourteenth National Symposium on Plant Tissue and Cell Cultures, entitled: *The Conservation of Plant Vitrocultures*; symposium organizers and editors of the volume: Cachiţă, C.D. and Sand, C., Ed. Alma Mater, Sibiu, 276 p.

15. 7 June 2006, Iaşi: The Fifteenth National Symposium on Plant Tissue and Cell Cultures, entitled: *The Micropropagation of Plant Species*; symposium organizer and volume editor: Cachiţă C.D. Ed. Risoprint, Cluj-Napoca, 2007, 266 p.

16. 8 June 2007, Bucharest: The Sixteenth National Symposium on Plant Tissue and Cell Cultures, entitled: *Plant Biotechnologies for the 21st Century*; symposium organizers and editors of the volume: Cachiţă, C.D., Brezeanu, A., and Ardelean, A; Ed. Risoprint, Cluj-Napoca, 2008, 214 p.

In 1991, the *Romanian Association for Plant Tissue and Cell Cultures* (ARCTCV) was founded in Cluj-Napoca. It is a professional, non-profit, juridical attested association. The Association elected Dr. Dorina Cachiţă as its Chairperson; at that time she was Scientific Researcher, qualification level I, at the Biological Research Centre in Cluj-Napoca. At the time of its foundation, the Association had 130 members. Until 1995, the Association edited its biannual publication – the ARCTCV *Bulletin*. At the same time, the Association was involved directly in the regular organization of national symposia on plant tissue and cell cultures, as well as

in the editing and publication of the volumes of proceedings, including the scientific papers and communications presented in plenary or in poster sessions. The main purpose of this association was, and still is, to ensure, at the national level, closer relations between the specialists in the field of plant biotechnology in the country, with a view to enhancing collaboration between such laboratories in Romania, as well as facilitating contacts with experts from other countries. Because of lack of funds, the *Bulletin* of the Association had a limited number of issues, insufficiently compensated by the success of the publication of the papers presented at the national symposia on plant tissue and cell cultures. Since it was founded, ARCTCV has affiliated itself to its correspondent international association: the International Association for Plant Tissue Culture & Biotechnology (IAPTC & B), whose members we are and whose specialized journals have published reports or informative papers on the studies conducted by the Romanian scientists in the field of plant biotechnology. A part of the Romanian specialists in the field of plant biotechnology have presented their scientific papers at the IAPTC congresses (we should mention here *D. Cachiţă*, *Pamfil*, *Palada*, *Roşu*, *Onisei*, *Ghiorghiţă*, *Toth*, *Zăpârţan*, *Cristea*, *the Corneanus, Petrescu*, *Rakosy-Tican*, and others).

The efforts undertaken at national level for the popularization of this new field of plant biology led to plant biotechnology gaining new followers in our country, assisting biologists in Romania in becoming familiar with this type of literature. Mention should also be made of the monographic works and popularization books published over the course of time in our country, in the field of plant tissue and cell cultures, as well as the undergraduate and graduate courses on this topic that have been published in Romania.

In 1984 Ceres Publishing House in Bucharest printed the first monograph in the field of vitro-cultures, entitled *Culturi de celule şi ţesuturi vegetale – aplicaţii în agricultură* [Plant Cell and Tissue Cultures – Applications in Agriculture], authors: *Cachiţă, Raicu and Badea*. Subsequently, in 1987, Ceres Publishing House in Bucharest printed another monographic work on plant tissue and cell cultures, entitled *Metode in vitro la plantele de cultură – baze teoretice şi practice* [*In Vitro* Methods for Crop Plants – Theoretical and Practical Foundations], author: Dr. Cachiţă, a work that was awarded the "E. Teodorescu" Prize by the Romanian Academy.

In 1985-1986, the "N. Bălcescu" Agronomic Institute in Bucharest organized the first (postgraduate) *course* on plant biotechnology for the purpose of training specialists (biologists and agronomists) in the field of plant vitro-cultures, making available to the students, from the very beginning of the course (1985), the volume entitled *Curs practic de culturi de ţesuturi* "in vitro"*, cu aplicaţii în legumicultură şi floricultură* [A Practical Course on *In Vitro* Tissue Cultures, with Applications in Horticulture and Floriculture], authors: Professors *Cachiţă* and *Petrescu*. To these was added the textbook *Ingineria Genetică – note de curs* [Genetic Engineering – Lecture Notes], edited by Professor *Anghel* and published by the University of Bucharest in 1988.

We should also mention the publication by the *Raicu* and *Badea* team (1986) and, separately, by *Ungureanu* (1990), of booklets for the popularization of plant biotechnology, these works being intended to familiarize especially the lay readers with the subject of plant vitro-cultures, as well as with their theoretical and practical implications in everyday life.

In the sphere of higher education, with a focus on plant biotechnology, we should mention the publication in 1993 of the volume entitled *Curs de Biotehnologie – Culturi de ţesuturi in vitro*, *cu aplicaţii în horticultură* [A Course on Biotechnology – *In Vitro* Tissue Cultures, with Applications in Horticulture], authors: Professors *Petrescu* and *Cachiţă*, a volume edited by the Academy of "Athenaeum" University (the Faculty of Horticultural Science and Bioengineering at the University of Bucharest).

In 1994, Ceres Publishing House in Bucharest published the volume entitled *Înmulţirea vegetativă a arborilor forestieri. Metode convenţionale. Culturi de ţesuturi in vitro* [The Vegetative Propagation of Forest Trees. Conventional Methods. *In Vitro* Tissue Cultures], authors: *Enescu*, *Ioniţă* and *Palada*. This monograph detailed mainly the theoretical and practical aspects relating to the vegetative propagation, both natural and *in vitro*, of woody plants.

Special mention is deserved by the summer courses organized from 1995 to 1998 by the Department of Biology-Geology, "Babeş-Bolyai" University, in Cluj-Napoca, the Department of Genetics (led at that time by Professor Dr. *Nicolae Coman)*. Financed through a TEMPUS-PHARE programme and enjoying international participation, these summer courses were focused on the genetic implications of vitro-cultures, in general, and of protoplast cultures, in particular. These courses led to the publication, in 1998, of the volumes *Utilizarea tehnicilor de electrofuziune în hibridarea somatică a plantelor* [Using Electrofusion Techniques for the Somatic Hybridization of Plants] and *Plant Genetic Engineering – Lab Manual* by Associate Professor Dr. *Rákosy-Tican* Elena, at Cluj University Press.

In 1999, Associate Professor Dr. *Ana Roşu* (teaching at the Agricultural University of Bucharest, the Faculty of Biotechnology), published at Amethyst-92 Press in the capital the monograph entitled *Elemente de biotehnologii vegetale, aplicaţii în ameliorare* [Elements of Plant Biotechnologies: Applications in Breeding], which was mainly addressed to students and specialists in the field of plant tissue and cell cultures, as well as to those interested in genetics and plant improvement.

In 2000, Prof. Dr. *Cachiţă* and *Sand* published the monograph entitled: *Biotehnologie Vegetală* [Plant Biotechnology], vol. I, addressed in particular to young university students specializing in this area.

A year later (2001), Dr. *Badea* and Dr. *Săndulescu* (both scientific researchers, qualification level I, from the Biology Institute of the Romanian Academy in Bucharest) published the monograph *Biotehnologii vegetale* [Plant Biotechnologies] with the help of the BIOTECH Foundation.

In 2004, Prof. Dr. *Cachiţă* and her collaborators published at the Cluj-based Dacia Press, in the biology series, the Universitaria Collection, volume I of the monograph: *Tratat de biotehnologie vegetală* [A Treatise on Plant Biotechnology], while in 2009 *Cachiţă* and *Ardelean* published – at the same press – volume II from the aforementioned monograph.

Subsequently, in 2005, Prof. Dr. *Ghiorghiţă* and Dr. *Petrescu* published a treatise entitled "Biotehnologiile azi" [Biotechnologies Today] at the "Junimea" Publishing House in Iaşi, about one-third of the book referring to the biotechnologies used in agriculture.

Information on publications in the field of plant tissue and cell cultures would not be complete if we did not mention the fact that several specialized articles focusing on the issue of plant biotechnology have been published by researchers from Romania in academic journals around the world.

In this category we should mention the publication by Prof. Dr. D. *Cachiţă* and C. *Crăciun* (1990) of a chapter about research on the ultrastructure of hyperhydric phyto-inoculi cells, included in volume 5 of the first encyclopaedia of plant tissue and cell cultures, which was entitled: *Handbook of Plant Cell Culture*, printed in the USA, by the McGraw-Hill Publishing Company, the editors of the aforementioned volume being *Ammirato* et al. The title of the chapter was: "Ultrastructural Studies on Some Ornamentals". This subchapter was mentioned by the editors in the foreword to the treatise. It should be noted that Prof. Dr. *Cachiţă* also published a chapter entitled "The Effect of the Nature and Origin of Explant on Micropropagation" in the encyclopaedia *Biotechnology in Agriculture and Forestry* (vol. 17), edited by Bajaj Y.P.S. at Springer-Verlag Press in Germany in 1991. In 1995, Prof. Dr. *Cachiţă* and *Crăciun* published another chapter entitled "Cryopreservation of Alfalfa (*Medicago sativa* L.) and Clovers (*Trifolium* Species)" in vol. 32 of the same encyclopaedia.

Although in recent years the economic recession in Romania has caused a sharp decline in scientific research, in general, and, implicitly, in research on plant cell and tissue cultures, in the higher education institutions from Bucharest, Cluj-Napoca, Timişoara, Iaşi, Oradea, Craiova, Arad, Bacău and Sibiu, as well as at the *Gene Bank* in Suceava, plant biotechnology laboratories have formed units in which biology, agronomy and forestry students have practised modern vitro-culture techniques, with explants or inocula derived from higher plants, as regards *micropropagation* (the clonal proliferation of valuable species), the steered *genetic modification* of certain plant species, or the *conservation* of phyto-inoculi in *gene banks*, so much so that, gradually, this discipline has been included in the curricula of several higher education institutions in our country.

More details about the beginnings of plant biotechnology research in Romania can be found in the book entitled: *Aniversarea a patru decenii – 1976-2016 – de la iniţierea cercetărilor de biotehnologie vegetală* [The Anniversary of Four Decades – 1976-2016 – since the First Research on Plant Biotechnology], co-authors: Prof. Dr. Cachiţă and Prof. Dr. Sava-Sand, published by "Lucian Blaga" University Press in

Sibiu, in 2016, the volume being launched on the occasion of the events that were hosted by Babeş-Bolyai University in Cluj-Napoca on 19 March 2016, at the Faculty of Biology and Geology, the Department of Genetic Engineering, an event organized by Prof. Dr. *Rakosy-Tican* and her team, under the title: *Sesiune Jubiliară și celebrativă 40 de ani de culturi de țesuturi și celule vegetale în România și celebrarea a peste 40 de ani de activitate în domeniu și de președinte al ARCTV*, a d-nei prof.dr. *Dorina Cachiță-Cosma*" [Jubilee Session celebrating of 40 years of plant tissue and cell cultures in Romania and over 40 years of work in the field and of ARCTV presidency, dedicated to Prof. dr. *Dorina Cachiță-Cosma*].

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Plant Cell and Tissue Cultures – A Page in the History of Biology in Romania

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In honorem Dr. Dorina Cachiță, "*The Lady of in vitro plant culture*"

SUMMARY. Four decades have passed since a new research field started being promoted in Romania: *in vitro* cell and tissue cultures of plants.This paper highlights the outstanding contribution to its development made by a reputed specialist – Dr. Dorina Cachiță, the pioneer in this field in our country. Moreover, the paper presents the social and economic context at the time this domain was launched, the difficulties in promoting it, it describes the author's beginnings and personal achievements together with the ones of his research group in the field of *in vitro* plant culture. On the whole, the article represents a page in the history of Romanian biology in Romania and is dedicated to the honour of PhD Prof. Dorina Cachiță.

Key words: achievements, beginnings, *in vitro* cultures, plants

Introduction

Some specialists in plant tissues and cell cultures in our country headed by Professor Elena Rakosy took the commendable initiative of organizing a jubilee session to mark the anniversary of four decades of research in this domain in Romania. A moment of assessment of contributions brought by a large number of compatriots who have devoted much of their work to this kind of research, but also an opportunity to celebrate a reputed researcher in the field, an emblematic figure of Professor Dorina Cachiță, who devoted so many decades of her professional life to *in vitro* cultures of plants. It is an area of research that this well-known specialist initiated and served with devotion.

I consider that such a moment was necessary, that the celebration of Professor Cachiță comes to recognize her undeniable merits in promoting this

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research area, because, when you say *in vitro* culture of plants in Romania, you almost automatically associate the field with a name – Dr. Dorina Cachiță. To attest this outstanding contribution, we can mention her publishing more than 270 scientific articles in various journals and books, 13 books, textbooks and book chapters devoted to the field, registering 12 patents related to *in vitro* culture, setting up (with colleagues) of the Romanian Association of Tissue and Cell Cultures (RATCC), organising (from 1981 to 2008, with collaborators) 15 editions of RATCC national symposium and the publication of papers presented at these meetings in proceedings volumes, organising and setting up specialised laboratories in several institutions (Institute of Biological Research in Cluj-Napoca, Oradea University, Biological Research Center in Jibou, "V. Goldis" University etc.), postgraduate (doctoral) supervising many young biologists from around the country, participating in numerous congresses, conferences and symposia organized in the country and abroad etc. It has been a remarkable effort and contribution to the development of science in Romania, which had to be honoured in some way, and this scientific event (19 March 2016) brings homage to the researcher, teacher and woman who was and is Dr. Dorina Cachiță. It was the reason for my decision to dedicate this article to her – a page in the history of Romanian biology – in sign of appreciation, respect and tribute to the personality of this colleague and friend.

Personal motivation to approach *in vitro* cultures of plants

It was in 1975. The first steps were taken in Romania to address a highly topical/ fashionable field in the world, but not to us –*in vitro* cell and tissue cultures of plants. I had been working for several years in an equally important area at the time – experimental mutagenesis in plants. The stake of mutagenesis research was not only knowledge of the cytogenetic, biochemical, physiological and chemical repercussions of physical and chemical mutagens in plants, but also their use in the induction of useful variations in the improvement of medicinal plants, all these representing the focal point of my research group. I knew something about the impact of *in vitro* cultures of plants, about some of their applications and we reckoned that an effective method of *in vitro* cloning of valuable genotypes isolated by us from the local populations of some species or populations mutagenized would have been extremely useful in their multiplication and obtaining seedlings necessary for the extension of these genotypes in field crops. So this is how I started.

At that time, I was conducting my research in a team in Pângăraţi (Neamt), at a Research Station established by "Al. I. Cuza" University, Iassy (1956); opened in 1970, for a short while it operated autonomously, before being affiliated to the Biological Research Centre (BRC) in Iassy (given that, in 1974, research centres were imposed a minimum of 60 members with higher education to preserve their status of legal entities). This centre along with a similar one in Cluj and Bucharest Institute of Biological Research constituted the Central Institute for Biological

Research (under the Ministry of Education). At the beginning of 1970, scientific research was required to function on contract, an action which was meant to streamline this sector. In retrospect, the measure had some positive effects, including empowering researchers, but the beginning was a real shock for us, researchers, because – on the one hand, we were not prepared to face this challenge, on the other hand, identifying sources of funding for research was far from being an easy task. In addition, great emphasis was placed exclusively on applied research, without taking into account the fact that, without major theoretical research, it is difficult to make progress in applicative research.

Hard times had come for research, in general, and for biological research, in particular, because, at a certain moment, it was on the brink of dissolution. Let me explain. Nicolae Ceausescu used to visit sites, enterprises, agricultural and research units, in order to witness the progress made in one area or another and feel "the devotion of the masses to the party". Such a visit took place in the mid 70s at the Institute of Biological Research in Bucharest. During that visit, some colleagues were rather uninspired and, instead of highlighting their achievements, their striving to make something out of nothing (because such were the times), they made the mistake to complain about various shortcomings faced by laboratories (some indeed ridiculously called labs), which was enough for Ceaușescu. He concluded (obviously being wrong) that biological research went the wrong way and that remedial measures had to be taken urgently. An immediate consequence of that visit was transferring some colleagues who had research interests common with agriculture to agricultural research units. Another measure imposed was that, every semester, the representatives of research teams from the Central Institute of Biology were called for meetings and had to draw up progress reports, addressing achievements and research perspectives. These meetings were usually attended by the vice president of the National Council of Science and Technology, Mihail Florescu (who had been appointed to monitor and improve the situation in biological research), and a deputy minister of education.

At Pângărați Research Station of the Centre for Biological Research in Iassy, this „earthquake" occurred somewhat later (in 1982) and it was even more painful than the episode previously reported, being caused more by subjective factors rather than by economic or professional ones. A representative centre, comprising more than 40 researchers, known and appreciated in the country (and abroad) for their achievements was broken into three smaller research groups (the one to which I belonged was about to disappear) subordinated to different ministries. After that unfortunate moment, about which I wrote on another occasion (Ghiorghiță, 2012) our mission to demonstrate our value and sustainability became more difficult, because we had to fight not only against material deprivation, but also against malevolence and prejudices of those patronising us.

Returning to *in vitro* cultures, I must say that, at that time, it was not easy to become initiated into this kind of work, because Romania faced major problems not only with the (poorly equipped) research laboratories, but with documentation and

scientific information (few subscriptions to magazines from abroad, and documentation and specialization in laboratories abroad was more a dream than reality). Fortunately, there were still Romanians who could benefit from documentation and specialized internships abroad, and those who returned to the country were also trying to capitalize experience. One such example is Professor Dorina Cachiță who worked at the Center for Biological Research in Cluj. As a young scientist, she received a grant to study in Belgium, in a laboratory of Free University of Brussels, which specialized in *in vitro* culture of plants. Once she returned home, she tried and managed to procure equipment and supplies necessary to practice these techniques and equipped a laboratory and a climate chamber to grow these cultures, which, in my opinion, turned her into a pioneer in this field in our country. Later on, a few such laboratories were established at other institutions in the country: Biology Institute in Bucharest, Bucharest University, Institute of Forest Research Ştefăneşti (Bucharest), ICCPT Fundulea, Agronomic Institute in Bucharest, Viticulture Research Station in Valea Călugărească and Stefanesti (Arges), 'Stejarul' Research Station Pângăraţi / Piatra Neamt, Vegetable Research Station in Bacau etc.

The urge to promote this kind of work in my institution more than four decades ago brought me in the position of getting to know Dr. Cachiță, who was the best suited specialist for this purpose. I obtained permission over the phone to visit her lab and obtain some information to help me to approach such research at my department. The visit took place in 1975, at a time when dr. Cachiță was in full swing organizing the lab, so I saw its foundation, the necessary equipment and was provided with some information in the field, along with the promise to support such concerns at my lab. Particularly important in this context was the experience obtained by my colleague Dr. Catherine Toth, who, during the 1970s benefited from several internships in biochemistry at the Biological Research Center Szeged of the Academy of Sciences of Hungary; it was a great opportunity to visit the plant tissue culture laboratory in their centre and obtain materials and information vital to the fulfilment of our dream.

The intention of approaching this area was salutary, but its putting into practice was more difficult because we lacked in our institution what was required for such an activity. Instead, we were young and enthusiastic, and Romanians are known to be inventive when their ambition is challenged. And the challenge was too great to resist. At Pângăraţi, we had already set up a room for growing plants in the basement (with walls more than 1m thick), where it was easier to maintain the temperature within variation limits in summer, the lighting was ensured by fluorescent lamps, and heating with electric heaters. It was a room intended to conduct experiments in plant physiology (growth tests, hydric behaviour etc.).

The laboratory was equipped with enough air ovens and ultraviolet lamps, we could find autoclaves at our colleagues, the microbiologists, but we missed an essential piece of equipment, namely a cell culture hood (with sterile air) in which to inoculate the biological material on nutrient media sterilized under aseptic

conditions. In addition, we had to procure some specific substances: growth regulators, agar, sucrose etc., included in culture media. The action to purchase the materials necessary for *in vitro* cultures of plants took us quite some time and many of them were thanks to those whom we asked for support. Not being able to obtain a cell culture hood (the investment chapter of the ministry for Pângărați being zero), we thought of ways to manage without it in order to perform inoculations of biological material in sterile conditions. The strategy we adopted to overcome this handicap may seem now a story, or madness, but it was all true.

One day (in 1980), we decided to get to work and try our fortune. As I said, having a Laboratory of Microbiology, we could benefit from the expertise of our colleagues to work under sterile conditions. Colleague Catherine Tóth prepared some hormonal variants of Murashige-Skoog culture media (1962), in which we included sucrose (as carbon source) and agar (for hardening), the medium being distributed then in Erlenmeyer flasks 100 ml, covered with cotton plugs shrouded in gauze. To create sterile conditions, we placed a lamp on a support above the work table whose UV radiation beam was directed to it, and, on this table, we lit several lamps with alcohol. These were the conditions in which we handled the nutrient medium and performed inoculations with explants, seeking for solutions to protect our hands and face against UV radiation (but we could not do anything against air ionization). In order not to put our lives in danger, we decided to work in sessions of 10-15 minutes at that table, followed by long pauses (pauses during which only the UV lamp was functional). Despite all protective measures, my colleague (more sensitive to UV) could not avoid some burns on her face. After inoculation, the flasks were placed in a growth chamber and exposed to a photoperiod of 12 hours.

Our first experimental subjects were *Daura innoxia* and *Vinca minor* species (from which were used as explants uninodal apices and fragments of young shoots). Even if some vials got infected, the success rate was quite good, given the poor working conditions. The results that we obtained in these investigations were presented by colleague E. T. Tóth on the occasion of the first symposium of this kind, held in 1981 in Cluj by Professor Cachiță, titled "*Tissue Cultures – a research tool in theoretical and practical plant biology*" and published in a proceedings volume elaborated on this occasion (Tóth and Ghiorghiță, 1981). We were proud of our success and I think we had every reason to be, because we had done something out of nothing and the beginning was promising.

Then came the dramatic moment at the end of 1982 when our institution was practically dismantled and undeservedly dissolved. We were forced to leave the premises from Pângărați, relocate in Piatra Neamt and make a fresh start. It was a very difficult and demanding task, but all 40 researchers, technicians and workers from the former "Stejarul" Research Station adjusted to this new formula. Our main concern in the new context was to maintain our status of specialists in the field and demonstrate that the decision about disbanding Pângărați research unit had been a great error. We also resumed our work in the field of *in vitro* cultures of plants. At

first, because we did not have a plant growth chamber any longer, we arranged a closet for this purpose. Fortunately, in the building where we had moved, we had a basement which was quite spacious (but found in an advanced state of decay), which could be set up as a semi-climatic room for plant growth. With great efforts, we managed to turn this space into one that was healthy and suitable for our purpose.

After what happened to Pângăraţi Department, the Minister Mihail Florescu (Vice President of CNST) paid us yearly visits to check how we complied with the requirements imposed at the time, if we recovered and made progress in our projects (which, in fact, I had to report anyway every semester at any general meeting – as the head of this research group –indicating the stage at which we were and what practical achievements we had). The Minister was accompanied on his visits by the Party Secretary for economic problems in Neamt County, Eng. Mihai Roman. When the Minister repeatedly observed the conditions under which we worked and how we struggled to achieve *in vitro* cultures, he must have been impressed, so he put in the charge of the party secretary to appeal to the management staff of the Complex of Synthetic Fibres from Săvineşti to assist us in arranging rooms for explants inoculation and culture incubation (including a climate control system) in our basement and to equip the lab with a more modern autoclave and a hood with sterile air, which was all achieved in a relatively short time. As an aside, despite other opinions, I believe that Minister Mihail Florescu's contribution to biological research was beneficial in those troubled times, as he was a man who proved much discernment, wisdom and tact in his actions. It was due to his intervention that we had better conditions in Piatra Neamt to assert ourselves in the field of *in vitro* cultures of plants.

It was a great achievement and satisfaction for us, but it had unintended consequences because Mihail Florescu Minister later visited Research Station for Medicinal and Aromatic Plants (SCPMA) Fundulea and reproached to the colleagues working there that there they had not developed the field of *in vitro* cultures, giving us as an example, a small group of research that was successful. Considering that some of our research contracts were financed by the Ministry of Education indirectly, through this station (to guarantee that our results in the field of medicinal plants are applied by them), the Minister's remark made the director of SCPMA Fundulea, dr. Emil Paun, take a grudge against us and, from that moment onwards, he would find every excuse to temper our enthusiasm and joy of success, always showing dissatisfied with our results.

A brief account of the objectives and accomplishments in this area

Our research group was formed in 1983 of seven researchers and four technicians and constituted the Group of Experimental Plant Biology and Genetics (which in 1990 acquired the rank of Research Laboratory). After suffering from the drama at the end of 1982, the expectations from us, the administrative and political

representatives, were very high. Every year, we made comments on dozens of lab and field experiences, planted experimental plots expanded on large areas, performed hundreds of tests etc. It was a hellish work. As there was so much field working which I was engaged together with my colleagues Florin Floria and Elvira Gille, I left *in vitro* cultures in charge of my colleague Catherine Tóth - to whom there joined in 1983 Tatiana Onisei, and in 1986 Doina Amariei.

After 1990, the external pressure which had been exerted on us (for seven years) disappeared, the field work diminished (because we had no lands in our property), which allowed me to return to *in vitro* cultures after that first attempt in the years 1980-1982. In February 1994, I became a professor at "V. Alecsandri " University of Bacau, but continued to work at my former institution in Piatra Neamt until 2007. I set up a properly equipped Laboratory of Genetics and Biotechnology in Bacau, including equipment (by means of research contracts) necessary to practice *in vitro* plant cultures, a laboratory which apprenticed many bachelor's, master's and doctoral students. I feel bound to make one more observation that may be surprising to many: in our work in the field of *in vitro* cultures of plants, we have been mostly self-taught, because we have not benefited from specializations in laboratories abroad. We told ourselves that, if others were successful in this area, so would we. And so it was. We learned from our own experience in the lab, from trial and error, and by participating in scientific meetings in the field how to overcome difficulties, how to progress.

The main objectives of the investigations in which I was involved were:

- Highlighting *in vitro* morphogenesis means for various explant types depending on explant nature, hormonal balance, growth conditions etc.;
- Regenerating plants through direct and indirect (via callus) organogenesis, analysing some morphological and physiological parameters for *in vitro* regenerants and studying their behaviour in the field;
- Analysing the biosynthesis capacity of secondary metabolites in *in vitro* and *ex vitro* cultures;
- Development of *in vitro* micropropagation technology to some of the species tested;
- Studying somaclonal variation and isolating potentially more valuable genotypes for culture;
- Haploid induction by experimental androgenesis and gynogenesis at some cultivated plants and observations on cytogenetic changes occurring in anther and ovary regenerants etc.

Some of the objectives previously mentioned were accomplished in investigating 26 medicinal and aromatic plant species: *Atropa belladonna, Vinca minor, Datura innoxia, Digitalis lanata, D. purpurea, Angelica archangelica, Anethum graveolens, Rosmarinus officinalis, Withania somnifera, Stevia rebaudiana, Stachys sieboldi, Catharanthus roseus, Trigonella foenum-graecum, Gentiana lutea, Mentha viridis, Mentha piperita, Melissa officinalis, Ocimum basilicum, Salvia*

officinalis, Hyssopus officinalis, Chrysanthemum balsamita, Rhodiola rosea, Sedum hybridum, Sedum fabaria, Rosa canina, Hippophae rhamnoides. For *Vinca minor*, *Stachys sieboldii*, *Datura innoxia*, *Mentha piperita*, *Mentha viridis*, *Rhodiola rosea,* there were elaborated *in vitro* micropropagation technologies.

A special achievement in this respect was obtained from *Rhodiola rosea*, which demonstrated that this technique can ensure their breeding and restocking with *in vitro* regenerated individuals in areas where the species is endangered (the Ceahlău Mountain) or disappeared (Ghiorghiță *et al.*, 2011). The investigations that followed the *in vitro* reaction of anthers and ovaries and the induction of experimental haploidy had as a subject some technical and vegetable crops: corn, sunflower, sugar beet, varieties of cabbage (for head, cauliflower, broccoli) tomatoes, potatoes, *Brassica juncea*.

I think that some technical contributions in the field of *in vitro* cultures of plants are not to be neglected either. My colleagues used as laboratory culture dishes Erlenmeyer flasks covered with cotton plug shrouded in gauze and accommodated neoplantlets regenerated *in vitro* in sterile potting soil. Both vials with nutritive medium and pots with soil (to accommodate *in vitro* regenerants) were sterilized in autoclaves. Since 1990, when I resumed my investigations in *in vitro* plant cultures, we no longer used this technique which facilitated contamination with bacteria and moulds. I decided to use two new strategies: a) I covered culture vessels (Erlenmeyer flasks) with double aluminium foil; b) to accommodate plants to septic conditions (*ex vitro*), I avoided using pots with sterile soil, finding it much easier and efficient to work in hydroponic environment. Proceeding this way, in addition to diminishing contamination hazards, the cost price decreased considerably. I shared these innovations with colleagues and many of them employed them in their research. I did not patent them as I did not consider it necessary.

The results obtained in investigations (in which I was personally involved) which aimed *in vitro* cultures of plants were the subject of 90 papers (in collaboration with some colleagues) published in various journals, but also in conference and symposia proceedings, in the country and abroad. In the *in vitro* Culture Laboratory of "Stejarul" Research Station in Piatra Neamt was conducted the doctoral research of 4 theses which included issues related to *in vitro* cultures authored by Tatiana Onisei (Onisei, 1995), Elvira Gille (Gille, 1996), Doina Amariei (Amariei, 2001), Mihaela Hârțan (Hârțan, 2009) and defended at "Al. I. Cuza" University of Iași. At the same university, as doctoral advisor, I supervised the doctoral theses elaborated by Daniela Nicuță (Nicuță, 2006), Diana-Elena Maftei (Maftei, 2007), Nicoleta Bădăluță (Bădăluță, 2011) whose research was conducted the Laboratory of Genetics and Biotechnology at the University "V. Alecsandri" of Bacau; Tina Oana Cristea (Cristea, 2008) – research undertaken in the laboratory for *in vitro* cultures of the Vegetable Research Station in Bacău; Daniela Ichim (Ichim, 2006) - research in the laboratories of the Faculty of Biology of "Al. I. Cuza " University); Justina-Brânduşa Ciobanu (Ciobanu, 2012) – thesis elaborated in the labs of the Gene Bank in Suceava.

Based on data from specialised literature and personal results obtained in investigations conducted on *in vitro* cultures and the regenerants of various plant species, we have developed and published (in collaboration) in 2002 a monograph entitled "*Experimental Haploidy in the Context of Modern Biotechnologies*" (Prisecaru and Ghiorghiță, 2002) and a book which comprises a large chapter on *in vitro* plant cultures, entitled "*Biotechnology today*" (Ghiorghiță and Nicuță-Petrescu, 2005). Moreover, some of the results obtained in this research were valued by their presentation at numerous scientific events organized in the country and abroad, including symposia and conferences organized by us in Piatra Neamt and Bacau.

I will always have a vivid recollection of attending the International Congress of Plant Tissue and cell Culture (ICPTCC) held in Amsterdam (Netherlands) in June 1990, shortly after the events of 1989 in Romania, a congress where, among other colleagues, I could meet Professor Dorina Cachiță. It was a great joy to meet her again after many years. I lived in full satisfaction of participating in a prestigious scientific event, as we were finally free to travel anywhere in the world. Over 8 years (in June 1998), we met again in Israel (in Jerusalem, where another edition of the Congress mentioned above was held), among whom was the organizer of the current event, Professor Elena Rakosy and we were proud that Romanians were more and more numerous attending scientific conferences organized outside the country.

Unfortunately, research remained a neglected area in our country after 1989. Before 1989, I was unhappy about not being allowed to go abroad, lack of provision of equipment, scientific information, insufficient budget and other sources to support research in Romania, but now things are much worse than then. And, in my opinion, *no research means no development*. Nevertheless, this elementary truth seems to be impossible to understand for our government, regardless of political regime, experience, training and efforts made by us, researchers.

Years have passed and here we are both (Professor Cachiță and I) at the age of retirement. In recent years, we have had the pleasure to meet more frequently than in the past for doctoral defences (as referees), at some scientific meetings and, for several years, at "V. Goldis" University in Arad, where the professor ended her academic career a year ago. As dean of the Faculty of Natural Sciences, she invited me to some activities organized at the university of Arad and I can say, without reserve, that I attended there scientific meetings organized at high academic standards, I met many personalities of science in the country and abroad, I spent unforgettable moments, I appreciated the kindness, openness and generosity of the hosts.

In the end, I would like to thank Professor Dorina Cachiță for being herself and permitting me to know her, for all she has achieved in this field, for the spirit of friendship, comradeship and mutual respect manifested between us whenever it was necessary, for the times – not many, but special – spent together.

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The Application of Combinatorial Biotechnology in Improving Potato Resistance to Biotic and Abiotic Stress

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SUMMARY. The concept of combinatorial biotechnology was introduced in previous presentations to international conferences in order to emphasize the importance to combine many tools of biotechnology, including phenomics, genomics and metabolomics, for the final goal to improve plant resistance to biotic and abiotic stress. This concept is exemplified here by a few examples in improving potato crop, one of the most important security crops worldwide and the third important crop as productivity at global scale. This crop is amenable for such improvement for some reasons: it responds well to tissue culture, somatic hybridization and transformation *in vitro*, its genome was sequenced and has got a rich resource of wild resistant relatives in the center of origin of potato crop. Moreover, potato is one of the crops facing great loses because of many diseases and pests, some of them causing total loss of production. The case studies presented in our work involve the use of sexually incompatible *Solanum bulbocastanum* and sexually compatible *S. chacoense* species as resources of multiple resistance genes, such as resistance to late blight caused by blight potato famine agent *Phytophthora infestans,* Colorado potato beetle and the abiotic stress caused by drought. Another example is genetic transformation with a marker free hair pin construct for PVY resistance combine with stress selection *in vitro* for tolerance to draught.

Keywords: Colorado potato beetle, DNA mismatch repair deficiency, draught tolerance, *Potato virus Y*, resistance to late blight, *Solanum bulbocastanum*, *S. chacoense*, Somatic hybridization

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Introduction

Potato ranks third in global crop productivity, and is recognized as an important security crop. Climate change and exponential growth of world population are posing new challenges to plant breeding (Vreugdenhil, 2007).

In order to obtain an increased crop production it is mandatory to use the most valuable cultivars, but such crops as potato with tetrasomic inheritance and vegetative propagation are lacking genetic diversity and might be difficult to improve further by classical breeding. Genetic resources which are useful to improve the quality of commercial species were exploited to a limited degree mainly because sexual incompatibility with many of the related wild species. Potato, in particular, has got a great diversity of tuber bearing wild *Solanum* species, distributed in South and North America, which represent a rich reservoir of resistance genes for potato improvement (Hawkes, 1990). The new technologies available today, mostly biotechnological tools and new data brought about by genomics, phenomics and metabolomics, allow us to find new possibilities to combine different methods toward the improvement of resistance traits in potato crop (Bradshaw et al. 2006). This is the reason why we introduced the concept of combinatorial biotechnology for crop improvement, exemplifying it in potato (Rakosy-Tican *et al*., 2012).

In this short review the goal is to present schematically some examples how one can combine somatic hybridization through protoplast fusion or genetic transformation, with molecular markers of resistance genes, molecular characterization of resistant wild species, the use of other tools of *in vitro* culture (embryo rescue, medium term conservation, stress selection), and different tools for somatic hybrids or transgenic lines characterization from molecular analysis, cytogenetic studies (flow cytometry, chromosomes counts, genomic *in situ* hybridization.GISH), resistance analysis, trichome analysis, up to phenomics studies. The final goal is to transfer and combine many resistance genes and traits in pre-breeding clones available for further plant breeding.

The use of *Solanum bulbocastanum* as a source of resistance to *Phytophthora infestans*, Colorado potato beetle and drought stress

The first example is the use of a diploid wild species which is sexually incompatible with potato, *Solanum bulbocastanum* (Rakosy-Tican *et al*., 2015). In this combinatorial biotechnology scheme for the transfer of: more resistance genes to *Phytophthora infestans* (*Pi*), the oomycete causing agent of late blight disease, also known as Irish potato famine disease; resistance to Colorado potato beetle (CPB) and

the tolerance to drought stress, many biotechnological tools were combined as illustrated in Fig. 1. In the first step, the accession of the wild species was selected by analyzing the presence of *Pi* resistance genes. From four resistance genes characterized to date, the genes *Rpi-blb*1, *Rpi-blb*2 and *Rpi-blb*3 were searched for by using gene specific markers (data not published). One accession coded *GLKS* 31741 (*blb* 41, Gross Lüsewitz Potato Collection of the IPK Gene Bank, Leibniz Institute of Plant Genetics and Crop Plant Research, Germany) was identified, which carries two resistance genes *Rpi-blb*1 and *Rpi-blb*3 (data under publication).

Potato cultivars (Agave, Baltica, Delikat, Quarta and Rasant) and *blb*41 were multiplied *in vitro* and used for protoplast isolation and electrofusion (Rakosy-Tican *et al*., 2015). Both symmetric and asymmetric somatic hybrids were regenerated as it was demonstrated by flow cytometry, molecular markers (SSR, AFLP) and cytogenetic studies (Rakosy-Tican *et al.*, 2015) (Fig. 1). Moreover, only two combinations of potato cultivars with *blb*41 i.e. Delikat and Rasant were fertile and by embryo rescue back-cross progenies were successfully produced (Fig. 1). Fertile somatic hybrids were further studied by using gene specific molecular markers and two resistance tests, leaf detached assay (DLA) and field resistance (area under disease progress curve – AUDPC) to identify resistant somatic hybrid clones. Finally, seven somatic hybrids and BC_1 clones were characterized as carrying both *Rpi-blb* genes transferred from the wild *blb*41 accession and expressing phenotypically the resistance trait in greenhouse and field (data under publication). Some of the somatic hybrids were also evaluated by a laboratory bioassay for CPB resistance. It was demonstrated that many of the somatic hybrids and their progenies are resistant to CPB (Thieme et al. 2014). Further on *in vitro* selection on PEG containing medium and on a phenotyping semi-automated platform (SSDS-HAS Szeged Hungary - http://www.plant-phenotyping-network.eu/eppn/has_hungary) for tolerance to mild drought stress, demonstrated drought tolerance of the wild species accession and some of derived somatic hybrids and BCs progenies (Dénes, 2015; Dénes *et al*., 2016 – abstract in this volume). These examples demonstrate the successful combination of somatic hybridization based on mesophyll protoplast electrofusion, with marker assisted selection (MAS), cytogenetics (classical and molecular techniques), *in vitro* micropropagation and cloning of resistant wild species accession, embryo rescue for back- crossing and further introgression of resistance genes, *in vitro* stress selection for drought tolerance and semi-automated phenotyping and finally but most important the analysis of resistance in the laboratory, greenhouse and field. All these tools allowed the selection of pre-breeding material combining multiple genes (two major *Rpi-blb* genes) for late blight resistance, with unknown factors inducing CPB resistance and biochemical strategies (data to be published) for drought tolerance.

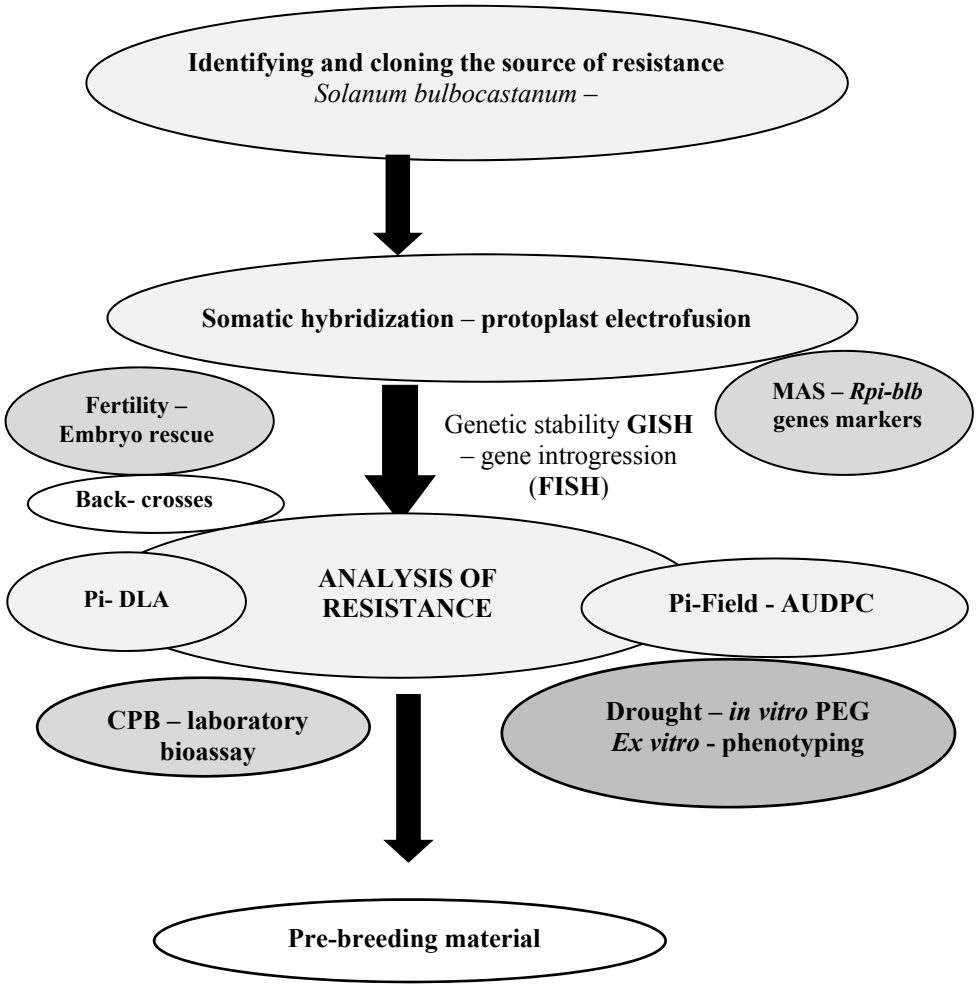

Figure 1. Schematic representation of combinatorial biotechnology applied to improve potato resistance to late blight, Colorado potato beetle and tolerance to drought stress, by using the wild incongruent species *Solanum bulbocastanum* (accession GLKS *blb*41)

The wild diploid species *Solanum chacoense*, sexually compatible with potato, used as a source of resistance to Colorado potato beetle – combining MMR deficiency with somatic hybridization

The second case study presented here involves another diploid wild species, *Solanum chacoense* (*chc* HL), which is a 2 EBN (endosperm balance number – Johnston *et al*., 1980), and hence sexually compatible with potato tetraploid cultivars, but the classical scheme takes a very long time and needs many steps to transfer only partially resistance from this species into potato gene pool.

One example of potato cultivar incorporating after many crosses genetic material from *chc* is cv. Pannonia (Cernák *et al.,* 2008). This is why we chose somatic hybridization by mesophyll protoplast electrofusion. In the first step the accession of the wild species was chosen i.e. the highest leptine producer (HL) accession PI 458310 (NPGS Sturgeon Bay, USA), and cloned *in vitro*. *S. chacoense* was also transformed, by *Agrobacterium*-mediated gene transfer, with the gene *Atmsh*2 – the key gene involved in mismatch repair of DNA (MMR) isolated from *Arabidopsis thaliana* and used as antisense (AS) or dominant negative mutant (DN) form (Rakosy-Tican *et al*., 2004). Both transgenic, MMR deficient, or wild type *chc* were used in the somatic hybridization experiments (Fig. 2; Rakosy-Tican *et al*. under publication). Regenerated plants were multiplied *in vitro*, analyzed for 'mutator' phenotype and microsatellite instability (MSI) (data under publication). The hybrid status of all somatic hybrids was analyzed by using flow cytometry, cytogenetic indirect (number of chloroplasts in guard cell) or direct methods (chromosome counts), molecular cytogenetics (GISH) and RAPD markers for leptine biosynthesis. Leptines I and II are very specific glycoalkaloids synthetized only in the green tissues of *S. chacoense*, from common glycoalkaloids as solanine and chaconine. These glycoalkaloids are well known as repellent and toxic for CPB (Flanders *et al*., 1992). Three RAPD markers were described by other authors (Bouarte-Medina *et al*., 2002), but only one marker was giving positive results in our studies, OPT-20 (Molnar *et al*., – data under publication). Moreover, to date there were two mechanisms known to interact with the voracious pest, CPB, leptines and glandular trichomes (Pelletier *et al*., 2011; Mărgineanu *et al.,* 2014; 2015). Besides leptines, we also analyzed the presence and density of the glandular trichomes considering them as the first level of interaction between the insect pest, in our case CPB, and the plant leaf (Fig. 3). Since, not only glandular trichomes but also non-glandular ones can interfere with the insect, at least as mechanical barrier, we also studied this type of trichomes (Fig. 3, Mărgineanu *et al.,* 2014; 2015). In order to reveal first the resistance of somatic hybrid plants with wild type *S. chacoense* to CPB, two assays were applied: the choice test and a laboratory bioassay.

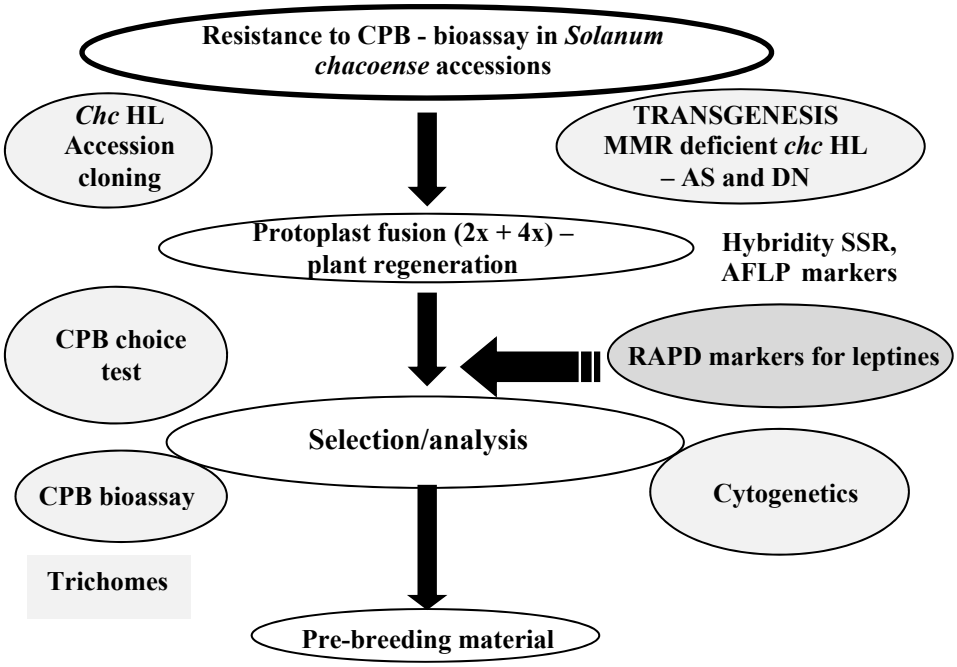

Figure 2. Schematic representation of the combinatorial biotechnology applied in improving resistance to Colorado potato beetle by using transgenesis for MMR deficiency, somatic hybridization and specific molecular markers for leptine biosynthesis along with different techniques for somatic hybrid plant characterization.

The choice test, also used by Cheng *et al*. (1995), allows the adult beetle to choose between one of the parent's leaf or one somatic hybrid leaf. After two hours the preference of the beetle can be quantified by measuring the quantity of leaf which was eaten by the beetle. The test shows the repellence of the wild species and its derived somatic hybrids. Moreover, the laboratory bioassay is done on 25 larvae, grown and fed with leaves of the parents and derived somatic hybrid plants. In this test, the viability, growth curve of larvae, the pupae formation and development of adults as well as adult fertility are assayed. By using those two tests, it was possible to identify somatic hybrids with both antibiosis and antixenosis effects on CPB (Molnar *et al.,* under publication) and it was shown that the proportion of resistant somatic hybrids involving *S. chacoense* with MMR deficiency is higher than when wild type *chc* was used. Those data sustain the hypothesis that MMR deficiency increases homeologous recombination between the two related species.

Moreover, the somatic hybrids with MMR deficiency were also phenotyped after a preselection *in vitro* on polyethylene glycol (PEG) 6000 containing media. It was surprising that although the parental species were not tolerant to drought, some of the somatic hybrids with MMR deficiency were tolerant and accumulated the same biomass as the control plants under normal watering. These results open new possibilities for the exploitation and integration in breeding of these somatic hybrid clones.

Figure 3. The interaction between Colorado potato beetle larvae and one somatic hybrid leaf shows the first level of defense based on glandular (black arrow) and non-glandular (open arrow) trichomes, as well as accumulation of anthocyanins on abaxial leaf surface (purple color).

Combining transgenesis with *in vitro* stress selection for the transfer of PVY resistance and tolerance to drought

Another example of combining genetic transformation with *in vitro* stress selection allowed us to select three clones integrating the marker free hairpin construct of coat protein gene from potato virus Y (PVY) with tolerance to drought,

after two steps of selection *in vitro* on media supplemented with PEG 6000, first at callus and then at plant level on increased concentrations of PEG (5, 10 and 15%) (Rakosy-Tican *et al.,* 2010; Mustaţă *et al.,* 2014). In this strategy, first the transformation procedure based on *Agrobacterium*-mediated protocols and using *gfp* reporter gene and *npt*II selectable marker gene were used to improve gene transfer and select the potato cultivars with the best regeneration abilities (Fig. 4, Rakosy-Tican *et al.*, 2007).

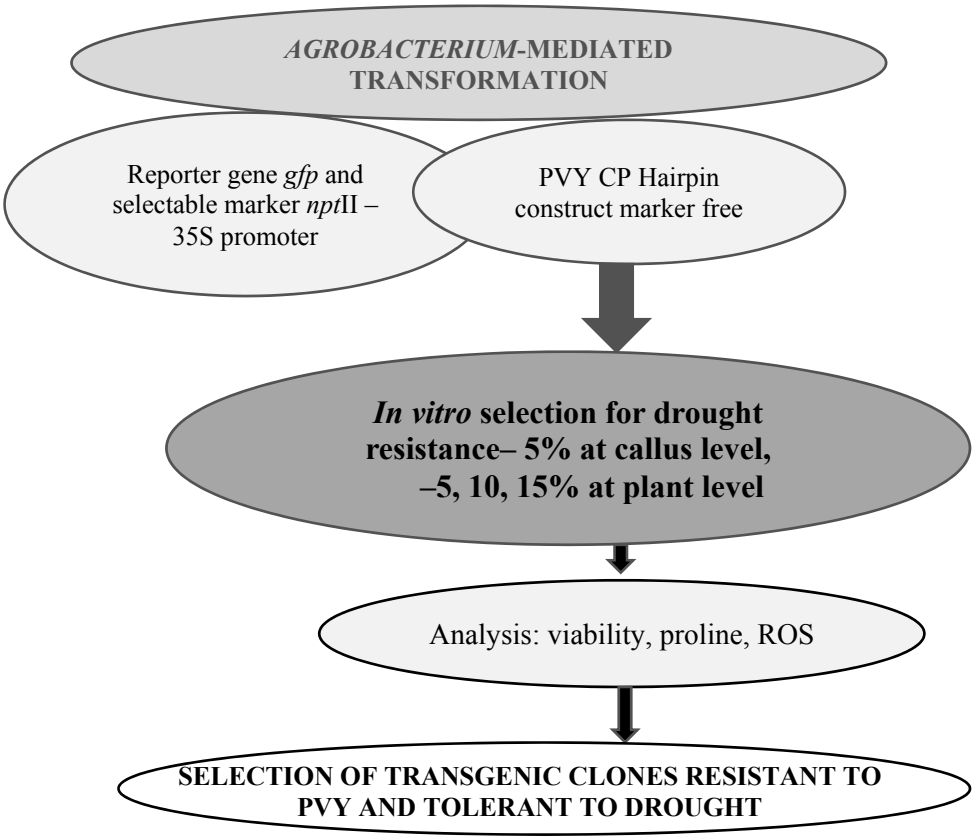

Figure 4. Combining transgenesis with a marker-free hairpun construct for PVY resistance and *in vitro* stress selection for drought tolerance in potato.

Then potato cvs. Baltica and Désirée were transformed by using a marker-free construct with hairpin structure containing PVY coat protein genes separated by an intron (Rakosy-Tican *et al.,* 2010). The transgenic clones were analyzed by PCR to confirm they integrated the hairpin marker-free construct and then were multiplied and stored *in vitro* as minitubers. The transgenic clones were then selected *in vitro* by

transferring internodes on media containing 5% PEG 6000 (Mustaţă *et al.*, 2014). The regenerated plants on PEG containing media, as well as controls were further selected on increased concentrations of PEG: 5, 10 and 15% PEG 6000. Plant regeneration and viability, proline biosynthesis and H_2O_2 were determined and three clones integrating PVY construct and showing tolerance to drought were selected (Mustaţă *et al*., 2014).

Conclusions

The case studies presented in this mini-review on the application of combinatorial biotechnology for potato improvement shows how different biotechnological tools like somatic hybridization or transgenesis can be combined with *in vitro* techniques, molecular analysis including marker assisted selection, cytogenetics and phenotyping (for resistance traits) for the transfer and integration of multiple genes and traits into potato crop. This new concept opens new ways for the integration of all modern tools of genomics, phenomics and metabolomics with *in vitro* technologies for better improvement of crops.

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Theoretical and Biotechnological Approaches in the Institute of Biology Bucharest Between 1975 - 2015 based on Plant Cell and Tissue Culture Technology

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SUMMARY. In the present paper we highlight the main research stages of the laboratory of plant cell and tissue culture at the Institute of Biology, Bucharest from the moment of its beginning until present.

Keywords: electrostimulation, *in vitro* plant conservation, plant transformation, protoplasts, secondary metabolites in *in vitro* cultures.

The year 1975 marked the beginning of research on plant biotechnology at the Institute of Biology Bucharest by the study of *in vitro* culture of plant cells. The team of researchers was coordinated by Dr. Aurelia Brezeanu within the newly created Laboratory of Plant Morphogenesis and Genetic Engineering, at the initiative of AcaD.G. Zarnea.

During the first stage (1975-1983) several basic research directions were started, that contributed to a better understanding of the biology of the plant development: plant *in vitro* cytodifferentiation and morphogenesis (Badea *et al*., 1982; Brezeanu and Davis, 1979; Davis *et al.,* 1976, Davis *et al.,* 1979; Brezeanu, 1980; Brezeanu, 1983; Brezeanu *et al*., 1980; Brezeanu *et al*., 1981; Brezeanu *et al*., 1982b; Mirancea and Brezeanu, 1986; Pătraşcu, 1981); protoplasts technology, a pioneer activity in Romania (Brezeanu *et al*., 1982a; ***, 1984). The studies were focused on microbial protoplasts (yeasts, bacteria) (Anghel *et al*., 1985; Anghel *et al*., 1989a; Zarnea *et al*., 1988) and plant protoplasts, including isolation of protoplasts (Brezeanu and Roşu, 1984; Cornea *et al*., 1992; Pătraşcu and Brezeanu, 1989), their chemical or electric fusion (Anghel *et al*., 1989b; Cornea *et*

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al., 1993). Another pioneering research direction during that period was the study of androgenesis/gynogenesis as tools for plant breeding (Badea and Raicu, 1982; Badea and Raicu, 1983; Badea *et al*., 1985; Raicu and Badea, 1979).

The 2nd stage (1984-1989) was more focused on biotechnological applications using plant cell and tissue cultures. A complex network of scientific cooperation was established with research institutes, experimental stations and universities. Important results were obtained regarding: clonal multiplication (Brezeanu *et al*., 1982b; Iordan *et al*., 1981a; Iordan *et al*., 1984; Roşu and Anghel, 1985), production of virus-free plants (Brezeanu *et al*., 1994a; Pătraşcu *et al*., 1985).

The 3rd stage (1990-1998) was mostly devoted to fundamental research topics including: gene transfer in plant cells mediated by bacterial plasmid vectors using direct (electroporation and electrotransfection) or indirect methods (co-cultivation) (Brezeanu *et al*., 1993b, Brezeanu *et al*., 1994b; Brezeanu *et al*., 1999a; Călin *et al*., 1996; Fologea *et al*., 1998; Iordan-Costache *et al*., 1996; Pătraşcu-Călin *et al*., 1991); electrostimulation in *in vitro* systems of cytodifferentiation and morphogenesis by using weak electric fields (Cogălniceanu *et al*, 1998b; Cogălniceanu *et al*, 1998c; Cogălniceanu *et al*., 2000b; Radu *et al*., 1994); the effect of hipersalinity (Holobiuc *et al*., 1999) and high aluminum concentration on plant genome (Badea *et al*., 1994b); the study of chimaera gene expression (Badea *et al*., 1999c) involved in pollinic (Badea *et al*., 1994a) and somatic embryogenesis (Badea *et al*., 2000) in *in vitro* plant cultures; the role of endo- and exogenous factors in expressing the androgenetic and/or gynogenetic potential thus regenerating haploid plants used in programs of genetic improvement production of haploid plants (Badea and Răduţoiu, 1999), somaclonal variability in *in vitro* cultures (Badea *et al*., 1991; Badea *et al*., 1999a) and *in vitro* stress selection (Badea *et al*., 1999b).

The 4th stage (1999-2005) continued fundamental research directions, such as apoptosis and senescence in plants using *crown gall* tissues infected by *Agrobacterium tumefaciens* (Brezeanu *et al*., 2001; Brezeanu *et al*., 2002; Maximilian *et al*., 2003; Maximilian and Brezeanu, 2004). In the international context focused on the phytohormonal control of plant development, our laboratory initiated studies on several factors that have a potential role as signals: aliphatic polyamines (Antofie and Brezeanu, 2000b; Brezeanu *et al*., 2000; Carasan and Brezeanu, 2002; Dumitrescu and Carasan, 2002), salicylic acid (Antofie *et al*., 2000; Antofie *et al*., 2003), external electric current (Cogălniceanu, 2006, Cogălniceanu *et al*., 2001b) and on biochemical markers (Carasan and Antofie, 2007; Carasan *et al.*, 2004a, Cogălniceanu *et al*., 1998a; Voichiţă *et al*., 2013; Voichiţă *et al*., 2014) involved in the morphogenetic processes in experimental *in vitro* systems, both in test and recalcitrant plant species (Badea and Săndulescu, 2001). The mechanisms

that induce stress tolerance (Antofie et al., 1999; Brezeanu, 2009; Carasan, 2009; Carasan *et al*., 2003; Cogălniceanu and Brezeanu, 2000) were studied by simulating in *in vitro* conditions using stress inductors like PEG (Dumitrescu, 2005), mannitol (Mitoi *et al*., 2009), sorbitol (Dumitrescu and Holobiuc, 2005), and specific mediators (ABA) (Carasan *et al*., 2004b; Carasan et al., 2005; Dumitrescu and Carasan, 2006), thus isolating cell line and/or tolerant individuals (Brezeanu, 2009, Brezeanu *et al*., 2002). Another major research direction was the potential utility of *in vitro* cultures to proliferate and to biosynthesize secondary metabolites of biotechnological interest. Thus, the study of *Vitis vinifera* callus resulted in the isolation of a *long-term* cell line highly proliferative and with a significant production of compounds valued by the pharmaceutical industry: anthocyanins, pycnogenol and resveratrol (Brezeanu *et al*., 1999b; Lupşea and Brezeanu, 1999; Cogălniceanu *et al*., 2000a; Matienco *et al*., 2004).

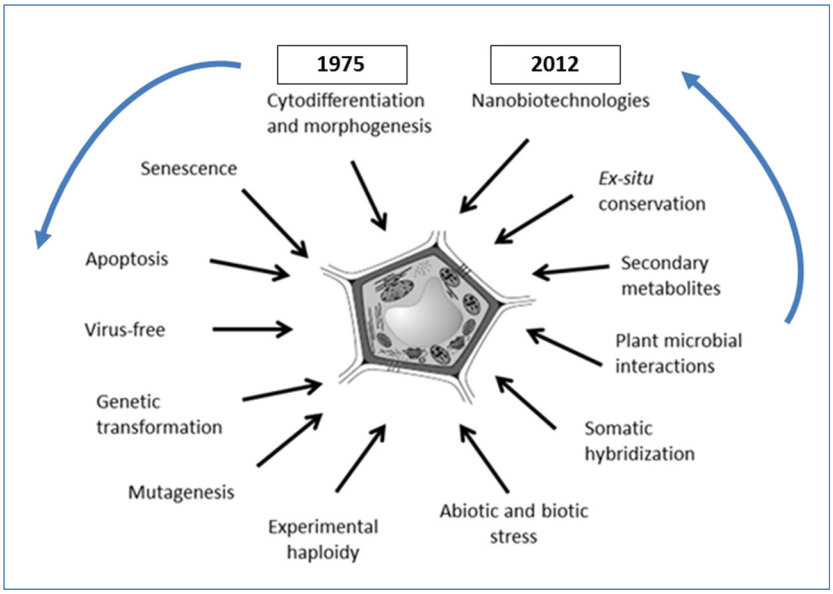

Figure 1. Schematic diagram of the main theoretical and biotechnological research topics at the Institute of Biology Bucharest, during the period 1975-2015 based on plant tissue and cell culture technologies.

The present stage includes several directions of research. One is focused on biodiversity conservation, by characterizing the *in vitro* development of protected plant species of Romania for their *ex situ* preservation (Blîndu *et al*., 2010; Cogălniceanu and Cogălniceanu, 2010; Holobiuc, 2007; Manole-Păunescu, 2014; Păunescu, 2008; Păunescu, 2009). Our studies have established micromultiplication procedures and

medium-term (Catană *et al*., 2010b; Holobiuc and Blîndu, 2006; Holobiuc *et al*., 2009c; Holobiuc *et al*., 2010) or long-term (Banciu and Cristian, 2015; Holobiuc and Catană, 2012; Holobiuc *et al.*, 2009a) storage conditions. A large variety of plant taxa have been introduced in vitrocultures: bryophyte (Brezeanu *et al.,* 2008; Brezeanu *et al.,* 2009; Cogălniceanu and Stoiculescu, 2007; Cogalniceanu, 2014), lichens (Banciu and Cristian, 2015; Cristian *et al*., 2013; Voicu and Brezeanu, 2008a; Voicu and Brezeanu, 2008b), ferns (Aldea *et al*., 2013; Banciu *et al.,* 2009a; Brezeanu and Banciu, 2009; ***, 2011) and a large number of vascular plants, from many families. Plant-microbe interactions (Cogălniceanu *et al*., 2010; Helepciuc *et al*., 2008; Helepciuc *et al*., 2014, Maximilian and Carasan, 1999; Maximilian *et al*., 1998; Maximilian *et al*., 2001, Maximilian *et al*., 2002), plant *in vitro* systems for secondary metabolites biosynthesis (Mihai *et al*., 2009; Mihai *et al*., 2010; Mihai *et al*., 2013) and plant *in vitro* systems involved in bio-nano-technologies (Matei *et al*., 2015; Mitoi *et al*., 2013) are our ongoing research topics.

Numerous plant species from different taxa have been studied over time in our lab from which we mention the most representative (species listed in alphabetical order): *Alyssum borzaeanum* Nyar. (Păunescu, 2007), *Andryala levitomentosa* (E. Nyar.) P.D. Sell. (Păunescu and Vântu, 2002), *Armeria maritima ssp. alpina* Willd (Blîndu and Holobiuc, 2006), *Arnica montana* L. (Panciu *et al*., 2014), *Artemisia tschernieviana* Besser (Holobiuc and Blîndu, 2006-2007), *Artemisia alba* Turra (Holobiuc and Blîndu, 2006-2007), *Astragalus pseudopurpureus* Guşuleac (Holobiuc *et al.*, 2005), *Bucegia romanica* Radian (Brezeanu *et al*., 2008; Cogălniceanu, 2014), *Campanula carpatica* Jacq. (Holobiuc and Blîndu, 2006-2007), *Campanula polymorpha* Witasek (Păunescu, 2010), *Campanula romanica* Săvul. (Manole and Banciu, 2014), *Centaurea pontica* Prodan & Nyar (Voichiţă *et al*., 2014), *Cerastium transsilvanicum* Schur. (Păunescu, 2005), *Cetraria islandica* (L.) Ach. (Voicu and Brezeanu, 2008b, Cristian *et al*., 2013), *Citrullus lanatus* (Thunb.) Matsum. & Nakai (Roşu *et al*., 1987), *Cladophora vagabunda* (L.) Hoek (Bavaru *et al.,* 2000), *Convolvulus persicus* L. (Holobiuc *et al*., 2015; Voichiţă *et al*., 2013) , *Datura inoxia* Mill. (Badea and Răduţoiu, 1999), *Dianthus callizonus* Schott et Kotschy (Păunescu and Holobiuc, 2003), *Dianthus glacialis* Haencke *subsp gelidus* (Schott, Nyman et Kotschy) Tutin (Holobiuc *et al*. 2009a; Holobiuc *et al*., 2010), *Dianthus nardiformis* Janka. (Holobiuc *et al*., 2009b), *Dianthus spiculifolius* Schur. (Holobiuc *et al*. 2009; Holobiuc *et al*., 2010), *Dianthus tenuifolius* Schur. (Păunescu and Holobiuc, 2005), *Dianthus trifasciculatus* Kit. (Holobiuc *et al*., 2014), *Doronicum carpaticum* (Griseb. et Schenk) Nyman (Holobiuc and Blîndu, 2006-2007), *Doronicum orientale* Hoffm. (Holobiuc and Blîndu, 2006-2007), *Draba dorneri* Heuff. (Catană *et al*., 2013), *Ecballium elaterium* (L.) A. Rich. (Voichiţă and Brezeanu, 2005), *Erigeron nanus* Schur. (Catană *et al.,* 2010a), *Fragaria X Ananassa* Duch. (Cogălniceanu *et al*., 2010), *Gardenia jasminoides* J. Ellis (Antofie and Brezeanu, 2000b; Antofie *et al*., 2000; Antofie *et al*., 2003), *Gentiana lutea* L.

(Holobiuc *et al*., 2008a; *Holobiuc* and Catană, 2012; Holobiuc, 2015), *Helianthus annuus* L. (Brezeanu, 1980; Brezeanu, 1983), *Hieracium pojoritense* Woloszczak (Holobiuc *et al*., 2004), *Iris halophila* Pallas (Holobiuc and Blîndu, 2006-2007), *Marsilea quadrifolia* L. (Banciu *et al*., 2009a; Brezeanu and Banciu, 2009), *Medicago sativa* L. (Badea *et al*., 1994b; Holobiuc *et al*., 1999), *Nicotiana tabacum* L. (Brezeanu *et al.,* 1982a; Cogălniceanu and Brezeanu, 1995; Cogălniceanu *et al*., 1998a; Mirancea and Brezeanu, 1986), *Ocimum basilicum* L. (Brezeanu and Cogălniceanu, 2005), *Oryza sativa* L. (Brezeanu and Davis, 1979), *Papaver alpinum* L. ssp. *corona-sancti-stefani* (Zapal.) Borza (Catană and Holobiuc, 2015), *Petunia x hybrida* Vilm. (Antofie *et al*., 2004; Carasan and Antofie, 2007), *Populus* (Iordan *et al*., 1984; Iordan *et al*., 1996), *Primula halleri* J.F. Gmelin (Holobiuc and Blîndu, 2006, Holobiuc and Blîndu, 2007), *Prosopis juliflora* (Sw.) DC. (Cogălniceanu *et al*., 2001a), *Pseudevernia furfuracea* L. (Banciu and Cristian, 2015), *Quercus robur* L. (Iordan *et al*., 1981b), *Ruscus aculeatus* L. (Banciu *et al*., 2009b; Manole and Banciu, 2015), *Salix* (Iordan and Brezeanu, 1985), *Scilla autumnalis* L. (Banciu *et al*., 2010), *Sequoia sempervirens* (Lamb. ex D. Don) Endl. (Stoiculescu *et al*., 2009), *Serratula bulgarica* Acht. et Stoj. (Manole-Aiftimie *et al.,* 2013), *Solanum melongena* L. (Roşu and Anghel, 1985), *Solanum tuberosum* L. (Blîndu and Holobiuc, 2005; Călin *et al*., 1996; Pătraşcu *et al*., 1985), *Spathiphyllum patinii* (R.Hogg) N.E.Br. (Antofie and Brezeanu, 2000a; Antofie and Brezeanu, 2003), *Stevia rebaudiana* (Bert.) Bertoni (Călin and Brezeanu, 1997), *Syngonium podophyllum* Schott (Antofie and Brezeanu, 2004; Antofie *et al*., 1999), *Triticale* (Verzea *et al*., 1994), *Triticum aestivum* L. (Angheluţă *et al*., 1997; Badea *et al*., 1991), *Triticum monococcum* L. (Davis *et al*., 1979), *Ulva rigida* C. Agardh (Bavaru *et al*., 2000), *Usnea barbata* L. (Voicu and Brezeanu, 2008a; Brezeanu and Voicu, 2008), *Veronica multifida ssp. capsellicarpa* (Dubovik) A. Jelen (Holobiuc *et al*., 2006; Holobiuc *et al*., 2008b), *Vitis vinifera* L. (Brezeanu *et al*., 1980; Brezeanu *et al*., 1993a), *Xanthoria parietina* (L.) Th. Fr. (Voicu and Brezeanu, 2008b), *Zea mays* L. (Maximilian and Carasan, 1999; Maximilian *et al.,* 2000).

Our laboratory has published during this period 15 books and book chapters and about 1000 scientific papers, both at national and international level. Under the scientific supervision of Dr. Aurelia Brezeanu over 42 doctoral theses in the field of cell biology and plant biotechnologies were finalized.

The scientific recognition and importance of our results was highlighted by the medals and prizes received:

International awards:

- **The Prize of the Academy of Sciences of the Moldova Republic**, 2004, for the book: *Carpoculture in vitro. Nonmorphogenetic pathway* (Matienco *et al*., 2004)

- **The Silver Medal at the Geneva Inventions Contest**, 2006, for the patent: *Carbonic material and obtaining procedure for anthocyanin pigment biosynthesis* (Hristea *et al*., 2005)

- **The highest Prize, *Fritzphil,* at the Scientific International Film Festival in Argentina**, 1986, for *Buds*, a scientific documentary film produced by Sahia Film Studios in 1985, with the director Mircea Popescu and scientific consultant Dr. Aurelia Brezeanu

National awards:

- ***Emil Racoviţă*** Prize *(Romanian Academy),* 1982, for the atlas: *Ultrastructure of the Plant Cell* (Anghel *et al*., 1981)

- ***Ion Ionescu de la Brad*** Prize *(Romanian Academy)*, 2012, for the book: *Conservarea Geo- şi Biodiversităţii şi dezvoltarea Durabilă în Ţara Haţegului-Retezat* (Mitoi and Blîndu, 2010)

- ***Ion Haşeganu*** Prize *(Romanian Horticulture Society),* 2013, for the book: *Utilizarea experimentală a elicitorilor fungici pentru imunizarea plantelor contra putregaiului cenuşiu* (Matei *et al*., 2011)

In the 40 years of scientific activity the following researchers worked in the field of plant biotechnology at the Institute of Biology Bucharest: ALDEA Florentina, ANGHELUŢĂ Rodica, ANTOFIE Maria Mihaela, AVRAM Dorina, BADEA Elena Marcela, BANCIU Crisitan, BREZEANU Aurelia, CATANĂ (BLÎNDU) Rodica, CĂLIN (PĂTRAŞCU) Alexandrina, CÂRCIUMĂRESCU Doina, COGĂLNICEANU Gina, COMAN Ion, CORNEA Petruţa Călina, CUCU (CIUCU) Natalia, DUMITRESCU Rodica, GREGORIAN Liliana, HELEPCIUC Florenţa Elena, HOLOBIUC (LUPUŞANSCHI) Irina Mihaela, IORDAN (COSTACHE) Margareta, LUPŞEA Simona, MANOLE (PĂUNESCU) Anca, MAXIMILIAN (VOICHIŢĂ) Carmen, MIHAI (STOICULESCU) Raluca, MIRANCEA Dorina, MITOI (CARASAN) Monica Elena, ROŞU Ana, SAVU Lorand, SCRIPCARU Atena, VASSU - DIMOC Tatiana, VĂTAFU Ion, VOICU (CRISTIAN) Diana, ZAMFIR Medana.

The results briefly presented in this paper highlight the main aspects of the research topics in the Institute of Biology Bucharest in the fascinating field of plant cell and tissue culture over the 40 years of existence.

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Genetic Stability Assessment of *in Vitro* Plants by Molecular Markers

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SUMMARY. Genetic stability of *in vitro* plants should be assessed in order to develop proper programs of conservation. Such conservation programs could be developed only after evaluation of genetic variability in the natural populations and the genetic stability or somaclonal variability after conservation. In case of valuable economic plants as fruit trees, vegetables, medicinal plants, the natural variability from populations should be preserved as sources of new cultivars or variations. In order to preserve endangered, vulnerable or endemic plants it is very important to evaluate the genetic variability in natural populations and to preserve this variability as well as individuals or habitats. Genetic variability or stability after plant conservation is usually assessed by DNA-based molecular markers as RAPD, SSR, ISSR, SRAP, RFLP and AFLP.

Keywords: conservation, genetic fidelity, *in vitro* plants, micropropagation, molecular markers.

Introduction

Plant cell and tissue culture became a versatile tool for rapid propagation and biomass production of valuable species. *In vitro* culture of plant cell, tissue and organ is associated with differences in physiological, epigenetic and genetic quality, namely, absence or lack of organogenic potential (recalcitrance), hyperhydricity (vitrification) and somaclonal variation. All of these phenomena are dependent of genotype and culture conditions and affect the practical application of tissue culture in plant propagation and genetic manipulation (Teixeira da Silva *et al.*, 2007).

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Usually by *in vitro* culture a huge number of somaclone could be obtained in short time and space. Multiplication of elite genotypes follows the preservation of the valuable genotype, thus the somaclonal variation is not a desired event. Despite of this, in some instances, somaclonal variation could be a valuable source of variability in order to select new genotypes. Genetic variations occur in undifferentiated cells, isolated protoplasts or calli. Recent studies have revealed that cell or tissue cultures undergo frequent genetic changes (polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplifications and mutations) and these are also expressed at biochemical or molecular levels (Teixeira da Silva *et al.*, 2007).

Somaclonal variation in regenerated plants is generated during dedifferentiation and is uncontrollable and unpredictable in nature and most variation is of no apparent use. The occurrence of cryptic genetic defects arising *via* somaclonal variation in the regenerants can seriously limit the broader utility of micropropagation systems.

Clonal propagation and preservation of elite genotypes, selected by their superior characteristics, require high degree of genetic uniformity among the regenerated plants. The occurrence of somaclonal variation is a disadvantage for both *in vitro* cloning as well as germplasm preservation method, therefore, the investigation of genetic variability/stability of *in vitro* plants is extremely important. There are several strategies to ascertain the genetic variability or stability, each of them having merits and limitations (Alizadeh *et al.*, 2015). Techniques based on morphophysiological, biochemical and cytological approaches are mainly based on characters which can be affected by the *in vitro* manipulation, environment, and types of plant tissue, thus the differentiation of somaclonal variation is difficult to achieve. Despite of this, DNA-based molecular markers are a versatile tool in various fields of biology. The major advantage offered by DNA molecular markers is objective analysis, thus the results could be easily repeated and shared between laboratories. Molecular markers are used to monitor somaclonal variation, verify the genetic fidelity of micropropagated plants and to identify genotypes with the desired response to *in vitro* culture conditions.

In this paper we summarize the most used DNA-based molecular markers for assessment of stability of *in vitro* plants.

Analysis of genetic stability of in vitro plants

Various molecular techniques are used to check genetic stability and the lack of somaclonal variation in tissue derived plants as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Inter Simple Sequence Repeat (ISSR) and Sequence-related Amplified Polymorphism (SRAP).

Molecular markers as RAPD and ISSR are easy to use, cheap and requiring no previous sequence information, thus most of the studies showing the genetic stability of *in vitro* plants used these markers. SSR requires previous information about region containing repeated sequences, but they are also used in many studies because they are cheap.

Nevertheless, technically more demanding AFLP are also used relatively frequently (Teixeira da Silva *et al.*, 2007). Application of RFLP markers with appropriately chosen probes gives the possibility to assess the genetic fidelity of micropropagated plants (Abe *et al.*, 2002; Devarumath *et al.*, 2002).

Several somaclonal variations is of epigenetic nature and it could not be detected with conventional structural molecular markers. In this cases, markers as Methylation Sensitive Amplified Polymorphism (MSAP) (Jaligot *et al.*, 2003; Hao *et al.*, 2004), methylation-sensitive RFLP (Jaligot *et al.*, 2002) or gene expression approaches (Morcillo *et al.*, 2006) need to be used. It has been shown that tissue culture induces transposition of several transposable elements as well (Courtial *et al.*, 2001).

Among the numerous molecular markers available, in the last years the most used molecular markers for different applications including assessment of *in vitro* plants fidelity are RAPD, SSR and ISSR markers because they are cost effective and require low amounts of DNA (Zietkiewicz *et al.*, 1994). These type of markers were suitable for establishing genetic stability of several micropropagated plants in crops such as wheat (Ateş Sönmezoğlu, 2012). In this study several wheat accessions were characterized, which in particular are not morphologically identifiable. In potato, RAPD, ISSR, SSR and AFLP markers proved that *in vitro* culture is a safe method for conservation of potato microtubers to produce true-to-type plants (Tiwari *et al.*, 2013). RAPD markers were also used for assessment of olive *in vitro* micropropagated plants (Peyvandi *et al.*, 2013).

SSR markers were applied in tree analysis as well, as for *in vitro* plants characterization of *Populus tremuloides* (Rahman and Rajora, 2001) and *Pinus pinea* (Cuesta *et al.*, 2010). In case of white or pedunculated oak (*Quercus robur)* seedlings, epicormic, crown and micropropagated shoots from mature trees were analysed by SSR and RAPD markers and no intraclonal or interclonal polymorphism was detected *(*Barrett *et al.*, 1997). *In vitro* plants of *Robinia pseudacaccia* multiplied by axillary buds showed no variations in RAPD banding but SSR markers showed high level of mutations in somatic tissues (Shu *et al.*, 2003). Two species of *Albizia in vitro* plants were analysed by RAPD markers regarding their genetic uniformity in comparison with mother plants (Tudor, 2012). In case of jojoba, a slow growing plant, vegetative propagation is the alternative for multiplication of this species. Genetic stability of *in vitro* regenerated plants was assessed by RAPD and ISSR markers (Bekheet *et al.*, 2015).

Several medicinal and aromatic plants were also analysed by molecular markers. RAPD and SSR markers proved the genetic stability in micropropagated *Achillea millefolium* group and other 6 related species (Wallner *et al.*, 1996). The genetic stability of the *in vitro* plants of *Artemisia absinthium* was assessed using ISSR and SSAP molecular markers. Both markers were able to detect the somaclonal variations in the callus regenerated plants, while no variation was detected in the plants regenerated from the nodal explants. SSAP has been found to be more useful in detection of variability as compared to ISSR (Kour *et al.*, 2014).

ISSR analysis was effective to eliminate the somaclonal variant in *in vitro* leaf-derived horseradish plants (Rostiana *et al.*, 1999). Somaclonal variation was detected in tissue culture of sugarbeet by RAPD markers (Munthali *et al.*, 1996), but in cauliflower *in vitro* plants obtained by somatic embryogenesis, no somaclonal variation was detected by ISSR banding (Leroy *et al.*, 2000). Numerous molecular markers were used for detection of somaclonal variation in eggplant obtained by somatic embryogenesis (Kantharajah and Golegaonkar, 2004). AFLP analysis detected no variation in sunflower plants obtained by regeneration from apical or axillary shoots originating from pre-existing meristems (Hewezi *et al.*, 2003)

Molecular markers are also valuable tools for characterization of *in vitro* plants of fruit trees. Thus, RAPD and ISSR markers were used to prove the genetic fidelity of clonally propagated apple from adventitious buds (Modgil *et al.*, 2005). RAPD markers were also used to prove the genetic stability of somatic embryos of peach (Hashmi *et al.*, 1997), or lemon (Deng *et al.*, 1995). The paternity of embryo culture-derived cherry plants was confirmed with RAPD markers (Hormaza, 1999). RAPD and SSR markers detected repeatable somaclonal variation in micropropagated kiwi plants (Palombi and Damiano, 2002). *In vitro* micropropagated plants of *Morus alba* were analysed by RAPD and ISSR markers for genetic stability assessment (Saha *et al.*, 2016).

Analysis of genetic stability of cryopreserved plants

In vitro plants of medicinal plants species *Dioscorea floribunda* were genetically stable, only 1 polymorphism band was obtained from over 5000 RAPD bands (Aruja *et al.*, 2002).

Genetic stability of several fruit tree plants cryopreserved was also proved by molecular markers. Thus, 15 cryopreserved plants of *Pyrus pyraster* derived from single buds were used for genetic analysis with RAPD and SSR and no polymorphism was detected between cryopreserved plants and the original genotype (Condello *et al.*, 2009). ISSR markers were used to prove genetic stability in apple (Yi *et al.*, 2015). In apricot, genetic stability of cryopreserved shoot tips was demonstrated by RAPD markers (Soliman, 2013).

Analysis of genetic stability of endangered and endemic plants preserved by in vitro culture

Molecular markers were used for analysis of genetic stability of several endangered or rare species. Thus, in *Zingiber rubens* the genetic uniformity of all regenerants was demonstrated by RAPD and ISSR markers (Mohanty *et al.*, 2011). *Swertia chirayita* a medicinal herb was multiplied for a span of three and a half years and the genetic fidelity of *in vitro* plants was analysed by ISSR and SSR markers (Joshi and Dhawan, 2007). These markers showed the homogenous amplification patterns in all regenerants. In *Dictyospermum ovalifolium* ISSR markers showed the genetic similarity of over 1650 plants obtained *in vitro* (Chandrika *et al.,* 2008). RAPD markers were also used for investigation of stability *in vitro* plants of endangered *Dendrobium nobile* orchid species (Bhattacharyya *et al.*, 2014).

Several endangered or rare species of *Caryophyllaceae* were preserved by *in vitro* culture. Genetic stability after *in vitro* conservation and cryopreservation was shown by SSR and ISSR markers in *Dianthus giganteus* subsp. *banaticus* (Jarda *et al.*, 20014) and *D. spiculifolius* (Cristea *et al.*, 2014).

RAPD markers were used to assess the genetic variability of critically endangered *Draba dorneri* for their conservation (Catană *et al.*, 2013).

Conclusions

Genetic fidelity or variability of *in vitro* plants is extremely important to evaluate in order to develop proper conservation programs.

The most valuable tools for analysis of *in vitro* plants are DNA-based molecular markers. Among these, RAPD, SSR and ISSR markers are cheap and easy to use.

RAPD and ISSR makers do not need previous information about the targeted sequence, the primers could be used in different plants species. Despite of these, SSR markers need previous information about repeated sequences targeted in this analysis.

For genetic variability and fidelity assessment it is strongly recommended to use different types of molecular markers.

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The Micropropagation Potential and Regenerative Ability of Somatic Embryos of *Vitis Vinifera* ssp. *Sylvestris* (Gmel.) Hegi

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SUMMARY. In this paper are presented the results obtained with *Vitis vinifera* ssp. *sylvestris* accessions subjected to the *in vitro* micropropagation. For clonal propagation were used meristematic tissues (apexes and axillary buds) aiming to establish an efficient protocol for long-term conservation or for virus-free plant recovery (if viruses were detected) applied to accessions belonging to seven different populations. The *in vitro* development showed particular aspects and significant differences among wild populations regarding their competence for differentiation, the moment of differentiation in inoculated explants, the aspect of proliferative structures and the rates of multiplication. The same accessions were tested also for their competence in plant regeneration by either organogenesis or embryogenesis starting from somatic tissues.

Shoot regeneration and normal plants were obtained from petiole explants derived from *in vitro* grown shoots of the seven accessions. Callus induction was obtained with all genotypes on MS media supplemented with BAP and 2,4-D, or IBA, and the best direct adventitious shoot formation was obtained after transfer on medium supplemented with BAP and IAA.

With anther culture, the genetic factors proved to be essential for callus induction and promoting the embryogenic process. Three, out of seven accessions, only male individuals, responded to anther culture by regeneration through embryogenesis on all used media with similar results, forming normal embryos, abnormal embryos and whole plants.

Keywords: embryogenesis, organogenesis, plant regeneration, *Vitis*, wild grapevine accessions.

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Introduction

During the last hundred years, the habitat of *V. vinifera* ssp. *sylvestris,* as wild grapevines, has been reduced due to pathogens or intensive forests and rivers management. Today small populations were identified only in certain ecosystems in Europe, including Romania. The recent papers underlined that *V. vinifera* ssp. *sylvestris* is one of the endangered subspecies and its conservation in germplasm collections could be an important source of plant material useful for the introgression of adaptive traits in cultivated grapevines (Ocete *et al.*, 2011). The ampelographic studies on wild populations have offered valuable information on evolution of cultivated grapevine varieties (Lacombe *et al.,* 2003). The analyses at the molecular level revealed important clues on genetic relationship among cultivated and wild grapevine accessions, and about the degree of genetic variability which could be of great interest for breeders (This *et al*., 2006; Schneider *et al.*, 2009; Arroyo-Garcia and Revilla, 2013).

In our country, many populations with few individuals of wild grapevines were identified in almost all provinces, especially along the main rivers, in areas with high humidity (Pop, 1931; Teodorescu *et al*., 1966; Popa *et al*. 2009). Due to their potential importance as source of valuable genes for improvement the cultivated grapevines, the collecting and establishing *ex situ* collections is essential to protect these wild populations in their native areas, and to prevent their extinction. In a previous paper, were presented the morphological characterization based on OIV descriptors of the individual plants collected from some wild grapevine populations growing along the Danube River, providing valuable information (Popescu *et al*., 2013). The aim was to establish an *ex situ* collection with virus-free plant material and to use it as possible starting plant material for further breeding of grapevine cultivars and rootstocks.

In this paper is presented a synthesis of *in vitro* results obtained with *V. vinifera* ssp. *sylvestris* accessions regarding: a) the regeneration competence of new structures by organogenesis from meristematic tissues, or by embryogenesis from somatic tissues; b) the specific aspects of evolution and multiplication of vegetative structures through the two types of regeneration.

Materials and methods

a. Meristematic tissues culture. Plant material from 7 accessions belonging to different populations of *Vitis vinifera* L. ssp. *sylvestris* was harvested from *ex situ* collection (Table 1). The young shoots of 2-3 cm in length, with apex and 1-2 axillary buds were sterilized by immersion for 3-5 min in 5.2% sodium hypochlorite

(v/v) with a few drops of Tween-20, followed by rinsing with sterile distilled water. From each accession, 50 apexes and 50 axillary buds were inoculated on media with different combinations of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). Apexes and axillary buds were initiated on Murashige and Skoog (M&S) media supplemented with 0.5, 1.0 or 1.2 mg/l BAP and 0.1 or 0.5 mg/l IAA. In all types of media were added: 10 mg/l ascorbic acid, 3% sucrose, and 5.5% agar. After 30 days on the initial medium, the new regenerative structures were periodically transferred on fresh medium with reduced sucrose concentration (2%) and modified growth regulators composition (1.0 mg/l BAP and 0.5 mg/l IAA). The culture medium was autoclaved for 20 min at 121°C and 1.2 bars, and the culture vessels were maintained under 16/8 hours' photoperiod, a light intensity of 3,000 lux and 22±2°C temperature. Evaluation of the regenerative processes involved observations on morphological aspects of the new structures and statistical analysis by polynomial regression of the data regarding the number of new explants generated after each transfer, average height of shoots, and the rate of multiplication.

b. The petiole culture was established by using petioles excised from *in vitro* multiplied shoots. The cultures were grown in the dark for the first 5 weeks, on M&S media containing BAP and indole-3 butyric acid (IBA). After that, the petioles were periodically transferred on fresh medium supplemented with 1.0 mg/l BAP and 0.5 mg/l IAA for further shoot regeneration and maintained under 16/8 hours' photoperiod and 22±2°C temperature.

c. For anther culture, the inflorescences in tetrad microsporogenesis phase, were collected before anthesis, kept at 4ºC for 48 h and then sterilized with 0.1% mercury chloride. Under binocular microscope, the anthers were separated and inoculated on solid M&S medium containing 1.0 mg/l BAP, 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 3% sucrose. After six weeks of maintenance in darkness at 20°C, the viable anthers were transferred on fresh medium, a modified M&S medium (double quantity of magnesium sulfate and triple amounts of manganese sulfate and copper sulfate) with the same growth regulators and sucrose. Subsequently, the anthers with calli, or anther-derived calli were sub-cultured every 30 days on fresh modified M&S medium supplemented with 1.0 mg/l BAP, 0.5 mg/l IAA, and 2% sucrose for embryogenesis induction, the maintenance of embryogenic potential and the somatic embryos germination. During these growing phases, the explants were maintained at 16/8 hours photoperiod, and 22±2°C. All the culture media were adjusted to pH 5.8 with NaOH before autoclaving.

Table 1.

Sampling locations, source of plant material, their symbols

Number and symbols	2 individuals: Vs8dm, Vs9dm	1 individual: Vs1	2 individuals: Vs10, Vs3eb	2 individuals: Letea 1, Letea 2
Location	Stârmina Forest	Hinova	Greaca	Letea Forest
Geographic coordinates	22º46'14'' Long.E 44º30'01'' Lat.N	22°46'36" Long.E 44°32'26" Lat.N	26°20'21" Long.E 44°6'33" Lat.N	29° 53'7" Long.E 45°34'24" Lat.N
Altitude (m)	118	100	60	12

Results and discussions

a) The regenerative competence of new structures from meristematic tissues in wild grapevine plants

The results of experiments in which apexes and axillary buds were used as meristematic tissues for the micropropagation of wild grapevine accessions showed that the tested media, similar to those used for cultivated grapevines, were efficient in promoting regenerative processes and ensured the development and growth of vegetative structures. The data collected before each transfer showed different responses induced by different combinations of BAP and IAA. In all tested genotypes, the best results were obtained from explants initiated on medium containing 1.0 mg/l BAP and 0.5 mg/l IAA (Fig. 1). After the fifth passage, considered to be reliable to keep the genetic accuracy of plant material, a similar growth of the shoots from both type of meristematic explants and a very significant higher rate of multiplication from axillary buds were registered (Table 2). While Vs8dm accession showed the lowest rate of multiplication and very short shoots, the Vs9dm accession, harvested from the same location, expressed the best response to *in vitro* culture conditions, giving the highest length of shoots and the best rate of multiplication.

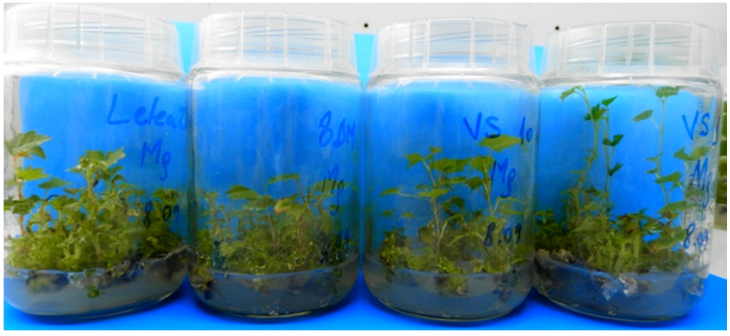


Figure 1. Expression of the rate of multiplication and the shoots raising, starting from axillary buds, with different *Vitis sylvestris* accessions: Letea 2, Vs8dm, Vs10 and Vs1.

Table 2.

Results data registered after five passages and seven months of *in vitro* culture.

Acce-ssion	Average height of shoots (cm) ± SD		Rate of multiplication after the 5th passage		Polynomial regression and coefficient of regresion	
	Apex (A)	Buds (B)	Apex (A)	Buds (B)		
Vs1	4.47±1.7	4.38±1.8	11.65	7.14	A: $y = 85,5x^2-408,6x+470,1$	$R^2 = 0,97$
					B: $y = 55,07x^2-272,7x+338,4$	$R^2 = 0,98$
Vs8dm	2.98±1.3	3.58±1.5	4.28	8.58	A: $y = 25,57x^2-114,7x+190,6$	$R^2 = 0,99$
					B: $y = 47x^2-189,9\ 263,6$	$R^2 = 0,97$
Vs9dm	4.35±1.3	4.11±1.9	12.32	31.04	A: $y = 22,45x^3-142,1x^2\ 256,1-48,33$	$R^2 = 0,99$
					B: $y = 56,1x^3-372,6x^2+796,4x-400,6$	$R^2 = 0,99$
Vs10	4.09±1.4	3.53±0.8	7.4	16.31	A: $y = 18,08x^2- 95,8x+172,1$	$R^2 = 0,98$
					B: $y = 17,8x^3-82,3x^2+116,1x+43,3$	$R^2 = 0,99$
Vs3eb	2.99±1.2	3.49±1.4	7.43	19.65	A: $y = 47,5x^2-213,6x+282,4$	$R^2 = 0,98$
					B: $y = 25,6x^3-138,9x^2+243,1x-38$	$R^2 = 0,99$
Letea 1	3.75±1.6	3.55±1.4	19.74	19.53	A: $y = 54,2x^3-421,5x^2+992x-560$	$R^2 = 0,98$
					B: $y = 36,6x^3-242,3x^2+490,8x-199,3$	$R^2 = 0,99$
Letea 2	2.41±1.1	3.70±1.1	16.32	21.12	A: $y = 27,9x^3-176,1x^2+338,1x-97,3$	$R^2 = 0,99$
					B: $y = 36,7x^3-236,1x^2+475,4x-187,3$	$R^2 = 0,99$
Mean	3.58±1.4	3.76±1.4	8.62	17.62		

The explants originated from the two individuals harvested from Greaca, responded to the *in vitro* culture either by differentiating of higher shoots from apexes (Vs10), or by a higher proliferation capacity from axillary buds (Vs3eb).

With the two accessions from Letea Forest were noticed differences regarding the moment of initiation the regenerative processes into the meristematic explants. Letea 2 showed a late response, more evident with apexes. After the third transfer on fresh media for multiplication of Letea 1 and Letea 2, the recorded data proved a similar behavior, these two wild grapevine accessions achieving almost the highest rates of proliferation.

These results confirmed previous observations about the *in vitro* development for wild grapevine accessions (Popescu *et al*., 2013) and proved the differences among investigated genotypes harvested from different populations, or from the same location, regarding the development of proliferative structures and the rate of multiplication. The two types of meristems (apical and lateral) contain cells with embryonic characteristics and expressed their cell totipotentiality by maintaining the cell division and by differentiating into shoots under *in vitro* culture (Cachiță-Cosma, 1987). The evolution of the two types of meristematic explants on the *in vitro* multiplication media, represented by polynomial regression, revealed a normal and adequate development for all tested wild grapevine accessions. The number of new buds and shoots increased progressively with each transfer on fresh medium, but each genotype responded by different rate of multiplication.

b) The competence of regeneration by organogenesis from petiole culture

The processes of regeneration from petiole were expressed in a similar way for all tested genotypes of wild grapevine, either by callus induction in a high proportion without any kind of differentiation on media with BAP and 2,4-D, or by necrosis of petioles on medium containing only BAP as growth regulator (data not shown). Tissue differentiation from the cut ends of petioles and *de novo* formation of meristematic structures from already differentiated tissues has proven to be dependent by genotype and interaction between genotypes and culture medium components. An interesting observation was that the colour and disposition of the calli on the inoculated petioles were the same for accessions harvested from the same location. The calli did not differentiate new structures and later on either necrosed, or maintained their proliferation.

The best percentages of new vegetative structures were obtained when the explants were inoculated on medium containing BAP and IBA. The callus induction was promoted during the first four weeks under darkness. The new regenerated structures (buds, or multiple small shoots) were noticed after transfer on a fresh medium and maintained under light photoperiod. In Table 3 are presented the results obtained after five months of *in vitro* culture of petiole explants.

Relative small amounts of IBA in combination with moderate quantities of BAP were efficient to induce the dedifferentiation at the cut ends and also to trigger the organogenesis processes. During the next four passages, new buds and multiple shoots were regenerated through direct organogenesis from one or both ends of the petioles. After six months from petiole initiation were obtained normal developed plants, able to be transferred to *ex vitro* conditions, from all tested accessions, and the recorded values varied between 5.3% for Vs10 and 17.2% for Letea 2.

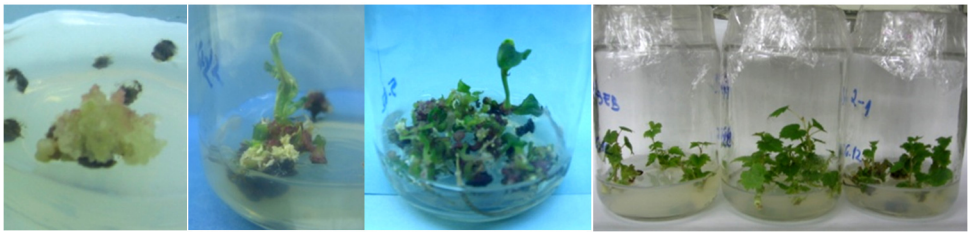
c) The competence for regeneration by somatic embryogenesis from anther tissues

The anther culture was tested three years consecutively using different media composition. The best results were obtained by applying the same procedure presented in Table 3. Somatic embryos and whole plants regeneration has been obtained only from individuals with male flowers, and only with three out of the seven tested genotypes (Fig. 2). Our results with wild grapevine confirmed those of Rajasekaran and Mullins (1983), who emphasized the importance of the type of flower for anther culture with grapevine and reported that genotypes with functionally male flowers express a higher embryogenic potential of anthers in comparison with genotypes having hermaphrodite or functionally female flowers.

Table 3.

The differentiation potential from somatic tissues obtained with tested wild grapevine accessions

Type of explant	Accession	Type of regeneration/ Specifications	% from initiated explants	Media composition
Petiole starting from 100 explants	Vs1	Brown calli at the cut ends Direct regeneration of buds	29.1 9.7	Initiation: M&S +1.5 mg/l BAP + 0.1 mg/l IBA + 3% sucrose Passages: M&S + 1.0 mg/l BAP + 0.5 mg/l IAA + 2% sucrose
	Vs8dm	White calli at one cut end Direct regeneration of shoots	87.5 6.3	
	Vs9dm	White calli at the cut ends Direct regeneration of shoots	44.2 10.5	
	Vs10	White calli at one or both cut ends Direct regeneration of shoots	87.2 5.3	
	Vs3eb	White calli at one or both cut ends Direct regeneration of shoots	32.9 7.9	
	Letea 1	White-brownish calli at the cut ends Direct regeneration of shoots	69.1 12.7	
	Letea 2	White-brownish calli at the cut ends Direct regeneration of shoots	79.3 17.2	
Anthers from indivi-duals with male flowers starting from 200 anthers	Vs1	Calli with embryogenic potential % Normal somatic embryos % Abnormal embryos Number of whole plant regenerated by embryogenesis / % of total somatic embryos	18 43.3 56.7 101 / 31.7	Initiation: M&S + 1.0 mg/l BAP + 0.5 mg/l 2,4-D + 3% sucrose Passages: modified M&S +1.0 mg/l BAP + 0.5 mg/l IAA + 2% sucrose
	Vs8dm	-	-	
	Vs9dm	Calli with embryogenic potential % Normal somatic embryos % Abnormal embryos Number of whole plant regenerated by embryogenesis / % of total somatic embryos	0.5 33.3 60.1 1 / 15	
	Vs10	-	-	
	Vs3eb	Calli with embryogenic potential % Normal somatic embryos % Abnormal embryos Number of whole plant regenerated by embryogenesis / % of total somatic embryos	3.5 73.2 26.8 23 / 56.1	
	Letea 1	-	-	
	Letea 2	-	-	



A B C D

Figure 2. Phases of somatic embryogenesis in anther culture with wild grapevine: A) callus formation; B) regenerated plant with secondary embryos; C) different type of somatic embryos; D) regenerated plant of Vs3eb, Vs9dm and Vs1.

Beside the genetic factors (genotype and type of flower), the induction of somatic embryogenesis from anther tissues could be optimized by media culture composition. Somatic embryogenesis appears to be dependent on the interaction between genotype, explants source and culture medium. Many experiments proved the necessary to develop specific regeneration protocols for each *Vitis* species and *V. vinifera* cultivar (López-Pérez *et al*., 2005). In our case, althought were tested different media composition (data not shown) for anthers initiation, callus induction and promoting the embryogenic process, only three accessions responded by regeneration through embryogenesis on all used media, and with similar results.

The callus derived from anther walls could maintain their proliferative capacity for a long period of time by periodic transfer on fresh media without any other result. But for further evolution is important only the embryogenic callus, which must be selected from the non-embryogenic one and transferred on specific media for embryogenesis induction, the maintenance of embryogenic potential and the somatic embryos germination.

From the total number of somatic embryos, a relative high proportion (between 56.7% for Vs1 and 73.2% for Vs3eb) was formed by abnormal embryos, without ability to develop into normal plants, such as: tube-shaped embryos, cup-shaped embryos, fused embryos, embryos with secondary embryos, or with more cotyledons (data not shown).

The normal somatic embryos became green and developed cotyledons and roots simultaneously. The number of whole plants regenerated from the embryos germinated on media containing moderate quantities of BAP and IAA depend on the number of clusters of normal embryos developed on transferred explants, and also on the proportion of cotyledonary embryos. Thus, a relative high proportion (between 15% for Vs9dm and 56.1% for Vs3eb) from the total somatic embryos regenerated from anther walls, developed into morphologically normal plants, physiologically able to be transferred to *ex vitro* condition (Table 3). The three genotypes, Vs1, Vs9dm and Vs3eb, showing embryogenic competence by somatic cells could be recommended for anther culture, and the other wild grapevine accessions as recalcitrant, or not able to respond to this type of *in vitro* culture.

Conclusions

Similar with cultivated grapevines, the wild grapevine (*Vitis vinifera* subsp. *sylvestris*) responded to *in vitro* culture by organogenesis (in the case of meristematic tissues, or petiole explants), or by embryogenesis (in the case of anther culture).

The genetic factors, culture media composition, added plant growth regulators, type of explants are important for regeneration from meristems and somatic tissues influencing the efficiency of these regenerative processes.

The meristematic structures (apexes and axillary buds) proved to be efficient for large-scale propagation of wild grapevine, aiming to regenerate healthy plants for long-term conservation.

The regenerative potential by organogenesis from petiole explants was comparable among tested genotypes, proving the strong correlation between regenerative competence of this type of explant and media composition supplemented with low quantities of auxin and moderate of citochinine.

The genetic factors (genotype and characteristics of sexual organs of the flowers) are essential in promoting the regenerative process into inoculated anthers. Only three accessions of *Vitis vinifera* subsp. *sylvestris* regenerated whole plants by embryogenesis and only from individuals with functionally male flowers.

The regenerated plants obtained from somatic embryos, either by direct germination on anther walls, or by indirect germination from anther-derived-calli, are valuable for further studies on their genetic uniformity or variability.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 125-131)

The First Clustering Centre on Plant Biotechnology in Romania

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SUMMARY. Plant biotechnology starts to develop worldwide in the beginning of the XXth century and it took more than 60 years to become recognized as an important pillar for supporting food security at the global level. Later on, after 1990 plant biotechnology become part of the bio-economy. Researchers from Romania recovered a lot in developing plant biotechnology as a science between 1970 and 1990. Still technology transfer from research to industry was pioneered with the support of researchers from the Biological Research Center Cluj-Napoca, Romania and implemented in Braşov County in 1988 when started the construction of the Plant Biotechnology Laboratory of Sere Codlea. In the next two years other three institutions have been developed clustering for the first time higher education, research and industry in 1990. The scope of this article is to emphasize relevant moments for the development of the cluster centre for plant biotechnology in Braşov County. Thus, the Biological Research Center Cluj-Napoca, three research institutions and one industry partner dedicated for plants biotechnology (e.g. ornamental plants, potato, sugar beet and grasses) implemented relevant mechanisms for technology transfer from research to industry. The Plant Biotechnology Laboratory of Sere Codlea, dedicated for ornamental and potato biotechnologies, collapsed due to the inconsistency of development policies before 2000. The other three research institutions joined together in a single one institute due to research policies of the time. That was one of the greatest losses of the country in terms of human and financial resources in supporting food security of the country and therefore Romania needs to recover these values for the future generations.

Keywords: clustering, crops technology, plant biotechnology policy, technology transfer, Romania.

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Introduction

The development of plant biotechnology became more than a necessity for the future of our country early in the ’80, due to the enormous economic gains promoted all over the world: quick development of new cultivars, access to a new generation of techniques and improve quality of life (Nisbet and Lewenstein, 2002). Thus, starting with that period Romania promoted the patenting in this domain and later in 1996, accessed the TRIPS (Trade-Related Aspects of Intellectual Property) (Boettiger *et al*., 2004), for supporting plant biotechnology for agriculture. Before 1980, the transfer of technology between research and industry with excellent results for the economy of the country was established (Lenaerts and Merlevede, 2015). After the communism fall, due to the rapid and dramatic political changes, Romania encountered a series of issues in continuing connecting research and industry with negative impact on biotechnology development (Borşa *et al*., 2011). After the national initiation of plant biotechnology at the industrial level that started in 1988 with the opening of the Plant Biotechnology Laboratory of Sere Codlea, we assisted 15 years later to a dramatic fall of many industrial platforms due to political incoherence in this domain (Sima *et al*., 2015). The Biological Research Center Cluj-Napoca through Professor Dorina Cachiță-Cosma played a central role in plant biotechnology development in our country and namely in clustering for the first time a series of five institutions belonging to higher education, research and industry (Cachiţă-Cosma and Vicol, 1990). The aim of this article is to present the major achievements of plant biotechnology clustering in Braşov County, Romania.

Materials and methods

This article is based on the historical analysis of the evolution of plant biotechnology in Romania, when the first clustering process in plant biotechnology became reality in Braşov County. Capacity building opportunities will be discussed based on a SWOT analysis.

Results and discussion

Plant biotechnology developed first based on recognizing defined principles of the Cell Theory published in 1838 by Schleiden and Schwann (Vasil, 2008). First experiments in plant biotechnology for cultivating plants in sterile conditions and accepted by the scientific community have been published in 1920 by Wilson. Step by steps these techniques become more and more accurate. Moreover they proved to become reliable parts of a new promising technology (White, 1934) if accessing

theoretical background of plant development according to theory on totipotency (Haberlandt, 1902). Between 1902 and 1934, the scientific community contributed to the generation of a new technology at the laboratory level and attempts for piloting their results have been published. After the Second World War the research is more and more oriented for proving the economic efficiency which finally acted in 1968 with the Green Revolution in Pakistan for fighting in ensuring food security for the poor (Nulty, 1972). After 1994, we assisted to a new era of plant biotechnology due to the production of first genetically modified crops species (Vasil, 2008) and today new techniques may further contribute to plants breeding (Lusser *et al*., 2012). Thus, between 1970 and 1990 at the global level it is a fertile period for the development of plant biotechnology solved with the successful transfer from the laboratory to industry level with strong economic rates. Unfortunately, Romania was not participating in the same period of time to the historical achievements of plant biotechnology as a new scientific emerging field. After 1970 the scientific community become aware about the benefits of these technologies that in the end of the '80 it is finalized with the development of a cluster centre on plant biotechnology. Brașov County is recognized for its huge traditional potential for potato and sugar beet production, for more than 100 years (Chiru *et al*., 2008a). Clean air, fertile soil, free viruses areas over 1000 m altitude and excellent communication are among the major features taken into account for setting this cluster for plant biotechnology development in close connection to the crops species, very popular in the region. The entire philosophy in behind this strategy was to connect higher education, research and industry for technology transfer, as a first step in the sustainable development of the country. Six focused PhD thesis, treating specific technology transfer, have been published in these cluster institutions in only 7 years (Sand, 1995; Sandu, 1998; Bălan, 1999; Constantinescu, 1999; Chiru, 1999 and Antofie, 2002).

The first industrial laboratory for obtaining free viruses plants. To be successful in coupling research and industry it is a great need of committed partners. Thus, the first largest laboratory for *in vitro* plants cultivation at industrial level was constructed in Romania between 1983 and 1987 with the commitment of the greenhouse state company Sere Codlea from Brașov County positioned at the foot of the mountain Măgura Codlei. The construction was supported by Eng. Ioan Băloiu the Director of this institution following the planning proposed by Professor Dorina Cachiță Cosma. This laboratory was placed in the neighbourhood of the decidual forests with strong descendent air currents all over the year. Such a place is important to keep away insects that may be virus vectors (Pop *et al*., 1992). The laboratory was projected to support a full activity of the green house (i.e. 200 ha of greenhouses) as well as other at least 3000 small producers distributed all over the country. The objectives of research conducted in Sere Codlea were to develop *in vitro* micropropagation technologies for free viruses plants (i.e. potato and ornamentals

species), to find the best technological solutions for *in vitro* inoculation, micro-propagation, pre-acclimation, acclimation and further for obtaining best results in super-elite plants. More than 40,000 carnation plantlets for more than 130 cultivars were transferred twice per year from the laboratory to the green house but in forced conditions it was possible to obtain up to 150,000 plantlets in 6 months. The technological flow was designed to include steps such as super elite and three levels of elite plants (i.e. elite I, II and III) for the commercial use (Petricele *et al*., 2009). A laboratory of over 800 m^2, respecting all international recommendations for biotechnology flow systems and equipped with the most modern infrastructure gave a constant financial gain of over 30% to the company. The lab complex comprises: i) a sterile part (i.e. an inoculation room equipped with 10 laminar flow hoods (each for two persons), 21 microscopes, a sterile lobby for sterile equipment, a deposit room, a room for culture media preparing and a sterilizing room), and ii) a non-sterile part (i.e. 6 growth chamber rooms for over 60,000 inoculate, a room for thermotherapy for up to 160 plants, a room for washing jars).

The personal was highly trained: 180 meristems/person/ 8h and 450 tubes of microporpagated plant material/8 h. Next to laboratory was constructed a 5,000 m^2 of greenhouse with couples of sections: acclimation (i.e. 6 greenhouses for 10,000 plantlets/each) pottery (i.e. 8 greenhouses for 600 each) and a sector for elite classes dedicated for commercial purpose. Highly instructed persons (1 per module) and a good management proved that such infrastructure may function properly. For almost 15 years this laboratory became the major supplier for ornamental species and potato plants free of viruses. Later, between 1996 and 1998, over 100,000 potato minitubers, viruses free, have been trade with a price 100 times lower compared to the market place (i.e. 100 minitubers/0.15 US dollars). The research was extended to gerbera free of pathogens with almost 10 meristems per plant (i.e. helical disposition into the apical meristem) and a rate of 30 meristems/person/8 h. At least 20 cultivars of gerbera were micropropagated in this lab for industrial purpose (Antofie *et al*., 1998). Over 30 cultivars of *Chrysanthemum sp. Saintpaulia* and tropical ornamental plants species were the subject of research for increasing the germplasm collection for indoor plants and for technology transfer as well.

Clustering plant breeding in Braşov. In 1990 also with the contribution of the Biological Research Center Cluj-Napoca, namely of professor Dorina Cachiţă-Cosma it was set up the Institute for Research and Development for Potato (IRDP), the Research Institute for Pastures (RIP) and the Research Institute for Sugar Beet (RISB) all three located in Braşov area. Thus, Sere Codlea contributed in personnel training and transfer in a highly organized research structure of IRDP closely working with research stations in the country for supporting the trade of the best potato cultivars in the country. This institute named today as the National Institute of Research and Development for Potato and Sugar Beet Brasov (NIRDPSBB) registered

in 25 years of existence 22 patents for 22 potato cultivars cultivated all over the country (Chiru *et al*., 2008b). Today the institute includes also the former RIP and RISB. Moreover 9 patents for sugar beet have been published by this institute supporting further their mission to connect research with industry according to the pioneer of the process (Mansfield, 1975). It is a fact that the Romanian policy was supporting the clustering of research institutions as a new mechanism for making more cost-efficient the process of technology transfer that is now, after 25 years, a new challenge at the global level (Palcic and Pandza, 2015). The current policy in research and development need further to support for the re-organization and development of these clusters on plant biotechnology as they will become more important in the future for supporting food security (Anderson *et al*. 2016) in a changing climate (Marcucci and Turton, 2015).

Conclusions

Four institutions, placed in Brașov County, have been created as a first cluster of plant biotechnology for supporting the Romanian bio-economy and for ensuring food security on long term.

A huge human resource has been trained and large financial resources have been invested into these institutions with constant revenue for industry of over 30%.

Despite of the positive results for technology transferring from research to industry, due to the political inconsistency in continuing the coupling between industry and research, this investment collapsed after 2004.

Important human resources remained in the NIRDPSBB with large implications from intellectual property rights due to 37 published patents on crops varieties.

Romania has a huge potential for developing clustering mechanisms in the transfer of technology and acting between research and industry that may be accessed for the support of food security policy in the context of the European Union.

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Biorecovery of a Model Oil-Polluted Soil after Exposure to Solutions of Typical Salts Found in Irrigation Water

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SUMMARY. The enhanced biorecovery of a model oil-polluted soil by soil wetting with solutions of typical salts found in irrigation waters was investigated. Garden soil was sampled from a selected location of predetermined weed composition for the purposes of determining soil seed bank composition. The air-dried soil was immediately polluted with spent lubricating oil (SLO) to obtain a constant 5% w/w concentration of oil in soil and emptied into wide bowls of 65 cm diameter, and 32 cm in height and set up in a screen house. Aliquots of 2.5 g of each Ca_2SO_4, (SCA) $MgSO_4$, (SMG) Na_2SO_4 (SNA) and K_2SO_4 (SKA) were weighed into distilled water to obtain constant 0.025 g/l salt solution. Distilled water served as the control (CTR). The oil-polluted soils were wetted with 1500 ml of control or salt solution. The experiment lasted for three months, after which study showed that there was reduction in total poly Aromatic volatile Hydrocarbon (24111.44 ppm) at the start of the experiment to 5.54 ppm. Compared to the control experiment, reduction in the total petroleum hydrocarbon (TPH), reduction in TPH was highest in SNA, being 97.02% remediation efficiency, compared to 72.44% in the SNS treatment. Bacterial species identified during the study included *Corynebacterium kutsceri, Streptococcus* spp*., Pseudomonas aeruginosa, Escherichia coli, Klebsiella* spp*., Bacillus lichenifomis* and *Staphylococcus* spp*.,* whereas fungi species included *Penicillium* spp., *Aspergillus niger*, and *Fusarium* spp. The abundance of the weed *Mariscus alterenifolios in* SCA (24), SMG (13), and CTR (20) may indicate a favoured environment for growth. Regeneration efficiency (RE) of weeds in the treated and control soils 62.5% by *Anelima aequinotiale* in CTR, 50% in SCA, and 12.5% in SNA.

Keywords: biorecovery, bioremediation, hydrocarbon, irrigation, salinity

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Introduction

As a primary recipient of waste products as well as diverse chemicals used to advance our technological development, the soil is constantly under threat in modern society. The fact that technology is basically powered by the petroleum industry, whether directly or indirectly leaves more to ponder. In most oil-producing economies like Nigeria, pollution caused by petroleum and its derivatives is the most prevalent problem, where it has led to the damage of the soil, water and both plants and animals (Essien and John, 2010). Soils are also rendered unproductive for years after spillage, reducing the growth performance of plants (Dale *et al.*, 2006). Odugwu and Onianwa (1987) demonstrated the effect of pollution on germination, growth and nutrient uptake of pawpaw. The chronic effects of oil on soil properties and microflora in a rainforest system was also investigated by (Amadi *et al.,* 1996). However, such several methods as physical (vapor extraction, stabilization, solidification), chemical (photo-oxidation, dissolution, detergent use), and biological methods (bioremediation), have been employed to remove oil wastes, its constituents as well as derivatives from soil and water. All these methods are useful depending on the priorities and circumstances of each oil pollution incident. Bioremediation, a biological method that uses microorganisms, plants and/or associated microorganisms to remove or render harmful material harmless is one of the promising cost and environmental effective approach (Merkl, 2005; Eman, 2008). The success of bioremediation of any oil-polluted soil depends upon a number of factors, including moisture as well as soil-nutrient status.

Biostimulation may have been less effective in accelerating the disappearance of oil on certain oil-contaminated ecosystems due to either the presence of high background nutrient concentrations or oxygen limitation. However, a few field studies did show enhanced oil biodegradation through nutrient addition (Lee and Levy, 1991). The implication, therefore, is that nutrient amendment may still be viable options the remediation hydrocarbons from ecosystems, especially when nutrients are limiting. Commonly used water-soluble nutrient products include mineral nutrient salts (e.g. KNO_3, $NaNO_3$, NH_3NO_3, K_2HPO_4, $MgNH_4PO_4$), and many commercial inorganic fertilizers (e.g. the 23:2 N:P garden fertilizer used in Exxon Valdez case). Some soils get nutrients indirectly from irrigation waters; examples being salts of nitrates and sulphates in irrigation waters. The present study hopes to investigate the effects some of the salts found in irrigation waters in the recovery of an oil-polluted soil.

Irrigation waters contain a significant amount of chemical substances in solution, varies according to the source and properties of the constituent chemical compounds. These chemicals including NaCl, Na_2SO_4, $NaHCO_3$, $MgSO_4$, $CaSO_4$ $2H_2O$, $CaCl_2$ $2H_2O$, KCl, and K_2SO_4 affects the growth of resident plants and soil microorganisms. Provin and Pitt in an undated report suggested that these compounds formed a list of characteristic salts found in irrigation waters.

These salts derive their source from the earth's crust from weathering. When they dissolve in surface of groundwater, they become available to plants particularly when such waters are used in irrigation purposes.

In remediation activities, the role of regular wetting of the soil to improve soil moisture has been reported in previous study to enhance remediation of oil-polluted soil (Ikhajiagbe *et al.*, 2013). The dilemma therefore is with the eventuality of having to inadvertently exposer the oil-polluted soil to saline irrigation waters. It is therefore the object of the present study to investigate the possibility for enhanced or retarded recovery a model oil-polluted soil upon exposure to solutions of typical salts found in irrigation waters.

Materials and methods

Preparation of soil

Topmost garden soil was sampled from a selected location marked 15 m x 15 m, and then air-dried to constant weight. The air-dried soil was immediately amended with spent lubricating oil (SLO) to obtain a constant 5 % w/w concentration of oil in soil. Prior to collection of soil, a survey of all weed species growing within the designated 15 m x 15 m partition was done. This would serve, in the study, as possible plants to make up the soil's seed bank. The polluted soils were distributed into wide bowls of 65 cm diameter, and 32 cm in height. The bowls were not saturated; this was to ensure that contents within the bowl remain within the bowl for the entire period of 3 months that the soils would be exposed to experimental conditions. Measured 25 kg of soil was originally measured into each bowl prior to amendment with SLO. The set up was laid out in a well-ventilated screen house with temperature ranges of $32.2 \pm 3.7\ ^{0}C$. The polluted soils wre eventually irrigated first with water (1000ml) and was left for 3 days for natural attenuation in the screen house.

Preparation of salt solution

Ca_2SO_4, $MgSO_4$, Na_2SO_4 and K_2SO_4 were required for preparation of each salt solution. Aliquots of 2.5 g of each salt were weighed into distilled water to obtain constant 0.025mg/l salt solution. Distilled water served as the control (CTR).

Wetting of oil-polluted soil bowls with salt solutions

Prior to pollution of garden soil with SLO, the water-holding capacity (WHC) of the garden soil was previously determined to be 218.92 ml/kg soil. Each 25 kg soil per bowl was wetted daily with 1500 ml of control or salt solution. The experiment lasted for three months.

Experimental parameters

The development and appearance of weeds in each bowl from soil seed bank was monitored. After 3 months soil was taken to the laboratory for microbial and PAH determinations. This was done by collecting soil from 10 random spots in each bowl, and an arbitrary uniform depth of 7.5 cm from soil surface.

Standard methods described by Dean and Xiong (2000) were used to determine aliphatic hydrocarbon fractions of the soil; whereas the methods of Cowan and Steele (1974) and Cheesebrough (1998) were used to isolate and characterize bacterial and fungal isolates.

Regeneration efficiency of each weed in remediated oil-polluted soil was determined at 3 months after application of treatments, using the formula developed below:

$$\frac{\text{No of weed at 3 months} \times 100}{\text{No of weed at } 1^{st} \text{ day}}$$

Tolerance index of weeds in the oil-polluted soil at 3 months was also determined as follows;

$$\frac{\text{Table 8 (3 MAP)} \times 100}{\text{Table 8 (STAT)}}$$

Where MAP – months after pollution and exposure

STAT - Concentration of contaminants in the oil-polluted soil just before soil was wetted with salts.

Results and discussion

This study therefore, was carried out to determine the recovery of oil-polluted soil exposed to saline waters for irrigation. Parameters used in the study to evaluate recovery were total hydrocarbons, weed regeneration, as well as soil microbial composition. The record of weeds that most likely constituted the soil seed bank of the soil used have been presented below (Table 1); the most predominant weed being *Mariscus alterenifolios* (Cyperaceae) with a composition of 13 individuals per square meter, compared to *Andropogon virginatus* (Poaceae) with 1 m^{-2}.

Results showed significant reduction in total petroleum hydrocarbons (TPH) of wetted soils, compared to the control. Fundamentally, soil wetted with Na_2SO_4 solution showed improved TPH remediation efficiency of 97.02%, compared to a range of 72.44 – 89.76% reductions in other wetted soils and 75.41% in the unwetted oil-polluted soil (Table 2).

Table 1.

Record of soil seed bank of the soil used in the present study.

Weeds	**Family**	***Total individual species m^2**	**Frequency of occurrence (%)**
Andropogon virginatus	Poaceae	1	0.826
Anelima aequinotiale	Commelinaceae	8	6.612
Asystasia gangetica	Poaceae	7	5.785
Croton hirtus	Euphorbiaceae	5	4.132
Centrosema pubscers	Fabaceae	6	4.959
Cyperus haspan	Cyperaceae	3	2.479
Chromolina benghanlensis	Commelinaceae	5	4.132
Eleusin indica	Poaceae	6	4.959
Fimbisstylis ferruginea	Cyperaceae	8	6.612
Gomphrina celosoides	Amaranthaceae	4	3.306
Kyllinga erecta	Cyperaceae	7	5.785
Mariscus alterenifolios	Cyperaceae	13	10.734
Pennisetum purpureum	Poaceae	6	4.959
Synedrella nodiflora	Asteraceae	4	3.306
Sporobolus pyramidalis	Poaceae	7	5.785
Tridax proambens	Asteraceae	4	3.306
Unidentified (< 5 cm tall)	-	27	22.314
Total		121	100.00

*The space within which plants were surveyed was 15 m x 15 m.

Table 2.

Total petroleum hydrocarbons after 3 months of exposure to various treatments.

	STAT	**After 3 months**				
		CTR	**SMG**	**SCA**	**SNA**	**SKS**
Nonane (C9)	3143.78	850.71	354.46	644.47	103.12	952.79
Decane (C10)	4326.36	1170.71	487.80	886.90	141.90	1311.20
Dodecane (C12)	5526.11	1495.37	623.07	1132.85	181.26	1674.81
Tetradecane (C14)	3276.43	886.60	369.42	671.67	107.47	992.99
Hexadecane (C16)	39.67	4.19	1.75	3.17	<0.005	4.69
Octadecane (C18)	2543.55	688.28	286.79	521.43	83.43	770.88
Nonadecane (C19)	835.84	226.18	94.24	171.35	27.42	253.32
Eicosane (C20)	2041.56	552.45	230.19	418.52	66.96	618.74
Docasane (C22)	1053.21	285.00	118.75	215.91	34.55	319.20
Tetracosane (C24)	783.49	212.01	88.34	160.62	25.70	237.45
Hexacosane (C26)	237.83	64.36	26.82	48.76	7.80	72.08
Tricosane (C30)	303.61	82.16	34.23	62.24	9.96	92.02
TAH (mg/kg)	24111.44	6518.01	2715.84	4937.89	789.55	7300.17
PAVH (mg/kg)	2412.32	5.54	1.43	3.98	0.95	8.48
TPH (mg/kg)	26523.76	6523.55	2717.27	4941.87	790.50	7308.65
TPH remediation efficiency (%)	-	75.405	89.755	81.368	97.019	72.444
Contamination factor	1562.05	6506.57	160.027	291.041	46.554	430.426

STAT - Concentration of contaminants in the oil-polluted soil just before soil was wetted with sulphate salts, TAH Total Aliphatic Hydrocarbon, PAVH Poly Aromatic volatile Hydrocarbon, TPH Total Petroleum Hydrocarbon, CTR soil wetted with water, SMG soil wetted with $MgSO_4$, SCA soil wetted with $CaSO_4$ solution, SNA soil wetted with Na_2SO_4 solution, SKS soil wetted with K_2SO_4 solution

Total heterotrophic bacterial count after three months of soil exposure to various sulphate salt solutions was 7.36 x 10^4 cfu/g in SNA and in 1.21 x 10^4 cfu/g SKS, compared to 2.39 × 10^4 cfu/g in CTR. Total Heterotrophic Fungi Count in the control was 0.29 x 10^4 cfu/g and 1.54 x 104 cfu/g in SNA. Hydrocarbon utilizing bacteria was highest in SNA (4.36 x 10^4 cfu/g), compared to 0.15 x 10^4 in SKS.

Comparatively, a look at soil microbial population (see Table 4) also showed that all microbial isolates present in soil prior to exposure to experimental conditions were recurrent three months after exposure. Bacterial species identified during the study included *Corynebacterium utsceri, Streptococcus* spp.*, Pseudomonas aeruginosa, Escherichia coli, Klebsiella* spp.*, Bacillus lichenifomis* and *Staphylococcus* spp.*,* whereas fungi species included *Penicillium* spp., *Aspergillus niger*, and *Fusarium* spp (Table 4). *Streptococcus* spp. and *Staphylococcus* spp. were both identified only in the soil wetted with Na_2SO_4 solution (SNA). The control soil, although oil-polluted, did not show presence of the bacteria. Perhaps, Na_2SO_4 affected soil conditions that enhanced performance of both soil microbes in the oil-polluted soil and also enhanced contaminant degradation.

One good reason for which salinity effects soil microbial population is because of the differences in tolerance of low osmotic potential by different soil microbial genotypes (Mandeel, 2006; Gennari *et al.*, 2007; Llamas *et al.*, 2008; Chowdhury *et al.*, 2011). In the present study, total fungal colony forming units was lower than total bacterial composition. Pankhurst *et al.* (2001); Sardinha *et al.* (2003); Wichern *et al.* (2006) earlier reported that fungi are more sensitive to osmotic stress than bacteria. Accordingly, while sensitive microbial cells may be impaired by the low osmotic potential necessitated by the saline condition of the irrigation water, Oren (2001) and Hagemann (2011) reported that some microorganisms, including fungi, can adapt by taking up osmolytes that enable them retain water (Beales, 2004). In this study, fungi species including *Penicillium* spp., *Aspergillus niger*, and *Fusarium* spp, which were initially isolated from the clean soil before exposure to saline water and oil, were recurrent three months after exposure (Table 4). It is perhaps suggested that these organisms have developed a strategy for survival in salt-treated oil-polluted soils. This is hereby presented for further study on possible survival mechanisms.

Table 3.

Total microbial count after three months of the oil-polluted soil to sulphate solutions

	THBC	HUB	THFC
		(x 10^4 cfu/g)	
CTR	2.39	1.23	0.29
SMG	4.14	2.76	0.51
SCA	2.06	1.52	0.25
SNA	7.36	4.35	1.54
SKS	1.21	1.06	0.15

THBC – Total Heterotrophic Bacteria Count, THFC - Total Heterotrophic Fungi Count, HUB – Hydrocarbon Utilizing Bacterial, cfu/g – Colony forming unit per gram, CTR soil wetted with water, SMG soil wetted with $MgSO_4$, SCA soil wetted with $CaSO_4$ solution, SNA soil wetted with Na_2SO_4 solution, SKS soil wetted with K_2SO_4 solution.

Andropogon Virginatus, Chromolina benghanlensis, Pennisetum purpureum, and *Tridax procumbens* were absent from soils 3 months after exposure to experimental conditions (Table 5). The abundance of *Mariscus alterenifolios in* SCA (24), SMG (13), CTR (20), and UCTR (23) may indicate a favoured environment for growth. However, lower presence of the weed was recorded in SKS (2) and SNA (1). The Table also shows that unidentified weed were 5cm below, with SMG having the highest number of unidentified weeds (30), compared to SNA (1). *Andropogon Virginatus, Asystasia gangetica, Croton hirtus, Chromolina benghanlensis, Fimbisstylis ferruginea, Gomphrina celosoides, Kyllinga erecta, Sporobolus pyramidalis, Tridax proambens* were absent in the salt-treated soils. There were a total of 48 plants per pot in SCA, 45 in SMG, 44 in CTR, 24 in SKS and 7 in SNA respectively.

Table 4.

Microorganism distribution of oil-polluted soil at 3 months after application of treatments

Isolates	**UCTR at Day 1**	**CTR**	**SMG**	**SCA**	**SNA**	**SKS**
		Oil-polluted soils after 3 months following salt exposure				
			Bacteria			
Corynebacterium kutsceri	+	+	+	+	+	+
Streptococcus spp.	+	-	-	-	+	-
Pseudomonas aeruginosa	+	+	+	+	+	+
Escherichia coli	+	-	+	-	+	-
Klebsiella spp.	+	+	+	+	+	-
Bacillus lichenifomis	+	+	+	+	+	+
Staphylococcus spp.	+	-	-	-	+	-
			Fungi			
Penicillium spp.	+	+	+	+	+	+
Aspergillus niger	+	+	+	+	+	+
Fusarium sp	+	+	+	+	+	+

+ present, - absent; UCTR unpolluted control Soil as used for the experiment. CTR soil wetted with water, SMG soil wetted with $MgSO_4$, SCA soil wetted with $CaSO_4$ solution, SNA soil wetted with Na_2SO_4 solution, SKS soil wetted with K_2SO_4 solution.

The regeneration efficiency (RE) of weeds in the treated and control soils have been presented on Table 6. RE of *Anelima aequinotiale* 62.5% in CTR, 50% in SCA, and 12.5% in SNA. The highest RE was obtained for *Mariscus alterenifolios* in SCA (218.2%). Generally, this weed had a significant regeneration capability compared to other weeds in the salt-treated oil-polluted soils. Significant regeneration of *Synedrella nodiflora* were also obtained in SNA and SKS, both being 100%. As observed earlier, Na_2SO_4-impacted soils showed enhanced performance of both soil microbes in the oil-polluted soil and also enhanced contaminant degradation; this may also be favourable for plant recovery. Plant recovery in SNA was comparatively lowest in the experiment. However, plants' tolerance index for recovered plants in SNA was better compared to other plants in the other wetted soils (Table 7).

Tolerant index of *Mariscus alterenifolios* in oil-polluted soil wetted with $MgSO_4$ was 28.88%, and 5.0 % in soil wetted with $CaSO_4$ solution, compared to 45.45% in the control oil-polluted soil (Table 7).

Table 5.

Abundance of weeds on remediated oil-polluted soil at 3 months after application of treatments (soil surface area in bowl is 2828.57 cm^2)

Weeds	UCTR	CTR	SMG	SCA	SNA	SKS
Andropogon Virginatus	0	0	0	0	0	0
Anelima aequinotiale	5	5	0	4	1	0
Asystasia gangetica	1	0	0	0	0	0
Croton hirtus	2	0	0	0	0	0
Centrosema pubscers	5	0	0	0	0	4
Cyperus haspan	0	2	0	0	0	0
Chromolina benghanlensis	0	0	0	0	0	0
Eleusin indica	5	3	0	1	0	0
Fimbisstylis ferruginea	2	0	0	0	0	0
Gomphrina celosoides	1	0	0	0	0	0
Kyllinga erecta	2	0	0	0	0	0
Mariscus alterenifolios	23	20	13	24	1	2
Pennisetum purpureum	0	0	0	0	0	0
Synedrella nodiflora	2	1	2	2	4	4
Sporobolus pyramidalis	3	0	0	0	0	0
Tridax procumbens	0	0	0	0	0	0
Unidentified (< 5 cm tall)	18	13	30	17	1	14
Total	48	44	45	48	7	24

Weeds accounted for on Table 5 comprise the soil seed bank of soil originally used for the experiment. UCTR unpolluted control Soil, CTR soil wetted with water, SMG soil wetted with $MgSO_4$, SCA soil wetted with $CaSO_4$ solution, SNA soil wetted with Na_2SO_4 solution, SKS soil wetted with K_2SO_4 solution.

Although the result shows that *Andropogon Virginatus, Asystasia gangetica, Croton hirtus, Cyperus haspan, Chromolina benghanlensis, Fimbisstylis ferruginea, Gomphrina celosoides, Kyllinga erecta, Sporobolus pyramidalis, Tridax proambens* had a tolerance index of 0%, the unidentified weed had a tolerance index of 29.54% in CTR, 66.66% in SMG 35.41% in SCA, 14.28% in SNA and 58.33% in SKS respectively. Comparatively, in the oil-polluted soils that wetted with salt solutions, average tolerance index was 5.88%, compared 5.88% in the control. However, average tolerance index in SCA was 3.23%.

The presence of salts in the soil negatively affects growth and development of resident plant species particularly owing to osmotic stress, ion toxicity or plants' reduced capability for essential nutrient absorption (Lauchli and Epstein, 1990). Essentially, increased salt concentrations in soils often lead to poor physicochemical condition of the soil which in turn inhibits seedling development and plant growth (Levy *et al.* 2002; Choudhary *et al.* 2004; Sharma and Minhas, 2005).

Table 6.
Regeneration efficiency of weeds in salt-wetted oil-polluted soils at 3 months after exposure to treatments. Soil surface area in bowl is 2828.57 cm^2.

Weeds	**CTR**	**SMG**	**SCA**	**SNA**	**SKS**
		(Regeneration efficiency, %)			
Andropogon Virginatus	0	0	0	0	0
Anelima aequinotiale	62.5	0	50	12.5	0
Asystasia gangetica	0	0	0	0	0
Croton hirtus	0	0	0	0	0
Centrosema pubscers	0	0	0	0	66.67
Cyperus haspan	66.67	0	0	0	0
Chromolina benghanlensis	0	0	0	0	0
Eleusin indica	50	0	6.67	0	0
Fimbisstylis ferruginea	0	0	0	0	0
Gomphrina celosoides	0	0	0	0	0
Kyllinga erecta	0	0	0	0	0
Mariscus alterenifolios	181.8	118.2	218.2	9.1	18.2
Pennisetum purpureum	0	0	0	0	0
Synedrella nodiflora	25	50	50	100	100
Sporobolus pyramidalis	0	0	0	0	0
Tridax proambens	0	0	0	0	0
Unidentified (< 5 cm tall)	48.15	111.11	62.96	3.70	51.85
Total	434.12	279.31	387.83	125.3	236.72

CTR soil wetted with water, SMG soil wetted with $MgSO_4$, SCA soil wetted with $CaSO_4$ solution, SNA soil wetted with Na_2SO_4 solution, SKS soil wetted with K_2SO_4 solution.

Increased salt concentrations in the soil results in osmotic stress, which in turn affects the microbiological properties of soil, reducing soil microbial biomass (Pathak and Rao, 1998; Oren, 1999). Incidentally, the soil microbial biomass and quality are significant soil properties for accessing the potentiality of the soil to remediate contaminants. Aside from osmotic stress, there are a number of other related factors necessitated by increased soil salinity; these include adverse pH changes, ion toxicities, as well a decline in potentially mineralizable N (Zahran, 1997).

Bandyopadhyay and Bandyopadhyay (1983) reported decreased mineralization and immobilization of soil nitrogen. The rates of nitrification and ammonification were also negatively impacted by saline soils (Wollenweber and Zechmeister-Boltenstern, 1989). These factors are sin-qua-non to successful microbial proliferation.

However, studies have shown the capability of *Pseudomonas* to significantly enhanced early plant growth in low fertility soil (Defreitas and Germida, 1992). In the present study, *Pseudomonas* was a prominently occurring bacterial species in the salt-treated oil-polluted soils. Invariably, there is improvement of resident plant development resulting from a concomitant compensation for soil nutrient deficiency by the bacteria, which may produce plant growth regulators within the rhizosphere.

Kloepper and Beauchamp (1992) and Wu *et al.* (2005) earlier reported improved root development and better water and nutrient absorption as a result of the microbial action. This also justifies the relative plant recovery percentages in the soil-polluted soils, where plants' ability to access water and nutrients were hitherto hindered (Defreitas and Germida, 1992; Lazarovits and Norwak, 1997; Burdman *et al.* 2000). Lindberg *et al.* (1985) and Frankenberger and Arshad (1995) earlier noted that root-colonizing bacteria may help stimulate plants growth and thus inhibit the damaging effects of environmental stressors by producing phytohormones when in association with the plant. Hasnain and Sabri (1996) also showed that *Pseudomonas* spp. initiated increases in plants auxin content as well as reduced accumulation of harmful ions in wheat plant.

Table 7.

Tolerance index of weeds on remediated oil-polluted soil at 3 months after application of treatments

Weeds	**CTR**	**SMG**	**SCA**	**SNA**	**SKS**
			(Weed tolerance index, %)		
Andropogon Virginatus	0	0	0	0	0
Anelima aequinotiale	11.36	0	8.33	14.28	0
Asystasia gangetica	0	0	0	0	0
Croton hirtus	0	0	0	0	0
Centrosema pubscers	0	0	0	0	16.66
Cyperus haspan	4.545	0	0	0	0
Chromolina benghanlensis	0	0	0	0	0
Eleusin indica	6.818	0	2.08	0	0
Fimbisstylis ferruginea	0	0	0	0	0
Gomphrina celosoides	0	0	0	0	0
Kyllinga erecta	0	0	0	0	0
Mariscus alterenifolios	45.45	28.88	5.0	14.28	8.33
Pennisetum purpureum	0	0	0	0	0
Synedrella nodiflora	2.272	4.44	4.16	57.14	16.66
Sporobolus pyramidalis	0	0	0	0	0
Tridax proambens	0	0	0	0	0
Unidentified (< 5 cm tall)	29.54	66.66	35.41	14.28	58.33
Average	5.88	5.88	3.23	5.88	5.88

CTR soil wetted with water, SMG soil wetted with $MgSO_4$, SCA soil wetted with $CaSO_4$ solution, SNA soil wetted with Na_2SO_4 solution, SKS soil wetted with K_2SO_4 solution.

The importance of soil microorganisms cannot be overemphasized. Apart from their prominent role in soil decontamination, they are also a central factor in nutrient cycling, soil organic content, as well as in sustaining plant production. Although a number of soil microorganisms exist that are tolerant to a number of environmental stress (Ikhajiagbe, 2010), however, stresses can be detrimental for sensitive microorganisms and decrease the activity of surviving cells, due to the

metabolic load imposed by the need for stress tolerance mechanisms (Schimel et al, 2007; Yuan *et al.*, 2007, Ibekwe *et al.*, 2010; Chowdhury *et al.*, 2011). This particularly informs the reduction in presence of some organisms in the present study, which were hitherto present in the soil prior to amendment with either saline solution or with oil. Most importantly, apart from the deleterious effects of oil on sensitive soil microbes, salinity also inhibits development of these microbial populations. The idea of this research is to investigate whether the imposition of salinization on the already stressed soils (with oil pollution) offers any respite for the remediation purpose of the soil microorganisms, particularly given the fact that these organisms may be inadvertently exposed to saline irrigation waters.

Conclusion

The impact of saline irrigation waters on the recovery of a model oil-polluted soil has been reported. Significant changes in soil TPH contents of the oil-polluted soils were reported, with enhanced remediation reported in the soil wetted with Na_2SO_4. Results also showed that for plants available three months after exposure to the experimental conditions showed below average tolerance indices, apart from which *Synedrella nodiflora* showed 57.14% tolerance index. Further study is therefore required to ascertain the mechanism of effects of these salts in both plants and the soil microorganisms in other to further clarify the results presented herein.

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Two *Protracheoniscus* Species (Crustacea, Isopoda, Oniscidea) in Romanian Fauna: Morphology, Ecology and Distribution

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SUMMARY. Our study is dedicated to two *Protracheoniscus* species from Romania, *Protracheoniscus major* and *Protracheoniscus politus*. The specific morphological characters of *Protracheoniscus major* Dolfuss 1903 and *Protracheoniscus* politus C. L. Kock 1841 are redescribed. The morphology of the male pleopod 1 in *P. major* and *Protracheoniscus asiaticus* Uljanin 1875 are presented comparatively. At *Protracheoniscus politus* C.L. Koch 1841 the specific morphological characters, the intraspecific morphological variation of some taxonomical characters and some morphological anomalies are detailed. Also, we presented the changes of the male pleopod 1 endopods morphology after the post reproductive moult, the chitinous lobes of the endopods` tip being removed together with the exuvia. The taxonomic confusions emerging from the remove of the chitinous lobes are presented. The phenomenon of the chitinous lobes` remove in the post reproductive moult was not taken into account by them, new isopod subspecies for science being described. The ecology and the geographic distribution are presented in both species.

Keywords: distributions, ecology, isopods, morphology, *Protracheoniscus*.

Introduction

In the Romanian specialty literature (Radu, 1985), there are confusions and errors in the presentation of the genus *Protracheoniscus*. These confusions were determined by the incomplete description of the morphological characters with

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taxonomical value, without many figures. The species *Protracheoniscus major* Dollfus 1903 was presented as *Protracheoniscus asiaticus* Uljanin 1875 (Radu, 1985). Only recently *P. major* was mentioned in western Romania (Ferenţi, 2013, Ferenţi *et al*., 2015). The confusion between the two species is also found in the papers of other researchers (Strouhal, 1929, Wächtler, 1937, Frankenberger, 1959, Schmolzer, 1965). Gruner (1966) was the first one who compared morphologically the male pleopod 1 and 2 and showed the obvious differences between the two species, *P. asiaticus* and *P. major*. Based on the research of Gruner, other authors also identified the species *P. major* in different European countries, in Hungary (Csordás *et al.*, 2005), in Poland (Jedryezkowski, 1981), in Romania (Ferenţi, 2013, Ferenţi *et al*., 2015). Schmalfuss (2003) mentions the taxonomical confusion between *P. asiaticus* and *P. major* specimens from European countries.

Referring to *P. politus*, Radu (1985) mentions three subspecies in the Romanian fauna: *P. politus politus* C.L. Koch 1841, *P. politus carpathicus* Verhoeff 1928, *P. politus similis* Radu 1951, and a fourth one, *P. politus slovakius* Strouhal 1940, which is present in Czech. Tomescu (1972) has studied the post-embryonic ontogenetic development in *P. politus*. He described the progressive development of the secondary sexual characters in males and their morphological variation. He found that the male endopods tips` chitinous lobes are removed with the exuviae in the post reproductive moult, which usually takes place in May and June. The lobes re-grow progressively until the next year, when they reach the characteristic size for this species. Probably, the researchers who described the above mentioned subspecies were misled by the fact that they captured males after moult. This fact will be demonstrated in the present paper, where firstly we will present the complete morphology of *P. major*, than we will re-describe the morphological characters of *P. politus*, will present the changes of the male pleopod endopod 1 due to the moult, and the variability of some characters and morphological anomalies.

Material and methods

The *P. major* specimens were collected from two localities. Fifteen males and 42 females were collected from Simeria, Hunedoara County, near the walls of an old building with partly fallen plaster, from under the debris and stones. The specimens were collected in May 1995. Two males were collected from the soil at the entrance of a vine cellar in Sălacea, Bihor County, on April 03, 2012. From the collected material five males were dissected and microscopic slides were made. The *P. politus* specimens were collected between 1991 and 2015 from different habitats: spruce forests, deciduous forests, riverside coppices, mountain meadows. In 11 habitats the collecting was realized with pitfall traps monthly and bi-monthly. The habitats were situated in different geographical units: 23 habitats from mountain

areas, seven habitats from hills and one habitat from plain area. The collected specimens from both species were preserved separately in alcohol 70^0. From *P. politus* 228 adult males and 117 females were collected. 93 males were dissected and microscopic slides were made using Canada balsam and Euparal. The preparations were studied at the stereomicroscope and microscope, the body of males and females and the appendages (antennae, pereiopods 1-3 and 7, male pleopods 1 and 2) were photographed. From the specimens collected monthly and bi-monthly with the pitfall traps were dissected and studied males from the same habitat but collected on different dates, from May to October. Thus, we could overtake the changes of the male pleopod 1 endopods after the post reproductive moult.

The re-description of the species *Protracheoniscus major* Dollfus 1903

Protracheoniscus major (Dollfus, 1903) Gruner 1966

Literature consulted for the description of the species: Strouhal, 1929, Wachtler, 1937, Frankenberger, 1959, Gruner, 1966, Schmolzer, 1965, Radu, 1985. In other papers is also mentioned the presence of the species *P. major* in different European countries, without the description of the specific morphological characters (Cochard *et al*., 2010, Csordas *et al*., 2005, Jedryckowski, 1981, Vilisics and Hornung, 2009). In Romania, Radu (1985) mentions incorrect the presence of the species *P. asiaticus*. In the present paper we detail the morphology of *P. major.*

Size: males 12 x 5 – 18 x 6.5 mm, females 12 x 5 – 18 x 7 mm.

Body color: males are dark brown (Fig. 1.1.a) and females light brown (Fig. 1.1.b). Both sexes present yellowish oblong spots at the coxal plates` base. The pleon is colored uniformly in dark brown (Fig. 1.1.a,b).

Somatic characters:

Cephalon. The lateral cephalic lobes are relatively low developed, with triunghiular shape, slightly inclined. The median lobe is reduced. The eyes are compound of 22-23 ommatidia (Fig. 1.1.c).

Pereion. The noduli laterales have different position on the segments 3 and four, compared with the other pereional segments (Fig. 1.1.d).

Pleotelson is short at both sexes, the lateral edges forming an oblique angle (Fig. 1.1.e,f).

Appendages:

Antennae` last article length represents half of the penultimate one's length (Fig. 1.2.a).

Pereiopods. The male pereiopod 7 ischium`s ventral side is slightly concave (Fig. 1.2.b). The male 1 pereiopod merus and carpus present numerous thin spines (Fig. 1.2.c). On the pereiopods 2 and 3 meros and carpus the spines are thicker and less in number (fig.1.2.d.e)

Pleopods. The male pleopod 1 exopods` exterior side is bended, forming and obtuse angle. The tip is slightly rounded. Both sides present short spines, more numerous in the internal side (Fig. 1.3.a). The endopods` basal half is slightly oblique (Fig. 1.3.b). The distal half's extremity is sharp, with fine hair in its internal side (Fig. 1.3.c). The pleopod 2 exopods are triangular with short spines at the external side (Fig. 1.3.d). The uropod exopods are long in males (Fig. 1.1.e), and short in females (Fig. 1.1.f). In figure 1.4. we present images with the pleopods 1 and 2 resulted in our research and drawings published by Grunner (1966) with the morphology of the pleopods 1 and 2 in *P. major* and *P. asiaticus*. The figures show obvious morphological differences between the two species.

Figure 1.1. *Protracheoniscus major* (Dolfus, 1903), male and female dorsal view:
a. ♂ 18 x 7 mm, , **b.** ♀ 15 x 7.5 mm, **c.** cephalic lobes, **d.** noduli laterales, **e.** ♂ pleotelson and uropods, **f.** ♀ pleotelson and uropods.

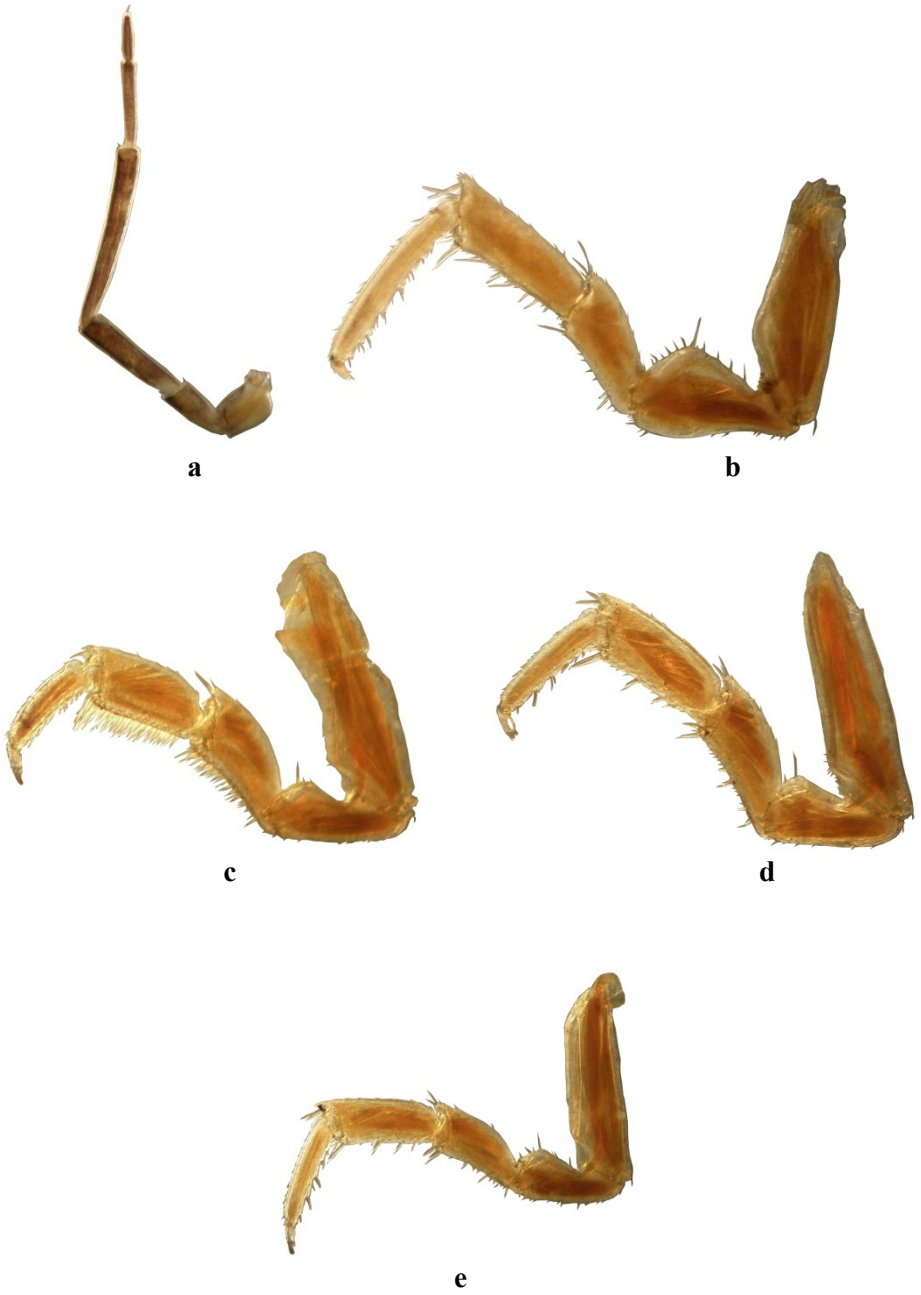

Figure 1.2. *Protracheoniscus major* (Dolfus, 1903), ♂ 16.5 x 6 mm, **a.** antenna, **b.** male pereiopods 7, **c.** pereiopods 1, **d.** pereiopods 2, **e.** pereiopods 3.

Figure 1.3. *Protracheoniscus major* (Dolfus, 1903), male pleopods 1 and 2: ♂ 16.5 x 6 mm, **a.** pleopods 1 exopods, **b.** pleopods 1 endopods, **c.** the apex of pleopods 1, **d.** pleopods 2.

Ecology. *P. major* is a synanthropic species in the European countries. It lives around buildings, preferring old houses with wet walls or cellars with vegetable and fruit deposits (Jedryckowski, 1981, Vilisics and Hornung, 2009, Ferenţi, 2013, Ferenţi *et al.*, 2015). Eshagi *et al*. (2015) affirms that in Iran *P. major* has numerous populations in agricultural areas.

Geographic distribution. *P. major* originates from Central Asia (Gruner, 1966). In Europe it is present in southern Russia, Romania, Hungary, Slovakia, Austria, Czech and Poland (Gruner 1966, Cochard *et al.,* 2010, Csordas *et al.*, 2005, Vilisics and Hornung, 2009, Jedryckowski, 1981, Ferenţi, 2013, Ferenţi *et al.,* 2015).

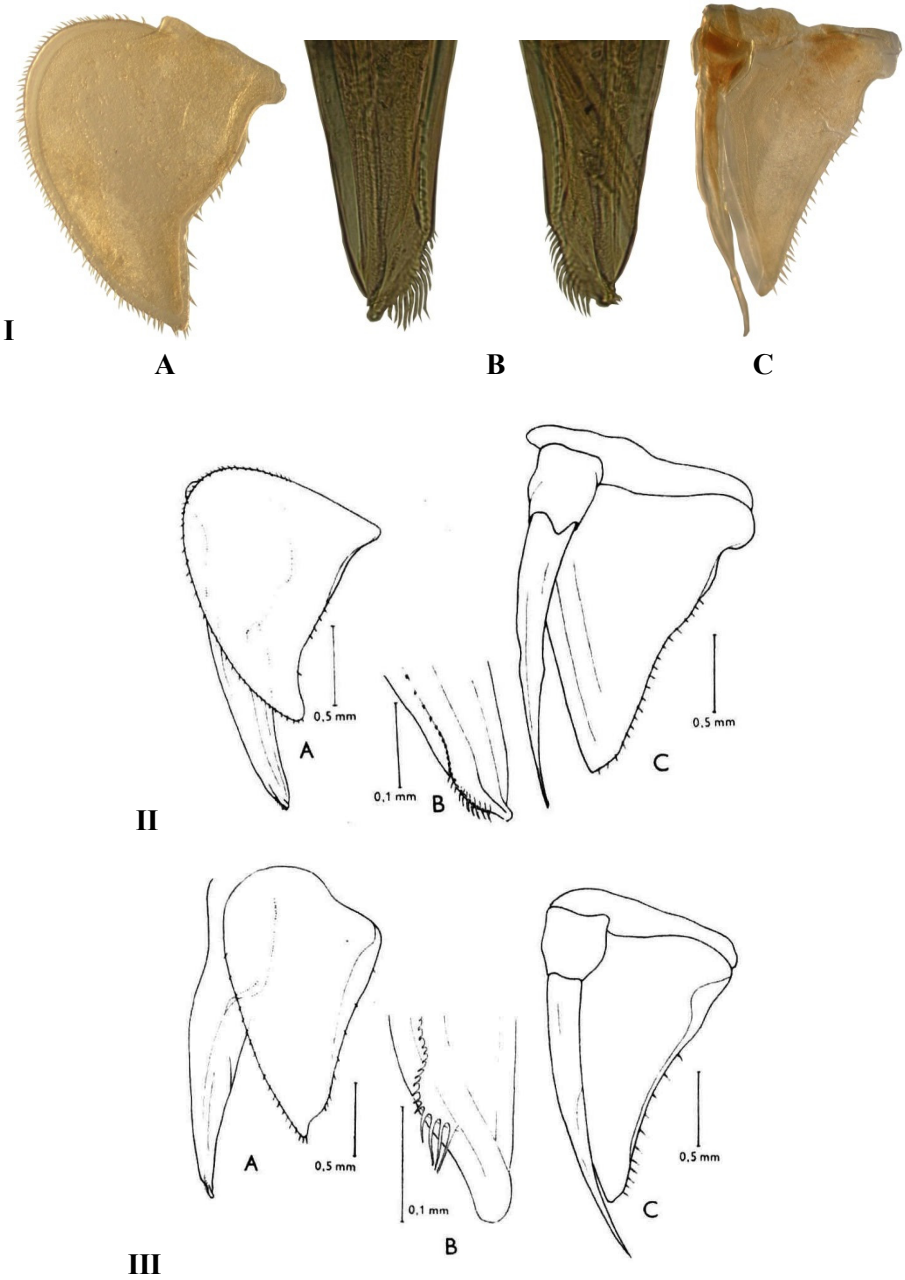

Figure 1.4. *Protracheoniscus major* (Dolfus, 1903), **I.** our research, **II.** after Grüner, 1966, **III.** *Protracheoniscus asiaticus* (Ulianin, 1875), after Grüner, 1966: **A.** pleopods 1 exopods, **B.** the apex of the pleopods 1 endopods, **C.** pleopods 2.

The re-description of the species *Protracheoniscus politus* C.L.Koch 1841

Protracheoniscus politus (C. L. Koch, 1841) Wachtler 1937
Protracheoniscus politus (C. L. Koch, 1841) Frankenberger 1959
Protracheoniscus politus (C. L. Koch, 1841) Schmolzer 1965
Protracheoniscus politus politus (C. L. Koch, 1841) Radu 1985
Protracheoniscus politus (C. L. Koch, 1841) Tomescu 1972

Literature consulted for the re-description of the species: Wachtler, 1937, Frankenberger, 1959, Schmolzer, 1965, Tomescu, 1972, Radu, 1985.

The re-description of the species *P. politus* is required because the main character with taxonomic value, the adult male pleopod 1 endopods changes after the post-reproductive moult, which usually takes place in May-June (Tomescu, 1972). In the biological material collected by us between the years 1990-2015 we have found some exceptions, like males collected in July-October with unmodified pleopod 1 endopods. The lack of data referring to the modifications of the morphology of *P. politus* male pleopod 1 endopods has contributed to taxonomic confusions, the males collected after the moult being described as new subspecies for science (*P. politus carpathicus* Verhoeff 1928, *P. politus slovakins* Strouhal, 1940 – cited by Radu, 1985).

Tomescu (1972) has studied and described the post-embryonic ontogenetic development in *P. politus*. He described the progressive show up of the male secondary sexual characters, as well the modification of the morphology of the endopods tip, the disappearance of the chitinous lobes after the post-reproductive moult. The rich and diverse samples, coming from a high number of habitats, situated in diverse geographical units has permitted a detailed study of adult *P. politus* males, with new results then was in the previous study (Tomescu, 1972), that was made on males collected from a single habitat.

Size: 93 males and 20 females were measured, the average size at males being 5 – 9 mm and at females 6 – 10 mm.

The body colour is dark brown; the median zone of the tergites is darker. Laterally of these zones there are fine yellowish marble shapes. At the base of the coxal plates there are yellow oblong spots (Fig. 2.1.a, b).

Somatic characters:

The cephalon have weak developed lobes, on the head there are numerous yellow-orange spots (Fig. 2.1.c).

The pleotelson has oblique angled lateral edges, the tip of the telson exceeding the tips of the last pleon segment (Fig. 2.1.d).

Appendages

Antennae: the penultimate antennary article's length is approximately equal with the half length of the last article (Fig. 2.2.a). The length ratio is a variable character.

The male 7 pereiopods ischium`s ventral side is slowly curved (Fig. 2.2.b). The 1-3 pereiopods carpus` and meros` ventral sides present dense rows of thin spines (Fig. 2.2.c,d,e).

The male 1 pleopods: The exopods are approximately triangular, their external edge being curved at their distal third (Fig. 2.3.a). The endopods` edges on their basal half are curved (Fig. 2.3.b). At the extremity of the endopods there are chitinous lobes, with fine and short spines on the external edge (Fig. 2.3.c). The chitinous lobes are removed with the exuviae in the post-reproductive moult, a process that was described by Tomescu (1972). The moult takes place generally in May-June. The male pleopods 2 have no taxonomical value (Fig. 2.3.d).

At males collected monthly (from April to October) from an oak forest from Livada locality (Ferenţi et al. 2012), in a hilly area (Satu-Mare County), we have noticed the development of these chitinous lobes before (Fig. 2.4.a,a') and after moult. In the first weeks after moult, the male pleopod 1 endopods resembles with the ones described by Strouhal in 1940 (cited by Radu, 1985), as being the subspecies named *P. politus slovakius* (Fig. 2.4.b,b',B). In males collected five months after the post-reproductive moult the newly formed chitinous lobes were very small, not having their initial size (Fig. 2.4.c,c'). They resemble with the chitinous lobes of the subspecies *P. politus carpathicus* (Fig. 2.4.c,c',C) described by Verhoeff in 1928 (cited by Radu, 1985). We consider that these two subspecies do not exist in reality, they being described on the basis of the morphological characters of *P. politus* males collected after the post-reproductive moult, after May-June. In males collected from mountain areas in July-October we have found males with normal sized chitinous lobes and males without these lobes, this fact proving that the moult process in a population does not take place in all males at the same time. In a sample collected on August 05, 2009 from the Călimani Mountains we also found males with and without chitinous lobes (Fig. 2.5.a,b). Thus, there is a possibility that not all males participate in the biological process of the reproduction.

At the 93 dissected males, of which microscopic slides were made, we have studied the variation of the pleotelson and the appendages morphology with taxonomical value, as well as the morphological anomaly cases. The length and the width of the pleotelson`s distal half vary (Fig. 2.6.a,b,c). The male pereiopod 7 ischiums ventral edge`s curve also vary, which can be more accentuated (Fig. 2.6.d), reduced (Fig. 2.6.e) or absent (Fig. 2.6.f). There is a high morphological variation in male pleopod 1 exopods (Fig. 2.7.a-f).

Morphological anomalies at the pleon segments were observed in three males, in which the segments 1 and 2 were longer. Between the coxal plates of the last pereion segment and the epimeres of the 3rd pleon segment there is a bigger distance than normal (Fig. 2.7.g). In two males anomalies were observed at the antennae, one of the antennae being smaller and depigmented compared with the pair, at antenna (Fig. 2.7.h). In a single case of the 93 dissected males we found the

anomaly of the pleopod 1 endopod, which was smaller and probably nonfunctional (Fig. 2.7.i). In the case of 12 *Trachelipus* species this kind of anomaly was not noticed (Tomescu *et al.,* 2015a).

Figure 2.1. *Protracheoniscus politus* C. L. Koch 1841, male and female dorsal view: **a.** ♂ 6.5 x 2.8 mm, **b.** ♀ 7 x 2.8 mm, **c.** cephalic lobes, **d.** pleotelson and uropods.

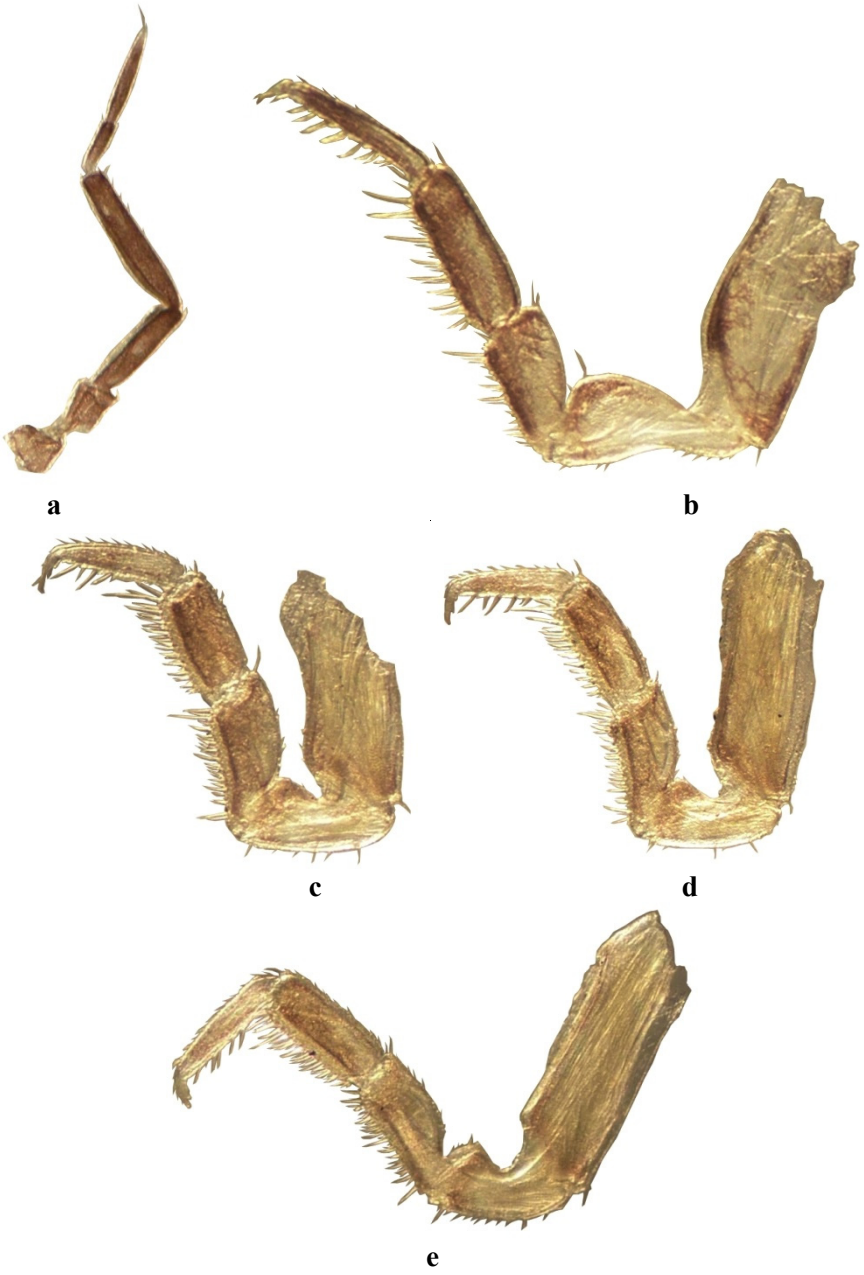

Figure 2.2. *Protracheoniscus politus* C. L. Koch 1841, ♂ 6.5 x 2.8 mm, **a.** antenna, **b.** male pereiopods 7, **c.** pereiopods 1, **d.** pereiopods 2, **e.** pereiopods 3.

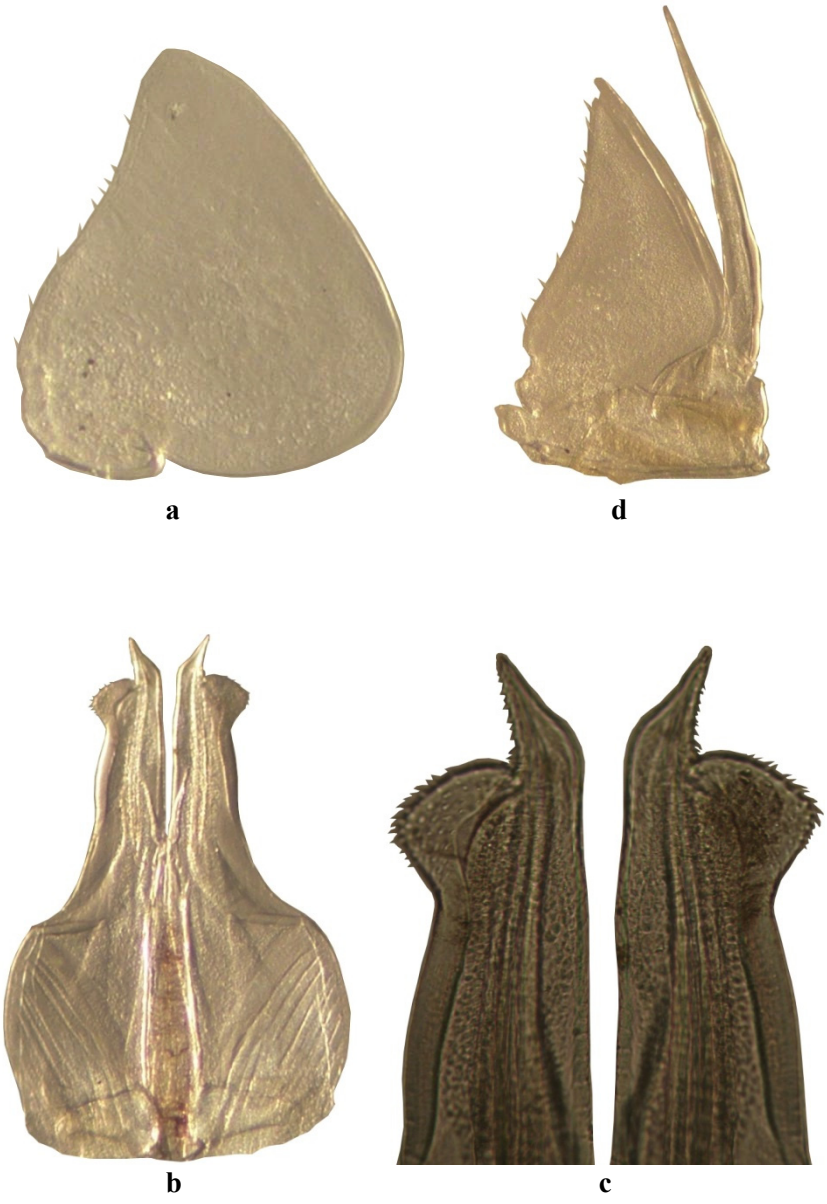

Figure 2.3. *Protracheoniscus politus* C. L. Koch 1841, male pleopods 1 and 2: ♂ 6.5 x 2.8 mm, **a.** pleopod 1 exopods, **b.** pleopod 1 endopods, **c.** the apex of the pelopod 1 endopods, **d.** pleopods 2.

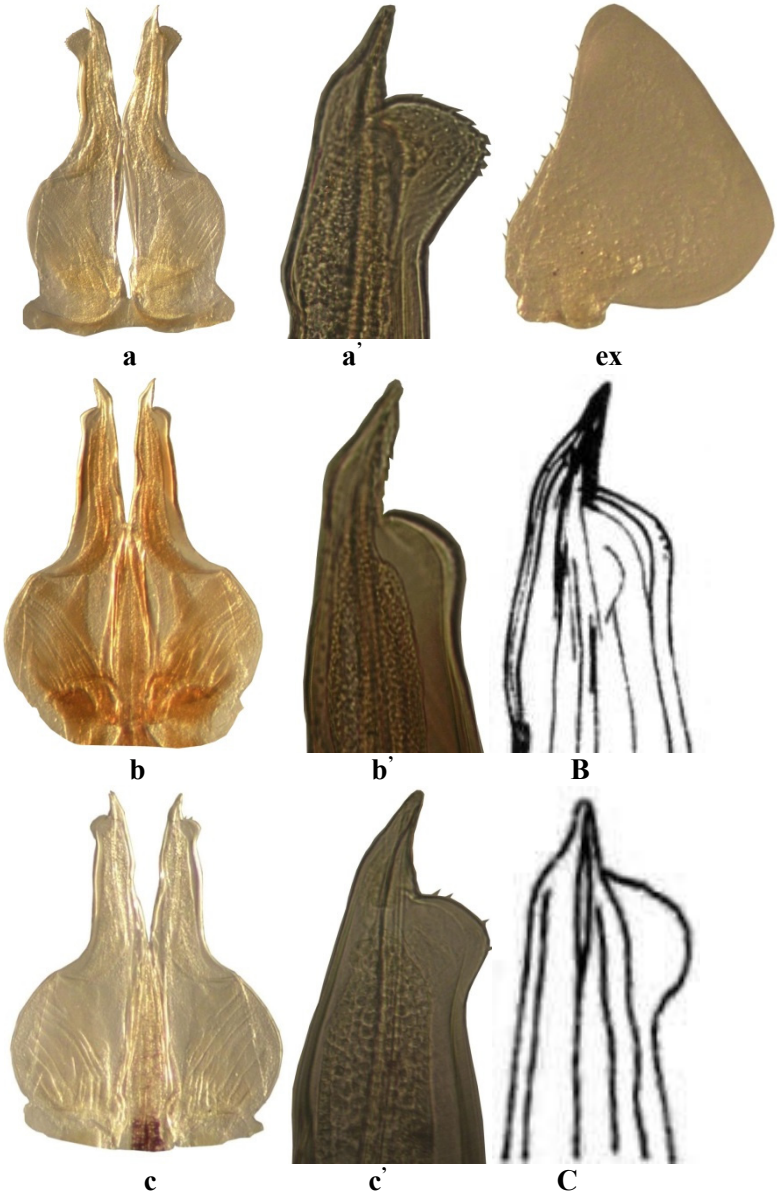

Figure 2.4. *Protracheoniscus politus* C. L. Koch 1841, male pleopods 1: **a, a'.** endopods of male 7.2 x 2.1 mm, collected in 11.May.2008 before molt, **ex.** exopods; **b, b'.** endopods of male 7.5 x 3 mm, collected in 06.July.2008, two months after molt; **B.** *P. politus slovakius* described by Strouhal, 1940 (cited by Radu, 1985); **c, c'.** endopods of male 6 x 2 mm, colectat in 03.October.2008, five months after moult; **C.** *P. politus carpathicus* described by Verhoeff 1928 (cited by Radu, 1985).

Ecology. *P. politus* is a typically sylvan species, living in the litter of regular and floodplain forests from all geographical units (mountains, hills, plains) (Hotea *et al*., 2003, Mureşan *et al.*, 2003, Radu and Tomescu, 1972, 1976, 1980/1981, Tomescu, 2010, Tomescu *et al*., 1979a,b, 2008, 2010, 2011a,b). In the research performed with pitfall traps in mountain pastures, we captured a high number of *P. politus* specimens, which proves that the ecological conditions of these pastures are optimal for this typically sylvan species (Tomescu *et al*., 2001, 2002, 2005). The species was absent in the pastures situated in hilly and lowland areas (Tomescu *et al.*, 1979b, 1995, 2011b). *P. politus* ecological valences are wide, this particularity being constant both in experimental studies and natural habitats. In laboratory we studied the superior lethal temperatures and found that all specimens died at temperatures over 30^0C (Tomescu and Radu, 1971a).

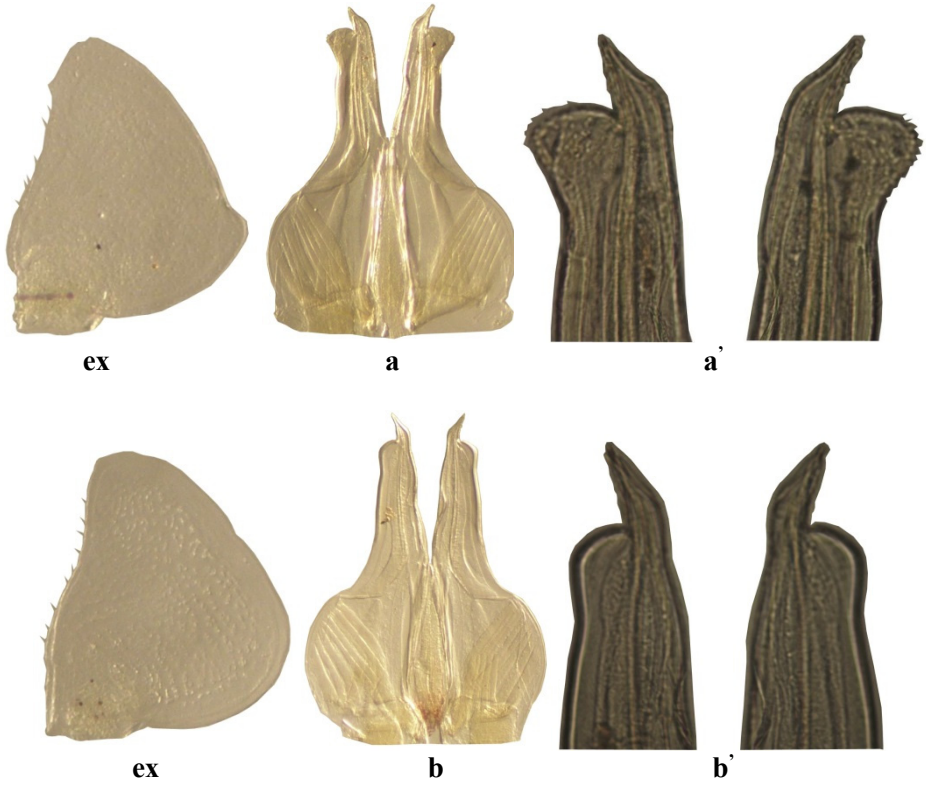

Figure 2.5. *Protracheoniscus politus* C. L. Koch 1841, males from the same sample collected in 05.August.2009, in Călimani Mountains, **a, a'.** male 6.5 x 2.5 mm, **b, b'.** male 7.2 x 3 mm, **ex.** pleopode 1 exopodites, ; **a, a'., b, b'.** pleopode 1 endopodites.

Figure 2.6. *Protracheoniscus politus* C. L. Koch 1841. **Morphological variation in males pleotelson**: **a.** ♂ 6.5 x 2.8 mm, **b.** ♂ 8 x 3 mm, **c.** ♂ 7 x 3 mm; **Pereiopod 7 d.** ♂ 6.5 x 2.8 mm, **e.** ♂ 7.8 x 3 mm, **f.** ♂ 6 x 5 mm.

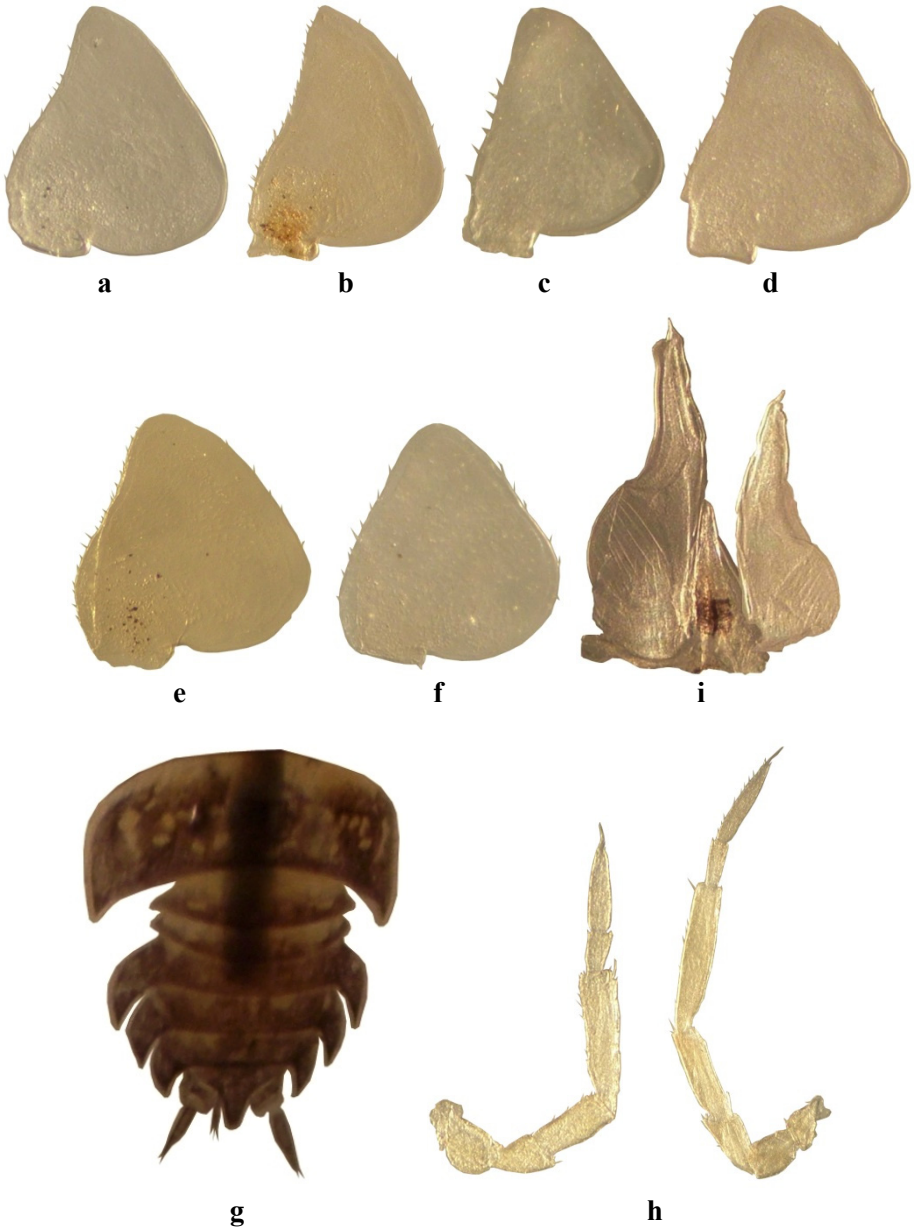

Figure 2.7. *Protracheoniscus politus* C. L. Koch 1841, **the variation of the pleopod 1 exopods: a.** ♂ 6.5 x 2.8 mm, **b.** ♂ 7.5 x 3 mm, **c.** ♂ 7 x 2.5 mm, **d.** ♂ 7 x 3 mm, **e.** ♂ 8 x 3 mm, **f.** ♂ 6 x 2.8 mm; **morphological anomalies: g.** ♂ 7 x 2.5 mm 1 and 2 pleonal segments, **h.** ♂ 6 x 2.5 mm antennae, **i.** ♂ 6 x 2.5 mm pleopod 1 endopod.

In field we studied the *P. politus* populations from two forested hills, with southern and northern exposition (Tomescu *et al.,* 1971b). The temperature under the litter and the humidity on the soil surface were measure. There were differences in temperature and humidity. At the southern exposure the temperature was 28^0C and the humidity 40%, and 16^0C and 65% at the northern exposure. The quantitative researches has shown the existence of some numerous populations of *P. politus* in forests of both hills (Tomescu *et al*., 1971b), which proves the relatively high tolerance of the species to the temperature and the humidity of the soil of the habitats it lives in. *P. politus* also has numerous and stable populations in peat bogs, where the humidity of the soil surface is 100% (Tomescu *et al.,* 2015b).

Geographic distribution. The species *P. politus* is present in eastern Germany, Poland, Czech, Slovakia, Austria, Serbia, Montenegro, Hungary and Romania (Schmalfuss, 2003). In Romania we have found *P. politus* populations in all forest types from mountain, hilly and plain areas, as well in mountain pastures with moderate soil humidity.

Discussion

The taxonomical status of the species *P. major* was clarified by Grunner (1966), who studied comparatively the male 1st pleopods in five *Protracheoniscus* species. He described comparatively the shape of male 1st pleopods in *P. major* and *P. asiaticus*, showing the obvious morphological differences between the two species. In our research we described additionally other morphological characters, the male and female cephalon morphology, the cephalic lobes, antennae, the pereiopods 1, 2, 3 and 7. In Romania, Radu (1985) mentions wrong *P. asiaticus* as being present in the country. *P. major* is present in many European countries, an exclusively synanthropic species, which lives near old houses and cellars (Jedryckowski, 1981, Vilisics and Hornung, 2009, Ferenţi, 2013, Ferenţi *et al*., 2015). We have collected individuals from two Transylvanian localities (Simeria and Salacea), but we consider that it is also distributed in other parts of the country. Recently it was encountered in another small town (Salonta) from western Romania (Ferenţi *et al.,* 2015). Further researches are necessary for know its exact distribution in Romania. Under the name of *P. asiaticus* it was recorded by Radu in different localities from Moldova, but without any precise indication and in Oradea (Radu, 1985).

In *P. politus* there are intraspecific morphological variations of the specific characters. At the pleotelson the length and the width of the distal half vary. Also, variations do exist at the length ratio of the last and the penultimate antennary articles. At male pereiopod 7 ischium the concavity of the ventral edge varies. Significant variations also exist at male 1st pleopods. The exopods shape varies. At

endopods there are variations related to the post-reproductive moult. At adult males, before moult there are chitinous lobes at the endopods` tip. After the reproduction period the post-reproductive moult takes place, the lobes being removed together with the exuviae. The presence of the chitinous lobes represents the main specific character for *P. politus*. After the chitinous lobes loss the endopods` morphology is modified, fact which determined the confusions of some researchers, which did not know the phenomenon. They studied *P. politus* individuals captures in certain intervals after moult and had described the subspecies *P. politus carpathicus* and *P. politus slovakius* (cited by Radu, 1985). The present and the previous (Tomescu, 1972) research emphasized the modifications of the male pleopod 1 endopods` tips related to the post-reproductive moult. The described subspecies are not valid; they represent taxonomical mistakes of the specialty literature.

In the material collected by pitfall traps between April and October, in 11 habitats, we found males of which endopods without chitinous lobes, but also males in which these were present. We consider that the moult is influenced by the males' participation in the reproduction activity, which triggers the moult process. Analyzing the monthly collected males, we could observe the process of the chitinous lobes` regeneration. In males collected in July, after the moult, the endopods` tip without lobes resembles the ones described by Strouhal (1940) in *P. politus slovakius*. In males collected in October, the lobes were small; resembling the ones described by Verhoeff (1928) in *P. politus carpathicus*. Analyzing the males collected in the next year, in May, we observed that the chitinous lobes were developed at their normal dimensions, specific for all males. Anomalies were observed in a reduced number of males of the 93 dissected males in pleon, antennae and in a male at the pleopod 1 endopods.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LXI, 1, 2016 (p. 167-175)

Long Term Consumption of red Bull and Alcohol can Affect Rat Skeletal Muscle Metabolism

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SUMMARY. In the past few years, Red Bull rapidly became a popular energy drink and this increase in popularity is the result of targeted advertising. The effects of short term consumption of Red Bull are known and they are beneficial, however the effects of chronic consumption may lead to serious health conditions. The purpose of the study presented in this work is to investigate if long term consumption of Red Bull affects the physiological and functional integrity of the skeletal muscles. The study was conducted on 28 albino male Wistar rats, weighing 182.11±4.7 g, divided into four groups. The Control (C) group received a standard diet and tap water. The Red Bull/Ethanol (RB+E) group were orally administrated 1.5 ml/100 g b.wt. of Red Bull and 0.6 ml/100 g b.wt. of ethanol daily, for 30 days. The same concentrations were administered to the groups that received the individual drinks. In the last six days of the experiment the animals were tested for physical performance using a weight-loaded forced swim test. After 30 days of treatment, immediately after exhaustion, the animals were killed under anesthesia and samples from the gastrocnemius muscle were harvested for biochemical parameters analysis and enzymatic activity assays. A significant decrease in glucose and glycogen concentration was registered in E and RB+E groups. Total protein concentration as well as AST and LDH activities remained unchanged in all groups. According to these results we can say that long term consumption of Red Bull energy drink, especially when combined with alcohol, may lead to significant changes in biochemical parameters strictly related to the carbohydrate metabolism. Red Bull and ethanol did not affect the physiological integrity of the skeletal muscle, although a transition probably occurred from type 2 (glycolytic, fast twitch) to type 1 (oxidative, slow-twitch) muscle fibers.

Keywords: energy drink, Red Bull, skeletal muscle

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Introduction

In the past few years, Red Bull rapidly became a popular energy drink and this increase in popularity is the result of targeted advertising. It is marketed as an enhancer of physical performance and as a stimulant for attention, concentration and the level of alertness ("Company" Red Bull, 2016). This makes it an appealing beverage for teenagers, athletes and people who undertake physical activities for recreational purposes.

One other way individuals choose to consume Red Bull is in combination with alcohol. The main reason for this habit is the sweetening of the alcoholic beverages and a diminished perception of alcohol intoxication (Pennay *et al.*, 2011). As stated in the aforementioned article, Red Bull can mask the signs of alcohol intoxication leading to greater levels of alcohol intake and consequently alcohol poisoning.

The short term effects of Red Bull consumption are beneficial for the organism because of its ergogenic role while engaging in physical activities, as observed in previous studies (Forbes *et al.*, 2007; Verma and Biswas, 2014).

However, the effects of chronic consumption are not yet studied in detail. Results from our past experiments show that these effects are of concern, especially on cardiac and hepatic biochemical parameters and enzymatic activities (Crișan *et al*., 2013; Crișan *et al*., 2014). In the studies performed by Seifert *et al.* (2011) and Rath (2012), a long term consumption of energy drinks has been associated with convulsions, diabetes and changes in behavior and disposition, especially in adolescents and young adults. Other possible effects, observed by Waguih *et al.* (2012) and Pennington *et al.* (2010) are cardiovascular diseases, obesity, insomnia, anxiety, dehydration and difficulty in concentration.

The present study is part of a larger research project targeting the effects of long term consumption of Red Bull and alcohol, in combination and separately, on male Wistar rats. With the majority of studies focusing on the physical performance of human subjects, there is little to no evidence of the effects of Red Bull on rat skeletal muscle. The purpose of this study was to investigate the effects of long term consumption of Red Bull and alcohol, in combination and separately, on the skeletal muscle in physically trained rats.

Materials and methods

All reagents used in this study were of analytical grade and were purchased from Sigma-Aldrich Chemie GmbH, Germany, Nordic Invest S.R.L., Romania and S.C. BioZyme S.R.L, Romania. The Red Bull energy drink was bought from the local market.

The study was conducted on 28 albino male Wistar rats, weighing 182.11±4.7 g, divided into four groups, Control (C), Red Bull (RB), Ethanol (E) and Red Bull/Ethanol (RB+E). All animals received a standard diet (S.C. Siamond Prod. S.R.L., Cluj Napoca, Romania). The C group (n=7) had *ad libitum* access to tap water, RB group (n=7) were orally administrated 1.5 ml/100 g b.wt. of Red Bull daily, for 30 days and EtOH group (n=7) received 0.6 ml/100 g b.wt. of ethanol daily. The same concentrations were administered to the RB+E group (n=7).

In the last six days of the experiment the animals were tested for physical performance using a weight-loaded forced swim test. The rats were forced to swim to exhaustion with a load of 10% of their body weight attached to their tails. Each rat was considered to have reached exhaustion when it remained submerged for longer than 5 seconds. Water temperature varied between 28 and 30^{o} C and none of the animals were affected by hypotermia.

After 30 days of treatment, immediately after exhaustion, the animals were killed by exsanguination under anesthesia. Samples from the gastrocnemius muscle were harvested for the following biochemical analysis: total muscle glucose, glycogen and proteins; enzymatic activities of: ALT, AST, and LDH.

Total glucose concentration was determined with the Somogy-Nelson colorimetric assay (Nelson, 1944). Glycogen concentration was determined using the Montgomery (1957) method modified by Lo *et al*. (1970). Total protein concentration was determined by Bradford (1976) colorimetric assay using the Bradford "ready-to-use" reagent. Reitman and Frankel (1957) photocolorimetric assay was used for the determination of AST and ALT enzymatic activities. The activity of LDH was determined spectrophotometrically by measuring the oxidation rate of NADH (nicotinamide adenin dinucleotide, reduced) at 365 nm (Bergmeyer and Bernt, 1974).

Results were analyzed using the two tailed *t* test and considered statistically significant at $p \leq 0.05$. Multiple comparisons were made (more details are presented in the Figure 1 description).

Results and discussion

The purpose of this study was to investigate the effects of long term consumption of Red Bull and alcohol, in combination and separately, on the skeletal muscle in physically trained rats.

Red Bull and ethanol, administered alone or combined, caused a decrease of skeletal muscle glucose concentration as seen in Figure 1a and Table 1. It has been previously shown that oral administration of niacin, one of the vitamins found in high concentration in Red Bull, or intense physical activity causes a muscle fiber transition from type 2 (glycolytic, fast twitch) to type 1 (oxidative, slow-twitch) (Khan *et al.*, 2013). Type 1 fibers are rich in mitochondria and mainly use fatty acids as an energy

source (Ringseis *et al.*, 2013); at the same time, glucose uptake by the skeletal muscle is inhibited. One explanation for the decrease in glucose concentration could be the result of these changes in muscle fiber phenotype and metabolism.

Xu *et al.* (1996) showed that alcohol has an inhibitory effect on the uptake of glucose by the skeletal muscle. Despite the consistent observation that acute and chronic alcohol intake impairs the *in vivo*-determined insulin-mediated glucose uptake by skeletal muscle cells (Spolarics *et al.*, 1994; Lang *et al.*, 2014; Wan *et al.*, 2005), there is little knowledge about the mechanism by which this happens. In theory, as described by Steiner *et al.* (2015), alcohol can affect insulin signaling at a number of key regulatory steps such as PI3K/AKT signal transduction and/or GLUT4 translocation.

The combined treatment caused a significant decrease in glucose concentration in the RB+E group, which was probably determined by the complementary action of Red Bull and ethanol.

As seen in Figure 1b and Table 1, glycogen concentration decreases significantly after Red Bull and ethanol consumption. As stated previously, type 1 fibers are rich in mitochondria and mainly use fatty acids as an energy source, therefore this type of muscle fibers usually have fewer glycogen deposits (Ringseis *et al.*, 2013). Similarly to the glucose concentration, glycogen deposits in rats treated with Red Bull may have decreased due to the changes in muscle fiber phenotype and metabolism.

A study by Peters *et al.* (1996) demonstrated that ethanol inhibits glycogen accumulation in type 1 fibers during the immediate recovery from high-intensity exercise. Other groups proposed that alcohol inhibits the activation of glycogen synthase, which leads to a low level of glycogen in the skeletal muscle (Xu *et al.*, 1992; Xu *et al.*, 1996). The animals in our study were sacrificed just after exhaustion. This fact, alongside the evidence that intense physical exercise facilitates the transition between muscle fibers, offered by Khan *et al.* (2013), can explain the decrease in glycogen concentration in the E group.

When administered together, the action of Red Bull and ethanol was complementary, determining the depletion of the glycogen deposits in the skeletal muscle (Fig. 1b, Table 1).

Interestingly, although we expected a decrease of the total protein concentration in each of the treatment groups, such an effect was not observed (Figure 2a, Table 1). Both ethanol and some ingredients from Red Bull are known to cause muscle injury (Martin *et al.*, 1985; Campana *et al.*, 2014), hence our expectations. In cases dating from 1989 to the present, patients were hospitalized presenting rhabdomyolysis and other severe affections caused by intoxication with large amounts of caffeine (Wrenn and Oschner, 1989; Chakraborty and Rajeswaran, 2007; Campana *et al.*, 2014), one of the main ingredients of RB.

Table 1.

Effects of Red Bull, ethanol and the combined drink on biochemical parameters and enzymes of the skeletal muscle in phisycally trained rats

Parameter	Control	RB	E	RB+E
Glucose concentration (mg/g tissue)	1,086±0,20	0,83±0,175	0,32 ±0,14 *	0,21 ±0,07 ** *
Glycogen concentration (mg/g tissue)	3,43±0,41	2,06±0,25 *	2,14 ±0,24 *	0,70±0,19 *** ** **
Total protein concentration (mg/g tissue)	46,05±2,73	47,45±1,47	47±2,11	47,86±0,92
LDH activity (µmol pyr/g tissue/min)	0,0133±0,0012	0,0131±0,0005	0,013±0,0013	0,0125±0,0005
AST activity (µg pyruvate/g tissue/ hour)	3269±151	3367±82	3347±84	3357±63
ALT activity (µg pyruvate/g tissue/ hour)	10061±166	9653±307	9570±298	9441±350

RB-Red Bull; E-Ethanol; RB+E-Red Bull+Ethanol; LDH-lactate dehydrogenase; AST-aspartate aminotransferase; ALT-alanine aminotransferase. Multiple comparisons were made: black - *vs* Control group; red - *vs* RB group; blue - vs E group; * p<0.05; ** p<0.01; *** p<0.001.

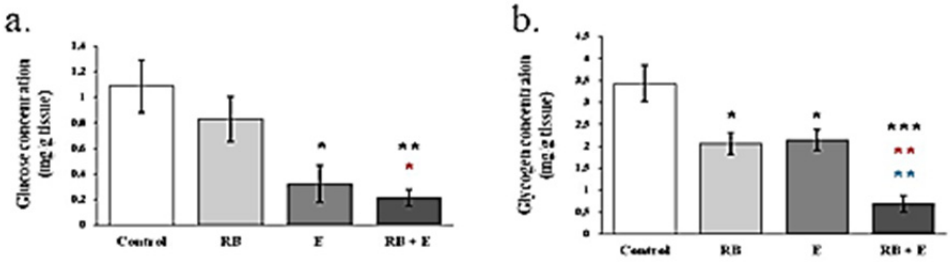

Figure 1. Effects of Red Bull, ethanol and the combined drink on skeletal muscle (a) glucose concentration and (b) glycogen concentration, of physically trained rats. n=7 in each group. The results are expressed as mean ± SE. Multiple comparisons were made: black - *vs* Control group; red - *vs* RB group; blue - vs E group; * p<0.05; ** p<0.01; *** p<0.001.

Although the amount of caffeine contained in the energy drink is high, it is unlikely to cause rhabdomyolysis. Even so, after 30 days of treatment we expected a decrease of the total protein concentration in the skeletal muscle of rats treated

with Red Bull. A plausible explanation can be the membrane stabilizing effect of taurine on the skeletal muscle (Huxtable and Bressler, 1973). The process is not yet fully understood but it is related to the ability of taurine to control the function of ion channels and consequently membrane excitability. Taurine also influences calcium homeostasis and excitation-contraction coupling (De Luca *et al.*, 2015).

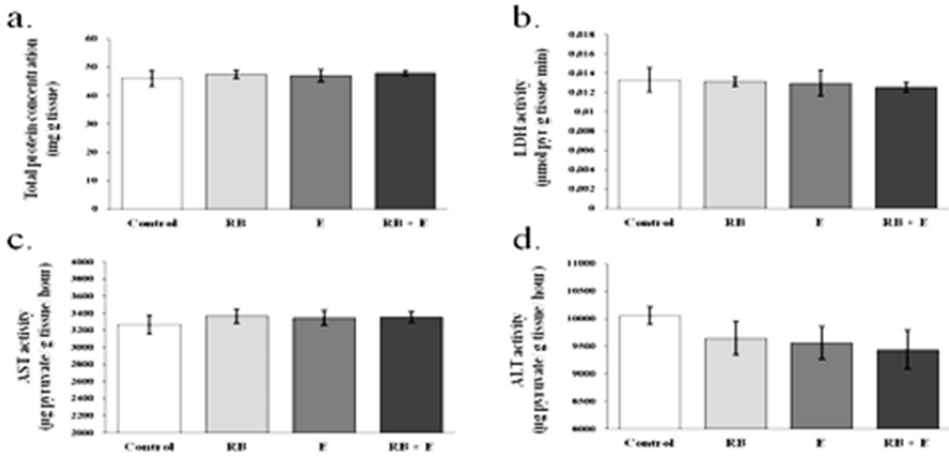

Figure 2. Skeletal muscle (a) total protein concentration; (b) LDH, (c) AST and (d) ALT activity of physically trained rats treated with Red Bull, ethanol and the combined drink. n=7 in each group. The results are expressed as mean ± SE

Considering the beneficial effects of taurine on the skeletal muscle, we assume that the markers for cell integrity will not vary between groups. LDH together with AST and ALT activities are enzymatic markers which, if found in high amount in the blood stream, indicate muscle injury and leaking of the enzymes from the tissue (Janssen *et al.*, 1989).

LDH is also a marker for glycolytic metabolism (Spriet *et al.*, 2000) and the administration of Red Bull and ethanol, separately or combined, did not affect its activity (Figure 2b, Table 1). These results confirm the fact that the skeletal muscle of the rats in all groups has undergone a change regarding the type of muscle fibers and consequently a transition from glycolytic to oxidative metabolism.

Similarly to LDH, AST activity did not suffer any noteworthy changes (Figure 2c, Table 1) and judging by these results we can assume that the physiological integrity of the skeletal muscle was not affected.

Interestingly, although not statistically significant, ALT activity was affected by the treatment and suffered a slight decrease in all groups (Figure 2d, Table 1). The decline in glucose utilization may have led to a low pyruvate concentration in the skeletal muscle and consequently to the decreased activity of ALT (Felig, 1973).

Conclusions

According to our results, we can say that long term consumption of Red Bull energy drink, especially when combined with alcohol, may lead to significant changes in biochemical parameters strictly related to the carbohydrate metabolism. Red Bull and ethanol did not affect the physiological integrity of the skeletal muscle, although a transition probably occurred from type 2 (glycolytic, fast twitch) to type 1 (oxidative, slow-twitch) muscle fibers.

The health conditions that Red Bull can lead to are dose-dependent, which necessitates the proper labeling of the product. This way, customers can make a conscious decision if they choose to consume the energy drink or not.

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Survey upon the Living Habits of Romanian but Hungarian Nationality Students Major in Biology and Physical Education

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SUMMARY. The present research paper had both a pedagogical type of work and also an investigative, assessing character, which was prepared with educative intention. The idea was the following: we have to know the reality concerning the lifestyle of our students, in order to decide the direction of further health education. The survey was performed by a questionnaire. The questionnaire applied for the research included 25 basic questions and further 33 questions with a segmented character. The results of the questionnaires prove that the hygienic knowledge of the students is insufficient, or they are not able to apply it properly. The majority of the interviewees feel the need to develop their hygienic knowledge. Most of the youths know the essence of health preservation, rules, but they do not apply them in practice, as they do not strive to prevention by their behavior and attitude.

Summarizing the ideas formulated in the present research paper, it is evident that the importance of health education has increased. The knowledge of the youths broaden during their high school and university studies, but these classes are insufficient. In the future it is essential to initiate modifications concerning the whole society on a higher level and united in order to change the health condition of society in the short run.

Keywords: food pyramid, healthy nutrition, narcotics

Introduction

"Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity"- this is the definition of health according to the WHO* (1948). Health is not an external factor, but the internal,

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essential accessory of our own physical and psychical being, which can be preserved only by a purposeful continuous activity. Our way of living and living habits is essential in preserving our health. It is in great part the responsibility of each person to maintain his/her own health. Families and different educational and pedagogical institutions are mainly in charge with the education for the proper lifestyle (Reilly and McDowell, 2003). At home and in the educational institutions those physical activities and competencies can be built in the system of knowledge and habits of the youngsters, which becoming elements of their lifestyle, will generate health, work capacity and vim.

Among the factors that imperil our health, the harmful habits are extremely significant. They appear decisively during young age, while such habits as smoking, consuming alcohol or drugs can evolve, endangering one's health on the long term. This is also the age when sexuality comes forward. Inappropriate forms of behavior imperil one's physical and psychical health, perturbing one's development, the evolution of the personality and the integration into society. The behavior that is destructive for health may not only bring the person into a life threatening situation, but also by its multitudinous character it is maleficent for the whole society (Ayliffe and Glanville, 2010, Szakály *et al.*, 2013).

The necessity of health education in our country is justified by the statistical analysis according to which the number of the Hungarian population in Romania decreased significantly by the data of the latest census. Among the main causes of the decrease of the population can be identified the significant emigration due to unemployment, which affects not only the Hungarian minority, but also the number of Romanian population, too. Another factor is the decrease of the number of live-births and last but not least there is the list of the diseases causing death, as related to some of these we are at the head of the statistics. The diseases connected with nutrition and lifestyle (such as cardiovascular diseases, evolution of tumors, diabetes and obesity) are the most frequent among the worldwide population (2005**). The diseases connected with nutrition and lifestyle are those ones caused in a substantial part by consuming too much, or too little energy, introducing some nutriments excessively such as too much salt or animal fat, or insufficiently as deficient amount of calcium. These diseases could be prevented through healthy nutrition and lifestyle.

The main goal of current research is the assessment and analysis of the habits of the students' community studying at the Biology and Physical education Faculties, from the point of view of hygiene. Considering the complex nature of this study, our goals can be formulated as follows: survey healthy nutrition (the frequency of consuming vegetables, fruit, meat, nutriments enriched with carbohydrates, and dairy products); judging the healthy lifestyle (doing exercise, sports, frequency of participating at competitions); racing the use of harmful substances (cigarettes, drugs and alcohol); determine the frequency of chronic (cardiovascular, metabolic) diseases occurring in the family.

Materials and methods

The hygienic survey was conducted with the contribution of 152 university students. The students were chosen from two faculties, biology and physical education. The surveying was performed by a questionnaire. The questionnaire applied for the research included 25 basic questions and further 33 questions with a segmented character. It includes the following questions: age, gender, data of birth, weight, height, BMI, nutritional behavior (vegetables, fruits, milk, sugar added foods, animal protein) physical activity, smoking, alcohol consumption, family history of metabolic and cardiovascular disease. Data was presented using descriptive statistics in the form of percentages. The results were processed by the means of the *Excel* program.

Results and discussion

The students were born within the 1992-1994 period. 94.74% of the students participating in the research were born in Romania and 5.26% in Hungary, but these latter ones accomplished their high school studies in Romania as their parents had moved back to their hometowns.

The parameter of healthy lifestyle that can be traced easiest, is the body mass index (BMI), which can be calculated by the square ratio of weight and height. Normal body weight is characterized by values between 18.5 and 24.99. BMI values under 18.5 are characteristic for emaciation and malnutrition, while values above 25 are characteristic for obesity, overweight.

Childhood obesity is a global epidemic and rising trends in overweight and obesity are apparent in both developed and developing countries (Flynn *et.al.* 2006). Several clinical data prove that obesity increases the probability of the emergence of not only metabolic and cardiovascular diseases, but also of different forms of cancer (Chang *et al.,* 2006, de Greeff *et.al.*, 2016, Halmi, 2008, Koponen *et al.,* 2013, Roberts *et al*., 2010, Wolin *et al*., 2010). The most frequent type of cancer among women is the endometrial carcinoma (Kis *et al*., 2015), which evolves in most cases in obese individuals with BMI over 30. According to WHO, obesity is rising by 30 million cases per year whereas the overall number of new cancer cases will increase by 300.000 cases per year. Both obesity and cancer contribute to increased worldwide mortality and healthcare costs. They are now both recognized as global healthcare concerns and have been the subject of worldwide calls to action (Ashrafian *et al.,* 2011)

The BMI between the interviewed students was higher than 25 for 10.52 % of them, belonging to the overweight category (Fig. 1). The highest BMI value was around 30. 82.89% of the students have normal BMI values, while 6.57% of the students can be rated in the undernourished group as their BMI was under 18.5 and the lowest BMI value was around 15. This group is also a category at risk as undernourishment increases mainly the risk of diseases of the vascular system.

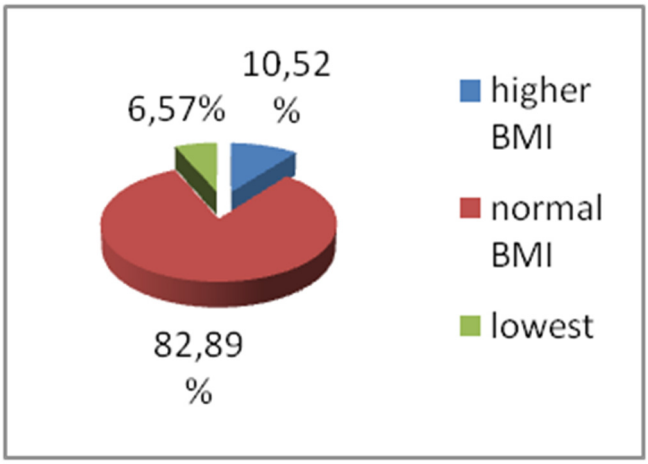

Figure 1. The body mass index (BMI) of the studied students

According to the results of this study, three-quarters of the students disposed to obesity are male though more than half of the interviewees are female.

As Fig. 2 shows, analyzing the habits of consuming vegetables of the interviewees, 51.97% of them consume vegetables daily, 19.07% three times a week, 17.76% twice a week, 5.92% four times a week, 2.63% rarely, less than once a week.

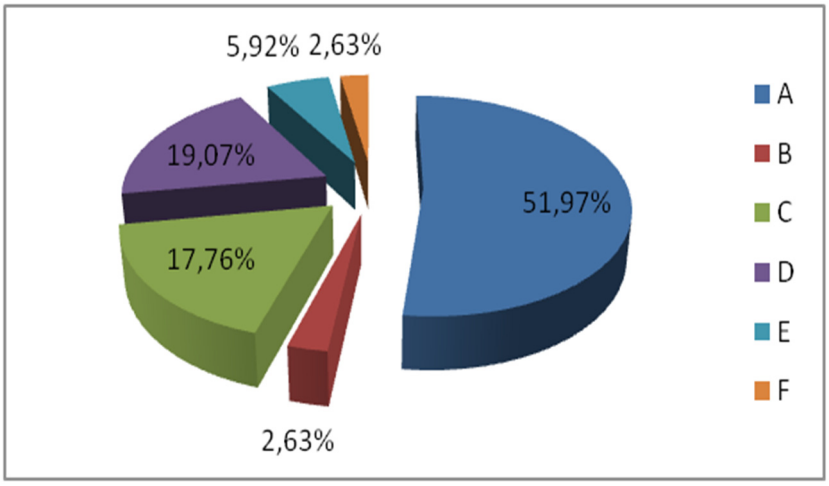

Figure 2. The proportion of vegetables consumers among the students; A-daily, B-ocasionaly, C-twice a week, D-three times a week, E-four times a week, F-less than once a week

The vegetables are consumed raw, or the majority of the students consume them steamed, boiled. 48% of the interviewees consume vegetables on a weekly basis. More than half of the students consuming vegetables daily are women (53.16%). Analyzing the habits of fruit consuming, 61.84% of the interviewees consume fruit on a daily basis, 18.42% of them three times a week, 7.23% twice a week, 6.57% four times a week, and 5.92% rarely (Fig. 3). The proportion of the students consuming fruit daily is much higher than the vegetable consumers'. Analyzing the distribution by gender, women consume fruit in a much greater number (53.16%). Similarly to the habits of consuming vegetables, 38% of the interviewees consume fruit on a weekly basis.

If we examine the bibliographic sources of dietetics it is evident that vegetables and fruit constitute the base of the food pyramid (Horváth, 2003).

Vegetables provide an important intake of fibers, minerals, glucose and vitamins, while fruit provide especially an intake of glucose, vitamins and minerals (Vanczák *et al.*, 2004). The consumption of vegetables and fruit among the interviewees correspond to the food pyramid***.

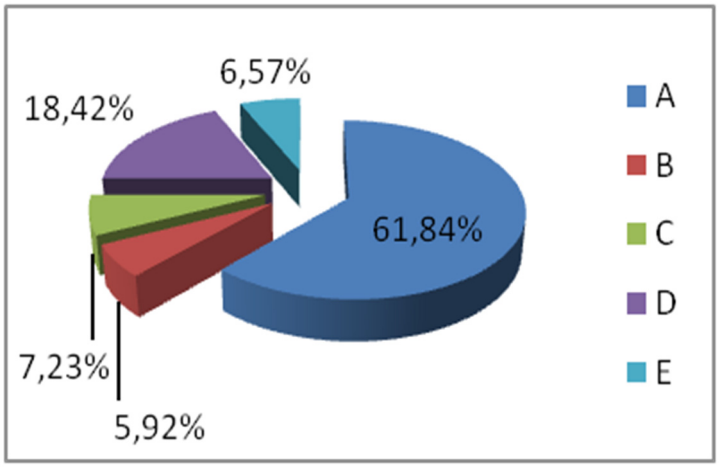

Figure 3. The fruit consumption on a weekly scaling, A-daily, B-rarely, C-twice a week, D- three time a week, E-four time a week

The proportion of people consuming sugar added food is high: 93.43%. Only 6.57% of the interviewees said that they do not consume sweets at all. The majority of the consumers of sweets also consume sweetened foods (Fig.4.) such as cakes, sweet soft drinks and sorts of chocolate on a daily basis. Merely 16.19% of the interviewees consume sweets rarely (less than once a week).

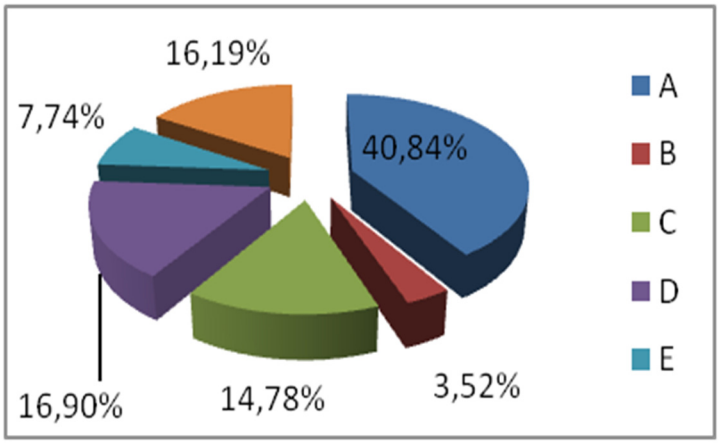

Figure 4. The frequency of sweet consumption on a weekly scaling;
A- daily, B- four times a week, C- three times a week,
D- twice a week, E- once a week, F -rarely

80% of the persons who do not consume sweets are male and 20% female. 94.5% of the sweets consumers are women and 5.5% are men. However, according to the BMI data men are more disposed to obesity. One possible explanation is the fact that the majority of the female students do sports on a daily basis, so the excess of energy ensuing from the sweets consuming is burnt this way.

The sweets consumption among the interviewees shows a high value. The perpetual sweets consumption on the long term puts a strain on the organism, which can cause different diseases. From this point of view 94.5% of the interviewees can be at risk.

44.73% of the interviewees consume meat on a daily basis, more than half of them weekly, and 2% do not consume meat at all.

More than half (55.88%) of the persons consuming animal protein on a daily basis are male. According to our results, women are leading in sweets consumption, men prefer animal proteins. Both of these are among the factors which increase the risk of obesity when they are consumed excessively. The different sorts of meat (pork, beef, venison) contain a lot of fat therefore increase the fat storage of the organism.

Despite the fact, that the energy value of fats is higher compared to carbohydrates, the organism degrades mostly carbohydrates and starts degrading the lipids accumulated in the adipose tissue only after the exhaustion of carbohydrates. The human body has always been used to storage, therefore those persons who want to achieve weight loss through an inappropriate diet, could gain weight despite low energy consumption.

As we could see, more than half of the interviewees are women; still the higher BMI is more frequent at men as they consume more animal protein than the interviewed women. If we represented the food pyramid of the interviewees, it would be widely different from the healthy food pyramid of a young adult organism. In a food pyramid that represents healthy nutrition the energy-rich foods are at the top of the pyramid, while by the own admission of the interviewees these foods are at the basis of their pyramids.

The answers to the inquiry related to the consumption of milk and dairy products, reveal that this type of food are more popular than the above mentioned ones. More than half of the interviewees consume milk and dairy products on a daily basis, while 2% do not consume them at all (Fig.5). On a distribution by gender there are no significant differences between the two sexes, since they both consume dairy products almost equally. Dairy products provide a significant intake of protein, minerals and liquids.

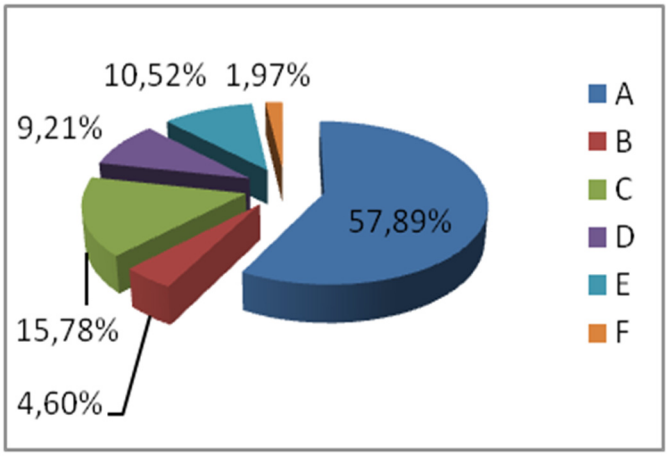

Figure 5. The consumers of milk and dairy products on a weekly scaling; A- daily, B- four times a week, C- three times a week, D- twice a week, E- once a week, F- does not consume milk or dairy products

Another requirement of healthy lifestyle is regular exercise, training. Physical inactivity affects metabolism and all major body systems, exerting powerful positive influences on the brain and spinal cord, consequently, on physical health, and motivation and ability to learn (Basch, 2011).

Physical inactivity has been identified as a risk factor of diabetes mellitus independently of its effects on body size, and dietary patterns. Physical activity of moderate to vigorous intensity and duration decreases the risk of conversion of impaired glucose tolerance into diabetes even in the absence of significant weight loss, and independently of other risk factors (Alberti *et.al*., 2007). Diabetes mellitus is a growing public health problem affecting people worldwide both in developing and developed countries, and poses a major socio-economic challenge. (Dahiru et.al., 2008).

In 2000, 171 million people were estimated to have diabetes around the world, and this figure is expected to rise to 366 million by 2030 (Wild et.al., 2004).The answers given to the questions related to doing exercise (fig.6), sports regularly reveal that 20% of the interviewees do not do any sports or exercise at all, while 80% do sports regularly. 33% of the regularly sporting students train does sports daily. Most of the students who do exercise on a daily basis train daily for several hours, but these students study mainly at sports departments. 22% of the interviewees do sports three or four times a week, 9% twice a week, 5% once a week, and 10% less than once a week.

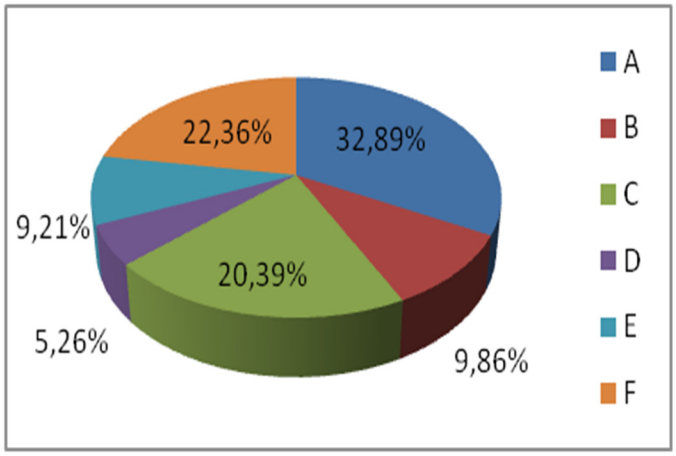

Figure 6. The frequency of doing sports, exercise on a weekly scaling; A- daily, B- rarely, C- not do sport, D- once a week, E- twice a week, F- three or four times a week

Half of the interviewees participate at sports competitions as well (51.14%). This higher proportion is due to the fact that three-quarters of the interviewees study at sports departments where daily trainings are obligatory, respectively participating at sports competitions is more popular than among the students studying at the Biology department.

Exercise raises the energy traffic of the human body and ensures a higher consumption of oxygen. Properly done exercise trains not only the muscles but also the vascular and respiratory system. Due to regular sporting the capacity of the lungs and heart increases, a greater amount of air enters the body that provides the oxygen necessary for the activity of the cells. Besides a good oxygen supply of the tissues it provides the evacuation of the decomposition products. Furthermore, regular exercise speeds up metabolism, prevents the storage of excessive nutriments, and hence prevents obesity, diabetes, hypertension, and the premature evolution of heart attack (Mahfouz *et al.,* 2015). Hence balanced sporting, training can be important tools of the health-preserving programs (Basch, 2011, Stamatakis *et al.*, 2009, Fair and Montgomery, 2009).

The consumption of health impairing substances (alcohol, cigarettes) is getting more and more popular among Romanian youths, too. 7% of the interviewees did not answer to the questions related to smoking, 67% do not smoke and 27% smoke. 32.25% of the smokers, smoke occasionally, while 67.75% of them smoke daily. The number of smokers is relatively low, but more than half of them smoke on a daily basis.

Smoking is the most dangerous among the health impairing risk factors (Wura *et al.*, 2016). According to the statistics of the WHO annual mortality due to smoking exceeds mortality due to drugs, alcohol, contagions and accidents altogether. In 1999 there were 1.25 billion smokers worldwide. In the developed countries 42% of men and approximately 24% of women are smokers, while in developing countries 48% of men and 12% of women smoke (Rydin, 2012). In developed countries smoking has decreased over the last years. This can be the result of the more and more successful health education, anti-smoking campaigns, restriction of the advertising of tobacco products, furthermore the increase of the smoke-free workplaces, means of transport and places of amusement.

According to the data of this research, two-third of the students does not smoke, but 17% did not answer to the question. It can be supposed that those who did not answer either do not know sufficiently the harmful effects of smoking, or they know it well and thus they do not want to admit it. Therefore it is essential to raise awareness among the interviewees related to the harmful effects of smoking.

While the proportion of smokers and users of narcotics is low, the consumption of alcohol is very popular among the interviewees. The alcohol consumption of the Hungarian population is high both within Hungary and beyond its borders. 3% of the interviewees do not answer, about 80% consume alcohol and only 17% do not consume alcohol at all. 41% of the alcohol consumers drink alcohol rarely but on a monthly basis, while 59% consume alcohol more often, on a weekly or even daily basis. Those who consume alcohol weekly drink alcoholic beverages, mainly beer or wine, usually twice or three times a week.

According to the results of this research those who consume alcohol regularly, happen to have an alcohol consumer family member. 11% of the interviewees did not answer to this question, whereas in the case of 20% there are alcoholic predecessors. 90% of those who have alcoholic predecessors consume alcohol regularly. According to the results of this research it can be concluded that the popularity of alcohol consumption within the family influences in a great deal the habits of young people concerning alcohol consumption. 14% of the interviewees have friends who consume alcohol on a regular basis. According to the hygienic survey, 43% of the students with alcohol-consumer friends also consume alcohol on a weekly basis. Based on the data of current survey next to the family, the frequency of alcohol consumption can be greatly influenced by the circle of friends.

Alcoholism is mainly considered the consequence of the "civilized" lifestyle, which is more frequent among persons with psychically traumatized personalities. Overwork, unsolved lifestyle, unsatisfied ambitions can all lead to such symptoms, behavior patterns (neurosis), for what the alcohol with its anxiolytic effect seems to be a solution. First it starts with a little daily amount of alcohol consumption which moderates the anxiety caused by discomfort in society. Nevertheless regular alcohol consumption causes addiction. Regarding their alcohol consumption habits, 80% of the interviewed students are at risk. Informative work is needed to raise their awareness of the harmful effects of regular alcohol consumption.

A person's health is greatly influenced by the "genetic health" inherited from the parents, therefore we asked about different metabolic and vascular diseases occurring within the family, too. The question specifically concerned the state of health of the close family members (parents, grandparents, siblings). More than half of the interviewees had someone within the family suffering from a chronic disease. Most students (52.63%) indicate diabetes and cardiovascular diseases.

Based on our survey 53% of the students are at risk, they can show tendency to the emergence of diseases occurring within the family.

Family history is an important risk factor for developing diabetes type 2. First-degree relatives of diabetic patients have long been known to have an increased risk of developing. Recent studies in genetic research have also identified the genetic variants linked with diabetes (Lyssenko *et al.,* 2008, Sladek *et al*., 2007).

Conclusions

Most of the students answer all the questions of the questionnaire.

The majority of the interviewees have BMI between 18.5 and 24.99, but there are also overweight and undernourished students as well.

The students consume vegetables and fruit regularly, the proportion of fruit consumption is higher at women. The excessive sweets consumption is characteristic for women; men consume less or do not consume sweets at all. The meat consumption is higher at men than at women. The proportion of consuming milk and dairy products is high, without any difference between men and women.

The culture of doing exercise of the students is undeveloped, they are unaware of its importance, only one-third of them do sports on a daily basis despite studying at biology or physical education specialization, where they encounter the notion of health daily.

A minor part of the students smoke, but they smoke on a daily basis. The proportion of alcohol consumers is high; they consume alcohol regularly, on a daily or weekly basis. The alcohol consumption is also popular in the families and circle of friends of those students who consume alcohol very often. More than half of the interviewees have precedents concerning diabetes and vascular diseases in their families.

On the basis of the results of the research the final conclusion is that regular sweetened food, alcohol consumption, and smoking can be considered as the greatest danger for the interviewees. Besides the rejection of the consumption of health-impairing substances, it is also essential to form the need to aspire to a healthy lifestyle. It is not enough to expound the health-impairing factors, but we have to strive to form a health preserving, sustaining and transmitting behavior.

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