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Volume dedicated to the anniversary of 200 years from the birth of Professor Anastasie Fătu and celebration of 160 years since the foundation of the first botanical garden from Romanian Principalities.

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ANASTASIE FĂTU – FOUNDER OF THE BOTANICAL GARDEN OF IASI

Cătălin TĂNASE[1*], Constantin TOMA[2], Tiberius BALAEȘ[3]

Remarkable personality, Anastasie Fătu, has organized the medical and social assistance in Moldova. The development of science in the first modern university of Romania and the establishment of the first botanical garden in Iasi (Fig. 1), recommend him as the founder of the school of medicine and natural sciences in the United Principalities.

Academician Emil Pop (1967) considers that Anastasie Fătu "*...he is in the history of Romanian medicine as a famous physician of the poor and the main organizer of hospitals in Moldova, tasks that have taken more time and energy, probably most of his professional activity. And yet, we the botanists, consider Fătu as one of us, as an evolved botanist and extremely progressive for its time, which created solid foundations for botanic education at university and which sacrificed on the altar of science with a rare generosity and a strong love for national culture not only his entire working power, but the savings acquired with sorrow. Fătu is, during the cultural renaissance of the young Romania, an extraordinary example of the great private initiatives, on the basis of which he could call the intellectualists of his time for big collective actions for the progress of the delayed scientific research*…"

Its scientific work recommend him as a brilliantly representative of advanced social aspirations of his time, being one of the most remarkable figures in the history of medicine, science and culture of Moldova. This spiritual legacy is embodied in approximately 3000 pages, published in more than 40 years of activity [MAFTEI, 1972].

Life and professional development. Anastasie Fătu was born on 2nd of January, 1816 in the Muşata village, the former county Fălciu (now Vaslui). His father Vasile, a priest, and his mother Ana, always wished that their son, who proved outstanding qualities since childhood, to be admitted as a Fellow of the state public schools. He attended primary school in Episcopal School of Huși, and then Vasilian Gymnasium in Iasi.

In 1834, as a distinguished prized of the Vasilian Gymnasium receives a scholarship to study in Vienna, where in 1841 he defended his doctorate in philosophy and law. During his studies understands (as will be noted later by V. A. Urechia in response to reception speech of doctor Anastasie Fătu at the Romanian Academy) that in a "*country widow of freedom, in the land of the whip and unjust privileges*" could not assert his conception of law and freedom, conception learned from books and from the courses followed at the Faculty of Law, University of Vienna.

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Gives up a profitable career, but without clean soul enjoyments, and since 1839, enrolls also in Vienna at the Faculty of Medicine.

Between 1841 and 1846 he continued his medical studies at the Sorbonne University in Paris, where he received his title of doctor in medicine, with the thesis: "*Des signes des maladies du cœur en général fournis par l'auscultation, la percusion, l'inspection et la mensuration*".

The activity of the physicians, but especially professor and academician Fătu, shows that at the two outstanding universities, was not satisfied with just the acquisition of knowledge necessary for professional career. In the two institutions Fătu closely surveyed cultural phenomena and scientific research results, comparing them to what might represent for his country. During his studies, has acquired principles that could help at organizing public health and promote natural sciences.

Returned home with the firm determination to devote his entire life to initiatives for the establishment of a humanitarian and civilized ordinances in the organization of public health, on 1st of December 1847, requests to Moldovan Health Committee to approve the free practice right to perform as a physician. After a written and oral exam, the Committee concluded that Anastasie Fătu: "*...is endowed with science worthy of appreciation and may be consented for free practice as doctor in medicine*…"

As a result, from 1848 to 1852 is appointed district physician and later physician of the 2nd Regiment of Militia (1849), becoming as the historian V. A. Urechia will appreciate "*...doctor of the poor and consoler of the sufferer...*"

As a doctor of the 2nd Regiment of Police, publishes in 1853 a *Project for police organization in Romania*.

In 1852 doctor Fătu is appointed professor and administrator of the *Gregorian Institute* by the prince Grigore Al. Ghica, where he will found a midwife school. Along with doctor Gheorghe Cuciureanu will offer pediatric assistance and will found the first nursery for abandoned children. As a professor he initiate the first official midwifes in Moldova, and for preparing them, wrote in 1852 *The manual for midwifes* (in Romanian: *Manualul pentru învăţătura moaşelor*).

Since 1873, after professor Dimitrie Brândză went to the University of Bucharest, he occupied the department of Botany and Zoology from the Faculty of Science at the University of Iasi.

In 1879, along with doctors Gheorghe Cuciureanu and Nicolae Negură, he contributed to the foundation of the Faculty of Medicine of the University of Iasi.

Anastasie Fătu died in Iasi, 130 year ago, on 3rd of March 1886, resting at the Eternitatea Cemetery [MITITIUC & TONIUC, 2006].

The personality of the great erudite was evoked in volumes [AIFTINCĂ, 2014; BOTNARIUC, 1961; BUDA, 2013; MITITIUC & TONIUC, 2006; POP, 1967; MAFTEI, 1972; POP & CODREANU, 1975; TOMA, 1986; TOMA, 2015], dictionaries [anonymous, 1982; NECULA, 2001], specialized journals [ANGELESCU & DIMA, 2006; BURDUJA & al., 1960; BURDUJA & TOMA, 1979; CONSTANTINESCU, 2009; LEOCOV, 1979, 1982; PAPP, BURDUJA & DOBRESCU, 1955; RESMERIŢĂ, 1982; TOMA, 1974, 1975, 1987, 1996], or within scientific meetings (1981, to commemorate 125 years since the establishment in Iasi of the first botanical garden, was unveiled, in the roundabout in front of the administrative building, the bust of doctor Anastasie Fătu, accomplished and donated by the sculptor Eftimie Bârleanu; in 1986, at the third Symposium of the Botanic Garden,

Mandache Leocov, Gheorghe Zamfir, Petru Jitaru and Constantin Toma evoked the personality of Anastasie Fătu.

The name of the great erudite is kept with gratitude at the Secondary School in commune Berezeni (Vaslui County) and in the dendrological park realized in mixed style.

Founder of the botanical garden. 160 years ago, in 1856, Doctor Anastasie Fătu became renown by founding oneself the first botanical garden from Romania in Iasi, with his own financial resources, this action being considered in society as a remarkable cultural event.

Like the Natural History Museum, established in Iasi in 1834, the Botanical Garden of Anastasie Fătu will contribute significantly to the natural sciences development [MITITIUC & TONIUC, 2006], the cultivated plants being used also to illustrate the botanic lessons taught in different schools and at the Academia Mihaileană, founded in 1835.

This achievements constitutes favorable premises for the establishment in Iasi in 1860, of the first modern University in Romania. Science domain was based from the beginning on the two previously established prestigious institutions: Natural History Museum and the Botanical Garden, whose scientific and educational activity in training youth was coordinated by Professor Anastasie Fătu.

In the period when Anastasie Fătu was attended the botanical garden development, he was collaborating with prestigious botanists known at that period: Dimitrie Brândză, Dimitrie Grecescu and Florian Porcius.

Moreover, Dimitrie Brândză presented to the students of the university, at the courses of botany, the plants from "Fătu's garden".

This garden organized on his property, was situated on a hillside, located near the Criminalu Palace, and near the historical monument *Râpa Galbenă*, delimitated by Butucului street (nowadays Anastasie Fătu street), Begiului Street (nowadays Florilor Street) and the road that connected with Bohotineului Street (nowadays Arcu Street).

The mission of the botanical garden was remarked by some of his contemporaries and some institutions. Thereby, Neculai Istrati, director of the Ministry of Public Instructions (Education) donated his salary for three months, and then proposed to depose an annual subvention from the public fund (exchequer). Afterward, the Town Hall of Iasi offered a subvention to the botanical garden, but those initiatives stopped in 1872, and the garden was close down under the pretext that it would block the urban development of the area [PAPP & al. 1955].

Like professor Fătu was mentioning, the purpose of this enterprise was "…*to improve sanitation in the city of Iasi and to induce young people studying plant biology and to give natural sciences lovers, the occasion to contemplate, in their resting moments, at the natural beauties*…" [POP, 1967].

In the press of the time, Gheorghe Asachi – the organizer of national schools in Moldova- acclaims the initiative and the activity of Anastasie Fătu to support instruction and education of youth, but also the Romanian patriotism of those who sustained by various means the maintenance and development of different institutions established in Iasi, after the model of most renown education and scientific institutions of Europe.

In the catalogue published, who contains more than 2500 of plant species cultivated in his garden, one may find that apart from the native species, which are growing spontaneous, the presence of some exotic gymnosperms, but also some species belonging to *Ficus, Acacia* and *Mesembryanthemum* genera.

Anastasie Fătu states about this garden that "…*the distribution of the plants was made after the natural method…the nomenclature is the Latin, adopted by the masters of Science, which enrolled people known Romanian words* ..." [POP, 1967].

In his manuscript entitled *Catalogus herbarii vivi et seminum ex horto 1870*, plants cultivated in the garden are listed as follows: in the first part the species are listed in alphabetic order, on two columns on each page, one with acclimatized native plants and one with exotic species. In the second part of the catalogue there are registered some groups of ornamental plants, vegetables, herbs and fruit trees. Thereby, Professor Anastasie Fătu gives the first contribution to the knowledge of flora in the Romanian, especially Moldovan territory.

The endemic plant species were obtained with the help of the botanist Iosif Szabó, and the exotic species came from Vienna or by exchanges with botanical gardens from Germany and from Chișinău. He built in his garden two greenhouses, one for the tropical species and one for the temperate ones. At that time, with his garden, Fătu achieves a true experimental field for exotic species acclimatization.

The university rector, professor Nicolae Leon stated: "…*the garden had a nice look, the ground was injured; we were descending into on a scale from Florilor Street, we were passing under the Fătu's window, from where we were arriving immediately in front of the greenhouses. Indescribable pleasure for us was to meet the pleasant botanic professor in the garden – and we found him very often among the tablets that indicates genera and species names. With a great pleasure he was putting himself at our disposal, he was carrying out a clasp knife and a pocket lens and started, I can hear him: this plant, gentleman, takes part of the family…, then showing us how the carpels meet each other, how ovules are inserted, different types of placentation etc.*"

Since October 1996, the Botanical Garden of Iasi, in carrying the name of the founder Anastasie Fătu, which is mentioned on the commemorative plaque on the façade of the administrative building.

Medical activity. Between 1863-1885, Anastasie Fătu will became physician at Sfântu Spiridon Hospital, trustee at Sfântu Spiridon House (1877 and 1880) and professor of popular medicine at the Theological Seminary "Veniamin Costachi" from Socola (1872), where he elaborated the *Popular medicine manual* (in Romanian: *Manual de medicină populară*), and on the inside cover is inserted immediately beneath his name the following mention: "…*Dr. in medicine and laws, substitute teacher at the Seminary of Socola, actual member of the Romanian Academy and several local or foreign scientific societies*…"

He mentioned in the preface of this work, that when he wrote it he was thinking: "…*in the absence of doctors in villages, priests to advice people. Priests must know the diseases causes and symptoms, to beware of them but also to teach the people*…" [LEOCOV, 1979].

Because he was convinced of the necessity to establish hospitals for children in Moldova, Anastasie Fătu was considered to be the first pediatric physician in Moldova, and with this attribute he found the first pediatric section and assesses some scientific criteria in forensic medicine in Romania.

On 1st of November 1876, Anastasie Fătu gives Sfântu Spiridon House, 17.000 "franci", necessary for the maintenance of five beds for sick children under the age of 10 years.

Doctor Fătu elaborated also rules (which unfortunately have not been preserved) for receiving children in this hospital, who worked in the building on the Pașcanu Street.

In this perspective, he printed at Iasi, in 1863, a *Project for organization of health and public hygiene police in Romania,* that could be considered a truly sanitary code, in which were mentioned all of the rules and medical regulations in that period.

In collaboration with Iacob Felix, coordinated the medical commission that elaborated the law for organization of health service, which was approved by the Senate in March 1872 and then, in May 1872 by the Chamber of Deputies. This first sanitary law of Romania highlighted the dependency of the administration of public health on the interior ministry [BUDA, 2013].

Academic and social activity. He was an excellent professor and patriot devoted to national progress of culture. Professor Fătu and the agronomist Ion Ionescu de la Brad had the initiative of founding in 1855, the *Society for encouraging Romanian youth to study abroad*, having the obligation to specialize in applied sciences, in domains such as chemistry, physics, agronomy or engineering.

As a result of financial support given by Anastasie Fătu, some young students, among them the future philosopher Vasile Conta, had the opportunity of studying at renowned universities from Europe [AIFTINCA, 2014].

In the work *The list of plant species cultivated in the Botanical Garden up to the year 1870* (*Enumerațiunea specieloru de plante cultivate în Grădina Botanică din Iassy până în anul 1870*, Fig. 2), Anastasie Fătu implement the use of a proper nomenclature, consisting of neologisms borrowed from Latin language and various words used in popular language. In this sense, he includes a many of well-defined terms which are kept in the scientific literature, thereby contributing to the creation of botanical Romanian language.

After four years of didactic activity, Fătu completed in 1877 the manuscript for the first Romanian manual of botany for universities, entitled *Botanic notions* (in Romanian: *Elemente de botanică*, Fig. 3). The first part of the manual, which is 482 pages large, will be published at Iasi in 1880, containing the cytology, histology, organography and plant physiology. The second part which contains aspects referring to taxonomy, phytography and plant geography will remain as manuscript since 1920, at the Romanian Academy Bibliotheca. In addition with the original publication in medical domain, this manual recommends Anastasie Fătu as an erudite knower of the plant biology.

This manual will open new perspectives and research directions less known in plant biology domain and for the last two decades of 19th century will represent the principal source for training students from the natural sciences faculties, medicine and agriculture.

Professor Fătu appreciated in the manual preface that "*…the main wish I had in the publication of this work was to spare, as possible, the precious time of students that is lost by copying the manuscripts…*" [POP, 1967].

Concurrently is the first work in which are popularized aspects regarding the Romanian nomenclature used in plant biology. In this manual, Fătu is giving a great attention on *Plant Physiology*, subject which does not had previous tackling [POP, 1967]. In carrying out this work he used the most famous branch treatises of the time (Jean Baptiste Payer, Achille Richard, Pierre Étienne Simon Duchartre, Julius von Sachs) but also the work of Dimitrie Brândză – *Elementary course of natural history*, in three parts, Iasi, 1873 – and the figures were reproduced from plates purchased from the universal exhibition in Vienna, organized in 1873.

Academician Emil Pop appreciate Fătu's work as epochal, as it opens new perspectives of the Romanian academic literature, in a specialization of biological sciences which will contribute especially in forming of entire generations of students from faculties

of sciences, medicine, agronomy, from last decades of 19th century. In this work Anastasie Fătu popularized the Romanian nomenclature in the botanical domain, some of the terms being still valid.

Manual content include the following divisions: Part I – *General Anatomy* and *Plant histology*: cytology, fiber tissues, vascular tissues, about epidermis, about chemical composition of plants; Part II – *Descriptive anatomy* or *Plant organography*: Section I A – *Cotyledon plants* (seeding plants); *Reproduction organs* (flower, inflorescence and their parts, fecundation, fruit and seed, fruit classification, seeds germination); Section II B – *Sporophytes* and *Cryptogams*; Part III – *Plant physiology* (absorption, sap circulation, transpiration, respiration, assimilation, growth, germination).

This manual highlights an issue considered topical in the botanical domain, an excellent documentation and especially an evolved scientific conception of Professor Fătu, in a period in which *vitalism* was getting biological shades, and its philosophy was contrary with "teaching about the vital force", seen supernatural in organisms. Fătu considered the plant as the unit, and the functions independent.

Some of the terms are present in the specialty literature today: referring on roots: vivacious; for strain: articulated, cladodes, tight, axillar tendrils, fastigiated, geniculate, pricks, hispid, axillary, floral, mixed, terminal buds, soiled, clocks, patents, proliferating, nauseating; for leafs: alternate, acuminate, cuspidate, caduceus, crenate, cordiform, emersed, ensiform, fidate, filodii, hastate, devolve, ligules, mucrone, mesophilic, obcordate, obovate, ochree, orbiculare, petioles, peltate, pinnate, palmate, partite, reniform recurved, runcinate, sagittal, sessile, spatulate, stipele, subulate, truncate, vaginule; the flowers - epigine, hypogyne, inflorescence, involucre, corymb, perigyne, raceme; on fruit: hesperides, sorozat, sicon.

Permanently preoccupied of social aspects, he studied also the curative role of mineral waters, publishing in 1851 the work *Description and use of simple water and mineral waters in Moldova* (Fig. 4), work translated in Russian in 1854 of Doctor Isidor Copeniki (Fig. 5) [MITITIUC & TONIUC, 2006].

Also he collaborated by publishing some articles with medical interest in *Gazeta de Moldavia* and other periodicals.

In the year 1857, Anastasie Fătu published in the *Journal of Agriculture*, edited by Ion Ionescu de la Brad, a series of articles that highlighted his concern regarding the good organization of the Greogorian Institute and the public hygiene.

Doctor Anastasie Fătu started his political activity since the unionist period, being elected deputy of Iasi in the ad-hoc Divan of Moldavia (Fig. 6), which proposed the unification of Romanian Principalities. He was part of the Elective Assembly of Moldavia, which chooses Alexandru Ioan Cuza as Domnitor (prince) on 5th of January 1859. In 1868 was elected president of the Assembly of Deputies and senator in 1869.

Recognition of scientific activity. On 11th of September 1871, Doctor Anastasie Fătu was elected for his rich activity as "actual" member (titular) of the Romanian Academic Society (originally Romanian Literary Society – on 1st of April 1866, became the Romanian Academy in 1879), at Natural Sciences section, recent constituted by choosing two days earlier along with Petrache Poenaru, mathematician (inventor of the frame tank) of the physician Nicolae Kretzukescu and the economist Petre S. Aurelian.

We have noticed the assessments of the economist Petre S. Aurelian, member of Romanian Academy, in the answer to the request of the erudite of Iasi, to publish in 1871 the first *Catalogue of the Botanical Garden*: "…*how fast would go the development of science*

in Romania, how quickly would be studied the natural history of our country when all of those who have had interests of science, have occupied important positions at a university department, would imitate the tireless Anastasie Fătu, but, what can we say! Many are called, few are chosen…" Petre S. Aurelian acclaimed [TOMA, 1987].

In *The speech of reception* sustained in the front of the members of the Society *Attempts to developments of natural sciences in Romania* (in Romanian: *Încercările pentru desvoltarea scientieloru naturali în România*, Fig. 7) [FĂTU, 1873]. he presented the situation of sciences in Romania and the contributions on fields (geology, mineral waters, flora and fauna of Romania), of the "*illustrious men who take active part to the propagation of natural sciences*", treatises and periodic publications elaborated by them, the public and private means which contributes at the evolution of Romanian sciences, and proposes the realization of a "*scientific map of the country*" [CONSTANTINESCU, 2009].

This speech presented in the meeting on 27th of August 1872 dignify the cultural dimension of the personality of the great erudite and can be considered a document with exceptional history and scientific values [CONSTANTINESCU, 2009]. In speech' text, written on 22 pages, Fătu realizes an argued diagnoses regarding the scientific research at the time. The document is accompanied by documented appendices elaborated on 150 pages, where are mentioned historical and statistic details related to all of the scientific societies, periodicals and the bibliography in natural sciences, the situation of museums and educational institution of all levels.

The speech highlighted the remarkable education of the grand erudite, but also the special interest and preoccupation of the patriot regarding the Romanian research perspectives. Noticing the relevant analysis of the dissemination of scientific results, he insists on the directions for a fast development of them. The great culture man appreciated that the prosperity of people and the progress of national civilization are not possible without the substantial input of science and culture [AIFTINCĂ, 2014].

Professor Fătu eulogize the activity and the important role of some personalities and private associations in the development of natural sciences, but considers that this initiative must be sustained by the state. This idea is emphasized by the fact that "…*if the private initiative is a commendable thing for those who undertake scientific investigations, it does not have sufficient financial resources to make them give all the fruits that enlightened people are expecting from the science prosperity. The role of the state has a great significance in this situation. This role and the responsibility are even greater as it dispose of the society collective means and as it can use powerful resources that neither the individual and even voluntary associations can ever have*…."

The vision of great erudite on the present and future of Romanian society are remarkable through next directions: scientific activity of the researchers; private associations; public instruction for forming young specialists in areas that are considered to be lean.

Also he considers that the scientific research must be "national", pretending that the results obtained may be applied for a good evolution and economic development of Romania. In this regard, he appreciated the importance of practical application of the researches in natural sciences domain and the realization of "*...a complete scientific map of the country which comprehends all the braches of our geological, zoological, botanical and agricultural wealth; in it to indicate the different climatic zones with the characteristic plants and meteorological observations for each county. It must also contain the statistical notices on the population and the agriculture product of each region. From this study we gain an accurate knowledge of our*

ore, of all species of plants, of animals and our agricultural development by regions and the influence of climate in plant's and animal life…" [POP, 1967].

Because all of his life denoted, beside a great character, that is an action man, in the final of his speech mentioned "*...to assert how deep I am convinced of the necessity and the usefulness of the scientific map of our country, from the modest resources that I have accumulated working for 25 years, I will deposit as fund the amount of ten thousand* «*franci*»…" [POP, 1967].

In that period the amount was equivalent with the state support for one year, the money will be used by the Natural Science Section for the subvention of the project [AIFTINCĂ, 2014].

Regarding the Academy, this institution had the mission of "regulator" and "propeller" for the scientific movement. Also he considers that "…*shall be the headquarters of all the procedures for the scientific development*…" [AIFTINCĂ, 2014].

In his speech in the chapter dedicated "Private means for science popularization" exhibited alongside with the *Society of Physicians and Naturalists* of Iasi, *Society of Natural Sciences* of Bucharest, the *Romanian Academic Society*, the *Society for encouraging Romanian youth to study abroad* and *Junimea Society*.

He considers that "…*The objectives of the Junimea Society are to knowing the truth trough open discussions that "mutually enlightens" the members and illustrates with some of the themes that were touched within the meetings regarding issues with national character. Their reach is to find the truth, and for these all of the opinions must be respected, for from their meeting and counteracting one to be able to separate*..."

The historian V.A. Urechia stated in the response, according to the academic usance, that "…*here is a fountain filled with precious notes regarding the development between the Romanians of natural sciences, notably in the current century. There is nothing left to say for a specialists, for me not even as much, to this speech*..." [POP, 1967] Further, the founder of the Romanian Academy made the following greeting to the great erudite and patriot Anastasie Fătu: "…*live long Mr. Doctor, because you have proved that your years are the country and Romanian science's years!...*" [POP, 1967].

From 1872 till 1886, Anastasie Fătu was the president of the *Society of Physicians and Naturalists* of Iasi, organizing it in sections: medicine, natural sciences and agronomy.

In the year 1872 he contributed to the organization of a small botanic garden in the yard of the *Society of Physicians and Naturalists* of Iasi. For this purpose, some species from the old garden were planted and with the help of the botanist Iosif Szabó brings in seeds from different parts of the country.

Furthermore he was corresponding member of the *Medico-Surgical Society of Bucharest*, president of the department from Iasi of the *Society for encouraging Romanian youth to study abroad*. Because he was known abroad, he was named member of the *Society of Natural Sciences of Frankfurt* and *Silesian Society for science development.*

Contemporaneous personalities surrounded with unanimous respect and with high appreciation for the physician, doctor and erudite professor, who devoted his life for the common weal.

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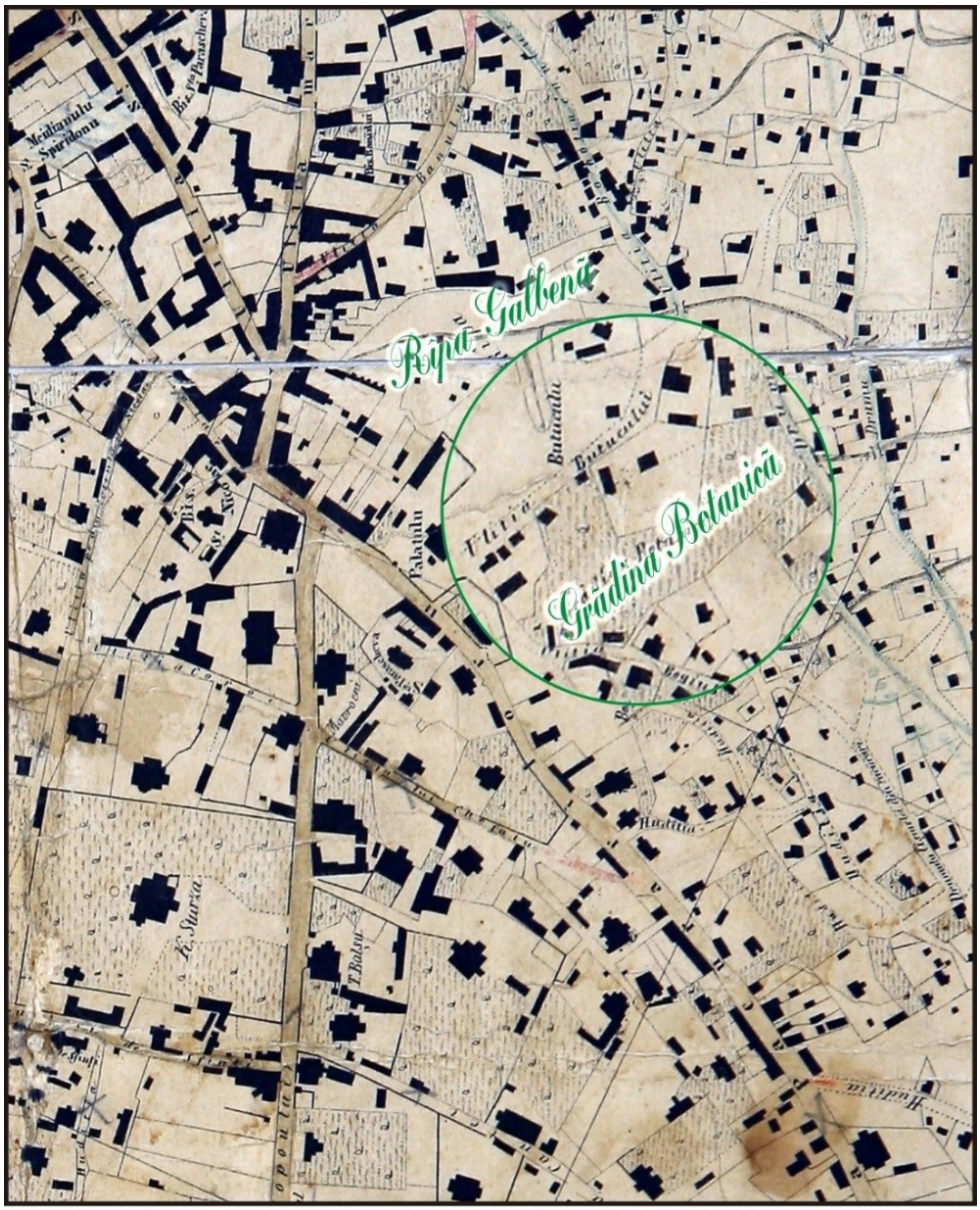
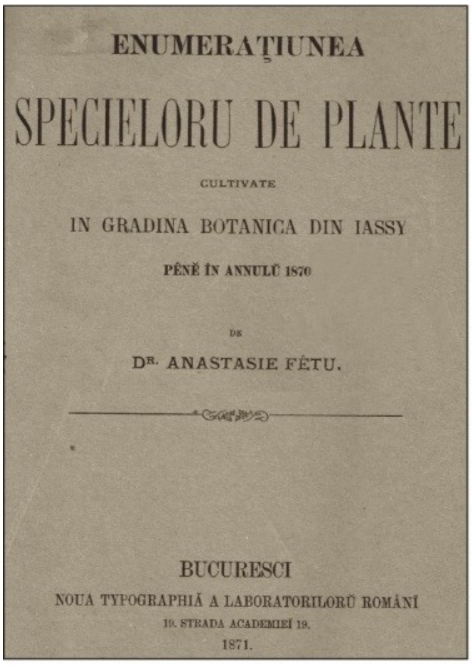
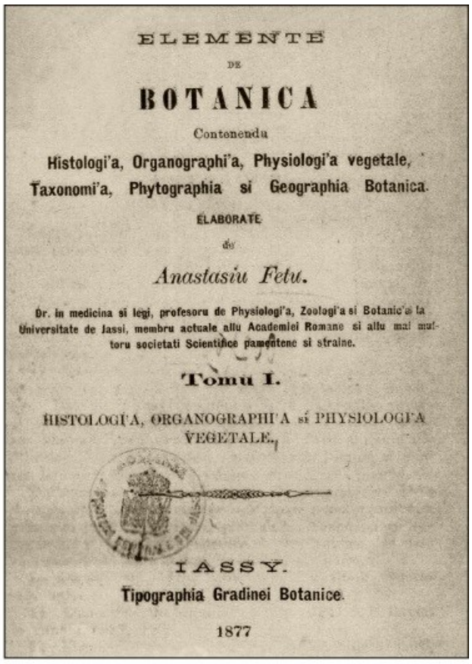


Fig. 1. Place of the Fătu's Botanical Garden in city plan made by engineer Fred Peytavin in 1857 (National Archives of Iasi, section Plans and Maps, no. 522)


ENUMERAŢIUNEA
SPECIELORU DE PLANTE
CULTIVATE
IN GRADINA BOTANICA DIN IASSY
PÊNĚ ÎN ANNULŬ 1870
DE
DR. ANASTASIE FĔTU.

BUCURESCI
NOUA TYPOGRAPHIĂ A LABORATORILORŬ ROMÂNÎ
19. STRADA ACADEMIEÎ 19.
1871.

Fig. 2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DE
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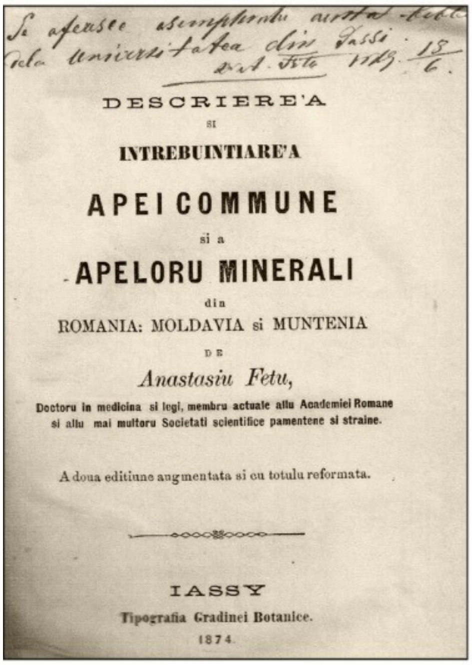
Contenendu
Histologi'a, Organographi'a, Physiologi'a vegetale,
Taxonomi'a, Phytographia si Geographia Botanica.
ELABORATE
de
Anastasiu Fetu.
Dr. in medicina si legi, profesoru de Physiologi'a, Zoologi'a si Botanic'a la Universitate de Jassi, membru actuale allu Academiei Romane si allu mai multoru societati Scientifice pamentene si straine.
Tomu I.
HISTOLOGI'A, ORGANOGRAPHI'A si PHYSIOLOGI'A VEGETALE.
IASSY.
Tipographia Gradinei Botanice.
1877

Fig. 3


DESCRIERE'A
SI
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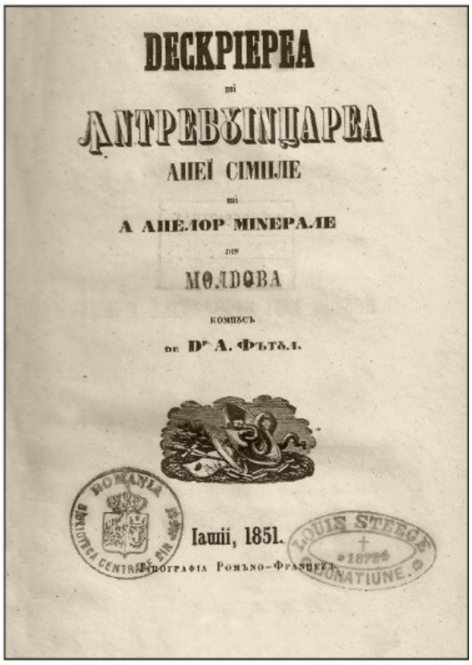
si a
APELORU MINERALI
din
ROMANIA: MOLDAVIA si MUNTENIA
DE
Anastasiu Fetu,
Doctoru in medicina si legi, membru actuale allu Academiei Romane si allu mai multoru Societati scientifice pamentene si straine.
A doua editiune augmentata si cu totulu reformata.
IASSY
Tipografia Gradinei Botanice.
1874

Fig. 4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КОМПУСЪ
ДЕ Dr A. ФЪТЪ.Л.
Iaшii, 1851.

Fig. 5

Fig. 6. Anastasie Fătu, deputy in Ad-hoc Divan of Moldova (1858)

J. Plant Develop.
23(2016): *15-25*

MORPHOGENESIS AND DEVELOPMENTAL BIOLOGY OF AFRICAN VIOLET (*SAINTPAULIA IONANTHA* H. WENDL.)

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Abstract: African violet (*Saintpaulia ionantha* H. Wendl.) has been domesticated, bred and commercialized. It is the most famous and popular of the *Saintpaulia* species, its ornamental value arising from its attractive leaves and flowers. African violet plants are easy to propagate by adventitious organ regeneration and are very sensitive to environmental factors including light, temperature, humidity, CO_2 concentration and photoperiod. This review offers a short synthesis on advances made in conventional vegetative propagation by adventitious organ regeneration, select early historical *in vitro* developmental perspectives, and vegetative and reproductive development of African violet.

Keywords: development; Gesneriaceae; thin cell layers; vegetative propagation.

Introduction

African violet (*Saintpaulia ionantha* H. Wendl.; Gesneriaceae) has attractive leaves and flowers (Fig. 1) that are typical of many members of the Gesneriaceae. African violet is mainly used for ornamental purposes, thus aspects related to flower color, leaf patterning, or yield are of interest to horticulturalists, plant breeders, molecular biologists, physiologists, biotechnologists, and for the floriculture industry. Thus, the improvement of flower- and leaf-related traits via vegetative propagation, the creation of somaclonal variation, and mutation breeding are fundamental aspects of African violet research.

Plant morphogenesis is regulated by complex genetic networks in a synchronized manner, and these serve as determining factors for numerous crop traits. Many constituents of these networks have been extensively studied in the model plant, *Arabidopsis thaliana* L. [VANHAEREN & al. 2016]. Multicellular plants have a unique group of cells that form new organs and replenish the daily loss of cells, or regenerate organs after injury, the pluripotent stem cells [AICHINGER & al. 2012]. Stem cells are located in stem cell niches that provide

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intercellular signals for differentiation such as hormonal control [ZHAO & al. 2010], or transcription factors [STAHL & SIMON, 2010; SABLOWSKI, 2011]. This review provides several details about traits related to development that could make this ornamental plant a possible model plant for the study of morphogenesis, differentiation and organ formation. It also addresses the environmental factors regulating vegetative growth and flowering, inflorescence development, specific conditions for propagation of African violet and biotechnological methods to improve flowering.

Fig. 1. A flowering African violet (*Saintpaulia ionantha* H. Wendl) (Gesneriaceae) plant. Initially redrawn from EASTWOOD & al. 1998 (p. 50), then modified.

Conventional vegetative propagation: possible model plant for adventitious organ regeneration (historical perspective)

FIGDOR (1907) may have been the first official report on adventitious regeneration in the Gesneriaceae, including African violet adventitious regeneration. FAIRBURN (1936) studied the propagation of African violet and other vegetatively propagated plants using leaf cuttings, noting how the inclusion of petioles was essential to induce roots. NAYLOR & JOHNSON (1937) described how portions of the leaf blade or petiole of African violet could regenerate one or more plants within 6-15 weeks if placed on wet sand or in water in Petri dishes when placed in a moist chamber. Even *ex vitro*, NAYLOR & JOHNSON (1937) noted the development of callus from the cut surface, with root initials forming within as little as 10 days, emerging from, but not derived from, the callus, i.e., originating from cells within the orginal explant. Their histological evidence showed that roots formed from thin-walled cells found underneath the leaf epidermis, while shoots formed from exogenous cells of the epidermal layer, i.e., shoots originated from epidermal cells.

Concept of thin cell layer and morphogenesis

Basic findings within earlier attempts made by FIGDOR (1907), FAIRBURN (1936) and NAYLOR & JOHNSON (1937) would later turn out to be an important reason for the success of thin cell layers (TCLs) [TEIXEIRA DA SILVA & al. 2007; TEIXEIRA DA SILVA & DOBRÁNSZKI, 2013, 2014; TEIXEIRA DA SILVA & al. 2015], especially longitudinal TCLs, in African violet tissue culture, making African violet an important ornamental plant model for developmental studies, like tobacco, and making it a possible viable form of *in vitro* regeneration for other *Saintpaulia* species [TEIXEIRA DA SILVA & al. 2015]. To date, only

two studies employed TCLs, but even so, the term TCL was not used for the earlier study. MURCH & al. (2003) used 0.25 mm thick petiole sections (i.e., transverse TCLs or tTCLs) to regenerate somatic embryos while KHOSARI-NASAB & al. (2014) induced callus and shoots from petiole and pedicel tTCLs. The ability to regenerate a large number of plantlets from a limited amount of tissue (in terms of surface area and volume) underlies the basic success of the TCL [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2014], and thus the success of the tissue culture of African violet. The origin of knowledge about this development came from early studies on vegetative propagation in the 1930's. Later, BROERTJES (1968), ENGELS & al. (1980), GEIER (1983), BROERTJES & VAN HARTEN (1985), Ohki (1994) and HOSOKAWA & al. (1998) confirmed similar histological evidence showing the involvement of epidermal cells (Fig. 2) in the formation of adventitious shoots from the petioles of African violet, noting, using chimeric tissue to base their assumption, the single cell origin of shoots, and thus annulling the claim by NORRIS & al. (1983) that shoots are derived from multiple cells. FINER & SMITH (1983) noticed that plastids that were in an arrested state of development in the epidermis underwent changes after placing leaf cuttings on kinetin-supplemented medium. REDWAY (1991) showed the epidermal origin of shoot primordia, after callus formed, but also showed the formation of shoot primordia from palisade tissue, which lies about 10 cell layers below the epidermal surface. This single cell origin of shoots would allow solid (i.e., non-chimeric) mutants to be produced, forming an important basis for mutation breeding studies. In *Saintpaulia*, some pinweel flower color cultivars are considered to be periclinal chimeras, with the petal margin arising from the L1 layer but the center of the petal from the L1+L2 layers. If shoot regeneration occurs from epidermal layers, then the flower color phenotype is identical to that of the flower margin of the mother plant. LINEBERGER & DRUCKENBROD (1985), ANDO & al. (1986) and PEARY & al. (1988) discuss shoot regeneration from the epidermal layer using pinweel-flowered periclinal cultivars in more detail. LINEBERGER & DRUCKENBROD (1985) found that whereas leaves, petioles, peduncles, sepals and inflorescences formed plantlets within 3-5 months, it took 8-10 months from subepidermal tissues, suggesting that the organogenic outcome reported in that study, and possibly in all other African violet studies, depends on the timing of sampling, which is also a function of the type of explant used [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2013]. In essence, regeneration from TCLs may be faster than from regular explants since a greater area and volume of cells is exposed to the exogenously applied plant growth regulators in the *in vitro* medium.

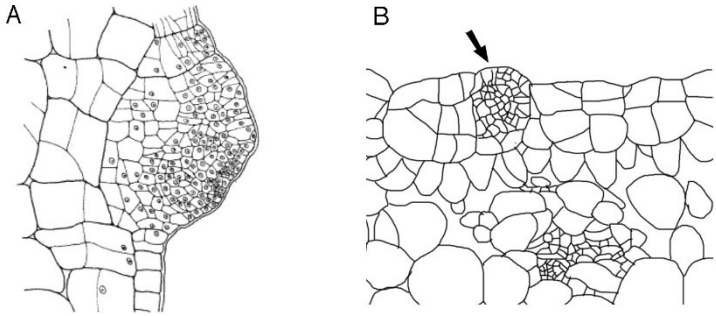

Fig. 2. Shoot development from epidermal tissue, as suggested by histological analyses by GEIER (1983), LO & al. (1997), and NAYLOR & JOHNSON (1937).
Arrow indicates a meristematic center.

***In vitro* morphogenesis and variability, sports and variegation**

ANDO & al. (1986) cultured 19 edged-type cultivars with a white corolla and a pigmented outer edge, and 9 Geneva edged-type cultivars with a pigmented corolla and a white outer edge, either as leaf cuttings, or in tissue culture. They found that single-colour cultivars did not form sports whereas single-colour sports, except for two cultivars, were produced in the remaing (edged-type and Geneva edged-type) cultivars at an average of 22%, but with a wide range of frequencies (0.9% to 68.8%), as a result of mutations. In contrast, the sports from edged-type cultivars had single-coloured corollas, and those from Geneva edged-type cultivars had white or extremely pale corollas, except for a single cultivar. Whereas the edged form ('Ms. Pretty') could produce single colour sports through tissue culture (i.e., reversion was possible), single colour sports of the same cultivar could never produce an edged form. PEARY & al. (1988) found that the variegated pattern of a foliar variegated cultivar ('Tommie Lou') was not caused by periclinal chimeras because the pattern of leaf variegation did not change in potted plants derived from the tissue culture of leaf and petal sections, or from subepidermal tissue. In contrast, tissue cultured plants of a variegated flower cultivar ('Candy Lou') segregated into the chimeral components, i.e., the chimera could not be stably propagated by tissue culture. Curiously, DÜMMER (1912) observed the phenomenon of peloria (i.e., reversion of asymmetric flowers into symmetric flowers) in *S. ionantha*.

Morphogenetic efficiency of explants: effects of age, season, and position

SCOTT & MARSTON (1967) noted that 24 °C and misting were suitable for the development of *S. ionantha* plantlets from leaf cuttings. According to HENTIG (1976), reflecting studies conducted in the early 1970's by this author, and using 'Rhapsodie in Blau' Typ 32 as the experimental material, there is a relationship between the length of the petiole of leaf cuttings and adventitious shoot formation. HENTIG (1976) formulated three important claims: 1) tissue from the fifth to tenth month of growth are most receptive; 2) leaves of the middle leaf zone and youngest leaves from the upper zone regenerate earlier (by 4-5 days) than older leaves (i.e., in the lower zone), forming within 56 days; 3) shorter petioles (1-3 cm) formed more adventitious shoots (and faster) than longer petioles (4-6 cm). In HENTIG's study, there were significant differences in the regeneration potential of young, middle-aged and old leaves before 56 days, but after 56 days, leaves from all three age categories were able to form adventitious shoots in 100% of explants. Even when leaves of all three age categories with petioles of different lengths were used, no less than 60% of all leaves were responsive, fortifying the notion that this is an ornamental plant that can be easily propagated vegetatively. Depending on the age of the leaf and on the length of the petiole, anything from 1 to 4 shoots could be produced per leaf.

Vegetative and reproductive development: role of environmental factors

JOHANSSON (1978) described members of the *Saintpaulia* genus as typically being shade plants while POST (1942) and STROMME (1985) described African violet as being day-neutral with respect to flower initiation and development. Inflorescences arise from leaf axils. During plug production, daily integrated photosynthetic photon flux (PPF_{DI}) reaching African violet plants in summer range from 10-15 mol/m^2/day but are as low as < 2 mol/m^2/day in winter. However, temperature and PPF_{DI} can influence the vegetative growth of African violet. For example, HANCHEY (1955) noted that by increasing PPF_{DI} from 0.31 to 1.9 mol/m^2/day, the number of leaves doubled from 22 to 44. HILDRUM &

KRISTOFFERSEN (1969) noted that the number of flowers, buds, and inflorescences per plant and flowers and buds per inflorescence of 'Biedermeier Rhapsodie' and 'Biedermeier Rosa' increased when PPF_{DI} was augmented from 3.1 to 9.3 mol/m^2/day. This treatment induced a maximum of 6.8 flower stalks/plant at 18 °C, but a maximum number of flowers and buds/flower stalk (9.1) at 24 °C. A maximum of 42 flowers/plant could be produced when daylight (i.e., most likely fluence rate) was cut by 75%, and when plants were exposed to 27 °C. In contrast, flower initiation and development was inhibited when plants grown in a greenhouse were exposed to < 2 mol/m^2/day [STINSON & LAURIE, 1954]. HILDRUM & KRISTOFFERSEN (1969) showed that both day and night temperature must be high to stimulate a greater number of flower stalks, and that flower production is linked to leaf production, given the source of inflorescences from leaf axils. This indicates that African violet is not only highly sensitive to environmental variables such as tempearture and PPF_{DI}, but that these variables can be used to manipulate plant growth and development, both of leaves and floral organs.

KWACK & KIM (1969) noted that sunlight reduced by half in the greenhouse was the best condition in terms of the growth and ornamental quality of African violet plants. As light intensity was decreased from full sunlight (5000-8000 foot-candles (f.c.)) to half sunlight (1000-2000 f.c.) and shade (500 f.c.), leaf area increased, i.e. 9.26, 12.44 and 13.38 cm^2, respectively. Full sunlight induced solarization, chlorosis in leaves or backward leaf curling, even though it produced higher dry weight (1.29, 1.21 and 0.90 g for full sunlight, half sunlight and shade, respectively). KIM & SANG (1982) argued that light intensity was critical for four varieties ('Monique', 'Robert O', 'Julianne' and an undefined local variety) of *S. ionantha*, since it is a semi-shaded plant. They confirmed that a light intensity of 5,000-10,000 lux (6.25-12.50% of natural sunlight in a plastic house) was the best condition for growth and ornamental value, i.e., photosynthesis, flowering percentage, number of peduncles, number of florets/peduncle, petiole length and leaf area.

In an experiment assessing the effect of illumination and culture medium on *S. ionantha* 'Oriental Red', LEE (1986) noted that 7000-8000 lux combined with a substrate composed of 40% peatmoss, 25% sand, 20% perlite, 10% vermiculite, and 5% compost was the best for producing wider leaves with greater leaf area (34-36 cm^2), best petiole length (5.8-6.2 cm), number of lateral shoots/explant (3.2), flowering rate (100%) and earliest flowering (number of days to flowering = 70 days), and number of peduncles (8.2-12.0).

PARK (2008) examined the effect of nitrogen fertilizer and light intensity on three varieties of *S. ionantha* and found that half-sunlight (4000 lux) and 200 mg/L $NaNO_3$ was the most effective combination for obtaining maximum fresh weight (FW) (e.g., for var. 'Narita' 1.9 g/cutting vs 0.9 g/cutting in full-sunlight and 0 mg/L $NaNO_3$). The combination of 4000 lux and 500 mg/L $NaNO_3$ increased the number of roots, the number of shoots (4.7/cutting vs 0/cutting), and chlorophyll content (52.4 mg/g FW vs 28.2 mg/g FW).

Using 'Utah' as the model cultivar combined with linear modeling to base their assumptions, FAUST & HEINS (1993) determined maximum leaf unfolding rate (LUR) to be 0.27 leaves/day when temperature was 25 °C while PPF_{DI} was 10 mol/m^2/day by using shadecloth to cut natural PPF_{DI} from a maximum of around 300 mol/m^2/day. When they decreased PPF_{DI} from 10 to 1 mol/m^2/day, optimal temperature was 23 °C, but this resulted in a lower LUR (0.18 leaves/day). Only when the leaf blade extended from 38-46 mm could an inflorescence begin to develop in the leaf axis [FAUST & HEINS, 1994]. Being able to predict and balance temperature, PPF_{DI} and thus LUR, would allow growers to plan their greenhouse cultures for the market. For example, FAUST & HEINS (1993), by applying a

PPF_{DI} of 7 mol/m^2/day at 22 °C, with a LUR of 0.244 leaves/day, formed 10.5 unfolded leaves/plant within 45 days (R^2=0.99). Also using non-linear models, FAUST & HEINS (1994) showed that as daily temperature was increased from 18 to 26 °C, days from leaf emergence to first open flower on the inflorescence of 'Utah' decreased from 86 to 55. They concluded that PPF is the primary factor influencing flower initiation while average daily temperature is the primary factor influencing the rate of inflorescence development. The three cardinal temperatures (T_{min}, T_{opt}, and T_{max}) for leaf appearance rate [FAUST & HEINS, 1993] are 8 °C, variable (as this depends on PPFD) and 30.8 °C, respectively and for leaf elongation rate [FAUST & HEINS, 1994], the values are 13.8 °C, 24 °C and 29 °C, respectively. In contrast, STRECK (2004) found that T_{min} was 10 °C and T_{max} was 33 °C for both leaf appearance rate and leaf elongation rate, and T_{opt} was 24 °C, as also modeled by FAUST & HEINS. CONOVER & POOLE (1981) were able to increase the percentage of inflorescences that formed in African violet 'Inge' within 9 months from 6% to 62%, and then to 100%, by exposing plants to 0.8, 1.6, and 3.2 mol/m^2/day, respectively. However, as FAUST & HEINS (1994) cautioned, even if LUR increases, a PPF of < 4 mol/m^2/day will negatively affect the number of developing inflorescences. Inflorescence development is acropetal [HASTON & DE CRAENE, 2007] (Fig. 3). Consequently, for African violet, the production of potted plants for season-dependent events such as Valentine's Day, Mother's Day, or Christmas, can be perfectly timed with the desired number of leaves and flowers.

The leaves of African violet are very sensitive to light, temperature, humidity and photoperiod [CHEN & HENNY, 2009], and minor changes in any of these factors can cause yellow or brown leaf spots [ELLIOT, 1946; YUN & al. 1997a; YANG & al. 2001]. Leaf spot is a cellular response confined to palisade cells in leaves, whose ultrastructure is destroyed [YUN & al. 1996a, 1996b], caused by a rapid drop in temperature, as may occur in overhead irrigation, especially at night, or by transferring plants from indoor to outdoor conditions, as was shown for 'Ritali' plants [YUN & al. 1997a]. Unlike chilling injury, leaf spot is irreversible. Outer leaves, which are more exposed to the surrounding environment, are more susceptible to leaf spot, and even exposure to 20 °C water can induce this disorder [MAEKAWA & al. 1987], which results from electrolyte leakage [MAEKAWA & al. 1990]. Leaf spot is a stress response caused by the rapid production of reactive oxygen species (ROS) following sudden temperature shifts [YASUDA & al. 1997], and an increase in the activity of antioxidant enzymes (superoxide dismutase and catalase). However, this response may be cultivar-dependent since 'Ritali' and 'Tamiko' (with about 75% and 95% incidence of leaf spot, respectively) were more susceptible to a drop from 30 °C to 15 °C than 'Maui' and 'New Jersey' (about <5% and 22% incidence, respectively) [YANG & al. 2001]. Sudden drops in temperature causing leaf spot are also characteristic of 'Ritali' leaves that display additional physiological disorders, namely a decrease in photosynthetic activity (especially a negative effect on PSI and PSII activity and a decrease in chlorophyll fluorescence) [YUN & al. 1997b, 1998], and plasmolysis [YUN & al. 1996a]. Recently, OHNISHI & al. (2015) revealed evidence of the involvement of calcium ions (specifically Ca^{2+} channels) after the degradation of the vacuolar membrane of palisade cells.

In a practical greenhouse trial to examine the response of stock plants and leaf cuttings (with 15 mm long petioles, according to HENTIG, 1976) to different commercial light sources (Grolux-low, Grolux-high, Verilux-low, Verilux-high, Fluora 77, Cool White 20, Cool White 30, Warm-White 30, Warm-White de Luxe 32, Interna 39 and Natura 36), SCHNEIDER-MOLDRICKX & AMBERGER (1982) discovered significant differences in the number of shoots that could be regenerated and in shoot FW. In their experiment, bulbs emitted 36-72 µmol/m^2/s, all test material was grown under a 16-h photoperiod, and data was pooled for three cultivars ('RH-26/74', 'Meta', and 'OP-50/75'). Cuttings cultivated under

light bulbs emitting higher radiant energy formed more and heavier shoots than those exposed to light bulbs emitting low levels of light energy. Grolux (emitting 63 µmol/m^2/s) was the most effective light source, forming 81 and 89 shoots/plot from stock plants and cuttings, respectively, and 598 mg/shoot. The response was strongly cultivar-dependent, with 'RH-26/74', 'Meta', and 'OP-50/75' forming 56, 94 and 80 shoots/plot and 589, 235 and 598 mg/shoot, respectively. A separate trial by the same authors showed that Warm-White 30 bulbs could induce the formation of as many as 122.1 shoots/plot in a cultivar-independent manner. The use by BOSCHI & al. (2000) of red and blue spectral filters, with different red, blue and far red ratios, on greenhouse African violet (cultivar unspecified) plants reduced most growth parameters (shoot and root dry weight, chlorophyll content, chlorophyll/protein ratio, net carbon dioxide (CO_2) exchange), in some cases significantly, compared to control polyethylene film, but did not alter the transpiration rate.

African violet plants ('Rosa Roccoco' and 'Big Star') exposed to continuous CO_2 enrichment, in which CO_2 concentration was increased from 330 to 1000-15,000 ppm, showed 50-100% higher net photosynthetic rate and 23-30% higher relative growth rate, most likely caused by the 39.6% increase in the number of formed leaves [MORTENSEN, 1984]. More refined experiments by MORTENSEN (1986) on 'Nicole', 'Lena' and 'Rosa Roccoco' showed that CO_2 enrichment (900 µL/L) had a more profound and/or significant effect in all three cultivars and in all parameters measured (greater dry weight, relative growth rate, number of leaves, leaf diameter, and number of flowers and flower buds, but reduced number of days to flowering) than ambient CO_2 (335 µL/L), a 1-h daily pulse at 900 µL/L, or a morning plus an evening pulse, each at 900 µL/L. For example, in 'Nicole', CO_2 enrichment resulted in 7.95 g/plant, a relative growth rate of 37.1 mg/g/day, 51.1 leaves/plant, 19.3 cm wide leaves, 56.1 days to flowering, and 168.7 flowers and flower buds/plant. The equivalent values for ambient (control) CO_2 levels were 3.56, 28.4, 35.3, 17.6, 66.4 and 66.8, respectively.

Vegetative growth can also be stimulated by the application of maleic hydrazide (MH). LEMATTRE (1977) found that spraying three African violet cultivars ('Rhapsodie', '2000', and '2738') with 0.1 to 0.2% MH resulted in a 47- to 176-fold increase in the number of vegetative shoots that formed, depending on the concentration and the cultivar. For example, in '2000', while control plants formed only an average of 0.1 vegetaive buds per plant, the application of 0.2% MH resulted in the formation of 17.6 vegetative buds, which could in essence be used as clones, and to inhibit early flowering, although the genetic stability of such clones was not tested. By applying 10 mg/L gibberellic acid (GA_3) at two-week intervals to four-month-old 'Rhapsody blue' plants, DVORSKÁ (1979) was able to increase the number of flower stalks by about 25% (10-12 vs 6-9 in controls). Furthermore, in the same study, a constant temperature of 22 °C resulted in the formation of 4-6 flower stalks in control plants, whereas a 14 °C/22 °C (day/night) temperature gradient resulted in the formation of 7-11 flower stalks. HERKLOTZ (1964) found that a day and night temperature of 25 °C favored regeneration from leaf cuttings than all other day/night temperature combinations ranging from 15 °C to 30 °C. MARTÍN-MEX & al. (2005) found that the application of 0.001 µM salicylic acid significantly increased the number of leaves (19 vs 16 in the control) and floral buds (14 vs 8 in the control), as well as rosette diameter (177 mm vs 139 mm in the control), and shortened the days to flowering (74 vs 89 in the control). A similar finding by JABBARZADEH & al. (2009) also confirmed the positive effects of a foliar application of 1×10^5 M salicylic acid on vegetative growth and flowering of African violet in terms of the number of leaves, rosette diameter, the number of flower buds and the number of days from planting to anthesis. These chemical methods provide means of delaying and/or enhancing vegetative growth and/or flowering in African violet to meet market needs.

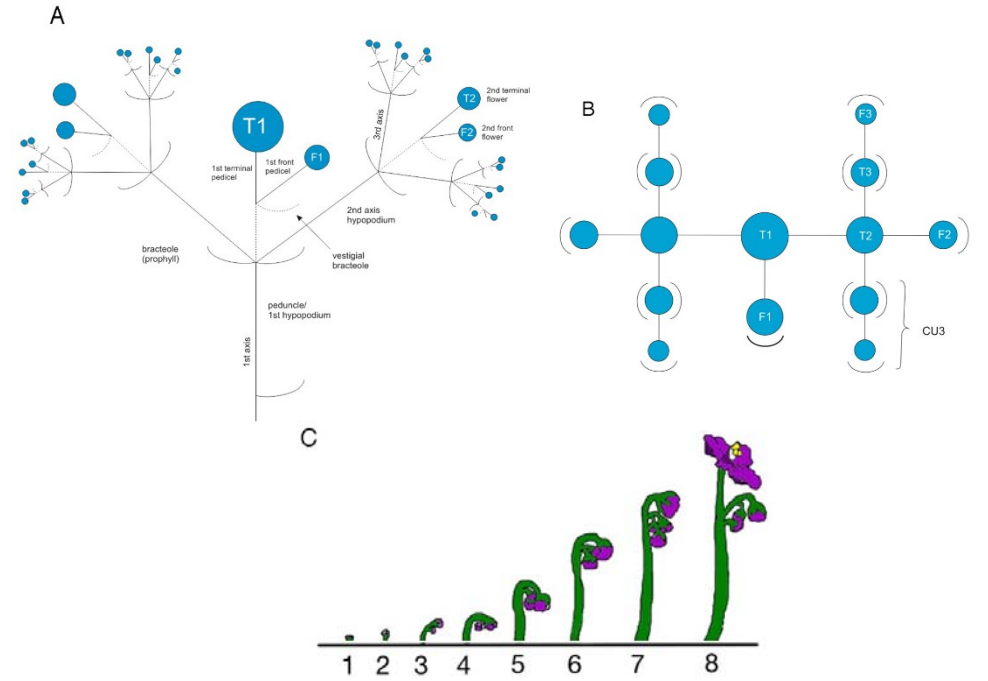

Fig. 3. Inflorescence development typical for the Gesneriaceae. (A) A pair-flowered inflorescence displays dichasial branching. (B) A plan view of the same inflorescence represented in (A). In A and B, T = terminal flower, F = front flower, parentheses indicate directions of growth / flower expansion, and numbers indicate the level of branching on the inflorescence axis. In B, CU represents a single cyme unit with a hypopodium (peduncle supporting the cyme unit) with a terminal pair of lateral bracteoles, subtending a terminal and an associated front flower. (C) The development of a *Saintpaulia ionantha* inflorescence, divided into 8 stages (according to FAUST & HEINS 1994): 1) visible reproductive bud (2 mm long) in the leaf axil; 2) visible peduncle subtending the primary bud; 3) peduncle starts to curve; 4) pedicel curves 90° relative to the peduncle; 5) pedicel curvature relative to the peduncle <90° and secondary buds are at the top of the inflorescence; 6) angle between peduncle and primary bud and pedicel increases, the pedicel is at the top of the inflorescence, and the primary bud emerges from the leaf canopy; 7) upper half of pedicel and primary bud are perpendicular to lower half of pedicel (i.e., at 90° angle); 8) the petals are perpendicular to the pedicel. (A and B) retraced, redrawn and modified from HASTON & DE CRAENE (2007) Fig. 1A and 1B (p. 15); (C) redrawn and modified from FAUST & HEINS (1994) Fig. 1 (p. 728).

Conclusions and future perspectives

The easy regeneration potential of African violet, as evident from as far back as the NAYLOR & JOHNSON (1937) study, alongside the debate about the single or multiple cell orgin of shoot buds in earlier work, are some interesting aspects that require additional attention from molecular cell biologists to address the factors that regulate morphogenesis in African violet. African violet can be easily and rapidly cloned. This, together with its ability to regenerate organs from different tissues, has facilitated several studies with the aim of understanding organogenesis, organ development and its related *in vitro* physiology. Hence,

African violet is valued as a model plant at the research/laboratory level and this potential can and should be further explored. Vegetative growth, flowering and floral organ development of African violet can be manipulated in a greenhouse or growth chamber by adjusting environmental variables including light source, intensity and quality, temperature, CO_2 enrichment, as well as chemical applications. Therefore, commercially, the production of potted African violet plants can be planned for the market to meet consumer demands.

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Author contribution statement and conflicts of interest statement

The authors contributed equally to all aspects of review development and writing. The authors have no conflicts of interest to declare.

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ECONOMIC VALUE OF SOME LEGUMINOUS PLANT SPECIES OF THE COLLECTIONS FROM THE BOTANICAL GARDEN (INSTITUTE) OF THE ACADEMY OF SCIENCES OF MOLDOVA

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Abstract: The results of the evaluation of the growth and development rates, the seed productivity, the green mass yield, the biochemical composition and the content of amino acids, phosphorous and calcium, the nutritive and energy value of the forage, as well as the biomethane productivity of local ecotypes of the leguminous species maintained in monoculture, in the collection of the Botanical Garden (Institute) of the Academy of Sciences of Moldova (BG ASM): *Astragalus ponticus*, *Coronilla varia*, *Lotus corniculatus*, *Medicago falcata*, *Onobrychis arenaria* and *Trifolium repens* are presented in this article. Control variants – the traditional forage crops: *Medicago sativa* and *Onobrychis viciifolia.*
The local ecotypes of the studied leguminous species were characterized by different growth and development rates. *Coronilla varia* and *Lotus corniculatus*, in the 2nd-3rd years, could be harvested, for the first time, 5 days earlier than *Medicago sativa*, but *Medicago falcata* and *Onobrychis viciifolia* – 18 days later. The green mass yield varied from 0.83 kg/m^2 to 4.08 kg/m^2. The studied ecotypes reached amounts of 0.60-0.89 nutritive units/kg and metabolizable energy 8.05-9.90 MJ/kg of dry matter, the content of digestible protein, of 106.28-225.09 g/nutritive unit, met the zootechnical standards; seed production: 19.12-83.00 g/m^2; the biomethane yield ranged from 692 to 3197 m^3/ha.
Higher yield of natural forage, dry matter and biomethane was produced by *Onobrychis arenaria* and *Coronilla varia*.

Keywords: biochemical composition, biological peculiarities, economic value, perennial leguminous species.

Introduction

Legumes from pastures and meadows contribute nitrogen to a complex and dynamic recycling system, organic matter containing legume proteins may be mineralized in soil, liberating N as nitrates (NO_3) and NH_4 that may be used by grasses and other species of plants. They are important in livestock feeding systems because they have the potential to extend the grazing season, increase the quantity of grazed forage and hay, and reduce the amount of N fertilizer needed. Legume feed not only improves forage quality but also increases the intake of the ration, hence, gives better performance in terms of livestock production. Many legume crops are also excellent honey plants, other plants can be used as raw material in various branches of the national economy, as they are for cosmetology, pharmaceutics and bioenergetics industry [DUKE, 1981; STODDARD, 2013].

The spontaneous flora of the Republic of Moldova (RM) is relatively rich and includes 5568 species of plants (superior plants – 2044 species, inferior plants – 3524 species), family *Fabaceae* Lindl. – 25 genera and 120 species [NEGRU, 2007]. The spontaneous flora of our country includes over 700 species of fodder plants and 71 species of them are leguminous plants [TELEUȚĂ, 2010]. The grasslands from the Republic of Moldova cover about 14% of the

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territory, they are in a deplorable condition and have very low productivity, with a share of leguminous plants decreasing from year to year [BANCIVANJI & al. 2012].

The collection of non-traditional forage plants of the Botanical Garden (Institute) of the Academy of Sciences of Moldova have 320 botanical taxa (species, varieties), including 73 leguminous plants [TELEUȚĂ, 2010; TELEUȚĂ & ȚÎȚEI, 2012; 2016].

This research was aimed at evaluating the biological peculiarities, the biochemical composition of the local ecotype of leguminous plant species and the possibility to use them as forage for ruminant animals or as biogas substrate.

Materials and methods

The seeds of the native leguminous species (*Astragalus ponticus* Pall., *Coronilla varia* L., *Lotus corniculatus* L., *Medicago falcata* L., *Onobrychis arenaria* (Kit.) DC., *Trifolium repens* L.) served as subjects of study and were collected from the spontaneous flora of the main botanical district from RM (region of plains of Balti and Bugeac steppe), maintained in monoculture in the collection of new and non-traditional forage plants of the BG ASM. The traditional forage crops (*Medicago sativa* L. cv. *Ilan* and *Onobrychis viciifolia* Scop. cv. *Adam*) served as control variants. The green mass and the samples for biochemical analysis were gathered after the first mowing, in the budding-flowering period. The scientific research on growth and development, yield and biochemical composition of the plants, was carried out according to the methodical indications [NOVOSIOLOV & al. 1983; PETUKHOV & al. 1989].

The carbon content of the substrates was determined by the data on volatile solids (organic dry matter), using an empirical equation [BADGER & al. 1979]. The biogas production potential and the specific methane yields were evaluated by the parameter "content of fermentable organic matter" [WEISSBACH, 2008].

Results and discussion

As a result of the phenological observations, it has been found that, in the first growing season, the studied perennial leguminous species are characterised by different growth and development rates. Thus, it has been determined that the plantlets of *Trifolium repens* and *Medicago falcata* emerge uniformly at the soil surface, 14 days after sowing, 3 days earlier as compared with the control, *Medicago sativa*, and 9 days earlier as compared with *Onobrychis viciifolia*. The plantlets of the species: *Astragalus ponticus*, *Lotus corniculatus* and *Onobrychis arenaria* emerge after 20 days, so, they need the same period of time as *Onobrychis viciifolia*, but the plantlets of *Coronilla varia* emerge the latest, that is, about 26 days after sowing or 12 days later as compared with *Medicago sativa.* The plants of *Astragalus ponticus* and *Coronilla varia* are distinguished by a very slow growth and development of the aerial part. By the end of the growing season, *Astragalus ponticus* develops the rosette with leaves, and *Coronilla varia* reaches the budding stage and the beginning of the flowering stage. The species *Lotus corniculatus*, *Medicago falcata*, *Onobrychis arenaria* and *Trifolium repens* pass through all the ontogenetic stages. The species *Lotus corniculatus* and *Onobrychis arenaria* are characterised by faster development rates, the development and the ripening of seeds start 7 days earlier, in comparison with *Onobrychis viciifolia* and *Trifolium repens*, 32 days earlier, in comparison with *Medicago sativa* and 39 days earlier than *Medicago falcata.*

In the following years, the leguminous species, studied by us, resume their growth and development in spring, when temperatures above 3-5 °C are established. The species *Trifolium repens* and *Medicago falcata* resume growth 7 days earlier than *Astragalus ponticus* and 3 days earlier than other species. The species: *Lotus corniculatus, Astragalus ponticus* and *Trifolium repens* are characterised by a faster development during the whole growing season, but they don't grow as tall as the traditional leguminous crops. So, by the end of April (Tab. 1), the plants reach 22.00-27.20 cm high, while the control species – about 35.90-38.10 cm. The shoots of *Coronilla varia*, in this period, reach a height of 47.20 cm.

Tab. 1. Duration of periods of growth and plant height of the studied species

Indicators	***Astragalus ponticus***	***Coronilla varia***	***Lotus corniculatus***	***Medicago falcata***		***Medicago sativa***	***Onobrychis arenaria***	***Onobrychis viciifolia***	***Trifolium repens***
	17.03	13.03	13.03	10.03		13.03	13.03	13.03	10.03
Resumed vegetation up to:	66	70	63	78		75	73	75	71
- budding	77	77	77	101		82	80	99	82
- flowering	117	141	121	158		143	128	133	119
- seed ripening									
Plant height, cm	27.20	47.20	26.70	31.03		38.10	37.00	35.90	22.00
- at the end of April	76.0	122.1	42.31	72.00		83.20	95.07	85.50	31.30
- at flowering		0							

It has been determined that the period of time from the restart of active growth until the flower bud formation, in *Lotus corniculatus*, constitutes 63 days, in *Medicago falcata* – 78 days, but in the control species – 75 days. The flowering stage of *Astragalus ponticus*, *Coronilla varia*, *Lotus corniculatus* and *Trifolium repens* starts 22 days earlier in comparison with *Onobrychis viciifolia* and 5 days – in comparison with *Medicago sativa*. The plants of *Medicago falcata* begin to bloom the last.

During the flowering stage, the shoots of *Coronilla varia* and *Onobrychis arenaria* reach 95.07-122.10 cm high, *Trifolium repens* and *Lotus corniculatus* – 31.30-42.31 cm, but the control – 83.20-85.50 cm.

The studied species need a different period of time from the beginning of flowering until the full ripening of seeds. So, *Trifolium repens, Astragalus ponticus* and *Lotus corniculatus* need 37-44 days, *Medicago falcata* and *Coronilla varia* – 57-64 days, while *Onobrychis viciifolia* needs 34 days and *Medicago sativa* – 61 days.

Seed production is a key pillar in the capacity of maintenance and expansion of the species. Analysing the seed productivity (Tab. 2), we conclude that the studied leguminous forage species differ from *Medicago sativa* and *Onobrychis viciifolia* in the quantity of produced seeds and in the weight of 1000 seeds. The plants of the genus *Onobrychis* (83.00-112.23 g/m^2) and *Astragalus ponticus* (40.90 g/m^2) are characterised by high seed productivity, the mass of 1000 seeds of these species is also quite high (8.44-14.09 g). The plants of *Lotus corniculatus* and *Trifolium repens*, even if they have lower seed productivity in comparison with the control species, produce the highest number of seeds – about 21 000-27 000 seeds/m^2.

We have also found that *Medicago falcata* produces more seeds than *Medicago sativa*, and *Onobrychis arenaria* – fewer than *Onobrychis viciifolia*.

The total yield, the quality and the seasonal distribution of forage may be of greater importance to the livestock producer. As mentioned above, the studied leguminous forage species have different growth and development rates that influence the productivity of natural forage and the dry matter content. Thus, a higher yield of natural forage and dry matter, in comparison with the controls, was produced by *Onobrychis arenaria* (4.08 kg/m^2 or 1.11 kg/m^2) and *Coronilla varia* (3.92 kg/m^2 or 0.87 kg/m^2), a lower one – by *Trifolium repens*, *Astragalus ponticus* and *Lotus corniculatus* (0.83-1.58 kg/m^2 or 0.28-0.55 kg/m^2). *Onobrychis arenaria* is distinguished by a higher productivity of natural forage and dry matter as compared with *Onobrychis viciifolia*, and *Medicago falcata* – by a lower one as compared with *Medicago sativa.* In some papers, it was mentioned that the productivity of *Coronilla varia*, under the climatic conditions of Russia, reached 65 t/ha green mass [DRONOVA & al. 2009]. The ecotypes of *Onobrychis arenaria* from Russia are characterized by longer lifespan (5-7 years), in comparison with *Onobrychis viciifolia*, and by higher productivity of forage – with 20-70% [MEDVEDEV & SMETANNIKOVA, 1981].

The low productivity of local ecotypes of *Trifolium repens, Astragalus ponticus* and *Lotus corniculatus* may be explained by the arid environmental conditions (low humidity, rainfall under 400 mm and high temperatures in spring) in recent years. The varieties of *Trifolium repens* and *Lotus corniculatus*, created in Romania and cultivated in areas with 600-1000 mm of precipitation per year, have a production potential of 35-55 t/ha of green mass or 8-12 t/ha of hay [MARUȘCA & al. 2011].

It is well known that cattle eat mainly leaves, due to their high content of nutrients, and the ratio leaves/stems influences the forage value. The forage of *Coronilla varia* and *Astragalus ponticus* is characterized by a high content of leaves (56-63%), but *Medicago falcata* – by a lower one (37%).

Tab. 2. The productivity of the studied species of the family *Fabaceae*

Species	fresh mass, kg/m^2	dry matter, kg/m^2	leaf content, %	seed production, g/m^2	weight of 1000 seeds, g
1	2	3	4	5	6
1. *Astragalus ponticus*	1.48	0.40	56	40.90	8.44
2. *Coronilla varia*	3.92	0.87	63	19.00	3.54
3. *Lotus corniculatus*	1.58	0.55	49	22.30	1.05
4. *Medicago falcata*	2.04	0.66	37	17.00	1.33
5. *Medicago sativa*	3.11	0.82	44	27.14	2.67
6. *Onobrychis arenaria*	4.08	1.11	40	83.00	12.15
7. *Onobrychis viciifolia*	3.95	1.03	39	112.23	14.09
8. *Trifolium repens*	0.83	0.28	41	19.12	0.71

For growth, development, reproduction, as well as for the production of high quality milk or meat, cattle need many nutrients they receive from feed. Proteins are the most important and the largest group of natural macromolecular compounds, essential for life, are a source of nitrogen for the body and play a crucial role in the use of the genetic productive potential. The forage of *Astragalus ponticus* (Tab. 3), is characterised by a high content of raw protein, 23.40% of dry matter, in comparison with *Medicago sativa* and *Onobrychis viciifolia*, while *Trifolium repens* and *Lotus corniculatus* have low content of raw protein. *Astragalus ponticus* is distinguished by a very high content of raw protein (63.84 g/kg), which

is 67-75% higher compared to the species *Trifolium repens* and *Lotus corniculatus.* A high capacity of nitrogen accumulation by *Astragalus ponticus* is also recorded in other papers [DAVIS, 1982]. The species *Coronilla varia* and *Medicago falcata* are at the same level, regarding the content of raw protein. Other authors [ACAR & al. 2001], remarked that *Medicago falcata* and *Coronilla varia* contain 14.58-14.86% raw protein, but *Lotus corniculatus* and *Trifolium repens* – 17.87-18.93% raw protein.

Tab. 3. Biochemical composition and nutritional value of the dry matter of the studied species of the family *Fabaceae*

Indicators	***Astragalus ponticus***	***Coronilla varia***	***Lotus corniculatus***	***Medicago falcata***	***Medicago sativa***	***Onobrychis arenaria***	***Onobrychis viciifolia***	***Trifolium repens***
1	2	3	4	5	6	7	8	9
1. Raw protein, %	23.40	14.72	10.50	14.87	17.03	17.38	17.44	11.38
2. Raw fats, %	2.75	2.81	3.20	1.78	2.30	3.55	3.39	2.10
3. Raw cellulose, %	31.90	35.46	28.60	31.20	33.31	29.80	33.50	42.00
4. Nitrogen free extract, %	32.35	39.74	48.60	45.43	39.41	41.87	39.43	38.44
5. Minerals,%	9.60	7.27	9.10	6.72	8.01	7.40	6.24	6.08
6. Nutritive units, NU/kg	0.77	0.89	0.72	0.83	0.80	0.85	0.84	0.60
7. Metabolizable energy, MJ/kg	7.89	9.90	8.85	8.84	8.65	9.44	10.44	8.05
8. Calcium, g/kg s.a.u.	6.37	12.89	14.00	12.08	16.83	14.60	11.16	13.61
9. Phosphorus, g/ kg s.a.u.	4.52	5.66	2.19	1.00	4.39	5.33	7.52	2.51
10. Digestible protein, g/nutritive unit	225.09	132.10	106.28	135.30	164.29	154.13	156.00	144.80

The vegetable fats in forage are the main source of energy for the animals because they are necessary for the organism in order to ensure the normal development of vital processes and transportation of soluble vitamins in fatty acids and it also contributes to the accumulation of fat in milk [McDONALD & al. 2011]. The forage of *Lotus corniculatus* and *Onobrychis arenaria* contains a high amount of fats (3.20-3.55%), at the same level as *Onobrychis viciifolia*, greatly exceeding *Medicago sativa.* The *Medicago falcata* has a low content of fats -1.78% (Tab. 3, line 2, column 4, 5, 6, 7, 8).

The content of cellulose is quite low in the species *Lotus corniculatus*, *Onobrychis arenaria*, *Medicago falcata* and very high in *Trifolium repens* (Tab. 3, line 3, column 4, 5, 7, 9). We also mention that the optimal cellulose content has a beneficial effect on the synthesis of proteic substances in the rumen of animals and on the reduction of the nitrate content [BAHCIVANJI & al. 2012].

The nitrogen-free extract (NFE), along with fats, provides the necessary energetic material for vital processes, contributing to the formation and storage of fats [PÂRVU 1992; BAHCIVANJI & al. 2012]. The content of nitrogen-free extract varies from 32.35% to 48.60%, it is high in the species *Lotus corniculatus*, *Onobrychis arenaria*, *Medicago falcata* and very low in *Astragalus ponticus*, this fact influences the possibility of the forage to provide energy (Tab. 3, line 4, column 2, 4, 5, 6).

The vegetal forage contains minerals in variable quantities, regarding the type of the elements and the proportion between them and other chemical compounds. Minerals are essential components of all tissues and organs that maintain a constant osmotic pressure, participate in the regulation of acid-base balance, activate a number of enzymes, moderate neuromuscular activities and prevent the emergence and development of diseases in animals. The presence of minerals in animal feed is indispensable for their growth and health

[SUTTLE, 1982]. The forage of *Lotus corniculatus* and *Astragalus ponticus* is characterized by a high content of minerals (9.10-9.60%), but the forage of *Trifolium repens* – by a lower one (6.08%), in comparison with the control species (Tab. 3, line 5, column 2, 4, 9).

Calcium is the mineral element that is found in the highest quantity in the animal body, it is a structural component of bones and teeth; it plays an essential role in blood coagulation, tensing of muscles, activity of enzyme systems, at cell level. The normal assimilation of calcium by organism depends also on the quantity of phosphorus in the feed. Phosphorus plays a multiple role in the vital processes in body, and, being a constituent of nucleic acids, it participates in protein synthesis, cell multiplication and transmission of hereditary characters; it is a component of ATP (adenosine triphosphate) the main deposit of energy in tensing of muscles, nerve activity, vital processes of body; as phospholipids, it enters the structure of cells and cell membranes regulating the transportation of solutions through them; participates in the metabolization of carbohydrates, fatty acids and amino acids etc. [SUTTLE, 1982; McDONALD & al. 2011].

The content of calcium in the studied species varies from 6.37 g/kg (*Astragalus ponticus*) to 14.60 g/kg (*Onobrychis arenaria*), being lower than in *Medicago sativa* (16.83 g/kg). Very low content of calcium has been found in the forage of *Astragalus ponticus* (6.37 g/kg), the other species (*Coronila varia*, *Lotus corniculatus*, *Medicago falcata*, *Medicago sativa*, *Onobrichys arenaria*, *Trifolium repens*) have higher levels of calcium (12.08-16.83 g/kg) than *Onobrychis viciifolia* – 11.16 g/kg (Tab. 3, line 8, column 1, 6, 8). The studied species have lower content of phosphorous (1.00-5.56 g/kg) than *Onobrychis viciifolia* (7.52 g/kg). The species *Medicago falcata*, *Lotus corniculatus* and *Trifolium repens* have very low content of phosphorous (corresponding 1.0, 2.19 and 2.51 g/kg). The species *Coronilla varia* and *Onobrychis arenaria* are distinguished by high content of phosphorous (corresponding 5.66 and 7.52 g/kg) as compared with *Medicago sativa* – 4.39 g/kg (Tab. 3, line 9, column 1, 6, 7).

The main indicators of the nutritional value of the natural forage are the content of nutritive units and metabolizable energy (MJ). The nutritional and energy value is determined by the biochemical composition and the digestibility of the organic substances from the forage that influence the health and the productivity of animals [MEDVEDEV & SMETANNIKOVA, 1981; McDONALD & al. 2011]. The nutritive units (NU) of the natural forage of the studied species reaches amounts of 0.60 (*Trifolium repens*) up to 0.89 NU/kg (*Coronilla varia*). *Trifolium repens* and *Lotus corniculatus* species containing 0.20 and 0.08 NU/kg correspondingly less than the control *Medicago sativa*. At the level control (0.80 NU/kg) are species *Astragalus ponticus* (0.77), *Medicago falcata* (0.83), *Onobrychis arenaria* (0.85) and *Onobrychis viciifolia* (0.84 NU/ kg). According to this index, species *Coronilla varia* is the most valuable forage crop (0.89 NU/kg).

Metabolizable energy is an index assessing the capacity of the feed to support of the processes of the milk production. This indicator of the studied species ranges from 7.89 up to 10.44 MJ/kg dry matter. The energy value of the forage of *Coronilla varia* and *Onobrychis arenaria* is at the same level as *Onobrychis viciifolia* (corresponding 9.90, 9.44 and 10.44 MJ/kg). The value of the metabolize energy of *Trifolium repens* (8.05), *Lotus corniculatus* (8.85) and *Astragalus ponticus* (7.89) is same in comparison with *Medicago sativa* (8.65 NJ/kg) - Tab. 3, row 7, columns 2, 4, 6, 9.

An important indicator of forage value is the content of digestible protein per nutritive unit. The content of digestible protein (DP) per nutritive unit (NU) ranges from 106.28 g DP/NU (*Lotus corniculatus*) up to 225.09 g DP/NU (*Astragalus ponticus*) and meets the zootechnical standards. Also green fodder of *Onobrychis viciifolia*, *Onobrychis arenaria* and *Trifolium repens* have high level of digestible protein (Tab. 3).

The amino acid composition is the most important factor in defining the food protein quality, followed by the digestibility of the protein and the bioavailability of its amino acids. The protein quality is determined by the ratio of certain amino acids, which provide the biological value of the feed. The efficiency of using protein crops in animal feed production strongly depends on the content of essential amino acids (EAA) in the various crops and the composition of compound feedstuffs [PÂRVU, 1992; McDONALD & al. 2011]. An essential amino acid, or indispensable amino acid, is an amino acid that cannot be synthesized de novo (from scratch) by the organism, and thus must be supplied in its diet. The nine amino acids humans cannot synthesize are phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine [McDOUGALL, 2002].

By analysing the amino acid content, we have found that the forage obtained from the studied species contains different amounts of amino acids, including those essential. The total amount of amino acids ranging from 8.316 mg/100 mg (*Lotus corniculatus*) to 13.377 mg/100 mg (*Astragalus ponticus*). The species *Onobrychis arenaria* and *Astragalus ponticus* contain the highest amount of essential amino acids, but *Lotus corniculatus*, *Medicago falcata* and *Coronilla varia* contain a very low amount. It has been found that all the studied species have a lower content of methionine than *Medicago sativa*, but the species *Onobrychis arenaria*, *Coronilla varia* and *Lotus corniculatus* are distinguished by a higher content of methionine in comparison with *Onobrychis viciifolia* (Tab. 4).

Tab. 4. The content of amino acids in forage (mg/100 mg dry matter) of the studied species of the family *Fabaceae*

Amino acids	*Astragalus ponticus*	*Coronilla varia*	*Lotus corniculatus*	*Medicago falcata*	*Medicago sativa*	*Onobrychis arenaria*	*Onobrychis viciifolia*	*Trifolium repens*
1	2	3	4	5	6	7	8	9
1. asparagine	3.110	1.857	1.129	1.871	1.711	2.037	1.751	1.593
2. **threonine***	**0.678**	**0.551**	**0.633**	**0.611**	**0.564**	**0.624**	**0.565**	**0.559**
3. serine	0.767	0.678	0.767	0.810	0.687	0.747	0.685	0.663
4. glutamine	1.953	1.418	0.941	1.659	1.360	1.561	1.398	1.381
5. proline	0.765	1.480	0.355	0.767	0.922	1.063	1.154	1.062
6. glycine	0.676	0.852	0.477	0.470	0.550	0.638	0.557	0.613
7. alanine	0.570	0.712	0.607	0.750	0.674	0.687	0.672	0.665
8. **valine**	**0.649**	**0.459**	**0.369**	**0.193**	**0.559**	**0.703**	**0.654**	**0.621**
9**. methionine**	**0.058**	**0.101**	**0.117**	**0.073**	**0.139**	**0.098**	**0.091**	**0.052**
10. isoleucine	0.510	0.344	0.315	0.422	0.459	0.502	0.459	0.453
11**. leucine**	**0.914**	**0.898**	**0.816**	**0.704**	**0.913**	**1.014**	**0.920**	**0.929**
12. tyrosine	0.458	0.442	0.319	0.427	0.458	0.543	0.491	0.517
13**. phenylalanine**	**0.794**	**0.647**	**0.416**	**0.510**	**0.850**	**1.035**	**0.937**	**0.806**
14. **histidine**	**0.411**	**0.239**	**0.136**	**0.295**	**0.326**	**0.406**	**0.371**	**0.364**
15**. lysine**	**0.760**	**0.524**	**0.517**	**0.673**	**0.619**	**0.746**	**0.706**	**0.658**
16. arginine	0.306	0.517	0.402	0.577	0.655	0.564	0.587	0.584
17. *Total sum of AA*	*13.377*	*11.719*	*8.316*	*10.812*	*11.456*	*12.968*	*11.998*	*11.520*
18. *Total sum of EAA*	**4.264**	**3.419**	**3.004**	**3.059**	**3.970**	**4.626**	**4.244**	**3.989**

* - essential amino acids are in bold

The species *Astragalus ponticus* and *Onobrychis arenaria* contain a higher amount of lysine (0.746-0.760 mg/100 mg) than the traditional crops, but the species *Coronilla varia* and *Lotus corniculatus* – a lower one (0.517-0.524 mg/100 mg). In comparison with traditional forage crops, *Astragalus ponticus* is characterized by a higher content of threonine, glutamine, valine, isoleucine and histidine; *Coronilla varia* is very rich in proline and glycine, rich in asparagine, glutamine, glycine and alanine; *Lotus corniculatus* – rich in threonine and serine,

Medicago falcata – rich in asparagine, threonine, serine, glutamine and alanine. *Trifolium repens*, as compared with *Medicago sativa*, is richer in proline, glycine, valine, leucine, tyrosine, histidine and lysine. The content of essential amino acids varies from 3.004 mg/100 mg (*Lotus corniculatus*) up to 4.626 mg/kg (*Onobrychis arenaria*); *Medicago sativa* – 3.970 mg/100 mg (Tab. 4).

The use of forage legumes as biogas substrate contributes to an increase in the potential of bioenergy and can help reduce the greenhouse gas emissions. Through symbiotic nitrogen fixation, they compensate inorganic N fertilizer in conventional farms, if the digestate is applied as a fertilizer to the non-legume crops [STODDARD, 2013]. The content of organic matter, biochemical composition, biodegradability and ratio of carbon and nitrogen (C/N) of the raw material are essential in the production of biogas. The C/N ratio of the studied species varied from 13, in the biomass of *Astragalus ponticus*, to 29-30, in the biomass of *Trifolium repens* and *Lotus corniculatus* (Tab. 5). The optimal C/N ratio is expected to be in the range 15-25, when the anaerobic digestion process is carried out in a single stage, and for the situation when the process develops in two steps, the optimal C/N ratio will range: for step I: 10-45; for step II: 20-30 [DOBRE & al. 2014].

Fermentable organic matter represents the proportion of organic matter which can be biologically degraded under anaerobic conditions and, thus, can be potentially utilized in biogas facilities [WEISSBACH, 2008]. The calculated gas forming potential of the fermentable organic matter of the studied *Fabaceae* species varied from 470 to 544 litre/kg volatile solid matter (VS), *Trifolium repens*, *Coronilla varia* – lower than *Medicago sativa*, but *Onobrychis arenaria*, *Medicago falcata* and *Lotus corniculatus* – exceeding *Onobrychis viciifolia*, they have similar content of methane. The methane yield per ha of studied species of the family *Fabaceae* (first mowing) ranged from 692 to 3197 m^3/ha, *Onobrychis arenaria* exceeding *Onobrychis viciifolia*, but *Coronilla varia* exceeding *Medicago sativa*.

Tab. 5. Gas forming potential of the fermentable organic matter (FOM) from the studied *Fabaceae* species

Indicators	***Astragalus ponticus***	***Coronilla varia***	***Lotus corniculatus***	***Medicago falcata***	***Medicago sativa***	***Onobrychis arenaria***	***Onobrychis viciifolia***	***Trifolium repens***
1	2	3	4	5	6	7	8	9
1. Ratio carbon/nitrogen	13	22	30	22	19	18	19	29
2. FOM, g/kg VS	641	626	680	678	642	685	658	588
3. Biogas, litre/kg VS	513	501	544	542	514	542	526	470
4. Methane, litre/kg VS	269	263	286	285	270	288	276	247
5. Methane yield, m^3/ha	1076	2311	1571	1881	2214	3197	2843	692

Conclusions

The species *Coronilla varia* and *Lotus corniculatus* can be harvested, for the first time, 5 days earlier, but *Medicago falcata* and *Onobrychis viciifolia* – 18 days later than *Medicago sativa*. Due to this fact, fresh natural forage can be provided for animals for a longer period of time.

The green mass yield varied from 0.83 kg/m^2 to 4.08 kg/m^2. The species *Onobrychis arenaria* and *Coronilla varia* have a productivity of 3.92-4.08 kg/m^2 green mass, at the same level as *Onobrychis viciifolia*, but by 26-31% higher than *Medicago sativa*.

The natural forage of the studied species reaches amounts of 0.60-0.89 nutritive units and 8.05-9.90 MJ/kg dry matter. The methionine content in the species *Onobrychis arenaria*,

Coronilla varia and *Lotus corniculatus* reaches 0.098-0.117 mg/100 mg of dry matter, thus, it is higher than in *Onobrychis viciifolia*, but lower as compared with *Medicago sativa*.

The calculated methane yield from the green mass of *Onobrychis arenaria* and *Coronilla varia* at the first harvest may reach 2311-3197 m^3/ha, exceeding *Medicago sativa*.

The studied taxa can serve as starting material in improving and implementing new varieties leguminous species in the production of protein rich forage, as well feedstock for biogas production.

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STRUCTURE OF SALT GLANDS OF *PLUMBAGINACEAE*. REDISCOVERING OLD FINDINGS OF THE 19th CENTURY: 'METTENIUS' OR 'LICOPOLI' ORGANS?

Marius Nicușor GRIGORE[1*], Constantin TOMA[1]

Abstract: Salt (chalk) glands of *Plumbaginaceae* represent interesting structures involved in the excretion of calcium carbonate outside plants' organs, especially on leaves surfaces. These chalk-glands, nominated by some authors as 'Licopoli' or 'Mettenius' organs are also very important from taxonomical point of view. Their structure has been a matter of debate for decades and a historical analysis reveals that there are still some inconsistencies regarding the contributions of earlier botanists in discovering and describing chalk-glands. The present work tries to provide a picture of historical progress recorded in the 19th century related to investigation of these structures, focusing especially on the two important names usually mentioned in relation to them: Mettenius and Licopoli. In this respect, several useful clarifications are made, with emphasis on the role played by the two botanists in the stimulation of research interest for these glands among the generations of botanists to come.

Keywords: chalk-glands, Licopoli, Mettenius, *Plumbaginaceae*, secretion.

Introduction

Plumbaginaceae constitute a well-represented cosmopolitan family in the temperate zones of the Northern Hemisphere and showing preferences for arid or saline, often coastal, environments [KUBITZKI, 1993]. The Angiosperm Phylogeny Group classification of flowering plants [APG, 2003] included this family in the *Caryophyllales* order, together with other families adapted to extreme environments including oligotrophic soils, arid zones, and soils with high salt content. The taxonomy and taxonomical affinities of this striking family are still very problematic and controversial [CRONQUIST, 1981; LLEDO & al. 1998, 2001, 2005; REYES, 1997; SHORT & WIGHTMAN, 2011; TAKHTAJAN, 2009]. For this reason, the number of genera and species included in the *Plumbaginaceae* differ greatly from one author to another: from about 12 genera and 400-500 species [REYES, 1997] to 10-27 genera and about 1,000 species [SHORT & WIGHTMAN, 2011], *Plumbaginaceae* is a well-known halophytic family [GRIGORE, 2008, 2012; GRIGORE & TOMA, 2010; GRIGORE & al. 2014] a reality since long recognized in botanical research [ENDLICHER, 1836-1840; LINCEVSKII & CERNIAKOVSKOI, 1952; BENTHAM & HOOKER, 1876; VOLKENS, 1884; PAX, 1897; STRASBURGER & al. 1894; LINDLEY, 1846; RĂVĂRUȚ, 1960; MOORE, 1972; TAKHTAJAN, 2009].

When referring to the *Plumbaginaceae* family, one should emphasize that one of the most obvious anatomical traits of its representatives is the presence of *epidermal glands* (chalk-glands and mucilage glands) located on leaves and stems. Actually, these glands were closely integrated in the taxonomical characteristics of *Plumbaginaceae*, as a significant anatomical

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feature, a tendency adopted by both older [PAX, 1897; STRASBURGER & al. 1894; VOLKENS, 1884] and recent authors [KUBITZKI, 1993; TAKHTAJAN, 2009].

When describing the secreting glands of the *Plumbaginaceae* species, METCALFE & CHALK (1972) and, earlier, SOLEREDER (1908) classify them into two categories:

1. Chalk (chalk-secreting) glands, also known as Mettenius glands or Licopoli glands, which generally occur on or inside the cavities on the inner side of the leaves and stem, sometimes surrounded by groups of elongated epidermal cells or by simple hairs. Individual glands of this sort are made up of 4 or 8 epidermal cells arranged in palisade surrounded by 1 or 2 layers, each one made up of 4 "accessory" cells. The walls between the secreting cells of the gland and the surrounding ("accessory") cells are cutinized. The secreting "organs" of this sort have been generally described as chalk glands, because they exude calcium salt and water; calcium salts are sometimes scattered on the leaf or stem surface by rain drops. The amount of secreted calcium salts depends on the type of soil, although, for instance, the British *Limonium* species analyzed by de FRAINE (1916) do not secrete limestone-containing substances.

2. Mucilage glands occur in some representatives of the *Plumbaginaceae* family; those occurring in the axils of the upper side of the *Limonium bellidifolium* and *L. binervosum* basal leaf, described by de FRAINE (1916), evidence a head resting on a head borne on a base consisting of few cells with very thick cuticle-lined walls.

The present contribution will actually deal only with chalk-glands ('Mettenius' or 'Licopoli' organs), and not with mucilage glands of *Plumbaginaceae* [GRIGORE & TOMA, 2010], which are the other type of epidermal glands found in the species of this botanical family. It is worth mentioning that an interesting phenomenon occurs regarding the semantics of these glands. Sometimes, in older botanical papers, `Mettenian gland(s)` expression is being used [de FRAINE, 1916; JACKSON, 1928]. This word derivation may suggest that authors have attributed to Mettenius the discovering and description of these intriguing structures.

Throughout the present work, the authors maintained the nomenclature used in the papers consulted, without any intention to find and use instead updated synonyms.

Historical approach

Frequently, the structure of salt glands from *Plumbaginaceae* has been differently interpreted by some authors, although these controversies are related rather to details than to their basic structure. These formations drew botanists' attention as early as the second half of the 19th century, as we will describe herein.

The chalk secretion and deposit on the surface of these organs have been noted long time before the detection and description of these glands. Thus, the French chemist BRACONNOT (1836, consulted paper) (and not from 1830, as he is erroneously quoted by MAURY, 1886) was the first who tried to analyze this mineral substance secreted by glands of different species of *Statice*: *S. monopetala*, *S. pruinosa*, *S. aphylla* and others and of *Plumbago*: *P. zeylanica*, *P. auriculata*, *P. scandens* and *P. rosea*. He investigated the 'inorganic scales (*écailles de nature inorganique*) produced by species of *Plumbaginaceae* family'; when examined with a magnifier glass, these white deposits on the surface of leaves appeared to Braconnot as a 'small parasitic fungus embedded in the tissues of host plant'. He has also anticipated the existence of special secreting formations involved in the occurrence of these deposits, but he did not use a specific term to nominate them. However, he made an interesting anatomical-like observation: after washing the leaves of *Statice* species with acids, he observed on their surface 'visible cavities indicating the places where the stalks of these small scales were embedded'. After having treated the leaves of

several *Statice* species with hydrochloric acid, he performed the dissolution of the secreted substance, which he identified as calcium carbonate and which contained suspended transparent formations, which he assumed to be the "organs" considered to have secreted this carbon-containing substance. However, this finding remained unknown to many future botanists for a long time.

MAURY (1886) and VUILLEMIN (1887) believed that the Italian botanist LICOPOLI (1866) was the first researcher who made a histological description of these calcium carbonate-secreting "organs", ignoring the fact that METTENIUS had mentioned them since 1856. For this reason, even nowadays, the terms 'Licopoli' and 'Mettenius' organs are being used in parallel in botanical literature. The reason for this perception is perhaps explained by the fact that some authors knew only Mettenius's or only Licopoli's paper and not both of them, so that they could not have an accurate historical picture. For instance, neither MAURY nor VUILLEMIN do mention Mettenius's work, whereas, out of the two French botanists, only MAURY (1886) mentions Braconnot's earliest paper. One may assume that Mettenius's paper, published in German, was inaccessible to French botanists and thus it has not been consulted; however, METTENIUS (1856) does mention Braconnot's findings.

METTENIUS (1856) described chalk-glands in a very succinct, but quite precise manner, in the way that he did not hesitate at all in using correct terms related to the chalk secreting function of these glands: *Kalksecretion* (chalk secretion) and *Kalkschüppchen* (chalk scales). He described the chalk glands of *Goniolimon tataricum* (Fig. 1), *Limoniastrum monopetalum* (Fig. 2), *Plumbago europaea* (Fig. 3) and *P. zeylanica* (Fig. 4).

Nevertheless, Mettenius's work (1856) represents a significant progress in the research of chalk-glands as compared to earlier Braconnot's (1836) paper, assumed to be the first in signaling chalk-secreting process. In his brief considerations, Mettenius underlined several considerable aspects. For instance, he has correctly shown that chalk-glands belong to the epidermal complex, and that they are derived from epidermal cells divisions, and – most important – that they are not connected with stomata or the vascular system. However, the structure of gland was incorrectly described by Mettenius as consisting of a group of four cells; his mistake was maintained subsequently by LICOPOLI (1866) and MAURY (1886).

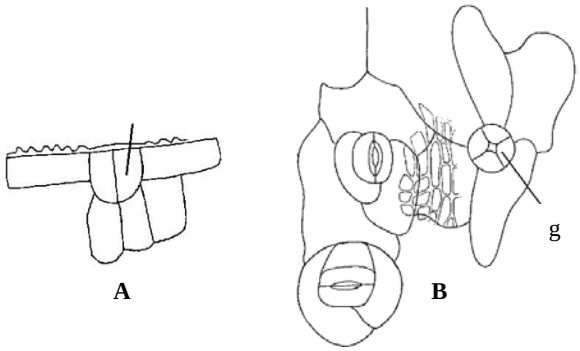

Fig. 1. Chalk glands (g) in the leaf of *Goniolimon tataricum* (A – cross section; B – surface view) [METTENIUS, 1856]

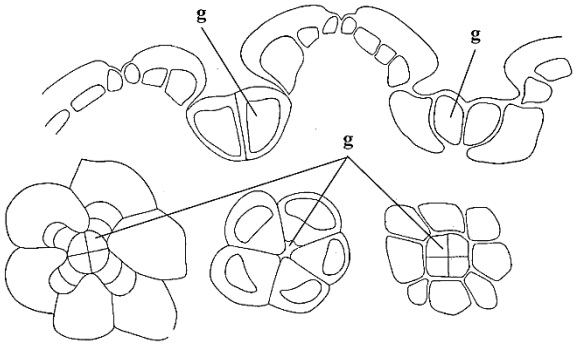

Fig. 2. Chalk glands (g) in the leaf of *Limoniastrum monopetalum* [METTENIUS, 1856]

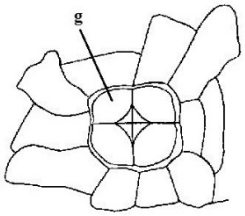

Fig. 3. Chalk glands (g) in the leaf of *Plumbago europaea*, surface view [METTENIUS, 1856]

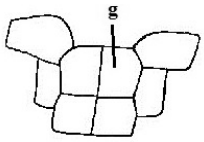

Fig. 4. Chalk glands (g) in the leaf of *Plumbago zeylanica*, cross section [METTENIUS, 1856]

The Italian botanist LICOPOLI, still considered the first who made a description of chalk glands of *Plumbaginaceae* species (*Statice monopetala*, 1866) provided a detailed analysis of these glands and depicted them in several drawings (Figs. 5-8). Indeed, his contribution is very extended and detailed; unfortunately, it has no references included, so that it is almost impossible to assert whether he knew Mettenius's paper or had other data in hand. Except for the fact that he did not nominate the exact types of gland-consisting cells, he was able however to distinguish them from an anatomical point of view and finally to deliver an accurate description of glands (known, as shown, as 'Licopoli organs'). In addition, he pointed out several important details with respect to these 'organs'; he correctly concluded that these glands are connected neither with the vascular system, nor with the stomata of plant leaf. Another important observation was that the excreted material of these glands is calcium carbonate; on his microscopical observations, he identified a chalk deposit at the top of the glands – clearly nominated as '*glandole*'.

LICOPOLI resumed his observations in a paper from 1879, where he used the term '*glandole calcifere*'. He states that: *these glands have an organization (structure) based on the type discovered and described in Statice monopetala in my previous work* – that from 1866. He added in the new paper several additional data and drawings; despite very detailed descriptions of these glands, he was not able to explicitly specify the eight-cell structure of these glands (1866; 1879). However, on a deeper text analysis, it could be foreseen that Licopoli may refer in 1879 on an eight-cell structure of these glands; for instance, when describing glands from *Statice splendens*, he referred to two distinct groups of four cells and even clearly depicted them in a surface view drawing (thus, eight cells).

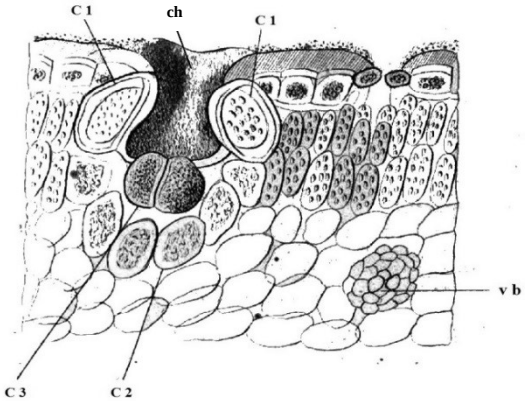

Fig. 5. Licopoli 'organs' in the lamina of *Statice monopetala* (C1, C2 – different types of cells; C3 – a complex of cells – *borsetta*, forming the bottom of the gland, ch – chalk deposit) [LICOPOLI, 1866]

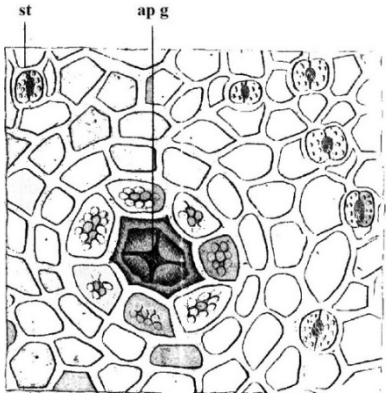

Fig. 6. Licopoli 'organ' in the lamina of *Statice monopetala*, front view (ap g - aperture of the gland; st – stomata) [LICOPOLI, 1866]

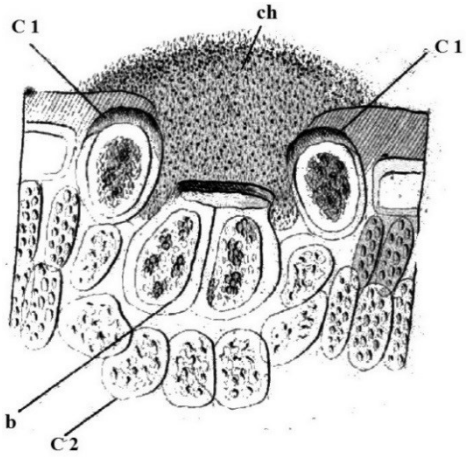

Fig. 7. Licopoli 'organ' in the lamina of *Statice monopetala*, cross section (C1, C2 – different types of cells; b – *borsetta*, forming the bottom of the gland; ch – chalk deposit) [LICOPOLI, 1866]

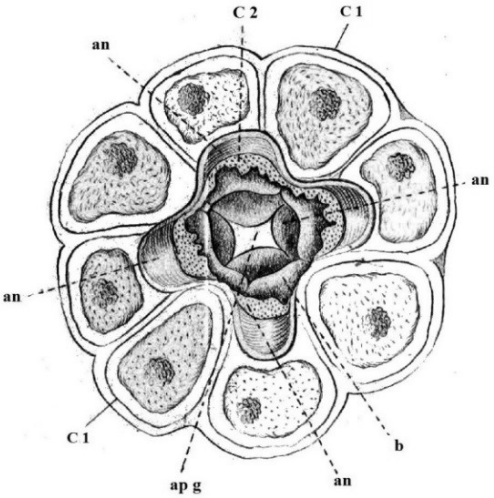

Fig. 8. Licopoli 'organ' in the lamina of *Statice monopetala*, detail in front view (C1, C2 – different types of cells; an – angles formed on the intersection of different types of cells; ap g - aperture of the gland; b – *borsetta*, forming the bottom of the gland) [LICOPOLI, 1866]

After Licopoli's findings – already known and commented by the botanists to come - the interest for the study of chalk-glands was intensified towards the end of the 19th century; the great majority of botanists recognize these glands as 'Licopoli' rather as 'Mettenius' glands.

MAURY (1886), in his extensive study on the structural organization of *Plumbaginaceae* species, evidenced and described Licopoli 'organs' in: *Plumbago europaea* (Figs. 9-10), *P. larpentae* (Fig. 11), *Statice limonium* (Figs. 12-13), *S. elata* (Fig. 14), and *S. lychnidifolia* (Fig. 15).

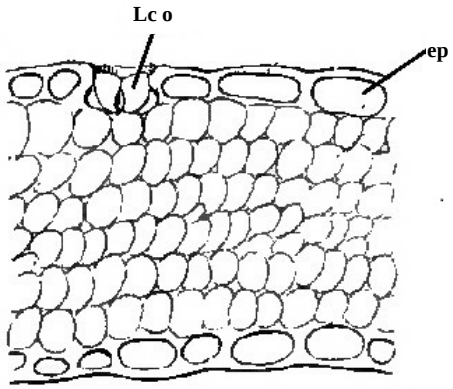

Fig. 9. Cross section through the lamina of *Plumbago europaea* (ep – epidermis; Lc o – Licopoli 'organ') [MAURY, 1886]

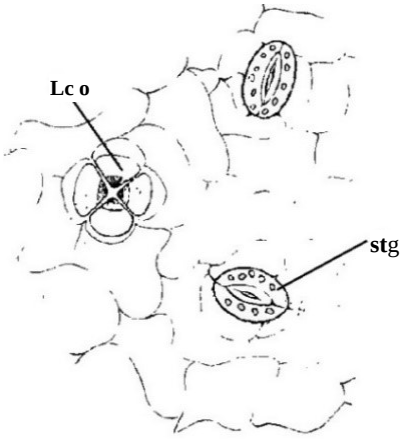

Fig. 10. Licopoli 'organs' (Lc o) in epidermis of *Plumbago europaea* (st – stomata) [MAURY, 1886]

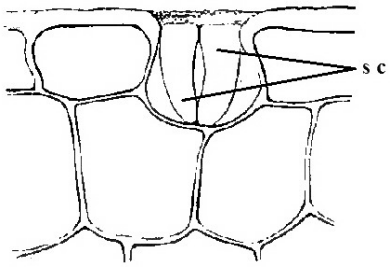

Fig. 11. Cross section through the lamina of *Plumbago larpentae* (s c – secretory cells of Licopoli 'organs') [MAURY, 1886]

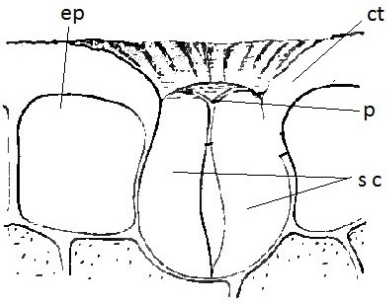

Fig. 12. Cross section through the lamina of *Statice limonium* (ep – epidermis; ct – cuticle; p – pore; s c – secretory cells of Licopoli 'organs') [MAURY, 1886]

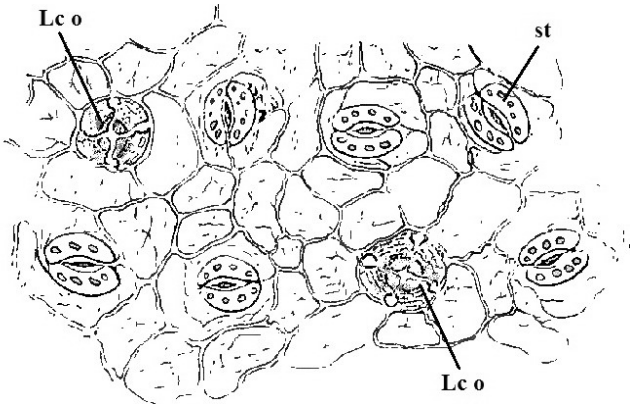

Fig. 13. Licopoli 'organs' (Lc o) at epidermis level of the lamina of *Statice limonium* (st – stomata) [MAURY, 1886]

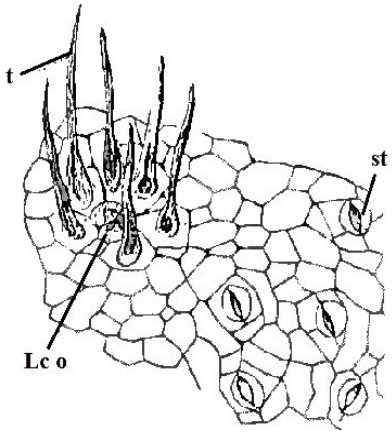

Fig. 14. Licopoli 'organs' (Lc o) at epidermis level of the lamina of *Statice elata* (st – stomata; t – trichome) [MAURY, 1886]

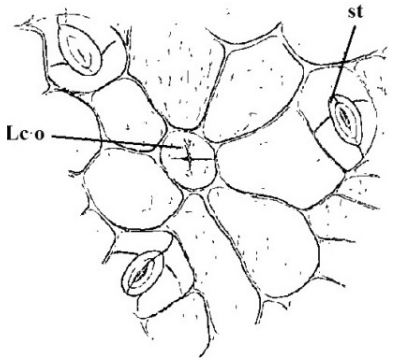

Fig. 15. Licopoli 'organs' (Lc o) at epidermis level of the lamina of *Statice lychnidifolia* (st – stomata) [MAURY, 1886]

De BARY (1877) described this secreting "organ" in a different manner; he stated that it included 8 cells originating in the divisions of a single primary mother cell, which is round or square in surface section. This cell is divided in four by two cell wall divisions, perpendicularly on the surface and on each other. In its turn, each of them is divided again, so that one of the new cells is triangular and internal, and the other is rectangular and peripheral.

VOLKENS (1884) and WORONIN (1885) adopted de BARY (1877) descriptions and interpretations. It seems that they were not aware of Licopoli's findings, since no mention is made of his interpretations. This is quite unexpectedly even for the papers of the 19th century, which are usually well documented and supported by literature, in the manner we know nowadays. Neither Volkens' nor Woronin's papers – written in German – have mentioned Licopoli's findings, while the Italian paper has no references, as already shown. VOLKENS (1884) maintained the basic 8-cell structure of these glands, and pointed out their irregular layout and their role in water elimination, seeing them as "safety valves" that start working when the absorption/transpiration ratio is altered. In his opinion, any excessive calcium salt is eliminated as carbonic acid. In *Statice limonium*, the cells adjacent to the gland become prominent and turn into conical protrusions.

Figures 16-21 show drawings of these glands in different *Plumbaginaceae* species, as VOLKENS (1884) depicted them. However, Volkens uses the terms: '*Sekretionsapparat*, *Kalkschuppe*', and '*drüse*' corresponding to secretory structures.

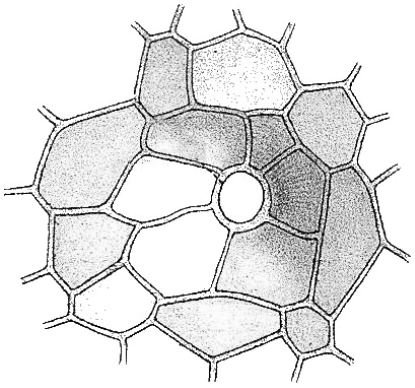

Fig. 16. Salt-secreting 'apparatus' of *Statice limonium* [VOLKENS, 1884]

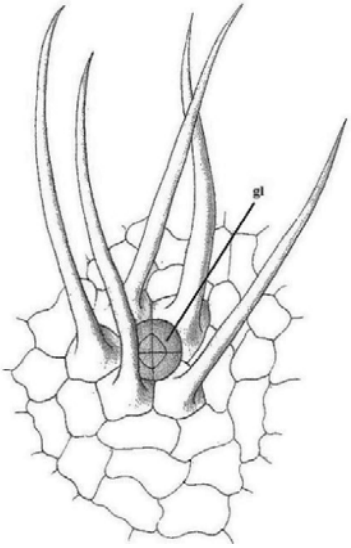

Fig. 17. Chalk gland (gl) at epidermis level of the lamina of *Statice latifolia* [VOLKENS, 1884]

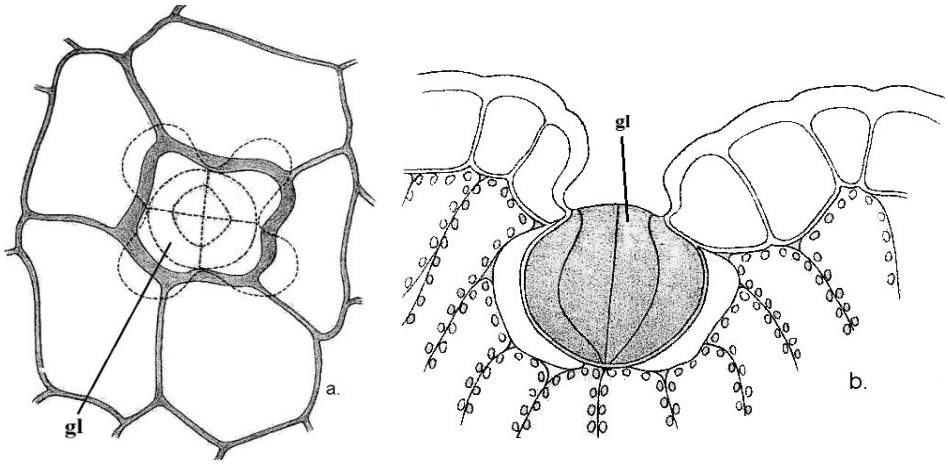

Fig. 18. Chalk glands (gl) of *Limoniastrum monopetalum* (a – surface view; b – cross section) [VOLKENS, 1884]

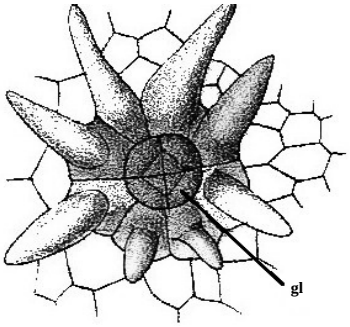

Fig. 19. Salt-secreting 'apparatus' (gl) of *Statice pruinosa* [VOLKENS, 1884]

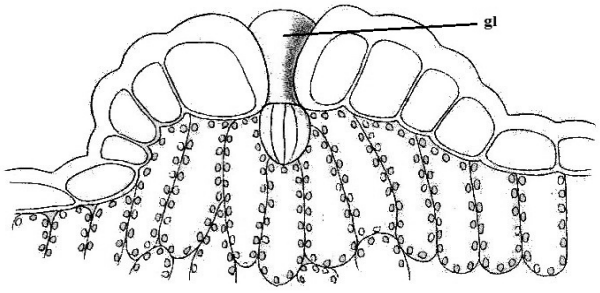

Fig. 20. Chalk gland (gl) of *Statice rhodia* [VOLKENS, 1884]

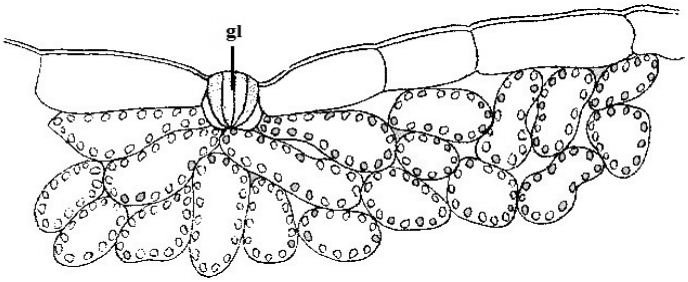

Fig. 21. Chalk gland of *Statice occidentalis* [VOLKENS, 1884]

WORONIN (1885) investigated the leaf structure of *Statice monopetala* and evidenced the chalk glands ('Kalkdrüse') (Figs. 22-24); he also made a drawing of these glands in *S. sareptana* (Fig. 25). In addition to the anatomical description of these glands, he made an interesting ecological observation: the secretion of calcium carbonate by species of *Plumbaginaceae* is conditioned by soil composition, precisely by its content in calcium carbonate. Woronin correctly stated that many species of this botanical family do not show an excretory process.

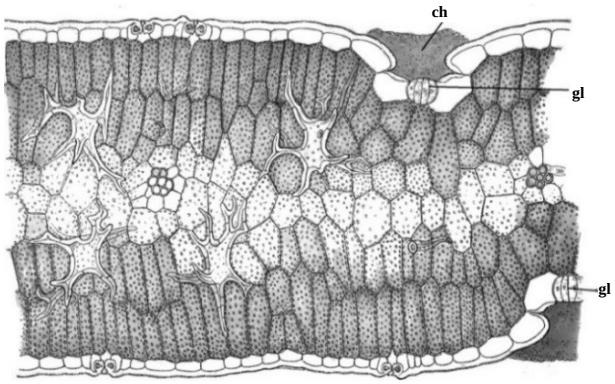

Fig. 22. Chalk glands (gl) in the lamina of *Statice monopetala* (cross section; ch – chalk deposit) [WORONIN, 1885]

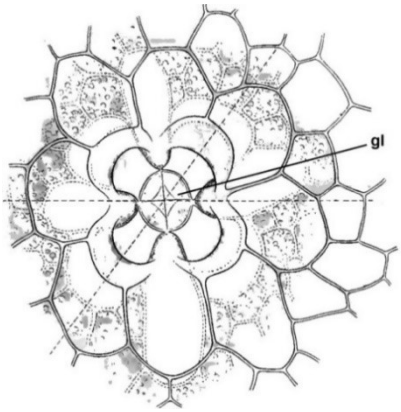

Fig. 23. Chalk-glands (gl) in the lamina of *Statice monopetala* (surface view) [WORONIN, 1885]

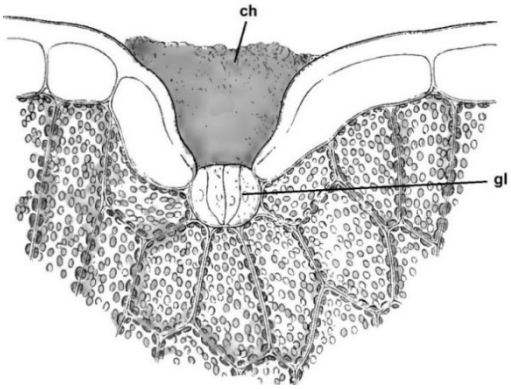

Fig. 24. Chalk-glands (gl) in the lamina of *Statice monopetala* (cross section, magnified image) [WORONIN, 1885]

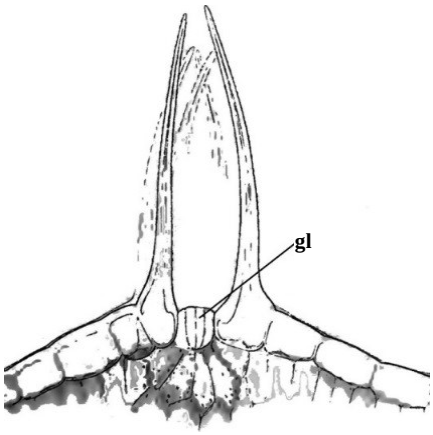

Fig. 25. Chalk-gland (gl) in the lamina of *Statice sareptana* (cross section) [WORONIN, 1885]

MAURY (1886) tried to elucidate the structure of the *Plumbaginaceae* glands, by pointing out the possible reasons for which other authors considered that these structures rely on 8 and not on 4 cells. When viewed from the top, on a small area of the epidermis, the "organ" looks like a circle divided into four sectors by two diameters perpendicular on each other. Each of these sectors *seems* (Maury's emphasis in the text) divided itself in two by a tangential line, which is more blurred than those of the other sectors. This is actually the inner wall of each secreting cell, which borders the central intercellular space; thus, it is this wall that corresponds to this line (which may be best seen on a longitudinal section of the "organ"). The secreting cells are curved, joined together at the bottom and then loosened along their whole length. Although the substance produced is mixed in this intercellular space, it expands at mid-cell height, the upper ends of which remain close to one another, so that the amount of secreted substance is not very large. The internal

pressure of these 4 cells made the product exit, due to the pressure put by the inner space walls on the fluid. This fluid removal mechanism is correlated by Maury exclusively with a structure built on 4 cells. In his opinion, if there were 8 cells, the substance would be simply exuded by the outer side of the "organ". In other words, de BARY (1877), VOLKENS (1884) and WORONIN (1885) argued that the calcium-containing fluid was eliminated by a mere osmotic phenomenon.

MAURY (1886) also conducted experiments on some *Plumbaginaceae* species, designed especially to analyze the formation and nature of efflorescences, made up of very fine salt filaments, occurring on the surface of *Plumbago capensis* and *P. zeylanica* organs. These experiments also permitted several conclusions:

1. The mineral substance secreted by the Licopoli "organs" are shaped like filaments, due to the pressure put on the central cavity of the organ by the 4 secreting cells;
2. Under humid conditions or in the presence of water (rain water, irrigation), the mineral substance becomes hydrated and the filaments turn into small discs on the epidermis;
3. The role of this mineral substance is similar to that played by hairs in other plants; the author argues that it regulates transpiration.

MAURY (1886) substantiates this last aspect in the following manner: the *Plumbaginaceae* living in arid or maritime environments should cope with the absence of hairs by accumulating a mineral substance on their surface. Species living in arid environments, *Limoniastrum* species and a specific number of *Statice* species are covered by a calcareous coating, which protects them against a too abundant transpiration. The data supporting his assumptions would be that the *Armeria*, *Acantholimon* species living in the uplands are less affected by these influences. The *Plumbago* species vegetate mostly in shady areas and, therefore, have a reduced number of Licopoli "organs".

Whereas MAURY (1886) was positively supporting the 4-cell structure of these Licopoli "organs", VUILLEMIN (1887) claimed that the 8-secretting-cell structure was very easy to prove. Although thin, the walls of these cells are easily dissolved in reagents; the accessory cells are persistent and their boundaries are hard and cutinized, and they are joined together at the bottom of the gland. These edges are carinated and followed by two side expansions applied directly on the connection line separating the accessory cells. The latter thus form a continuous barrier between the glandular cells, on one hand, and the parenchyma and epidermis, on the other; all substances that shift from one to the other have to pass through the accessory cells. The cutinized ridges have a rather constant layout in the various genera of the *Plumbaginaceae* family; each of them is made up of a lateral and a deep side. The lateral side makes up a triangle pointing towards the inside of the gland; the 4 deep sections, which form a cross, are almost parallel to the surface of the epidermis.

Unlike MAURY (1886), who claimed that the *Limoniastrum monopetalum* "organs" are full of limestone-containing substances, the analyses made by VUILLEMIN (1887) on the same species, did not reach a similar conclusion. Instead, he used another research method: he burned a piece of leaf in potassium; this action, even when lasting for a long time, does not modify the limestone-containing product. The epidermis is easily dissociated and each isolated gland remains stuck to the excreted mass. The dissolution process led to the disappearance of the thin walls separating the glandular cells; the accessory cells often persist with the cutinized ridges, which support and separate them. When one examines this type of "skeleton" (in *Limoniastrum monopetalum* – Fig. 26 and

Statice latifolia – Fig. 27), one may notice the completely loose and empty gland, despite the limestone covering the external side. The concretion stuck to the inner chamber (inner space) diverticula, which precedes the gland, is made up of two parts joined together by a constriction: the outer part, found on the surface of the epidermis, and the inner four-lobed part, which resembles the shape of the actual gland.

In *Statice imbricata* (Fig. 28), 6 cells, separated by very thin angled walls, can be noticed. There are actually 4 glandular cells flanked by two accessory cells. The thin cellulosic walls stretching between the accessory cells and the secreting components are almost always partially masked by cutinized borders. Glandular cells usually stick out from the surface of the leaf, since the accessory cells sink between the gland and the adjacent portions of the epidermis.

The parenchyma cells have an oblong shape and a palisade-like layout (with much reduced meatuses) in the gland (Fig. 28b). In the section joined to the epidermis, the accessory cells are often much thicker than in the deep section. The epidermal cells have punctuations both on their lateral sides and on their deep side. These punctuations are evenly scattered on the lateral sides and grouped on the deep one in round surfaces (corresponding to parenchyma cell insertions), whereas the opaque ones correspond to intercellular meatuses.

The cuticle is interrupted in the hypostomatic chambers (Fig. 26), and fenestrated outside these chambers.

Generally speaking, the basic structure of the glands detected and studied by VUILLEMIN in the *Plumbaginaceae* species remains constant. Only 4 of the 8 glandular cells are excretive. The two rows of cells are sometimes similar in terms of their dark and fine-grained content, which clearly differentiates them from the accessory cells and from epidermal or cortical elements. Exchanges occur easily among them, due to their thin walls. The external secreting cells communicate easily with the accessory cells through osmosis, along their walls, which are also thin, but separated from the latter by other leaf tissues. Cutinized ridges prevent any communication between the parenchyma and glandular cells in the interstice separating the accessory cells, as well as the formation of any meatus, by providing a proper sealing of the latter.

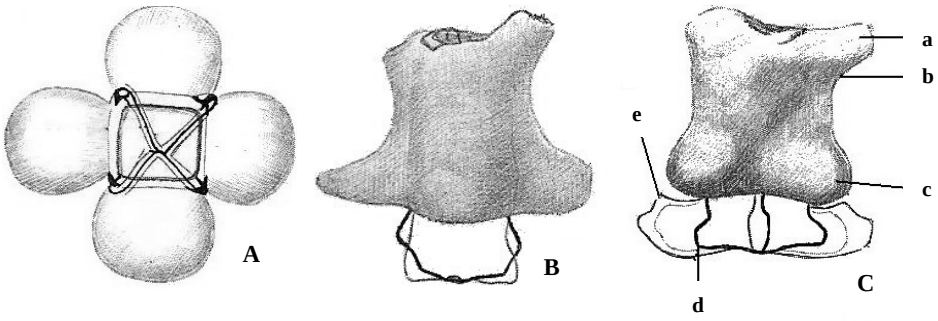

Fig. 26. Structure of chalk-gland in *Limoniastrum monopetalum* (A – gland observed in front view, without chalk mass; B – skeleton of gland, without accessory cells; C – a, external limit of cutinized frame that forms the edge of the internal chamber; b, orifice of the chamber in which basis gland opens; c, basis of chambers diverticula; e, extremity of free side of accessory cells) [VUILLEMIN, 1887]

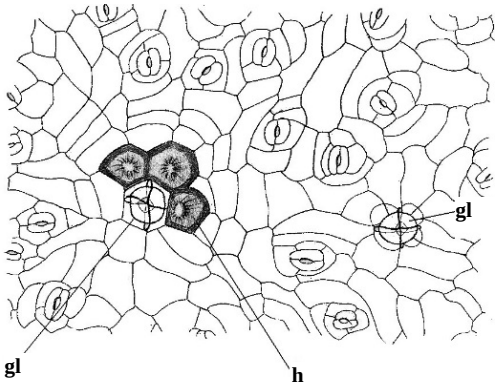

Fig. 27. *Statice latifolia.* Epidermis, surface view (gl – chalk glands; h – hairs) [VUILLEMIN, 1887]

In the species whose accessory cells are very well developed and partly sealed on their sides, like *Limoniastrum guyonianum* (Fig. 29), a cuticle sheet grows between them and bifurcates on their outer side, so that to prevent wall detachment. The accessory cells are connected with the epidermis and parenchyma cells, appearing as bridges connecting the leaf tissues with the gland; from this point of view, they behave like the basal cells of glandular hairs.

The above-cited author considers the two anatomic structures, *i.e.* gland and hair, as homologous. The accessory cells would correspond to the foot, whereas the secreting cells to the head of a glandular hair, yet one that underwent an extreme shortening.

The surface section of glandular cells differs from that of the other walls due to its complete cutinization. The cutinized plate was best noticed on the front view of an epidermis. In *Statice tatarica* (Fig. 30), the depth of the chamber preceding the gland (which is almost as thick as the epidermis) and the plate are located at the level of inner side of this layer. After having treated the epidermis with a chlorine-iodine solution, the author viewed it as a violet lamella covered with yellow discs (representing glands). Each disc still leaves the impression of two dividing walls in a cross-like layout and other four walls in a rhombus-like layout. The surface is also divided into 4 triangles close to the middle and 4 neighboring trapezoids close to the borders.

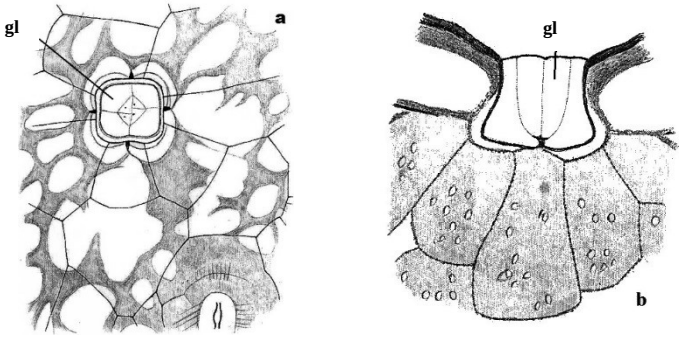

Fig. 28. *Statice imbricata.* Chalk-gland (a - cuticular network of the deep side of epidermis, continued in the proximity of a stoma; b – gland, in cross section, with 4 secretory cells and 2 accessory cells) [VUILLEMIN, 1887]

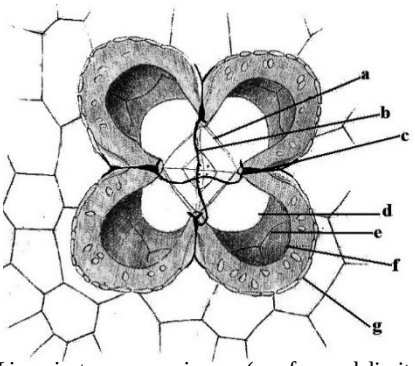

Fig. 29. *Limoniastrum guyonianum* (a – frame delimitating the free surface of secretory cells; b – cutinized edges supporting the gland; c – projection of edges between accessory cells; d – orifice at whose basis the gland opens; e – external limit of cutinized frame constituting the limit of the chamber; f – basis of diverticula of the chamber; g – the most external segment of the accessory cells) [VUILLEMIN, 1887]

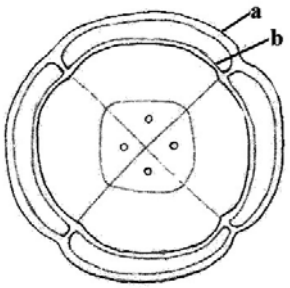

Fig. 30. *Statice tatarica* (a – orifice of excavation in the depth of which the gland opens; b – frame delimitating the free surface of secretory cells) [VUILLEMIN, 1887]

Conclusions

The salt glands (chalk-glands) of *Plumbaginaceae* represent striking structures involved in the excretion of calcium carbonate at the level of aerial organs (leaves, stems) of halophytes from arid and saline environments. According to our analysis, their secretion product has been evidenced about 20 years prior to their anatomical description. While many authors still consider that LICOPOLI (1866) was the first botanist who mentions them, it is by now obvious that, actually, METTENIUS (1856) did this prior to the Italian botanist. Indeed, Licopoli gave an extended and accurate description of them and his research could be considered as exclusively focused on the chalk-glands of *Statice monopetala*. As a matter of fact, all experiments developed by the plant anatomists of the 19th century in this direction were intense attempts at clarifying the structure and functions of these chalk-glands.

Acknowledgements

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CLONAL PROPAGATION AND EVALUATION OF PEROXIDASE ACTIVITY DURING *IN VITRO* RHIZOGENESIS IN *MENTHA ARVENSIS* L.

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Abstract: A rapid and efficient plant propagation system through nodal explants was developed for *in vitro* propagation of *Mentha arvensis.* Nodal explants exhibited high frequency shoot proliferation on Murashige and Skoog's (MS) basal medium supplemented with 0.5 mg l^{-1} BAP (6-benzylaminopurine). Microshoots were best rooted on ½ strength MS medium with 0.5 mg l^{-1} IBA (indole-3-butyric acid) and 2% (w/v) sucrose. Plantlets with high (≥ 0.8) Fv/Fm (φ_{P0} – maximum quantum yield of primary photochemistry) were successfully shifted to natural conditions. The overall survival rate from *in vitro* growth to field transfer was 74%. The developed micropropagation protocol can be successfully used for large-scale multiplication and genetic modification of this high value medicinal plant species. In addition, the results of this study also indicate the key role of Gpx (guaiacol-peroxidase) as a marker of *in vitro* rhizogenesis in *M. arvensis*.

Keywords: Fv/Fm, guaiacol-peroxidase, *in vitro* rhizogenesis, *Mentha arvensis*, Micropropagation.

Introduction

Mentha L. is a genus of aromatic perennial herbs belonging to the family Lamiaceae, distributed widely in temperate and sub-temperate areas of the world. Several *Mentha* species are considered industrial herbs as they are a source of essential oils enriched in certain monoterpenes, commonly used in food, flavor, cosmetic and pharmaceutical industries. *Mentha arvensis* L., known as common mint, is often used as a folk medicine to treat indigestion, nausea, sore throat, and toothache since prehistoric time. The fresh leaves contain essential oils consisting of monoterpenes like menthol, menthone, carvone and pulegone as major constituents, which are widely used by industries in food, pharmaceutical, flavor and/or fragrance formulations. The plant also contains anti-viral [ALI & al. 1996], anti-inflammatory [VERMA & al. 2003], anti-mycotic [PANDEY & al. 2003], anti-fungal [DUARTE & al. 2005], anti-oxidative [ZAKARIA & al. 2008] and anti-bacterial [WANNISSORN & al. 2008; COUTINHO & al. 2008; NASCIMENTO & al. 2010; JOHNSON & al. 2011] activities.

Owing to the diverse biological activities and increasing consumer interest, few efforts were carried out to propagate this valuable plant species through tissue culture techniques [CHISHTI & al. 2006; ISLAM & BARI, 2012]. In spite of earlier studies, more efficient and rapid *in vitro* propagation techniques are required for the genetic transformation and conservation of this medicinal plant. In the present investigation, we report a new plant

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regeneration method for *M. arvensis* through nodal explants. This efficient, rapid and simple regeneration system facilitates the application of plant tissue culture and genetic engineering approach in *M. arvensis*. In addition, efforts were also carried out to understand the involvement of guaiacol-peroxidase (Gpx) in rhizogenesis *in vitro* in this plant species.

Materials and methods

Culture media and growth conditions: The young shoots of *M. arvensis* were collected from plants grown at the botanical garden of Jaipur National University, Jaipur, India. The stem segments dissected and washed thoroughly under running tap water for 30 min to eliminate dust particles and then with 5% teepol for 8-10 min and rinsed several times in sterile distilled water. Thereafter, the explants were surface sterilized under a laminar flow chamber with aqueous solution of 0.1% (w/v) solution of $HgCl_2$ for 3 min and finally washed with sterile distilled water for 2–3 times.

After trimming both the cut ends, the sterilized stem explants were inoculated vertically on MS Medium [MURASHIGE & SKOOG, 1962] supplemented with 3% (w/v) sucrose and various concentrations (0.5 – 5.0 mg l^{-1}) of BAP and KIN (kinetin), singly or in combinations for multiple shoot induction. MS medium lacking plant growth regulators served as control. These cultures were transferred to a culture room and maintained at 25 ± 2 ºC and 65 – 70% relative humidity with photoperiod of 16 h using a photosynthetic photon flux density (PPFD) of 40 µmol/m^2 s^1 provided by cool white fluorescent tubes (Philips, India). To induce rhizogenesis, elongated shoots with 2–3 pairs of healthy leaves were cultured on MS supplemented with various auxins.

Hardening and acclimatization: Well rooted plantlets were gently washed in sterile water and transferred to plastic cups (10 cm × 8 cm) containing sterilized mixture of sterile soil, sand and coco peat (1:2:1). The plantlets covered in transparent polyethylene bags were kept for 3 weeks in growth chamber at 25 ± 2 ºC with 16 h photoperiod and 40 µmol/m^2 s^1 of irradiation. The plantlets were irrigated with tap water. The hardened plantlets were subsequently transferred to earthen pots containing normal garden soil and were maintained in greenhouse conditions.

Measurement of Fv/Fm (φ_{P0}): Photosynthetic screening during hardening and acclimatization process can help to improve the performance and survival of micropropagated plants. Thus, the maximum quantum yield of primary photochemistry (φ_{P0}= Fv/Fm) of plantlets growing under growth chamber and green house conditions was regularly measured using a Plant Efficiency Analyser, PEA (Hansatech Instruments, Kings Lynn, Norfolk, U.K.) according to HEBER & al. (2011). The leaf samples were dark adapted for 2 h before the fluorescence measurements. The plants with high φ_{P0} ($\geq$ 0.8) were subsequently transferred in natural conditions. The survival rate of plantlets was recorded after 1 month of transfer to natural conditions.

Guaiacol peroxidase (GPx) activity (1.11.1.7): To determine the Gpx activity during the rhizogenesis *in vitro*, the nodal explants were cultured on root-induction medium [½ strength MS medium + 2% (w/v) sucrose + 0.5 mg l^{-1} IBA] and withdrawn daily, until the emergence of root primordia, to homogenize in pre-chilled mortar and pestle using phosphate buffer (pH 7.0). The homogenate was centrifuged at 10,000 rpm for 20 min and used to essay GPx activity. GPx activity was measured spectrophotometrically at 25 ºC by following the method of RACUSEN & FOOTE (1965). The reaction mixture consisted of 1.5 ml of 50 mmol l^{-1} potassium phosphate buffer (pH 7.0), 0.2 ml of enzyme extract, and 1

ml of 1% guaiacol, and the absorbance was set zero at 470 nm. The reaction was started by the addition of 0.3% hydrogen peroxide (0.2 ml) and changes in absorbance were recorded for every 15 s up to 5 min. Enzyme activity was calculated in terms of units g^{-1} fresh wt. One unit of enzyme was defined as the amount of enzyme necessary to decompose 1 mmol of substrate per minute at 25 °C.

Experimental design and statistical analysis: MS medium without hormone was treated as control in all experiments. All experiments were conducted with a minimum of 30 replicates per treatment and each experiment was repeated thrice. The data were analyzed statistically by one-way analysis of variance (ANOVA) followed by Tukey's test at P = 5 % using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA) and data represented as mean ± standard error (SE).

Results and discussion

***In vitro* establishment and multiplication**: Among the two different cytokinins tested, BAP proved better than KIN for accelerating the multiple shoot induction in nodal explants of *M. arvensis*. KIN at low concentration (0.5 mg l^{-1}) could provoke the development of single shoot per node after 12 days of culture (Fig. 1 A). Regenerated shoots elongated further on same medium (Fig. 1 B). Higher concentrations of KIN could not alter the frequency of shoot bud proliferation *in vitro*. All concentrations of BAP (0.5-5.0 mg l^{-1}) induced multiple shoot induction in nodal explants of *M. arvensis* (Tab. 1). The best response in terms of explants response (%), mean number of shoots and mean shoot length was achieved on MS medium supplemented with 0.5 mg l^{-1} BAP (Fig. 1 C). Frequency of shoot induction was drastically decreased with increasing concentration of BAP. Low frequency of shoot bud induction and callus formation at basal part of nodal explants was noted on medium enriched with high concentration of BAP (Fig. 1 D). Similar, inhibitory effect of higher concentrations of BAP on *in vitro* shoot proliferation has also been reported earlier in *Pterocarpus marsupium* [ANIS & al. 2005], *Arachis hypogaea* [BANERJEE & al. 2007], *Doritis pulcherrima* [MONDAL & al. 2013] and *Salvia splendens* [SHARMA & al. 2014]. Combinations of BAP and KIN could not initiate any significant morphogenetic responses in nodal explants of *M. arvensis.*

Tab. 1. Influence of BAP on direct shoot organogenesis in *M. arvensis* L.
Values represent the mean of 30 replicates ± SE, n= 3.

BAP concentration (mg l^{-1})	Explant response (%)	Mean number of shoots per node	Mean shoot length (cm)
0.5	92.05 ± 4.1	8.20 ± 1.04	3.45 ± 0.33
1.0	81.84 ± 2.9	6.44 ± 0.81	3.31 ± 0.53
2.0	41.88 ± 4.3	2.83 ± 0.93	3.26 ± 0.44
3.0	33.68 ± 4.2	2.38 ± 0.49	0.92 ± 0.12
4.0	20.51 ± 2.7	2.24 ± 0.05	0.66 ± 0.08
5.0	11.30 ± 2.1	2.24 ± 0.09	0.66 ± 0.76

***In vitro* rhizogenesis:** The rooting characters like percentage of cultured shoots producing roots, mean number of root/shoot and mean root length (cm) have been significantly affected by the MS medium strength and IBA concentration. Overall, MS

medium of ½ strength proved better for induction of *in vitro* rhizogenesis as compared to full strength MS medium whether with or without IBA of any concentration (Tab. 2). ½ strength MS medium supplemented high concentrations of IBA (3.0-5.0 mg l^{-1}) could lead the development of aerial roots (Fig. 1 E). High frequency (96 ± 6.2) induction of rooting was obtained on ½ strength MS medium with 0.5 mg l^{-1} IBA and 2% (w/v) sucrose. Similarly, IBA-induced rhizogenesis *in vitro* has been reported in many plant species, *i.e. Tectona grandis* [MENDOZA DE GYVES & al. 2007], *Phaseolus vulgaris* [KWAPATA & al. 2010], *Gentiana kurroo* [SHARMA & al. 2014], *Cattleya* [DEWIR & al. 2015]; *Hemidesmus indicus* [SHEKHAWAT & MANOKARI, 2016].

Tab. 2. Influence of IBA and MS strength on *in vitro* rhizogenesis in *M. arvensis* L. after 4 weeks of culture. Values represent the mean of 30 replicates ± standard error (SE).

Media Combinations [(MS strength + IBA ($mg1^{-1}$)]	**% Rooting**	**Mean no. of root/shoot**	**Mean root length (cm)**
Full + 0.0	14.0 ± 2.1	1.43 ± 0.7	0.77 ± 0.2
Full + 0.5	28 ± 3.3	1.84 ± 0.3	0.97 ± 0.2
Full + 1.0	26 ± 4.1	1.89 ± 0.5	2.26 ± 0.7
Full + 2.0	21 ± 2.9	1.96 ± 0.4	2.29 ± 0.4
Half + 0.0	36 ± 3.7	3.58 ± 0.3	2.61 ± 0.8
Half + 0.5	96 ± 6.2	8.50 ± 1.1	6.28 ± 0.3^{e}
Half + 1.0	77 ± 4.9	6.50 ± 1.4	3.83 ± 0.5
Half + 2.0	48 ± 3.2	2.66 ± 0.5	2.91 ± 0.4

Hardening and acclimatization: The increased use and efficiency of plant tissue culture techniques is still limited by the high percentage of plants which are lost or damaged when transferred from *in vitro* to *in vivo* conditions [POSPÍŠILOVÁ & al. 1999]. It is due to a poor photosynthetic capacity of *in vitro*-cultured plantlets, caused by insufficient inflow of carbon dioxide and the sucrose added to growth medium causing negative feedback for photosynthesis. Therefore, screening of photosynthetic potential of *in vitro* raised plants is essential to optimize hardening and acclimatization duration to ensure their high survival rate in field conditions. Plantlets regenerated *in vitro* were successfully acclimatized in the growth chamber (84% survival) and then in the greenhouse (88% survival). The φ_{P0} value of *in vitro* developed plantlets was found 0.42, which was slightly increased during hardening process in culture chamber (Fig. 2 A). Micropropagated plants achieved φ_{P0} value 0.73 in first week of their transfer at green house conditions. All the plants gained φ_{P0} value $\geq$ 0.8 within three weeks of acclimatization process. Fully hardened plants having high PSII photochemical efficiency ($\varphi_{P0} \geq 0.8$) were shifted from green house to natural conditions with 100% survival rate (Fig. 2 B). The regeneration method standardized in the present investigation relies on the fact that it is efficient, quick and highly reproducible method for micropropagation and genetic transformation studies of *M. arvensis*.

GPx activity during *in vitro* rhizogenesis: The biochemical changes drive cellular differentiation and organization as tissues and finally growth as organs. As per GASPAR & al. (1992) rhizogenesis includes three biochemical phases: *1*) an induction phase, characterized by a sharp reduction in peroxidase activity, *2*) a phase of root initiation with corresponding increase in peroxidase activity, and *3*) an expression phase, characterized by a gradual decline in peroxidase activity, which is followed by the emergence of root

primordia. In this study, we determined the possible involvement of Gpx in rhizogenesis *in vitro* in *M. arvensis.* In the present study, nodal explants of *M. arvensis* showed a slight decline in the Gpx activity on the 2th day, a gradual increase till the emergence of root primordia (8th day) and a drastic reduction on the 10th day on the root-induction medium (½ strength MS medium with 0.5 mg l^{-1} IBA and 2% (w/v) sucrose). An increase followed by a decrease in peroxidase activity preceding root appearance has also been observed in *Psoralea corylifolia* [ROAT & al. 2000], *Plumbago zeylanica* [SAXENA & al. 2000] and *Petunia* × *hybrida* microshoots [KOTIS & al. 2009]. According to the GASPAR & HOFINGER (1988), the increase in Gpx activity during the induction phase is related with a reduction in the content of endogenous auxin level; the reduction of peroxidase activity during the initiation phase is associated to an increase in the endogenous auxin level, leading to the differentiation of root primordia. Increase in peroxidase activity could also be related to the lignification of root primordia cells [MARJAMAA & al. 2009; NOVO-UZAL & al. 2013]. The results of this study suggest a key role of Gpx in *in vitro* rooting of microshoots of *M. arvensis*.

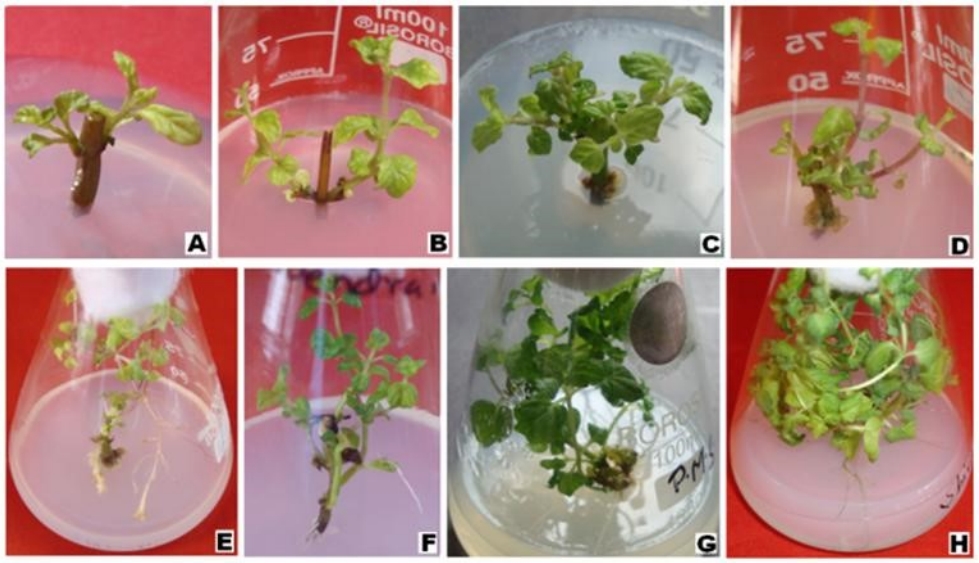

Fig. 1. *In vitro* propagation of *M. arvensis*. **A- B.** Shoot induction from nodal explant on MS medium fortified with 0.5 mg l^{-1} KIN. **C.** Multiple shoot proliferation on MS medium containing 0.5 mg l^{-1} BAP, 4-week-old culture. **D.** Shoot bud induction and callus formation at basal part of nodal explants on MS medium augmented with 5.0 mg l^{-1} BAP. **E.** *In vitro* development of aerial roots on MS medium supplemented with IBA (5.0 mg l^{-1}). **F.-H.** *In vitro* rhizogenesis on ½ strength MS medium with 0.5 mg l^{-1} IBA and 2% (w/v) sucrose.

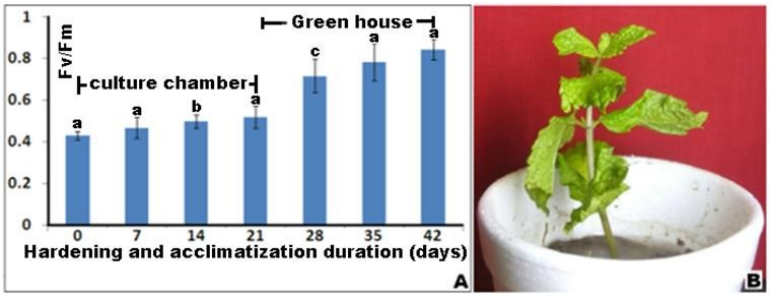

Fig. 2. A. Fv/Fm (φ_{P0}) values of *in vitro* raised plantlets during hardening and acclimatization [Different letters mean that there are significant differences among treatments. Tukey Test HSD ($p > 0.05$) n=30]. **B.** Acclimatized plantlet.

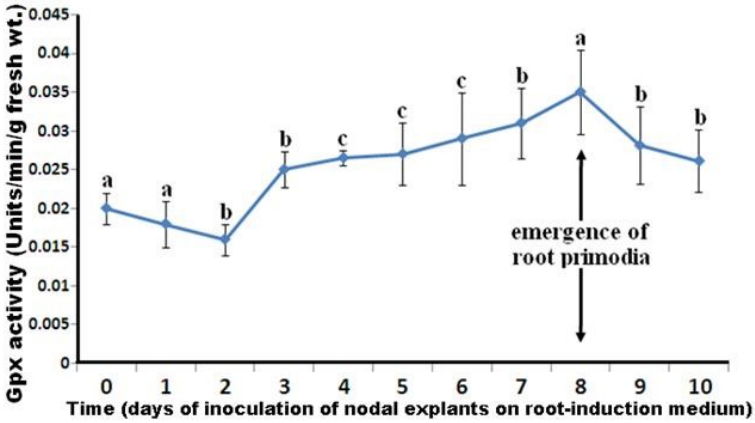

Fig. 3. Gpx activity during *in vitro* rhizogenesis in nodal explants of *M. arvensis.* [Different letters mean that there are significant differences among treatments. Tukey Test HSD ($p > 0.05$) n=30].

Conclusions

The present study describes an efficient and reproducible protocol for *in vitro* propagation of *M. arvensis* using nodal explants. The protocol can be used for clonal propagation and genetic modification of this high value medicinal plant species. In addition, biochemical study also indicates the key role of Gpx as a marker of rhizogenesis in *M. arvensis*.

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PHENOLIC CONTENT OF *ARTEMISIA ANNUA* L. FROM NATURAL HABITATS IN REPUBLIC OF MOLDOVA

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Abstract: The aim of our study was to assess the phenolic compounds content and profile of *A. annua* samples harvested from natural habitats in R. Moldova. The samples, consisting in leaves, were harvested in August 2014 (before flowering) from different growing sites in north, centre and south regions. Phenolic extracts were obtained by methanol extraction of the residual plant material resulted from artemisinin separation. The phenolic compounds were identified and quantified by TLC and HPLC-DAD analyses, respectively. In all samples, four phenolic acids (caffeic, p-coumaric and chlorogenic acids, cynarin) and two flavonoids (isoquercitrin and luteolin-7-glucoside) were identified and quantified, cynarin being the major compound. The variations in phenolic composition between samples harvested from the same growing site and also for the samples from different growing areas (south, centre, north), were mostly quantitative. Similar phenolic profiles were obtained for all samples, regardless of the growing site. Phenolic acids were the dominant components in the phenolic extracts.

Keywords: *A. annua* leaves, flavonoids, phenolic acids, HPLC, TLC.

Introduction

Artemisia annua L. is an annual species 30–200 (–250) cm in height. It has a pioneer strategy characterized by a high degree of morphological and reproductive plasticity and massive seed production. This species is native in East Asia, most probably Inner Mongolia in China, where it is part of the grassland and steppe vegetation. *A. annua* has become widespread in temperate regions worldwide [TUTIN & al. 1976; VALLES & al. 2001]. In the flora of the Republic of Moldova *A. annua* is present in all regions of the country, but the distribution is very uneven. It regularly occurs in association with human settlements, ruderal habitats associated with transport infrastructure like roads and railways, rarely occurs in fields, as well as in semi-natural habitats [NEGRU, 2007; TZVELEV, 1994; VISJULINA, 1962].

A. annua was widely studied due to the biological effects of its extracts. The chemical composition of *A. annua* consists of volatile and non-volatile constituents. The volatile components are mainly represented by essential oils (0.23-0.97%) [MOHAMMADREZA, 2008]. The main non-volatile compounds include sesquiterpenoids, phenolic acids, flavonoids and coumarins, steroids [WHO Monograph on GACP for

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Artemisia annua, 2006]. *A. annua* is the main source of artemisinin, a sesquiterpene lactone used for the treatment of *falciparum* type malaria in many countries [SUBERU & al. 2013]. In addition, phenolic compounds are an important group of bioactive molecules found in high amounts in *A. annua* plants.

According to the literature, the most representative phenolic compounds in *A. annua* are flavones and their glycosides (luteolin, luteolin-7-glucoside, apigenin), flavonols and their glycosides (kaempferol, quercetin, isoquercitrin, rutin, patuletin), coumarins (coumarin, 6,7-dimethoxy-coumarin) and phenolic acids (ferulic acid) [CAI & AL. 2004, IVANESCU & al. 2010].

The phenolic extracts of *A. annua* showed antioxidant and antitumor activity [IQBAL & al. 2012; ZHU & al. 2013]. It is well known that the radical scavenging capacities of plant extracts is correlated with the phenolic content [FERREIRA & al. 2010; SYTAR & al. 2016].

The antioxidant properties of *A. annua* phenolic extracts were reported in several *in vitro* tests such as ABTS, ORAC, ferric reducing antioxidant power and lipid peroxidation in emulsion model and also *in vivo* mouse models [SKOWYRA & al. 2014; KIM & al. 2014].

Regarding the role of phenolic compounds in the plant, it was demonstrated that they are important molecules in plant stress responses, thus having an adaptive role for environmental parameters such as altitude, temperature, evapotranspiration [BAUTISTA & al. 2016]. Flavonoids are involved in plants interactions with other organisms and their response to the environmental stress, mainly due to their strong antioxidant properties [MIERZIAK & al. 2014].

Thus, the synthesis and accumulation of phenolic compounds is influenced by the environmental factors (biotic and abiotic), which are characteristic to each growing site.

The study aimed at assessing the phenolic compounds content and composition for *A. annua* samples harvested from natural habitats in R. Moldova, in order to identify high yielding plants to be used in breeding programs.

Material and method

Plant material

The plant samples consisted in *A. annua* leaves harvested before flowering stage from several habitats in the southern, central and northern regions of the Republic of Moldova in August 2014. We harvested the samples at this plant development stage, when artemisinin content is higher, since our main goal was to isolate the phenolic compounds from the residual plant material resulted from artemisinin extraction.

For each growing site, a habitat assessment was performed, and the plant associations were described (Tab. 1). Description of the associations was done according to the phytosociological research method of the central European school, based on the traditional ecological-floristic systems developed by TÜXEN (1955) and J. BRAUN-BLANQUET (1964). Voucher specimens of identified species are deposited in the herbarium of the Botanical Garden (Institute) of ASM.

R. Moldova has a temperate-continental climate. The average yearly air temperature is 8-10 °C. The average annual amount of precipitation goes down from 620 mm at the north-west to 490 mm at the south-east (Ministry of Ecology, Constructions and Territorial Development of the Republic of Moldova, National Institute of Ecology. Republic of Moldova – State of the Environment Report 2002).

Chemicals and reagents

Methanol (for analysis and HPLC grade), acetonitril, dichloromethane, formic acid and 2-aminoethyldiphenyl borate (NP reagent) were from Merck (Darmstadt, Germany), ethyl acetate was from SC Chimreactiv SRL (Romania), Kollisolv PEG E 400 (Macrogol 400), quercetin, rutin and cynarin were from Sigma Aldrich (Steinheim, Germany), caffeic acid, p-coumaric acid, isoqercitrin and luteolin-7-glucoside were from Roth (Karlsruhe, Germany), hyperoside and chlorogenic acid were from Hwi Analytik GmbH (Ruelzheim, Germany).

Phytochemical analysis

Extract preparation

The dried and milled plant material was extracted with chloroform in order to isolate the sesquiterpene lactone fraction (especially artemisinin). Afterwards, the residual plant material was extracted 3 times at 40 °C in the ultrasonic bath (40 KHz) with methanol 100% for the isolation of phenolic compounds (phenolic acids and flavonoids).

The extracts were vacuum dried using a rotary evaporator, and stored at -20°C until analyzed. The extraction yields for phenolic compounds were calculated for each sample and the data is presented in Fig. 1.

Thin Layer Chromatography (TLC) analysis

For the TLC analysis the dried extract was re-dissolved in methanol, at a concentration of 35 mg dry extract/ mL. *Stationary phase*: HPTLC 20x10cm, silica gel 60 F_{254}, plates (Merck); *mobile phase*: ethyl acetate/formic acid/water (80/10/10, v/v/v); *development distance:* 8 cm; derivatization: NP solution (10 g/L, in ethylacetate) and PEG solution (Macrogol 400, 50 g/L, in dichloromethane); *visualization:* 366 nm.

High Performance Liquid Chromatography (HPLC) analysis

For the HPLC analysis, the dried extract was re-dissolved in methanol, at a concentration of 3.5 mg dry extract/mL.

The phytochemical analysis was performed using an Agilent 1200 HPLC system coupled with a DAD G1315D detector, G1311A quaternary Pump, G1329A autosampler and G1322A degasser.

The chromatographic conditions where: Nucleodur C18 Isis (250 x 4.6 mm, 5 µm) column; mobile phase water adjusted to pH 2.5 with phosphoric acid (A) and acetonitrile (B); elution gradient 2-14-20-26-43% B for 0-20-40-46-58 min. after which we switched back to the initial conditions for 10 min; flow 1 mL/min. Detection was performed at 320 nm for phenolic acids and 350 nm for flavonoids. The phenolic compounds were identified and quantified according to their UV-VIS spectra and available standards. The quantitative results were expressed as mg/100 g dry plant material (d.w.).

Results and discussion

Habitat assessment

In R. Moldova *Artemisia annua* is tending to populate some anthropogenic habitats with a high number of segetal and ruderalised vascular plants, such as: *Amaranthus deflexus* L., *Anagallis arvensis* L., *Anagallis foemina* Mill., *Anchusa pseudoochroleuca* Shost., *Atriplex tatarica* L., *Atriplex oblongifolia* Waldst. et Kit., *Ballota nigra* L., *Berteroa incana* (L.) DC., *Bidens tripartita* L., *Brachyactis ciliata* (Ledeb.) Ledeb., *Capsella bursa-pastoris* (L.) Medik., *Cuscuta campestris* Yunck, *Cyclachaena xanthiifolia* (Nutt.) Fresen., *Daucus carota* L., *Descurainia sophia* (L.) Webb ex Prantl, *Diplotaxis muralis* (L.) DC. etc., forming

sometimes pure vegetal associations (*Artemisietum annuae* Fijalkowski 1967) (Photo 1) where species becomes mono-dominant in some ruderal phytocoenoses or being a part of floristic component of other phytocoenoses: *Galinsogo-Euphorbietum pepli* Mititelu 1972, *Portulacetum oleracei* Felföldy 1942, *Portulacetum oleracei-Amaranthetosum deflexi* (Grigore 1968) Sanda et al. 2001, *Capsello-Descurainietum sophiae* Mucina 1993 (Photo 2), *Hordeetum murini* Libbert 1939, *Chenopodio vulvariae-Urticetum urens* (Slavnić 1951) Soó (Photo 3), etc.

The description of the samples and their harvest site is presented in Tab. 1.

Photo 1. *Artemisietum annuae* Fijalkowski 1967 pure vegetal association, Rascaieti, Stefan Voda district

Photo 2. *Capsello-Descurainietum sophiae* Mucina 1993 vegetal association (antropogenic habitat), Naslavcea, Ocnita district

Photo 3. *Chenopodio vulvariae-Urticetum urens* (Slavnić 1951) Soó vegetal association, Cosauti, Soroca district

Tab. 1. Description of *A. annua* samples

Samples	Growing site/district		Plant association
1-3	South	Ciumai, Taraclia	*Portulacetum oleracei-Amaranthetosum deflexi* (Grigore 1968) Sanda et al. 2001
4-6		Rascaieti, Stefan Voda	*Artemisietum annuae* Fijalkowski 1967
7-9		Colibasi, Cahul	*Galinsogo-Euphorbietum pepli* Mititelu 1972
10-12	Centre	Bacioi, Chisinau	*Portulacetum oleracei* Felföldy 1942,
13-15		Trebujeni, Orhei	*Artemisietum annuae* Fijalkowski 1967
16-18	North	Naslavcea, Ocnita	*Capsello-Descurainietum sophiae* Mucina 1993
19-21		Cosauti, Soroca	*Chenopodio vulvariae-Urticetum urens* (Slavnić 1951) Soó
22-24		Branzeni, Edinet	*Hordeetum murini* Libbert 1939

Phenolic compounds assessment

Extraction of phenolic compounds was made using 100% methanol under ultrasound assisted extraction. The extraction yield for was calculated for each sample and the data is presented in Fig. 1. It varied from 10.22% (sample 6) to 13.57% (sample 10), with lowest average values for the samples harvested from Rascaieti (Stefan Voda district) and Trebujeni (Orhei district) and the highest average values for the samples harvested from Bacioi (Chisinau).

Identification of phenolic compounds was performed by two chromatographic methods, namely TLC and HPLC.

The TLC fingerprint (Fig. 2) showed the presence of the following phenolic compounds: rutin (Rf=0.26), chlorogenic acid (Rf=0.46), hyperoside (Rf=0.50), luteolin-7-glucoside (Rf=0.54) and cynarin (Rf=0.86). In addition, two other flavonoids (green spot with Rf=0.12 and orange spot with Rf=0.18) and three phenolic acids were separated (blue spots with Rf=0.30; 0.58; 0.68).

By HPLC analysis, in all samples 25 phenolic acids and derivatives were separated (Fig. 3a), four of which being identified and quantified: caffeic acid in amounts of 1.70 – 4.31mg/100 g d.w., *p*-coumaric acid 0.50 – 4.35 mg/100 g d.w., chlorogenic acid 112.64 – 210.48 mg/100 g d.w. and cynarin 307.13 to 617.72 mg/100 g d.w. (Fig. 4).

Furthermore, five flavonoids were separated, among which isoquercitrin and luteolin-7-O-glucoside (Fig. 3b). Isoquercitrin was found in amounts of 5.24 – 30.33 mg/100 g d.w., while luteolin-7-glucoside content was 9.80 – 40.47 mg/100 g d.w. (Fig. 5).

Cynarin was the major phenolic compound in all *A. annua* samples, but in literature there are few reports on it regarding only its presence in *A. annua* species, with no quantitative data [ZAO & al. 2014; ZAO & al. 2015].

Phenolic compounds such as chlorogenic acid, *p*-coumaric acid, cynarin, caffeic acid, hyperoside, isoquercitrin, rutin and luteolin-7-glucoside were identified in previous studies on *A. annua* aerial parts and leaves [CAI & al. 2004; IVANESCU & al. 2010; ZHAO & al. 2015].

In contrast with our study, showing phenolic acids as dominant constituents, in the study of SONG & al. (2016) on fresh leaves harvested at flowering stage, the flavonoid composition was more diverse and the flavonoid content was higher. This difference in phenolic profile can be attributed to the plant development stage of *A. annua*, but further studies are needed to confirm this hypothesis.

The values of the phenolic compounds content for the samples harvested from the north region had a narrower variability range, compared with the samples from the centre

and south. The samples from north were characterized by a higher content of flavonoids. Interestingly, high variations of phenolic compounds content were obtained for the samples harvested from the south and centre regions (Fig. 4 and Fig. 5).

The lowest phenolic compounds contents were determined for the samples harvested from Rascaieti (Stefan Voda district) and Trebujeni (Orhei district) growing sites which are characterised by *Artemisietum annuae* Fijalkowski 1967 plant association.

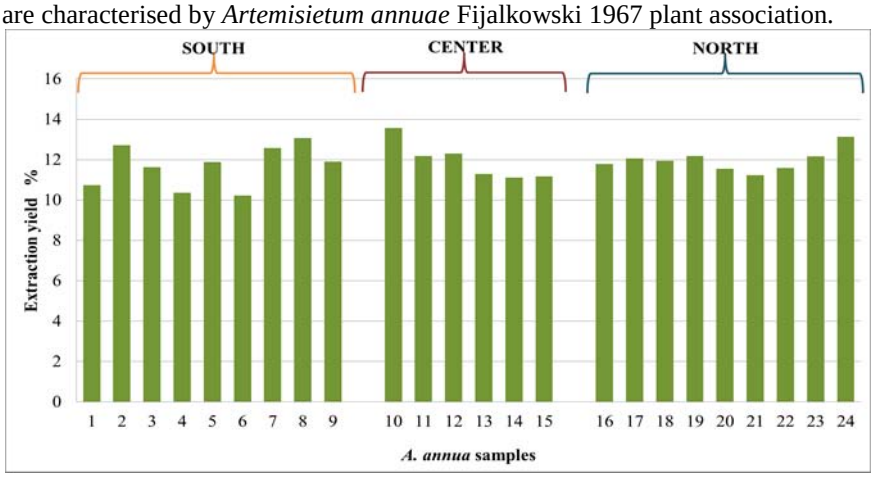

Fig. 1. Extraction yields for methanolic extracts of *A. annua* samples (% dry weight)

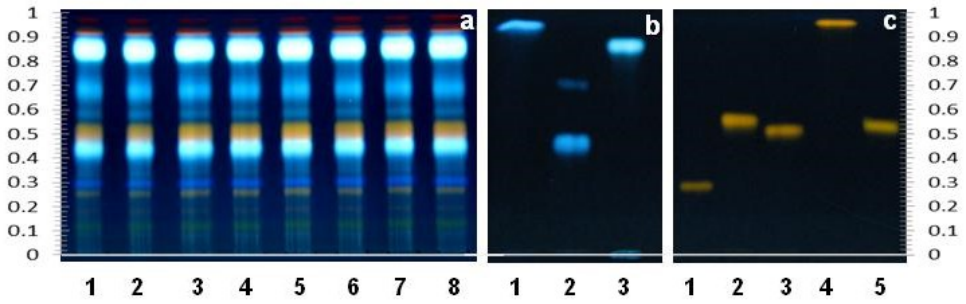

Fig. 2. TLC chromatogram for phenolic acids and flavonoids of *A. annua* samples.
a. *A. annua* samples 1 to 8; **b.** Standards for phenolic acids: 1) caffeic acid, 2) chlorogenic acid, 3) cynarin; **c:** Standards for flavonoids: 1) rutin; 2) isoquercitrin; 3) hyperoside; 4) quercetin; 5) luteolin-7-glucoside

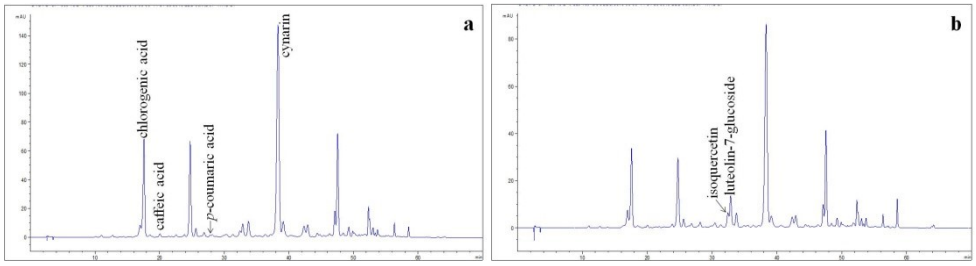

Fig. 3. Chromatographic profile of *A. annua* (sample 1). a. phenolic acids at 320 nm. b. flavonoids at 350 nm

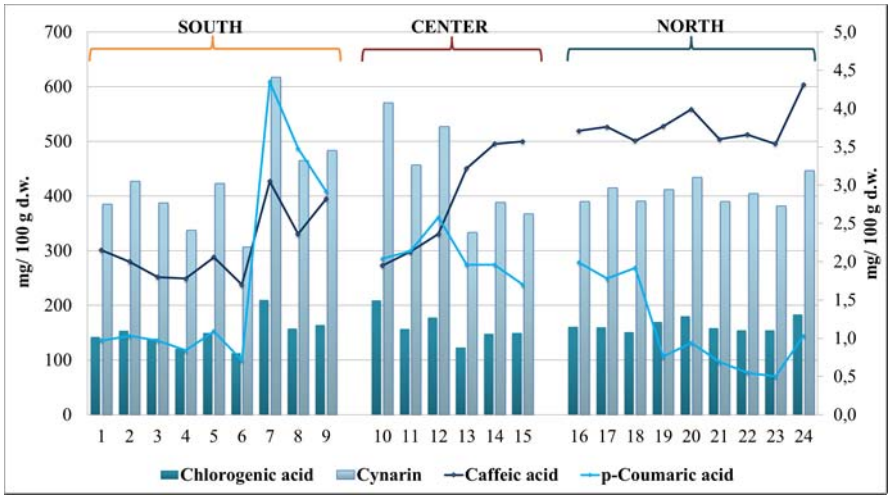

Fig. 4. Phenolic acids conent in *A. annua* samples

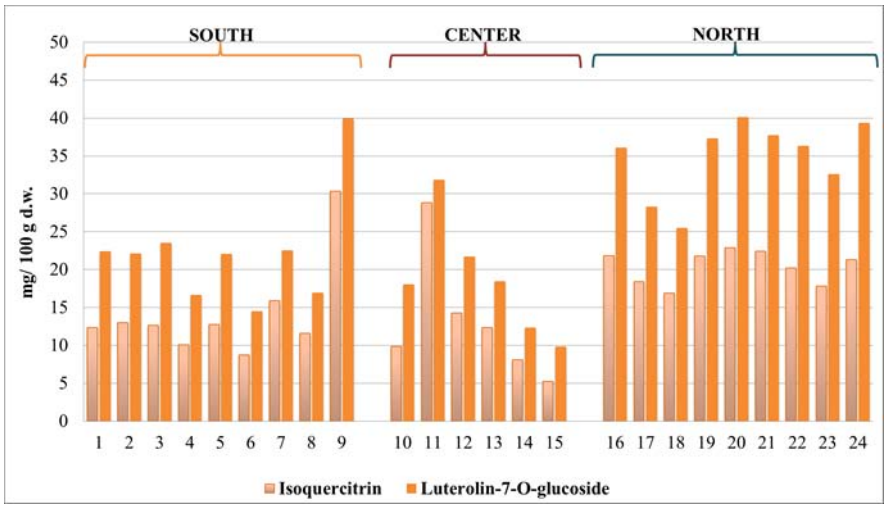

Fig. 5. Flavonoids content in *A. annua* samples

Conclusions

To our knowledge, this study presents the first data on phenolic profile of *A. annua* leaves harvested before flowering. Furthermore, it is the first time when quantitative data on cynarin in *A. annua* were obtained.

The variation on phenolic compound composition between samples, for the samples harvested from the same growing site and also for the samples from different growing areas (south, centre, north), were mostly quantitative. Similar phenolic profile was obtained for all samples, regardless of the growing site.

Phenolic acids were dominant components in the phenolic extracts, both qualitatively and quantitatively.

Several accessions had high amount of phenolic compounds, being promising candidates for breeding programs.

Considering the high amounts of phenolic compounds in our samples, it is feasible to use the residual plant material resulted from artemisinin extraction as a source of phenolic compounds, and thus achieving also the sustainable exploration of raw materials.

Acknowledgements

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ENZYMATIC ACTIVITY AND SOLUBLE PROTEIN CONTENT IN SEEDLINGS OF *CALENDULA OFFICINALIS* L. UNDER SALT STRESS

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Abstract: Enzymatic activity and soluble protein content in relation to salt stress tolerance were investigated in *Calendula officinalis* seedlings after 24 days of treatment with different salt treatments, including NaCl, $CaCl_2$, $MgCl_2$ and mixtures of them. The marigold seedlings were used in order to investigate the possible salt-inducible responses and the possible alleviative role of calcium and magnesium salts in respect with adverse salinity conditions. Activity of superoxide dismutase (SOD; EC 1.15.1.1) and peroxidase (POD; EC 1.11.1.7) generally slightly decreased under salt treatments, with minor variations from the value recorded for control series and within applied treatments. Catalase (CAT; EC 1.11.1.6) activity was stimulated by NaCl salinity and $MgCl_2$ addition; its activity was found to be lowest under calcium and magnesium chloride treatments solely.

Keywords: antioxidant system, calcium, flavonoids, magnesium, salinity, seedlings.

Introduction

Marigold (*Calendula officinalis* L.) is the common name for *Calendula* genus of *Asteraceae* family. *Calendula* is stated to possess antispasmodic, mild diaphoretic, anti-inflammatory styptic, antihemorrhagic, emmenagogue, vulnerary, and antiseptic properties [DUKE & al. 2002; BARNES & al. 2007; EBADI, 2007; YBERT & DE LAAGE, 2007]. Traditionally, it has been used to treat gastric and duodenal ulcers, amenorrhea, dysmenorrhea and epistaxis; crural ulcers, varicose veins, hemorrhoids, anal eczema, proctitis, lymphadenoma, inflamed cutaneous lesions (topically) and conjunctivitis (as an eye lotion) [EBADI, 2007; KHALID & al. 2012]. Phytochemical studies have reported four main groups of constituents, for *Calendula*, namely flavonoids, polysaccharides, volatile oil and triterpenes [AZZAZ & al. 2007; BARNES & al. 2007].

Animal studies have reported wound-healing and anti-inflammatory effects, supporting the traditional uses of calendula in various dermatological conditions. The anti-inflammatory effect is due to the triterpenoid constituents, although flavonoids may contribute to the activity. The reputed antispasmodic effect may be attributable to the volatile oil fraction. In addition, immunostimulant activity has been reported for high molecular weight polysaccharide components. Clinical research assessing the effects of calendula

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preparations is limited, and rigorous randomized controlled clinical trials are required [BARNES & al. 2007].

Salinity is one of the most severe environmental factors limiting the productivity of agricultural crops. Most crops are sensitive to salinity caused by high concentrations of salts in the soil [PITMAN & LAUCHLI, 2004]. The cost of salinity to agriculture is estimated conservatively to be about $US 12 billion a year, and is expected to increase as soils are further affected [GHASSEMI & al. 1995]. An alarming scenario has been described in this context, including a future dominated by salinity and aridity in agriculture, fresh water scarce and consequently food crisis [GRIGORE & al. 2014]. Hence, the necessity to elucidate the salt-mechanism tolerance in plants with medicinal properties is a condition of great interest.

There are several studies focused to salt stress in *C. officinalis*; they refer to influence on germination [GHARINEH & al. 2013; SEDGHI & al. 2010; TORBAGHAN, 2012], or impact of exogenous salicylic acid on growth parameters in marigold under salt stress [BAYAT & al. 2012]. Few studies refer on antioxidant activity under salt stress conditions [CHAPARZADEZ & al. 2004].

Therefore, the aim of this study is to investigate several biochemical responses of the medicinal plant *C. officinalis* subjected to different salt treatments; in addition, calcium and magnesium salts are discussed in relation to the possible role in alleviating NaCl effects.

Materials and methods

Plant material, treatment and growth conditions

In order to investigate the effect of salt solutions on several biochemical parameters in *C. officinalis* L. seedlings, an experiment was conducted in controlled laboratory conditions based on a completely randomized design. Marigold (*C. officinalis*) seeds were obtained from Agricultural Research and Development Station, Secuieni Neamt, Romania. Intact seeds, which were homogeneous and identical in size and colour, and free from wrinkles, were chosen. These seed were then sterilized with sodium hypochlorite 10% for 30 seconds and were washed with sterile distilled water. After that, 30 marigold seeds were sown and germinated in plastic pots. After 7 days (corresponding to a uniform seedlings emergence) salt treatments started and were carried out by adding 100 mL of salt solutions (or distilled water for the control treatments) to pots once per week. The follow treatments were applied: 50mM NaCl, 50mM NaCl + 10mM $CaCl_2$, 50mM NaCl + 20mM $MgCl_2$, 100mM NaCl, 100mM NaCl + 10mM $CaCl_2$, 100mM NaCl + 20 $MgCl_2$, 150mM NaCl, 150mM NaCl + 10mM $CaCl_2$, 150mM NaCl + 20mM $MgCl_2$, 10mM $CaCl_2$, 20mM $MgCl_2$. The NaCl concentrations were chosen as optimal after performing previous trial at different concentrations. Concentrations of calcium and magnesium chloride were preferred according to previous research experiments [GRIGORE & al. 2012]. The biochemical analyses were conducted at 24-days old seedlings; five different individuals from each treatment were selected to measure all the analyzed parameters.

Preparation of extracts and assay enzyme

Marigold seedling sample (0.3g) were homogenized with phosphate buffer (pH=7.5). After that the homogenates were centrifuged the supernatants were used for enzyme assays.

Superoxide dismutase (SOD) activity was estimated by recording the decrease in absorbance of superoxide-nitroblue tetrazolium complex by the enzyme [WINTERBOURN & al. 1975]. About 3 mL of reaction mixture, containing 0.1 mL of 1.5 mM nitroblue tetrazolium (NBT), 0.2 mL of 0.1 M EDTA, 2.55 mL of 0.067 M potassium phosphate buffer, and 0.01 mL of enzyme extraction, were taken in test tubes in duplicate from each enzyme sample. One tube without enzyme extract was taken as control. The reaction was started by adding 0.05 mL of 0.12 mM riboflavin and placing the tubes below a light source of 215 W florescent lamps for 5 min. The reaction was stopped by switching off the light and covering the tubes with black cloth. Tubes without enzyme developed maximal colour. A non-irradiated complete reaction mixture, which did not develop colour, served as blank. Absorbance was recorded at 560 nm and 1 unit of enzyme activity was taken as the quantity of enzyme that reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes.

Peroxidase (POD) activity was determined spectrophotometrically by measuring the oxidation of o-dianisidine at 540 nm [MOLLER & OTTOLENGHI, 1966] with slight modification. The reaction was started by adding 0.1 H_2O_2 0.05% on mixture reaction containing 0.2 mL of enzyme extraction, 0.8 mL distillated water and 1.5 mL 1% o-dianisidine. After 5 min. the reaction was stopped with 2.5 mL H_2SO_4 50%. One unit of POD activity was expressed as the amount of enzyme that produced a change of 1.0 absorbance per min.

Catalase (CAT) activity was measured according to the method described by Sinha, 1972. Briefly, the assay mixture consisted of 0.4 mL phosphate buffer (0.01 M, pH 7.0), 0.5 mL hydrogen peroxide (0.16 M) and 0.1 mL enzymatic extract in a final volume of 3.0 mL. About 2 mL dichromate acetic acid reagent was added in 1 mL of reaction mixture, boiled for 10 min, cooled. Changes in absorbance were records at 570 nm. CAT activity was expressed as the amount of enzyme needed to reduce 1 µmol of H_2O_2 per min. The activity of these enzymes (SOD, POD and CAT) was expressed as unit per mg proteins (U/mg protein).

The determination of **soluble protein content** was determined according to BRADFORD method (1976) with bovine serum albumin as standard. Thus, this assay is refers to the binding of Coomassie Brilliant Blue G-250 at aromatic amino acid radicals and measuring the colour at 595nm.

Statistical analysis. The statistical analysis was performed using Student *t*-test. Values with $p<0.005$ were considered as statistically significant.

Results and discussion

Enzymatic activity

There is no uniform response of *Calendula* seedlings with respect to salinity stress, regarding the investigated enzymatic activity: SOD, CAT, and POD (Fig. 1, Fig. 2 and Fig. 3).

Superoxide dismutase activity was registered to be visibly higher only in plants subjected to elevated concentration of 150 mM NaCl (14.27%), comparatively with un-stressed plants and other salt treatments. However, the other values remain only slightly lower than in control samples. In all cases, the addition of calcium and magnesium chloride to NaCl corresponds to lower values than those recorded for NaCl treatments solely. SOD

activity showed a significant decrease ($p<0.05$) in case of singular treatment with $CaCl_2$ and, respectively, $MgCl_2$.

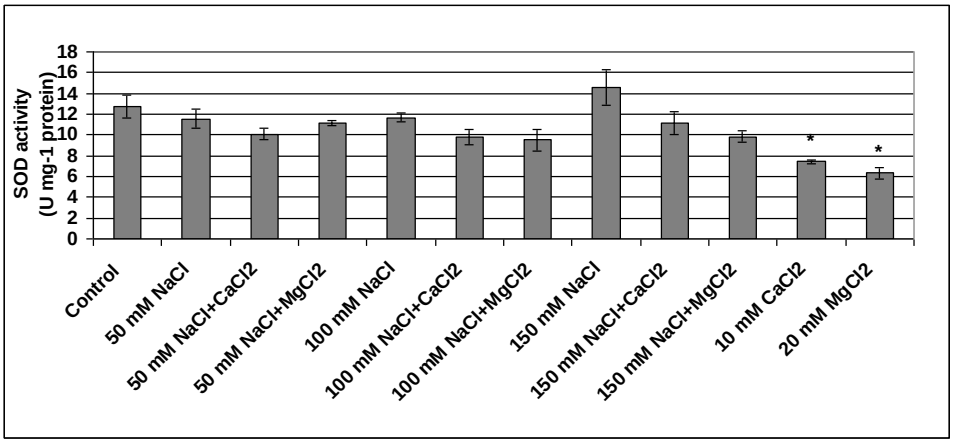

Fig. 1. Effect of NaCl, $CaCl_2$, $MgCl_2$ and their combination on SOD activity of *C. officinalis* (Values are means ± SE of 5 replicates); *significant at $p< 0.05$

SOD is an important antioxidant enzyme and is the first line of defence against oxidative stress in plants [ALSCHER & al. 2002; JITESH & al. 2006]. The production of toxic superoxide free radicals appears to be a universal problem in aerobic cells exposed to different type of stress. The elimination of superoxide by superoxide dismutase (SOD) produces H_2O_2 which in turn is removed by various peroxidase or catalase.

Usually, salt stress in cultivars differing in salt tolerance evidenced an increased SOD activity in salt-tolerant genotypes of pea, cotton, tomato and wheat; therefore, induction of SOD activity was suggested as a reason for improved tolerance to salinity in these species [HERNANDEZ & al. 1993, 1995, 1999; GOSSETT & al. 1994; MITTOVA & al. 2003; SAIRAM & al. 2005] and in general [SHARMA & al. 2011]. However, there are exceptions from this general statement; SOD activity may decrease in several species under salt conditions or it may vary in the same species in different investigated organs. For instance, in tobacco leaves, SOD activity increased, while in the roots of the same plant, SOD activity decreased [MYTINOVA & al. 2010]. In *Plantago major* exposed to NaCl stress, the roots were characterized, unlike leaves, by high constitutive activity of SOD [RADYUKINA & al. 2009]. In forage sorghum seedlings, salinity of 50 and 100 mM NaCl induced significant increase in SOD activity, in tolerant genotypes compared to sensitive group [HEFNY & ABDEL-KADER, 2009]. Other data report that under salinity conditions (50 and 100 mM NaCl), CAT (and POD) activity decreased in *Calendula officinalis* [CHAPARZADEH & al. 2004].

CAT activity has been found to be higher in NaCl stressed plants (50 and 100 mM NaCl), while under 150 mM NaCl, the activity was almost imperceptibly lower than the control (Fig. 2). Interestingly, the smallest value of CAT in plants under 150 mM NaCl salinity treatments (-1,6%) is inversely correlated to SOD activity under the same conditions (14.27%). The sodium chloride mixed to $CaCl_2$ and $MgCl_2$ induces different responses. The addition of magnesium chloride to all NaCl treatments implies higher values of CAT activity

than the control plants; addition of calcium chloride show lower and higher values comparatively with the control. However, only under elevated 150 mM NaCl salinity, the added calcium and magnesium chloride seem to increase the CAT activity compared to NaCl treatment alone. By application of the singular treatment with $CaCl_2$ the activity of CAT showed a significant decrease ($p < 0.01$).

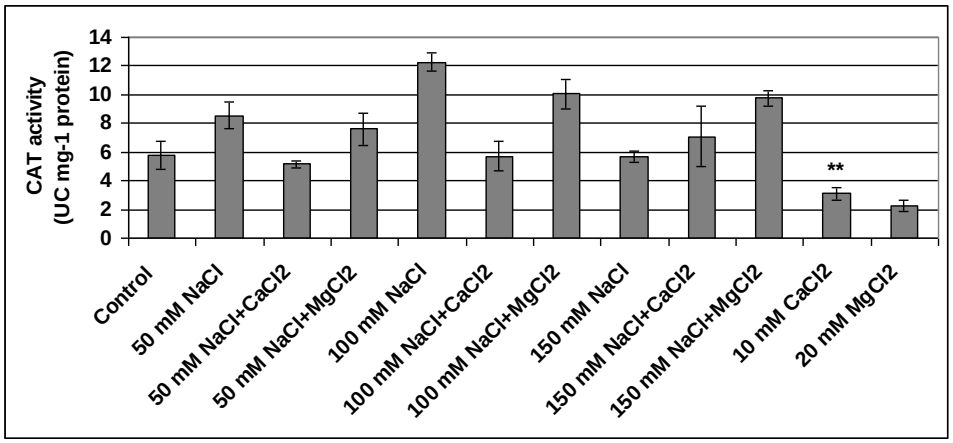

Fig. 2. Effect of NaCl, $CaCl_2$, $MgCl_2$ and their combination on CAT activity of *C. officinalis* (Values are means ± SE of 5 replicates); **significant at $p < 0.01$

Catalases are haem-containing tetrameric enzymes involved in the removal of H_2O_2 and during salt stress and other abiotic stress conditions [WILLEKENS & al. 1997]. Abiotic stresses cause either enhancement or depletion of CAT activity [EL-SHINTINAWY & al. 2004; SHARMA & DUBEY, 2005; NOREEN & ASHRAF, 2009]. For instance, in strawberry (*Fragaria* x *ananassa* Duch., cv. *Selva*) leaves under NaCl stress, there was an inhibition in CAT activity [TANOU & al. 2009]. In *Plantago major* roots subjected to NaCl stress a lower CAT activity has been reported [RADYUKINA & al. 2009]. In nine genetically diverse pea (*Pisum sativum*) cultivars exposed to salt stress, a decreased CAT activity has been found, while SOD activity was enhanced by salinity conditions [NOREEN & ASHRAF, 2009]. In tobacco leaves, CAT activity declined under salinity stress [MYTINOVA & al. 2010].

POD activity in NaCl stressed plants show fairly lower values when compared with control plants (Fig. 3). The mixed salts (NaCl with calcium and magnesium chloride) induced different values, as compared with control or series stressed with NaCl solely. For instance, $MgCl_2$ added to 50 and 150 mM NaCl imply slightly higher values than those recorded in plants subjected only to corresponding NaCl treatments.

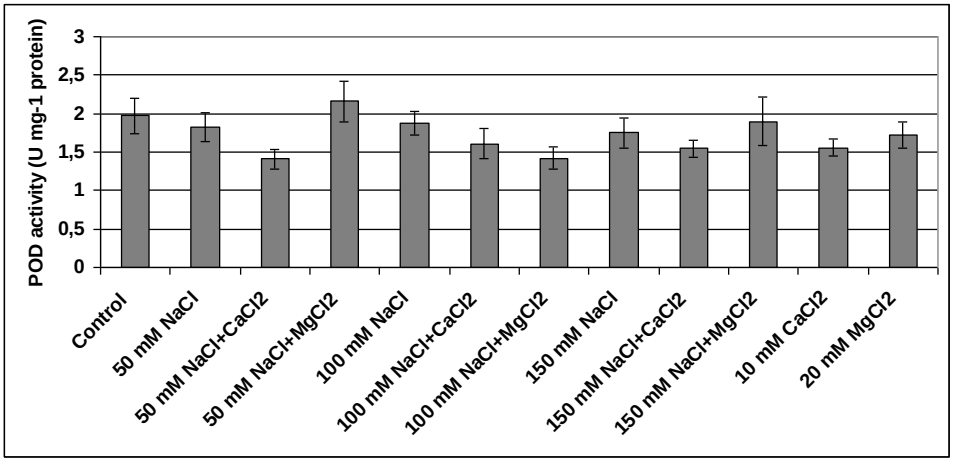

Fig. 3. Effect of NaCl, $CaCl_2$, $MgCl_2$ and their combination on POD activity of *C. officinalis* (Values are means ± SE of 5 replicates)

Plants contain abundant amounts of peroxidases that are involved in H_2O_2 scavenging; they are associated with the cell wall where they generate phenoxy compounds from cinnamic acids [JITESH & al. 2006]. Activity of POD has been markedly enhanced by salt stress in several genetically diverse pea cultivars [NOREEN & ASHRAF, 2009].

Soluble protein content

Under salinity conditions, the registered values are generally higher than in control (Fig. 4). The NaCl treatment induces values very closed to those registered for control; however, the mixture of NaCl with calcium and magnesium chloride increased the soluble protein content as compared with control series. Plants treated only with $CaCl_2$ and $MgCl_2$ have the highest values of soluble protein content. As regards the protein amount in stress condition, the data reported from literature is abundant numerous and greatly variable. Therefore, some authors [AGASTIAN & al. 2000] published that soluble protein content increases at low salinity and decreases at high salinity in *Morus* sp. Depending on NaCl concentrations, in some wheat varieties (Faur, Iasi, Fundulea) the protein content was reduced in 4 days old seedlings, but it tends to increase after 168 and 240h of salt treatment, as compared to control [OPRICĂ, 2011]. On the other hand soluble protein amounts of leaves significantly decreased in response to salinity at some species like *Paulownia imperialis* and *P. fortunei* [AYALA-ASTORGA & ALCAREZ-MELENDEZ, 2010; PARVAIZ & SATYAVATI, 2008; PARIDA & DAS, 2005]. However, in other study [FAYEZ & BAZAID, 2014], the soluble protein of barley leaves was slightly changed in response to salt and water deficit stresses as well as with the combined treatments (salicylic acid and KNO_3).

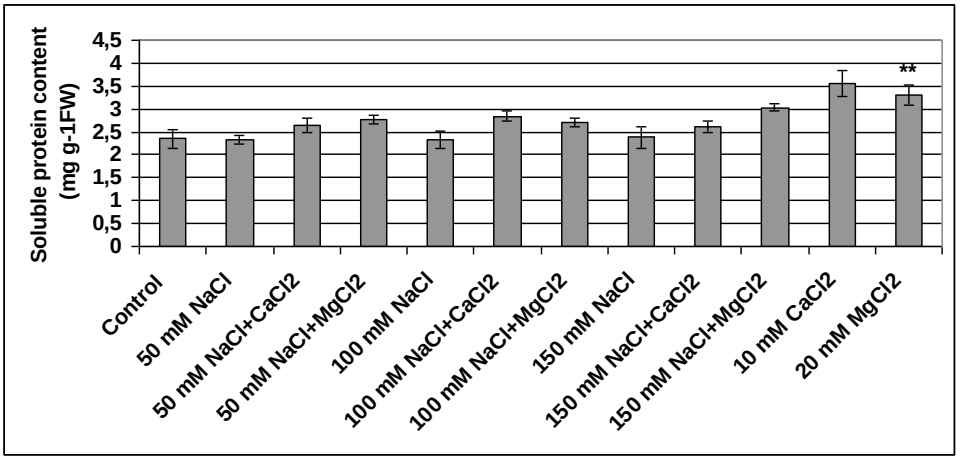

Fig. 4. Effect of NaCl, $CaCl_2$, $MgCl_2$ and their combination on soluble protein content of *C. officinalis* (Values are means ± SE of 5 replicates); **significant at $p < 0.01$

Conclusions

There is no uniform response of *Calendula* seedlings with respect to salinity stress, regarding the investigated enzymatic activity: SOD, CAT, and POD. SOD activity, usually considered as a first line of defense against oxidative stress, shows no important increase as a response of salt stress. The soluble protein content registered values generally higher than in control plants, with highest values recorded in the case of plant series subjected to calcium and magnesium chloride.

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MICROBIOLOGICAL AND CHEMICAL EVALUATION OF SEVERAL COMMERCIAL SAMPLES OF *TILIAE FLOS*

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Abstract: The aim of our study was to evaluate the chemical and microbiological composition of seven commercial samples of *Tiliae flos cum bracteis*. Sample S2 presents the maximum level of microbiological contamination with $1.2x10^4$ colony forming units (CFU) aerobic germs/g, $9.6x10^3$ CFU fungi/g, and 360 CFU *Salmonella sp.*/g. For all samples the number of colony forming units for each type of microorganisms was under the limits of European Pharmacopoeia, 8th edition. The amount of flavonoids ranges between 489±1.25 mg/100 g (S2) and 647±1.32 mg/100 g (S4). For polyphenols the quantities range between 663±2.12 mg/100 g (S2) and 1,169±2.76 mg/100 g (S3). The maximum levels of metals determined in mineralised extracts were: 10.35±0.22 mg Mn/100 g (S5), 22.66±0.23 mg Zn/100 g (S3), 1.36±0.12 mg Cu/100 g (S4), 9.92±0.12 mg Fe/100 g (S2), and 9.15±0.12 mg Pb/100 g (S3).

Keywords: flavonoids, metals, microorganisms, polyphenols, *Tiliae flos cum bracteis.*

Introduction

Tiliae flos cum bracteis, generally named lime flowers, represent the flowers and bracteis of different species of *Tilia*. Lime flowers are used in traditional medicine in feverish cold, respiratory diseases and migraine, but also for their sedative, antispasmodic, antioxidant and hepatoprotective effects [KARIOTI & al. 2014; YAYALACI & al. 2014].

The dichloromethane extract from *Tilia cordata* showed antiproliferative activity on lymphoma cell line, and the extract from *Tilia x viridis* proved antiproliferative effect on tumour lymphocytes and stimulated normal lymphocytes [MANUELE & al. 2008].

In cosmetics, extracts from lime flowers are used for hydrating and astringent effects. Aqueous extracts from *Tilia* are used in folk medicine to trait anxiety. Ethanol and dichloromethane extracts from *Tilia cordata* Mill. had antiproliferative activity on murine lymphoma cell line BW 5147 [BARREIRO ARCOS & al. 2006].

Tilia tomentosa Moench bud extracts (TTBEs) are used in folk medicine for their sedative effect. Compounds from these extracts have the capacity to fix on $GABA_A$ and benzodiazepine receptors from hippocampal neurons and to mimic agonist effects of GABA and benzodiazepines [ALLIO & al. 2015].

European Pharmacopoeia, 8th edition, sets rules for microbiological quality of medicinal plants that are used to prepare aqueous extracts, so the maximum limits are 10^7 colony forming units (CFU) aerobic germs/g vegetal product, and 10^5 CFU fungi/g vegetal product [EUROPEAN PHARMACOPOEIA, 2014].

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The aim of our study was to evaluate the chemical (polyphenols, flavonoids, heavy metals) and microbiological quality of lime flowers.

Material and method

In our study, we evaluated seven samples of commercial sorts of *Tiliae flos cum bracteis*.

Microbiological evaluation

For each sample, extracts have been prepared according to European Pharmacopoeia - 1 g sample was mixed with 10 mL peptone-phosphate buffer (pH 7). 1 mL of each extract and diluted extracts (1:10; 1:100) was inoculated on agar broth (aerobic germs), MacConkey broth (*Escherichia coli, Salmonella* sp.), and Sabouraud broth (fungi). The Petri dishes were incubated for 5 days at 37 °C (for bacteria) and 7 days at 25 °C (for fungi). The results were expressed in number of colony forming units/g sample (CFU/g) [EUROPEAN PHARMACOPOEIA, 2014].

Chemical evaluation

5 g of sample was extracted with 100 mL methanol at 80 °C and the extract was used for quantification of polyphenols and flavonoids.

Flavonoids were quantified by a spectrophotometric method based on the reaction of flavonoids with aluminium chloride and sodium nitrite resulting in a pink compound with a maximum absorbance at 510 nm. The results are expressed in mg rutoside/100 g sample [HATANO & al. 1988].

Polyphenols were determined by a spectrophotometric method based on the reaction with Folin-Ciocalteau reagent resulting in a blue compound with a maximum absorbance at 765 nm. The results are expressed in mg caffeic acid/100 g sample [SINGLETON & ROSSI, 1965].

Each determination was made in triplicate and the results represent the average ± standard deviation.

Metals content

1 g of sample was mineralized with nitric acid and the metals were determined by atomic absorption spectrophotometry. For each sample, an aqueous extract was prepared according to the rules for water extracts used as pharmaceutical solutions (6 g vegetal sample extracted with 100 mL of boiling water). The results are expressed in mg metal/100 g sample for each extract

Results and discussion

Firstly, we analysed the macroscopic quality of the samples. Because in our samples the vegetal product is minced, it is difficult to identify the species of linden that have been harvested. According to the European Pharmacopoeia, linden flowers are represented by inflorescence of *Tilia cordata* Miller, *Tilia platyphyllos* Scop., *Tilia* × *vulgaris* Heyne or a mixture of these [EUROPEAN PHARMACOPOEIA, 2014.]. Romanian producers indicate on the label of the box of medicinal product the presence of flowers from *Tilia cordata* and *Tilia platyphyllos* but it is very difficult to establish the species when samples are minced.

Microbiological quality

The results of the microbiological evaluation are presented in Tab. 1.

For all samples, the level of microbiological contamination is under the limits of European Pharmacopoeia, 8th edition [EUROPEAN PHARMACOPOEIA, 2014.]. Sample 2, having the highest level of microbiological contamination, also presented a lot of brown fragments indicating sample processing deficiencies during drying of vegetable product or during storage.

Tab. 1. Microbiological evaluation of *Tiliae flos cum bracteis* samples

Sample	Microorganisms (CFU/g)		
	Aerobic germs	*Salmonella* sp.	Fungi
S 1	$2.4x10^2$	0	$1.8x10^2$
S 2	$1.2x10^4$	$3.6x10^2$	$9.6x10^3$
S 3	4.510^3	0	$3.6x10^3$
S 4	$3.6x10^3$	20	$3x10^3$
S 5	$4.2x10^3$	10	$4x10^3$
S 6	$5.4x10^3$	10	$4.8x10^3$
S 7	$3.8x10^3$	16	$3.2x10^3$

Microbiological contamination is an important parameter for the quality of medicinal herbs because microorganisms, by their enzymes, could modify bioactive phytochemicals. On the other hand, the microorganisms could contaminate aqueous extracts prepared from medicinal herbs, modify their composition and influence the patient's health.

Fungal strains identified in tested samples are different but *Rhizopus nigricans* was found in all samples. Other fungal strains were identified: *Mucor sp.* in S 1 and S 3, *Aspergillus niger* in S 3 and S 5, *Penicillium* sp. and *Aspergillus niger* in S 2.

Chemical composition

The quantities of flavonoids and polyphenols determined in lime flower samples are presented in Fig. 1 and 2.

Flavonoids and polyphenols are compounds with antioxidant properties and could pass in water extracts. By HPLC-DAD and HPLC-ESI-MS analysis, Karioti and Ieri identified in water extracts from *Tilia platyphylos* different quantities of epicatechin, tiliroside, quercetin and kaempferol derivatives and ferulic acid [IERI & al. 2015; KARIOTI & al. 2014].

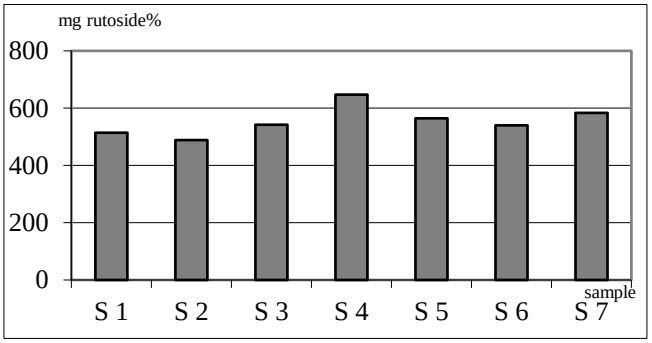

Fig. 1. Flavonoid content in *Tiliae flos cum bracteis* samples (mg rutoside/100 g sample)

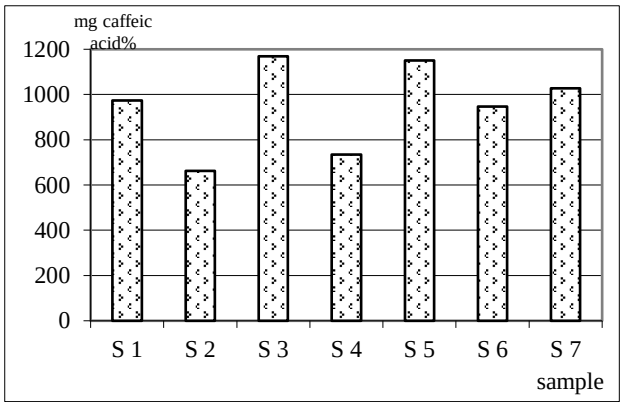

Fig. 2. Polyphenol content in *Tiliae flos cum bracteis* samples (mg caffeic acid/100 g sample)

Flavonoids and polyphenols present antioxidant properties. These compounds have hydroxyl groups that neutralize reactive species of oxygen or different radicals involved in oxidative processes.

Metals content

The metals content in samples depends on the type of metal and its properties. The results are presented in Tab. 2.

Tab. 2. The metals content of *Tiliae flos cum bracteis* samples

Sample	mg metal/100 g sample				
	Mn	Zn	Cu	Fe	Pb
S 1	3.17±0.15	4.56±0.08	0.93±0.03	3.76±0.09	0.93±0.02
S 2	6.52±0.18	4,41±0.10	0.99±0.07	9.92±0.12	0.19±0.02
S 3	6.10±0.19	22.66±0.23	0.79±0.08	5.99±0.10	9.15±0.12
S 4	9.83±0.24	5.61±0.12	1.36±0.12	4.81±0.19	4.52±0.11
S 5	10.35±0.22	8.59±0.15	0.90±0.06	7.86±0.18	0
S 6	7.45±0.18	6.24±0.09	0.82±0.05	6.42±0.12	0.35±0.04
S 7	7.01±0.15	7.93±0.22	0.91±0.04	5.91±0.13	0

European Pharmacopoeia indicates the necessity to evaluate the metals content of vegetal samples used as medicinal herbs but it does not specify limits for metals content.

In mineralised extracts, there have been identified some metals that could induce toxic effects on humans such as: manganese, copper, iron, zinc, and lead.

In water extracts, a small quantity of metals passes because these metals have low water solubility and the method used for extraction does not destroy the complexes between metals and other cell constituents.

The ratio of water extraction depends on metals: manganese - between 7.35% (S 2) and 25.92% (S 3); zinc between 4.53% (S 2) and 20.42% (S 5); copper between 9.33% (S 1) and 14.11% (S 5); iron between 0% (S 1, S 2) and 4.95% (S 4). Water extracts didn't contain lead.

In small quantities, these metals, excepting lead, are necessary for the body because they are involved in many biochemical processes. Romanian laws do not mention limits for the content of metals in medicinal plants, but limit the metals content in foods. Thus, the

limits are 0.3 mg% for lead, 5 mg% for zinc and 5 mg% for copper [REGULATION OF HEALTH MINISTRY, 1998].

Cells of the body could use metals present in aqueous extract in different biochemical processes. So, *zinc* is used for immunity, enzymes structure, hormones synthesis and insulin structure. Normal daily intake of zinc for adults is 7-10 mg. The excess of zinc (over 15 mg/day) might affect the nervous system and heart [DART, 2004].

CELECHOVSKA analysed samples of *Tiliae flos cum bracteis* and found zinc contents between 13.8 and 32.5 μg/g. Similar to our evaluation, the yield of water extraction for metals from vegetal samples was under 25% [CELECHOVSKA & al. 2004].

Copper is present in the structure of enzymes that catalyze dopamine and epinephrine synthesis, melanin synthesis and in enzymes of respiratory chain. Normal daily intake for adults is 1-1.5 mg and an intake over 4 mg/day for a long time could induce Wilson's disease.

Manganese is a metal used for enzymes structure, collagen synthesis and glucose synthesis. In excess, this metal will affect neurons that produce dopamine and, by this mechanism, could induce symptoms similar to Parkinson's disease [DART, 2004]. Most of countries have different limits for manganese in medicinal herbs with maximum value of 1 mg%.

Iron is used for haemoglobin synthesis and also for ATP synthesis. The normal daily intake is 10 mg for men and 15 mg for women. Iron excess induces oxidative processes and affects the structure of DNA and lipids. When iron is present in water extracts, it could modify the colour of these extracts or could precipitate active compounds in extracts. The yield of water extraction for iron is low because it reacts with polyphenols from vegetal samples or is present on vegetal samples as powder with low water solubility.

Lead is a toxic metal that blocks haemoglobin synthesis and affects neurons [DART, 2004]. Lead is present in samples but boiling water extraction is not enough to separate it. The WHO standard is 1 mg% for lead in raw materials for herbal medicines [WHO, 1998]. Samples S 3 and S 4 contain lead over this limit.

Heavy metals are present in plants that have been harvested from polluted area and sometimes are used like biomonitors to assess the level of atmospheric pollution [ANNAN & al. 2013; HÜLSMANN & al. 2005]. Tomašević and Marković analysed linden leaves and bark from different areas and they found a direct correlation between the level of heavy metals and air, respectively soil pollution of the area where these trees are growing [MARKOVIĆ & al. 2013; TOMAŠEVIĆ & al. 2004].

Comparing the results for metals, we observe differences between values depending on metal, so this could indicate the type of pollution of area from where flowers have been harvested.

Conclusions

The chemical composition will influence the quality of plant extracts obtained from them and ultimately, the therapeutic effects.

Microorganisms and metals present in plant samples can alter the biological properties of plant extracts or can cause toxicities in some patients.

By increasing the time and temperature for water extraction, more important quantities of metals could pass in herbal extracts.

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STUDIES ON ANTIOXIDANT, ANTIHYPERGLYCEMIC AND ANTIMICROBIAL EFFECTS OF EDIBLE MUSHROOMS *BOLETUS EDULIS* AND *CANTHARELLUS CIBARIUS*

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Abstract: The study evaluated the antioxidant, antihyperglycemic and antimicrobial effects of both ethanolic and hydromethanolic extracts of the fruiting bodies of wild edible mushrooms *Boletus edulis* (penny bun) and *Cantharellus cibarius* (golden chanterelle) sampled in Poiana Stampei (Suceava county, Romania). The total phenolic contents of extracts were also determined. *Boletus edulis* hydromethanolic extract showed the highest total phenolic content (72.78±0.29 mg/g). This extract was also the most active as scavenger of DPPH and ABTS radicals (EC_{50}=151.44±0.85 and 65.4±0.4 µg/mL, respectively) and reducing agent (EC_{50}=46.77±0.34 µg/mL). *Cantharellus cibarius* ethanolic extract showed high ferrous ion chelating (EC_{50}=82.9±0.6 µg/mL), 15-lipoxygenase (EC_{50}=236.7±1.5 µg/mL) and α-glucosidase (EC_{50}=9.77±0.06 µg/mL) inhibitory activities. For both mushrooms, the ethanolic extracts were more active against *Staphylococcus aureus* ATCC 25923 than the hydromethanolic ones. The antioxidant and antihyperglycemic effects revealed in this study support further investigations for a possible valorization of both mushrooms in the dietary supplement and pharmaceutical industries.

Keywords: *Boletus edulis, Cantharellus cibarius,* ferrous ion chelation, free radical scavenging, α-glucosidase, 15-lipoxygenase, reducing power.

Introduction

Edible mushrooms are consumed for their nutritional and functional properties in fresh or dried form [CHEUNG 2013; VALVERDE & al. 2015]. Bioactive compounds such as polysaccharides, proteins, triterpenoids, phenols and flavonoids have been isolated from edible mushroom species [LIU & al. 2016]. Moreover, numerous studies have reported that some edible mushrooms have antioxidant, antitumor, antiallergic, anti-inflammatory, anticholesterolemic, antiviral, antibacterial and immunomodulatory effects [CHANG & WASSER, 2012]. In oriental medicine, many edible mushrooms are widely used to prevent chronic diseases [SARIKURKCU & al. 2008]. Edible mushrooms are also known for their low glycemic index and high mannitol and dietary fibers content that recommend them for diabetic patients diet [CHANG & WASSER, 2012]. Evaluation of chemical composition and pharmacological activities of edible mushrooms is still an active research area.

Boletus edulis Bull. (*Boletaceae*, penny bun) is a widespread mushroom that grows in deciduous and coniferous forests in Europe, North America and Asia. Due to its nutritional value and unique taste, it is considered a culinary delicacy and a functional food [TSAI & al.

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2007; WANG & al. 2014]. Fruiting bodies of *Boletus edulis* are an important source of carbohydrates (mannose, rhamnose, glycans), lectins (boledulin A, B, C), organic acids (malic, oxalic, quinic, ketoglutaric acids), aminoacids (glutamine, alanine, serine, proline) and microelements (Co, Cu, Fe, Ni) [FAURE & al. 2014]. Polysaccharides isolated from *Boletus edulis* are responsible for many biological activities such as antitumor, anti-inflammatory and antioxidant effects. Oral administration of a water-soluble polysaccharide purified from *Boletus edulis* proved to have antitumor effect on renal cell carcinoma in mice [WANG & al. 2014]. Ethyl acetate fractions rich in boledulin A, B and C showed moderate cytotoxic activity against human myeloid leukemia HL-60, breast cancer MCF-7, hepatocellular carcinoma SMMC-7721, colon cancer SW480 and lung cancer A-549 cells [FENG & al. 2011]. Hot water extract of *Boletus edulis* (5-320 μg/mL) showed immunomodulatory activity due to a stimulatory effect on splenic lymphocytes proliferation [WANG & al. 2013]. *Boletus edulis* extracts exhibited antioxidant and antiviral effects. Strong free radical scavenging activity was reported for methanolic and hot-water extracts of *Boletus edulis* fruiting bodies [SARIKURKCU & al. 2008; TSAI & al. 2007]. Methanolic and water extracts rich in polyphenols and α,β-glycans exhibited antiviral activity on type-1 *Herpes simplex* virus (HSV-1), while hot-water extract rich in lectins showed antiviral properties against type-1 human immunodeficiency virus (HIV-1) [SANTOYO & al. 2012; ZHENG & al. 2007].

Cantharellus cibarius Fr. (*Cantharellaceae*, golden cantherelle) is the most common wild edible mushroom in European coniferous forests and hardwood forests that can be harvested from early spring to fall [DREWNOWSKA & FALANDYSZ, 2015; HONG & al. 2012]. *Cantharellus cibarius* mushrooms are rich in ergocalciferol but also carotenoids; the latter are responsible for the yellow-to gold pigmentation of the fruiting bodies [FALANDYSZ & al. 2012; DREWNOWSKA & FALANDYSZ, 2015]. Other constituents such as polysaccharides, lectins, phenolic acids, lipids, sterols and indolic compounds have been recently isolated from *Cantharellus cibarius* extracts. Similar to *Boletus edulis*, *Cantharellus cibarius* is of great interest due to its antitumor, anti-inflammatory and immunomodulatory effects but also for its antimicrobial and antigenotoxic potential [VALENTAO & al. 2005; DREWNOWSKA & FALANDYSZ, 2015]. The immunomodulatory effect is apparently related to acetylenic acid derivatives as these compounds, isolated from the methanolic extract of *Cantharellus cibarius*, were able to enhance gene expression of peroxisome proliferator-activated receptor gamma (PPAR-γ) [HONG & al. 2012]. At the same time, a polysaccharide-rich fraction of *Cantharellus cibarius* stimulated the proliferation of mouse splenocytes [HAN & al. 2013]. Regarding possible benefits of *Cantharellus cibarius* in Alzheimer's disease, a slight inhibition of acetylcholinesterase was reported for the methanolic extracts rich in polyphenols [ORHAN & USTUN, 2011]. This mushroom could also have a beneficial role in other chronic diseases as it contains phytochemicals with anti-inflammatory properties. MORO & al. (2012) investigated the anti-inflammatory mechanism of *Cantharellus cibarius* methanolic extracts and concluded that these extracts could reduce the expression of inducible nitric oxide synthase (iNOS), interleukins IL-1β and IL-6 in lipopolysaccharide-stimulated macrophages.

Further investigation is necessary to broaden the therapeutic applications of *Boletus edulis* and *Cantharellus cibarius* in pharmaceutical and functional food industries. The purpose of our study was to evaluate the polyphenolic content of edible mushrooms *Boletus edulis* and *Cantharellus cibarius* sampled in Suceava county, Romania. Our further objective

was to assess their antioxidant, antihyperglycemic and antimicrobial effects. In this respect, ethanolic and hydromethanolic extracts were prepared and investigated.

Materials and methods

Mushroom material

Fruiting bodies of *Boletus edulis* (Bull.) and *Cantharellus cibarius* Fr. were collected in September 2011 in Poiana Stampei, Suceava county, Romania. The mushroom material was cleaned and stored at -18 °C. For further analysis, the samples were defrosted and air dried in shade. Voucher specimens are deposited in the Laboratory of Pharmacognosy, Faculty of Pharmacy, Grigore T. Popa University of Medicine and Pharmacy, Iasi, Romania.

Ethanolic extracts preparation

Dried and powdered mushroom samples (50 g) were extracted twice with 500 mL of 96% ethanol at room temperature for 3 h under continuous stirring. The combined ethanolic extracts were evaporated at 40 °C under reduced pressure resulting in the final ethanolic extracts.

Hydromethanolic extracts preparation

After ethanolic extraction, the mushroom residue was further extracted with methanol:water mixture (1:1, v/v) using the same procedure. The extracts were evaporated at 40 °C under reduced pressure resulting in the final hydromethanolic extracts.

Total phenolic content

The total phenolic contents of both extracts were evaluated by Folin-Ciocalteu assay [WANGENSTEEN & al. 2004].

DPPH radical scavenging effect

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was assessed according to a previously described method [LIU & al. 2012; WANGENSTEEN & al. 2004] with slight modifications. Briefly, DPPH radical scavenging activity was determined after 60 min reaction time in darkness at room temperature.

ABTS radical cation scavenging effect

ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid] radical cation scavenging activity was determined using the method of RE & al. (1999).

Reducing power

Reducing power was evaluated according to the method described by ZHANG & al. (2011).

Ferrous ion chelating effect

Ferrous ion chelation assay was performed according to VENDITTI & al. (2010).

15-Lipoxygenase inhibition

The ability to inhibit the peroxidation of polyunsaturated fatty acids was investigated using 15-lipoxygenase inhibition assay as previously described [BITO & al. 2014; WANGENSTEEN & al. 2004].

α-Glucosidase inhibition

The capacity to inhibit α-glucosidase was performed as described by LIU & al. (2012) with slight modifications. α-Glucosidase from *Saccharomyces cerevisiae* was dissolved in phosphate buffer (67 mM, pH 6.8 at 37 °C) to a concentration of 0.86 IU/mL. An aliquot of 0.05 mL of each extract was mixed with 0.5 mL phosphate buffer, 0.02 mL glutathione (3 mM) and 0.02 mL α-glucosidase. After 5 min incubation at 37 °C, a volume

of 0.05 mL *p*-nitrophenyl α-D-glucopyranoside (10 mM) was added followed by 15 min incubation at 37 °C. The reaction was stopped with 2.36 mL sodium carbonate (0.1 M). The absorbance was determined at 400 nm.

Antibacterial and antifungal effects

Antibacterial activity was evaluated against Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 278523), while the antifungal activity was tested against *Candida albicans* ATCC 10231, *Candida glabrata* ATCC MYA 2950 and *Candida parapsilosis* ATCC 22019. The strains belonged to the Culture Collection of the Microbiology Department, Grigore T. Popa University of Medicine and Pharmacy, Iasi, Romania.

Antibacterial and antifungal effects were evaluated by agar diffusion assay [WAYNE, 2015].

Results and discussion

Extraction

The solvent used for extraction has a great influence on the phenolic content and consequently on the biological effects of vegetal extracts. In our study, the hydromethanolic extracts showed higher yields than the ethanolic ones (Tab. 1).

Total phenolic content

The highest phenolic contents were detected in the hydromethanolic extracts (Tab. 1). Regarding *Boletus edulis* extracts, the values found in our study were higher than those reported by TSAI & al. (2007) for *Boletus edulis* samples collected in Taiwan (5.73±0.05 and 5.81±0.10 mg/g for the ethanolic and hot water extracts, respectively). Lower phenolic contents were also reported for the methanolic extract of *Boletus edulis* from Portugal (5.03±0.11 mg/g) [BARROS & al. 2008]. *Cantharellus cibarius* extracts showed higher phenolic contents than those reported for the methanolic extract of *Cantharellus cibarius* from India (3.20±0.05 mg/g) [RAMESH & PATTAR, 2010] and Portugal (1.75±0.5 mg/g) [BARROS & al. 2009]. These different values reported in literature might be due to the harvest moment, substrate on which mushrooms grew, storing conditions and duration [HELENO & al. 2010]. Our results indicate that the total phenolic content depends on the mushroom species and solvent used for extraction.

Tab. 1. Extraction yields and total phenolic contents of *Boletus edulis* and *Cantharellus cibarius* extracts

Mushroom	Extract	Abbreviations	Yields (%)	Total phenolic content (mg/g)
Boletus edulis	ethanolic	Be-E	14.69	35.83±0.92
	hydromethanolic	Be-HM	24.31	72.78±0.29
Cantharellus cibarius	ethanolic	Cc-E	9.50	11.27±0.32
	hydromethanolic	Cc-HM	31.69	11.53±0.03

DPPH radical scavenging effect

For all tested concentrations, the hydromethanolic extracts showed higher scavenging activity against DPPH radical than the ethanolic extracts. As it can be concluded from the low effective concentrations 50% (EC_{50}) (μg/mL) (Tab. 2), *Boletus edulis* extracts exhibited stronger DPPH radical scavenging effects than *Cantharellus cibarius* extracts. In the same assay, an EC_{50} of 2.33±0.06 μg/mL was found for quercetin [ZAVASTIN & al.

2015]. In contrast to our study, TSAI & al. (2007) reported lower scavenging activity for the ethanolic and hot water extracts from commercial samples of *Boletus edulis* from Taiwan (EC_{50}=1.75±0.02 and 15.78±0.10 mg/mL, respectively). Moreover, other researchers reported higher EC_{50} values for the methanolic extracts of *Boletus edulis* from Portugal (EC_{50}=1.54±0.03 mg/mL) [FERNANDES & al. 2013] and Poland (EC_{50}=1.80±0.01 mg/mL) [HELENO & al. 2015].

With respect to *Cantharellus cibarius*, KOSANIC & al. (2013) found lower EC_{50} values for the methanolic and acetonic extracts of *Cantharellus cibarius* from Serbia (EC_{50}=192.57 and 158.4 µg/mL, respectively).

ABTS radical cation scavenging effect

In this assay, hydromethanolic extracts displayed higher ABTS radical cation scavenging effects than the ethanolic ones (Tab. 2). *Boletus edulis* hydromethanolic extract was the most active; at 250 µg/mL, it almost completely scavenged ABTS radical cation (90.62±0.15% scavenging activity). In the same assay, quercetin showed an EC_{50} value of 1±0 µg/mL [ZAVASTIN & al. 2015].

Reducing power

The highest reducing power was determined for *Boletus edulis* hydromethanolic extract. However, both *Boletus edulis* extracts were more active than the extracts of *Cantharellus cibarius* (Tab. 2). The reducing effects of the tested extracts were lower than that found for quercetin in our previous studies (EC_{50} =2.98±0.12 µg/mL) [ZAVASTIN & al. 2015]. In contrast to our study, other researchers reported a lower reducing power for the methanolic extracts of *Boletus edulis* from Portugal (EC_{50}=0.71±0.01 mg/mL) [FERNANDES & al. 2013] and Poland (EC_{50}=0.63±0.02 mg/mL) [HELENO & al. 2015]. However, the ethanolic extract of *Cantharellus cibarius* from Turkey had a similar reducing capacity (0.315±0.10 at 500 µg/mL) as the one found in our study (0.34±0.00 at 533.34 µg/mL) [ORHAN & USTUN, 2011].

Ferrous ion chelating effect

In this assay, the ethanolic extracts were more active than the hydromethanolic ones. The strongest ferrous ion chelating effect was exerted by *Cantharellus cibarius* ethanolic extract (Tab. 2). At 576 µg/mL, this extract chelated ferrous ions by 91.47±0.44%. At the same concentration, *Boletus edulis* ethanolic extract showed 55.32±0.26% chelating activity. In the same assay, an EC_{50} value of 6.34±0.06 µg/mL was determined for ethylenediaminetetraacetic acid (EDTA), a very potent metal chelator [ZAVASTIN & al. 2015]. KHALILI & al. (2015) also reported the capacity of the methanolic and ethyl acetate extracts of *Cantharellus cibarius* to chelate plasmatic ferrous ions in iron overloaded mice. In our study, *Boletus edulis* ethanolic extract showed a weaker capacity of chelating ferrous ions than the methanolic extract of *Boletus edulis* from Turkey (90.2±0.85% chelating activity at 500 µg/mL). Extracts with strong chelating capacity might be able to chelate the excess of pro-oxidant ferrous ions in the human body [SARIKURKCU & al. 2008].

15-Lipoxygenase inhibition

In contrast to the hydromethanolic extracts, the ethanolic ones exhibited a stronger inhibition of 15-lipoxygenase (Tab. 2). It is worth noting that at 833.34 µg/mL, both *Boletus edulis* and *Cantharellus cibarius* ethanolic extracts almost completely inhibited 15-lipoxygenase (100% and 97.53% inhibition, respectively). At the same concentration, the percentages of 15-lipoxygenase inhibition showed by the hydromethanolic extracts were very low (20.14±0.23% for *Boletus edulis* extract and 1.02±0.18% for *Cantharellus cibarius* extract). With respect to the EC_{50} values, all extracts were less effective than quercetin; the

latter was found to inhibit 15-lipoxygenase with an EC_{50} value of 19.5±0.7 µg/mL [ZAVASTIN & al. 2015]. The inhibition of 15-lipoxygenase could not be associated with the phenolic content; other compounds seem to be responsible for this effect. 15-Lipoxygenase inhibitors are able to restrain lipid peroxidation in the human body [BITO & al. 2014].

Antihyperglycemic activity

α-Glucosidase is an important enzyme involved in the hydrolysis of starch and disaccharides to glucose units. α-Glucosidase inhibitors slow the absorption of carbohydrates thus being able to control postprandial hyperglycemia [KUMAR & al. 2013]. The ethanolic extracts proved to be strong α-glucosidase inhibitors (Tab. 2). Their activity was significantly higher than that of acarbose (anti-diabetic drug) that inhibits α-glucosidase. In the same assay, acarbose inhibited the enzyme with an EC_{50} of 70.7±0.3 µg/mL, as determined previously [ZAVASTIN & al. 2015]. *Boletus edulis* ethanolic extract had a remarkable α-glucosidase inhibitory activity (84.27±0.19% at 83.34 µg/mL). Further studies should be done to identify the constituents responsible for this activity. Other researchers stated that polysaccharides from mushrooms are involved in the antihyperglicemic effect as they elevate insulin level in plasma, increase hepatic glycogen and reduce carbohydrates decomposition by restraining α-glucosidase [WANG & al. 2016].

Tab. 2. Antioxidant and antihyperglycemic effects of *Boletus edulis* and *Cantharellus cibarius* extracts

Type of activity	EC_{50} values (µg/mL)			
	Be-E	**Be-HM**	**Cc-E**	**Cc-HM**
DPPH radical scavenging effect	411.63±0.25	151.44±0.85	>833.34	730.37±3.05
ABTS radical cation scavenging effect	124.77±2.80	65.4±0.4	387.1±6.0	179.57±1.65
Reducing power	98.54±0.55	46.77±0.34	872.99±6.69	241.92±1.20
Ferrous ion chelating effect	449.13±5.15	7954.5±45.3	82.9±0.6	3752.57±35.65
15-Lipoxygenase inhibition	348.27±1.55	-	236.7±1.5	-
Antihyperglycemic activity	13.2±0.00	-	9.77±0.06	131.3±2.2

Antibacterial and antifungal effects

In the last decades, antibiotic resistance among microbial strains has dramatically increased. Since the current treatment often failed to overcome multidrug-resistance, researchers have investigated the antimicrobial activity of natural products [NOWACKA & al. 2014]. In our study, all extracts acted selectively against Gram-positive bacteria (*Sarcina lutea* ATCC 9341 and *Staphylococcus aureus* ATCC 25923). According to the inhibition zone diameter (IZD), the most sensitive bacteria was *Sarcina lutea* ATCC 9341 (Tab. 3). The ethanolic extracts showed the highest antimicrobial activity. According to IZD, *Boletus edulis* ethanolic extract had a slightly lower antibacterial activity against *Sarcina lutea* ATCC than chloramphenicol (20 *vs*. 25 mm). These results are in agreement with other data reporting antibacterial activity against *Staphylococcus aureus* ESA 7 (strain isolated from pus) for the methanolic extracts of *Boletus edulis* and *Cantharellus cibarius* showing minimum inhibitory concentrations (MIC) of 5 and 50 µg/mL, respectively [BARROS & al. 2008]. NOWACKA & al. (2015) found that the extracts of wild growing mushrooms from Poland were more active against Gram-positive bacteria than Gram-negative bacteria. Not only the activities of individual compounds in mushroom extracts, but also the interactions between them might be responsible for these differences in the antibacterial potencies [KOSANIC & al. 2016].

Our study also revealed that all extracts were inactive against Gram-negative bacteria although other studies found that *Cantharellus cibarius* ethanolic extract was active against *Escherichia coli* ATCC 25922 (MIC=15 µg/mL) and *Pseudomonas aeruginosa* ATCC 27853 (MIC=13 µg/mL) [RAMESH & PATTAR, 2010]. *Boletus edulis* ethanolic extract was the only extract with antifungal activity against *Candida parapsilosis* ATCC 22019 but its efficacy was lower than that of nystatin. In contrast to our results, KOSANIC & al. (2013) reported that the methanolic and acetonic extracts of *Cantharellus cibarius* were active against *Candida albicans* IPH 1316 (MIC=10 and 5 mg/mL, respectively).

Differences in the microbial cell wall structure might also explain, in part, the different antimicrobial effects of investigated mushroom extracts. Gram-positive bacteria cell wall is composed of several layers of peptidoglycans, Gram-negative bacteria cell wall consists of one peptidoglycan layer and an outer membrane containing phospholipids and lipopolysaccharides, whereas the fungal cell wall contains chitin and other polysaccharides [KOSANIC & al. 2016; SILHAVY & al. 2010].

Tab. 3. Antibacterial and antifungal activity of *Boletus edulis* and *Cantharellus cibarius* extracts

Extract/ Positive control	**Diameter of inhibition zone (mm)**						
	***S. aureus* ATCC 25923**	***S. lutea* ATCC 9341**	***E. coli* ATCC 25922**	***P. aeruginosa* ATCC 278523**	***C. albicans* ATCC 10231**	***C. glabrata* ATCC MYA 2950**	***C. parapsilosis* ATCC 22019**
Be-E	14	20	0	0	0	0	0
Cc-E	14	17	0	0	0	0	0
Be-HM	11	13	0	0	0	0	10
Cc-HM	11	12	0	0	0	0	0
Ampicillin (25 µg/disc)	23	30	17	0	n.d.	n.d.	n.d.
Chloram phenicol (30 µg/disc)	20	25	22	0	n.d.	n.d.	n.d.
Nystatin (100 µg/disc)	n.d.	n.d.	n.d.	0	20	20	21
	n.d. – not determined						

Conclusions

As far as we know, this is the first report that underlines the antioxidant, antihyperglycemic and antimicrobial effects of *Boletus edulis* and *Cantharellus cibarius* mushrooms from Suceava county, Romania. The present study demonstrates that these mushrooms are valuable sources for the development of antioxidant and antihyperglycemic dietary supplements. *Boletus edulis* hydromethanolic extract showed a remarkable free radical scavenging activity which is related to its high content in phenolic compounds, while *Cantharellus cibarius* ethanolic extract proved to be an important 15-lipoxygenase inhibitor and ferrous ion chelator. Furthermore, *Boletus edulis* and *Cantharellus cibarius* ethanolic extracts were effective α-glucosidase inhibitors. The components of both ethanolic extracts should be further investigated for antidiabetic activity. The ethanolic extracts showed a moderate antimicrobial activity against Gram-positive bacteria and therefore a possible synergism of these extracts with conventional antibiotics should be further evaluated. Taking into consideration the results of the present study, we can conclude that *Boletus edulis* and *Cantharellus cibarius* can bring important positive effects on the human health as functional foods.

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BOTANICAL AND PHYTOCHEMICAL APPROACH ON *PASSIFLORA* SPP. – NEW NUTRACEUTICAL CROP IN ROMANIA

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Abstract: It has been performed a complex investigation – morpho-anatomical, physiological, taxonomical and phytochemical one – of *Passiflora* nutraceutical plants from Hofigal S.A., in the frame of project PN-II-PCCA-2013-4-0995, contract 160 (MAIA)/2014. Anatomic analysis of leaf lamina, petiole and stem, provided data with taxonomical importance, leading to the conclusion that plant material belongs to *Passiflora caerulea* L., in concordance with world monographers of *Passiflora* genus: VANDERPLANK (2000) and ULMAN & MacDOUGAL (2004). Physiological investigation referred to the following parameters: coefficient k, leaf area index (LAI), chlorophyll fluorescence, stomatal conductance and yield of green plant biomass. Phytochemical investigation consisted in analyzing active principles (polyphenols, flavonoids) content, in correlation with their antioxidant activity and determination of cytotoxicity of *Passiflora* extracts in NCTC cell line. At 10-150 µg/ml concentrations, it was recorded a normal cell morphology. At concentrations over 250 µg/ml, the plant extract become cytotoxic, altering the cell membrane structure, cells viability and proliferation.

Key words: *Passiflora* plants, leaf (lamina, petiole) and stem structure, taxonomical considerations, physiological parameters, phytochemical aspects, cell viability, Romania.

Introduction

Known and cultivated in Romania at the beginning as an ornamental plant [GRINŢESCU, 1955], *Passiflora* L. (Fam. *Passifloraceae*) became a promising nutraceutical crop, relatively newly acclimatized for its benefic proprieties.

There are mentioned in the literature a large biodiversity of *Passiflora* spp. in the world, more than 95% originary from South America, and 5% from Asia, Australia, North America. *Passiflora* spp. are wild in North and South America, the West Indies, the Galapagos Islands, Africa, Australia, the Philippines, Asia and many Islands in the Pacific Ocean [VANDERPLANK, 2000; ULMAN & MacDOUGAL, 2004]. According to VANDERPLANK (2000), *Passiflora* comprises 18 genera and approximately 630 species, distributed in the tropical regions of America, Asia and Africa. The genus *Passiflora*, whose

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center of origin is South America and center of geographic distribution is the northern and central regions of Brazil, has the largest number of species in the family *Passifloraceae*, ≅ 400 [VITTA & BERNACCI, 2004; OLIVEIRA & al. 2013]. However, only approximately 60 species produce fruits with commercial values (SCHULTZ, 1968; SCHULTZ & HUBER, 1968; MANICA, 1997; OLIVEIRA & al. 2013], either for consumption or medicinal use [OLIVEIRA & al. 2013]. Approximately 90% of the species originated in the Americas [LOPES, 1991; OLIVEIRA & al. 2013] and more than 120 of them are native from Brazil [CERVI, 1997; SOUZA & LORENZI, 2005; OLIVEIRA & al. 2013]. Brazil is the largest world producer with approximately 650,000 tons/year, 83% of world production [GAMA & al. 2013]. Also, the literature refers to an extensive variety of *Passiflora* pharmacological proprieties [DHAWAN & al. 2004; PATEL & al. 2011; SINGH & al. 2012; MIRRODI & al. 2013; SAHELIAN, 2014; SHI & al. 2014; DEVESA & al. 2015 a.o.] (Fig. 2). *Passiflora* accumulation of biological active compounds depends significantly to the environmental conditions [CARNEVALLI DIAS & al. 2010; INGALE & HIVRALE, 2010; IZAGUIRRE & al. 2013; CHAGUT & al. 2014], that is why we had the following objectives to investigate the influence of *Passiflora* cultivation in Romania on: (1) the morpho-anatomical aspects of leaves and stems, with importance in the taxonomy of *Passiflora*; (2) physiological aspects mainly leaves and stems and on (3) the production and accumulation of biological active compounds in plants. Till now there were not performed histo-anatomical studies with taxonomic, ecological and technological importance on *Passiflora* spp. cultivated in Romania [TOMA & RUGINĂ, 1998; TOMA & TONIUC, 2008; TOMA, personal communication]. Also, the phytochemical investigations of *Passiflora* plants cultivated into Romania conditions represents a novelty. Our aim was to characterize botanically and phytochemically the local *Passiflora* population cultivated in the open-greenhouse on Hofigal experimental fields.

Material and methods

Biological material for morpho-anatomical evaluation consists in *Passiflora* L. leaves, stems and shoots, collected on 23 June 2015 from the Hofigal experimental field. These were preserved in 70% ethylic alcohol. For histological analysis, the usual methods used in plant anatomy [ȘERBĂNESCU-JITARIU & al. 1983] have been followed. *Passiflora* leaves, stems and shoots have been manually cross cut in the median zone of lamina lobs, petiole, stem and shoots. Paradermal sections were prepared for analyzing the characteristics of the epidermis in apical view. Differential and successive colorations of crossed material with Iodine green and Carmine Alum have been applied [ȘERBĂNESCU-JITARIU & al. 1983; ȘESAN & al. 2015]. All microscopic slides have been analyzed with a DOCUVAL optical microscope in normal and polarized lights (crystal study). Photomicrographs have obtained with a microscope incorporated Nikon D90 digital camera.

For **physiological evaluation**, *Passiflora* plants have been monitorized during the whole experiment and measured their morpho-physiological parameters, at the 7th day (10.06.2015), at the beginning of experiment and after 20 days (22.06.2015), at the end of our trials. *Passiflora* samples were prelevated for biochemical analysis of bioactive compounds and for alternative tests on cell cultures at the same dates. It has been evaluated finally the yield level (green biomass) for each plot/replicate (15 plant samples/replicate). Results have been evaluated statistically by the variance analysis. There were used the following methods: (a) classic and computational methods for evaluation of leaf area (cm^2), (b) fluorometry method for determining chlorophyll fluorescence [Y(II)] in arbitrary units

and (c) porometry method for the stomatal resistance (s/cm) and stomatal conductance (nmol/ m^{-2} s^{-1}).

(a) Method for leaf area determining (LA – Leaf Area; LAI – Leaf Area Index). This method have been suggested by MONTGOMERY in 1911 [CHANDA & SINGH, 2002], using formula: **LA (cm^2) = length (L cm) x width (l cm) x k (coefficient)**, **Coefficient k** for *Passiflora* leaves is known from the literature [REIMBERG & al. 2009; MORGADO & al. 2013]. We have been calculated it using the classic method of drawing of scanned fresh leaves on the A4 sheet of paper divided in square of 5 mm. For our experiment we have randomized collected 10 leaves/replicate, in total 30 leaves/plot/variant (Fig. 1). In order to calculate coefficient k, it has been used the formula **K = Leaf area calculated by another method / L x l**. Data of length, width and leaf area have been statistically discussed by test t. **The computational method** is based on different softwares [DOBRE & LAZĂR, 2014 a.o.], used in our researches, too [RĂUT & al. 2015; GHIUREA & al. 2015] for the scanned sampled leaves / plots. By this method the advantage is obtaining rapidly and precisely the value of leaf area (cm^2), after a proper calibration of equipment.

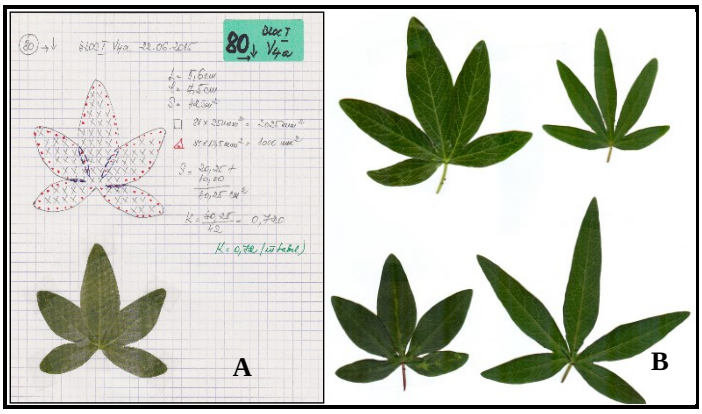


Fig. 1. A. Working A4 squared sheet of paper used for classical method of leaf area estimation; **B.** *Passiflora* simple, palmate-partite leaves (herborized and scanned) [ŞESAN, 2015]

(b) Fluorometry. The light energy absorbed by chlorophyll molecules on leaves follows 3 pathways: (i) it is used to produce reduced equivalents ($NADPH+H^+$) and metabolic energy (ATP), (ii) it is dissipated as heat or (iii) could be re-emitted as light - chlorophyll fluorescence, respectively. All three processes are in competition, thus increasing of efficiency of a certain pathway, will determine a reducing of output of the other two ways. Through evaluation of chlorophyll fluorescence output it is possible to obtain information on the changes in photochemical efficiency and in the dissipation of heat. The measurements of chlorophyll fluorescence of *Passiflora* plants have been performed with the fluorometer Walz Pam-2500 on different representative, healthy leaves of a plant (3 leaves/variant). It has been calculated the average value Y(II) of each plant, as well as the average value of replicates.

(c) Porometry. This method is based on the study of the gas diffusion through pores, especially through leaf stomata. Plant transpiration being adjusted by the opening and closing of stomata, these parameters are essential in many plant research domains. This is a measure of plant resistance to the loss of vapors through stomata and it is an indicator of plant physiological status. The used equipment is working through the measurement of the

necessary time for a leaf to release sufficient water vapors for changing the relative humidity in a certain chamber with a standard quantity. It has been used a porometer Delta-T Devices AP4 for the measurement of stomatal resistance (s/cm) and stomatal conductance (nmol/$m^{-2}s^{-1}$) of *Passiflora* leaves (Tab. 4).

Phytochemical investigations. Processing plant material: Fresh plants (leaves, sprouts with leaves and flowers) were dried at 50 °C for 24 hrs. and grinded. Each part was extracted in ethanol and petroleum ether 70 % (v/v) (Et-OH), in a ratio of 1.5:10 (v/v), for 10 days, filtered through vacuum filter and the filtrate was stored at 4 °C in dark until use. The dry weight (dw) was determined on a moisture analyzer (Radwag).

***Qualitative analysis*:** The extracts were qualitatively analyzed for different phytochemicals, like tannins ($FeCl_3$ test), polysaccharides (Molisch test), glycosides (Borntrager test), triterpenoides (Salkowski test), saponins (foam test) and alkaloids (Hager test) [OANCEA & al. 2013; SINGH & al. 2012].

Quantitative analysis*:** Quantitative analysis consisted in ***total polyphenolic content (determined by Folin-Ciocâlteu method), ***flavonoids content*** (assessed using the aluminum chloride colorimetric method) [POURMORAD, 2006], and ***the antioxidant capacity*** using two methods. The first of them which measures the scavenging of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), based on the decrease of the DPPH maximal absorbance at 516 nm in the presence of extracts [OLLANKETO, 2002; RICE-EVANS, 1996] and the second method which assesses the inhibition of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) cationic radical [KAVITHA & al. 2012].

Plant extracts preparation. To be tested in cell culture, hydro-alcoholic extracts were brought to dryness by evaporation of the solvent, and then were re-dissolved in PBS. The obtained extracts were sterilized using Millipore filters with a porosity of 0.22 mm. Dry substance for each sample was determined using a thermobalance (Moisture Analyser Balance, RADWAG Poland).

***In vitro* assays for vegetal material cytotoxicity determination** were realized using a stabilized line of mouse fibroblast L929 cells (ATCC, cell line, NCTC clone 929) provided from the European Collection of Cell Cultures (ECACC). The NCTC cells were cultured in Eagle's MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 mg/ml penicillin, 100 mg/l streptomycin and 500 mg/l neomycin), maintained at 37 °C in a humidified incubator with 5% CO_2, for 24 hrs. They were seeded in 24- well plates at a cell density of $5x10^4$ cells/ml for 24 hours to allow adherence and then were incubated in the presence of different dilutions (1, 10, 50, 100, 150, 250 and 500 µg / ml) of *Passiflora* extracts for 24 hrs., respectively 48 hrs. **The cell viability** was determined by colorimetric method with Neutral Red (NR). After removal of the plant extract from the wells, the solution of the NR (50 mg/ml) prepared in MEM medium supplemented with 10 % fetal bovine serum was added. After an incubation period of 3hrs at 37 °C in 5% CO_2 atmosphere, the NR solution was removed and it was added an equal volume of fixative solution. The absorbance of the solution in the wells was measured at 540 nm, using a plate reader Mithras LB 940 (Berthold Technologies). Results were reported as percent viability depending on the control sample (cells incubated without the plant extract) considered as 100 % viability [FOTAKIS, 2006]. **Cell morphology**: The L-929 line cultured in the presence of various dilutions of plant extracts for 48 hrs., were fixed in methanol and stained with Giemsa solution for 20 minutes, examined under the optical microscope Zeiss Observer D1 20X objective.

Statistical analysis. All phytochemical analyses were made in triplicate and the cell culture experiments were separated performed in tree replicates. Significant statistical differences were considered at $p<0.05$.

Results and discussion

Importance of *Passiflora* plants as medicinal and nutraceutical ones is determined by the effects on human (patho)physiology and is illustrated in Fig. 2.

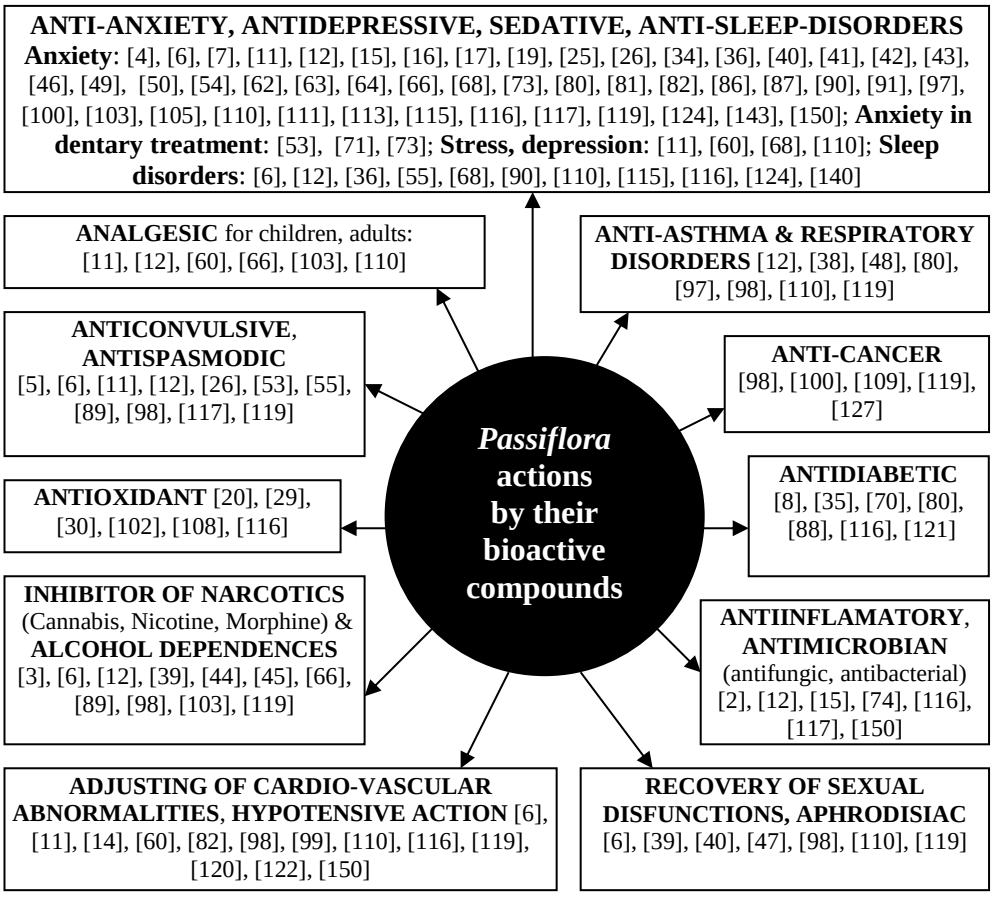


Fig. 2. Main medicinal and nutraceutical importance of *Passiflora* spp.
Numbers in square brackets are the numbers from the References Section.

Botanical evaluation. Morpho-anatomical investigations

Leaves (PLATES I-II, Figs. 3-12, Tab. 1). **Lamina** is simple, palmat-partite (Fig. 1A, 1B), until 12 cm length, with 5 entire inequally lobes, ovate-lanceolate, acute apex, with serrate lamina border and penate nervation. **Mediane nervure** of leaf lobe (350 μm width) presents the adaxiale part relatively plane or slightly convex and the abaxiale proeminent semi-circular

(Fig. 3). **Epidermis** (adaxiale and abaxiale) presents isodiametric cells in cross section, with the extern tangential wall secondary thickened (3.5 μm) and covered with a cuticula (1.20 μm) with clear cuticulare ridges. **Mechanical tissue** is an angular collenchyma, consisting in 3-4 layers adaxially and, respectively, 1-2 layers abaxially. **Conducting tissues** are represented by 1-2 conducting bundles of colateral type, disposed in a compact ground parenchyma. Xylem belt is adaxially oriented, and the phloem belt is abaxially located. **Lamina** of the leaf lobe is approximately 180-220 μm width, with dorsi-ventral structure and hypostomatic. In cross sections epidermal cells appear as rectangular, tangentially elongated. Adaxiale (superior) **epidermis** is formatted only by proper epidermal cells (20-30 μm width/60-70 μm length) (Fig. 5). Abaxiale epidermis (inferior) is formatted by proper epidermal cells (20-30 μm width/30-40 μm length), stomatal cells (17/22 μm) and stomatal annexes cells (Fig. 6). **Stomata** are as anomocitic and anisocitic types and they are differentiated only at the level of abaxiale epidermis (390 stomates/1 mm^2) (Figs. 5, 6). Tector and secretor trichoms are not present on the level of both epidermis. **Mesophyll** is differentiated in palisadic 1-layered tissue, with vertically elongated cells (80-90 μm), abaxially localized and in lacunous parenchyma sphaerical and board cells with small abaxial lacunes (Fig. 4). Calcium oxalate druses (10-15 μm diameter) are frequent in the lamina tissues. They were identified mainly along nervures, disposed in uniseriate strand (Figs. 7, 8).

Petiole (1.5 mm diameter) is circular in sectional view, with adaxial shallow groove and monosimetrical structure (Fig. 9). **Epidermis** presents thick rectangular cells in cross section, with thick cuticle. **Mechanical tissue** consists on 3 or 4 subepidermal layers of angular collenchyma. The remaining ground tissue is a parenchyma with thin walled cells. **Conducting tissues** are organized in about 8 collateral bundles of different size. Xylem elements are in endarch position and phloem elements forms compact mass on the outer part of the xylem. Calcium oxalate druses are abundant in the phloemic and ground parenchyima (Fig. 10).

Tab. 1. Analyzed *Passiflora caerulea* leaf parameters

Leaf parameters	Measurements / size
Median nervure of leaf lobe	350 μm thickness
Epidermis - extern wall	3.5 μm thickness
Cuticule	1.2 μm thickness
Leaf lamina	180-220 μm thickness
Cells in adaxiale (superior) epidermis	20-30 μm width x 60-70 μm length
Cells in abaxiale (inferior) epidermis	20-40 μm width x 40-80 μm length
Stomatal cells	10 μm width x 22 μm length
Stomatal index	390 stomata / 1 mm^2
Mesophyll cells	80-90 μm length
Petiole	1.5 mm diameter
Druses size	15-20 μm diameter

PLATE I

***Passiflora caerulea* – LAMINA**

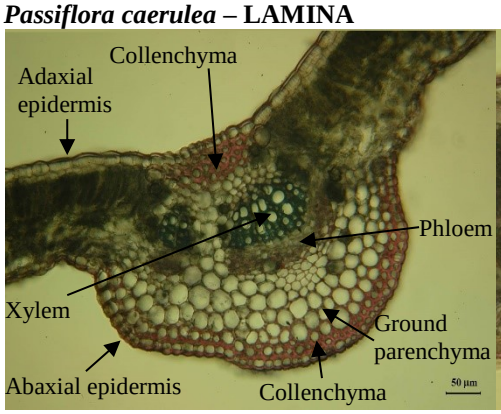


Fig. 3

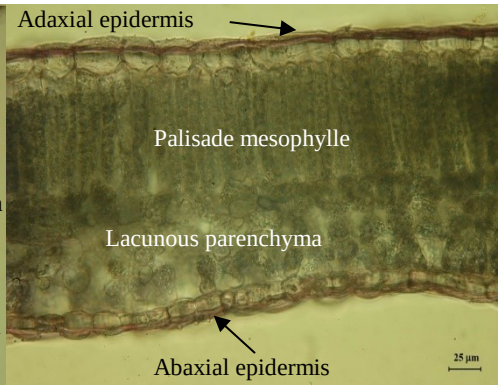


Fig. 4

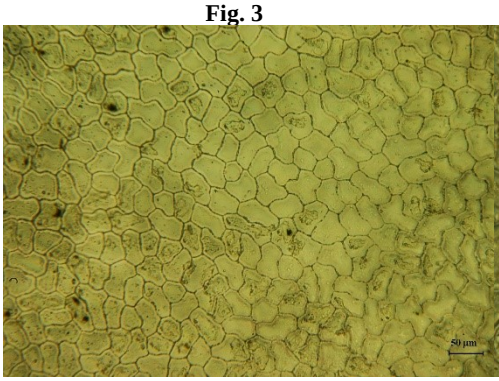


Fig. 5

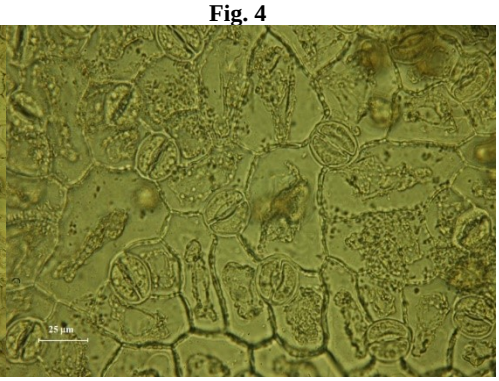


Fig. 6

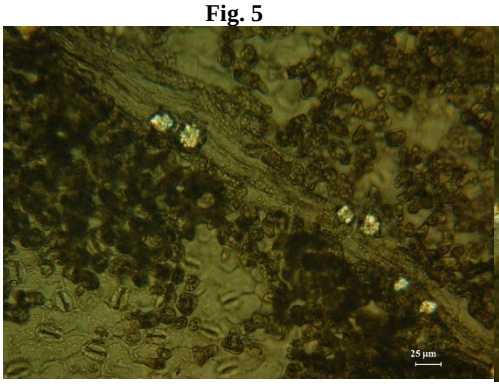


Fig. 7

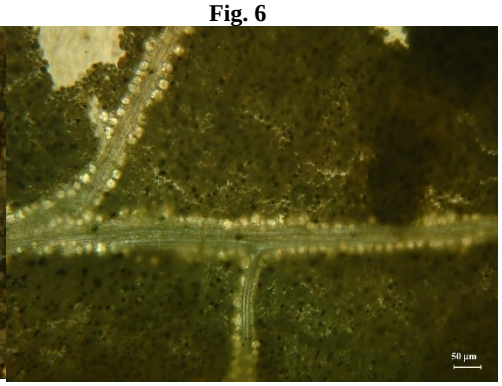


Fig. 8

PLATE II

***Passiflora caerulea* – PETIOLE**

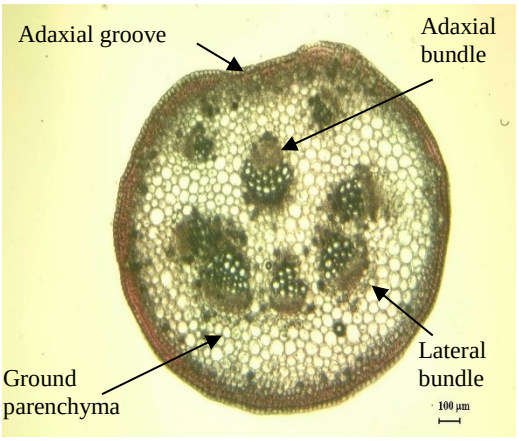


Fig. 9

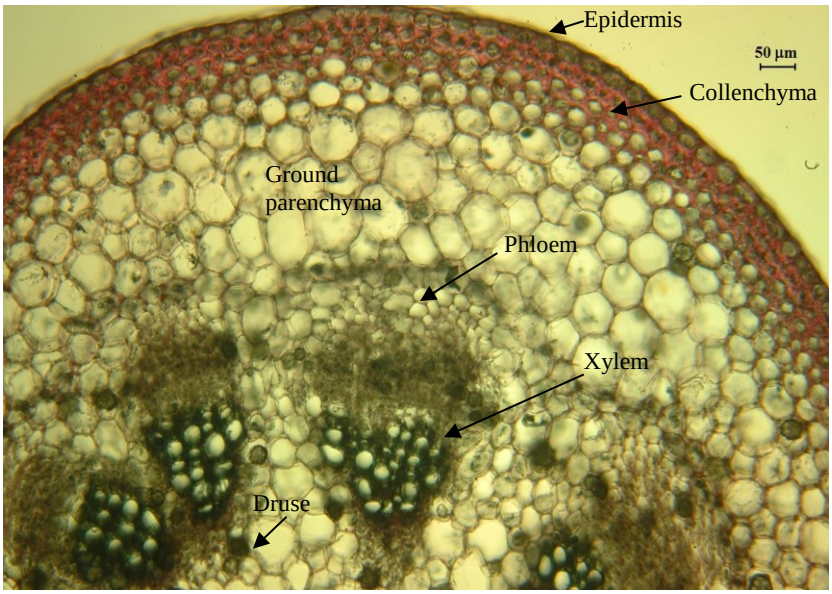


Fig. 10

Stem (PLATE III, Figs. 1, 11-12, Tab. 2) has a circular outline, irregular-ribbed, approximately 2.5-3.0 mm diameter and secondary structure (Fig. 11). **Epidermis** forms a continuous layer of cells, with slightly and uniform, regular thickened walls, covered with a thickened cuticule (6-7 μm). At the level of ribs some epidermal cells are divided. **Cortex** contains a subepidermal angular collenchyma, more developed at the level of ribs (3-4 cellular layers) and a cortical meatic (with small empty spaces) parenchyma (4-5 cellular layers). The central cylinder is voluminous (approximately 2 mm diameter). **Secondary vascular tissues** form a continuous vascular ring, consisting of an outer layer of secondary phloem and an inner layer of secondary xylem (xylem vessels up to 100 μm) (Fig. 12). The primary xylem is present in the inner part of the secondary xylem cylinder. The secondary phloem is accompanied by the packs of sclerenchymatic periphloemic fibres of different sizes. At the maturity of the organ, the ground parenchyma of central cylinder suffers a disorganization and forms a large pity cave. Calcium oxalate druses are abundant, being present in the epidermal cells, in the cortical cells and in the phloemic parenchyma, too (Fig. 11).

Tab. 2. Analyzed *Passiflora caerulea* stem parameters

Stem parameter	Measurements / Size
Stem	2.4-3.0 mm diameter
Central cylinder	2 mm diameter
Epidermis cells cuticule	6-7 μm thickness
Xylem vessels	10-100 μm diameter

The following **structural aspects** analyzed in our study are important in characterization of analyzed *Passiflora* samples:

i. Leaf lamina with a dorsi-ventral structure, hipostomatal and with lack of trichoms;
ii. Stomata of anomocitic and anizocitic types;
iii. Leaf petiole with an adaxial large kennel and a mono-symmetrical structure with vascular distinct fascicles;
iv. Irregular-ribbed stem which differentiates a secondary structures with concentric xylem and phloem rings, respectively;
v. Stem presenting collenchyma and sclerenchyma tissues;
vi. Leaves generating only collenchyma;
vii. Calcium oxalate crystals present in different cells from the leaf and stem structure of the druse type.

These aspects can be compared only partially with literature data [PÉREZ-CORTÉZ & al. 2005; GARCIA & PÉREZ, 2008; ZERPA & GOMEZ, 2014, DE FARIAS, 2014; CHINNIAH & THIAGARAJAN, 2015; WOSCH & al. 2015] because they have studied exotical species, non-cultivated in our country. In Romania there were not performed histo-anatomical studies with taxonomic importance up to the present [TOMA & RUGINĂ, 1998; TOMA & TONIUC, 2008; TOMA, personal communication], our investigations being the first ones with a significant highlighting to define more accurately the taxa of *Passiflora* genus.

PLATE III

***Passiflora caerulea* – STEM**

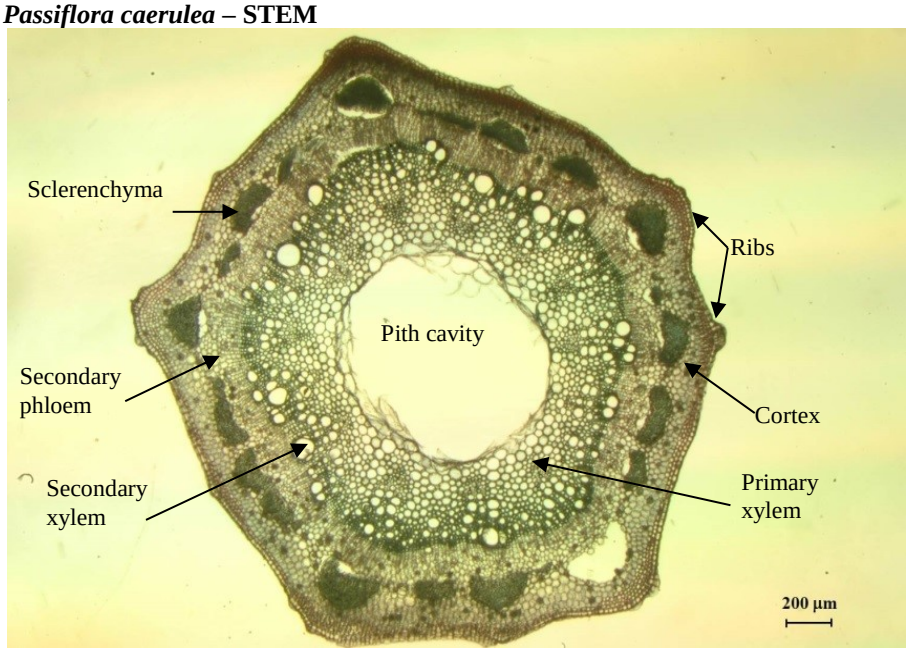


Fig. 11

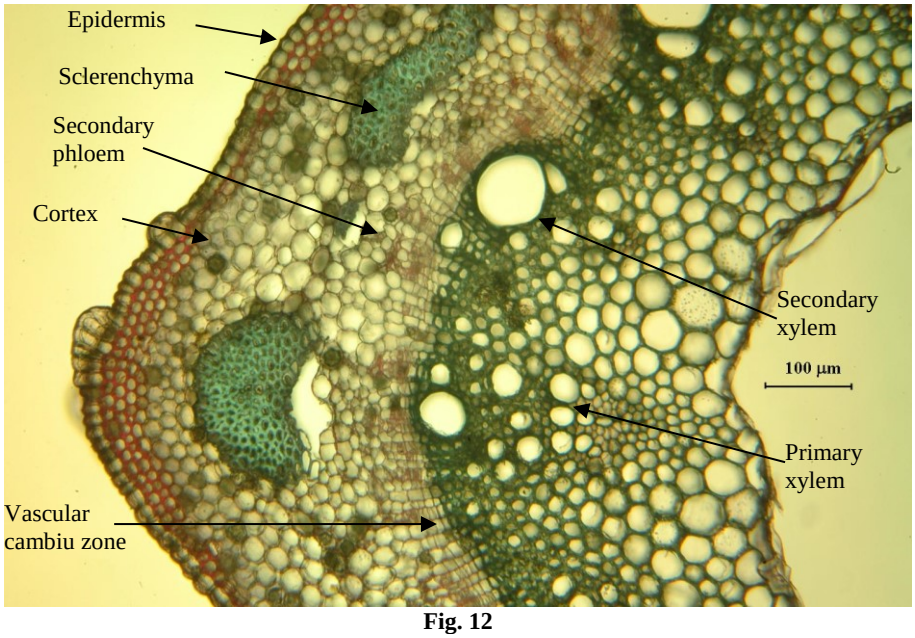


Fig. 12

Taxonomical considerations

All morpho-anatomical investigations have been performed in order to complete the botanical identification and description of the *Passiflora* material from Hofigal experimental field (Vouchers 404766-404767 are deposited in the Collection of Botanical Garden Bucharest). Data on this material is presented in the Tab. 3.

Tab. 3. Botanical identification characters of *Passiflora caerulea* L. [GRINȚESCU, 1955; SCHULTZ, 1968; TUTIN & al. 1968; CERVI, 1997; DEGIGNANI, 2001; VANDERPLANK, 2000, 2003; BERNACCI & al. 2003, 2008;ULMAN & MacDOUGAL, 2004; VITTA & BERNACCI, 2004; SOUZA & LORENZI, 2005; REGINATTO & al. 2006; REJMÁNEK & REJMÁNEKOVÁ, 2009; BLANKESPOOR, 2012; HUTCHINSON, 2012; DE FARIAS, 2014; SAHELIAN, 2014; FRANCO, 2014; Wikipedia, accesed 2016].

Common name: Watch, Passion flower.
Plant type: Woody climbing plant, with tendrils, growing to a height of 15 m height; glabrous vine.
Stem: subangular, striate, grooved, stout.
Leaves: Glabrous leaves, sectate-palmate, with oblong acuminate lobes; 3-, 7- or 9-lobate, generally 5-palmate-lobed, leaf 5-18 x 6-18 cm, with entire margin.
Petiole: 1.5-4(5) cm long.
Petiole glands: 2-4 petiole glands, occasionally 6, stipitate.
Stipules: falciforme; semi-ovate to subreniform, slightly dentate to subentire, 1-2 cm x 0.5-1 cm.
Peduncle: Slender, fine, 3-7 cm long, solitary.
Bracts: Broadly ovate bracts; 3 oval large bracts, green pale, 1.5-2.7 x 1-2.3 cm, pale green, entire, free.
Flowers: Blue and pink, white, blue, and blackish purple, (6-)7-9(-10) cm diameter. Hypanthium (floral tube) absent or saucer-shaped and inconspicuous, corolla white. Blue and white flowers, up to 10 cm diameter. **Sepals** 5, petaloid, with a gland subapical green, white to pale green, oblong, 2-3.5 x 1-1.5 cm, petals white inside, green outside, oblong, 2-3.5 x 1.3-1.8 cm, slightly keeled outside, keel terminating in a short awn. Calyx tube cup-shaped. **Petals (corolla)** 5, similar as form and size with sepals. Petals white inside and outside, sometimes tinged pink, oblong, 2-4 x 0.8-1.5 cm. Corolla disposed in 2 verticils, outer verticil consisting in many filiform apendices (radia), purple or blackish purple at the base, white in the middle and blue at the apex. Corona filaments in 4 series, outer 2, (0.8)1.5-2.5 cm, usually up to 2 cm long, purple at the base, white in the middle and blue towards apex; inner series 0.1-0.2 cm long. **Gynophore** well developed, 0.8-1 cm high. **Stamens** 5 green pale with yellow anthers. Unilocular green **ovary**, ovoid or subglobous, glabrous. **Styles** 3, reddish, unit at the base, with 3 reniform stigmats. **Flowering time**: V-X.
Fruit: ovoid pulpy berry, yellow at maturity; fruiting in greenhouse; fruit brightly orange when ripe, (4-)5-7 x 3-4 cm. Green fruit contain cyanhidric acid (0.0118-0.013% HCN.
Seeds: obcordate or cuneate, 0.5-0.6 x 0.4 cm, coarsely reticulate.
Place of origin: Brazil, Peru; introduced and spread in Europe at the beginning of XVIIIth century. Spread: South America in USA - California, New Zealand, Australia, South Africa.
Varieties: *Passiflora* Constance Elliot (The Garden I, 1887: 595) a.o.
Propagation: Best propagation by cuttings of a reliable source.
Uses: Ornamental plant, covering fences, walls, kiosks, and to decorate windows.
Bioactive compounds: Alkaloid *passiflorine* with therapeutical proprieties. Fruits contain glucose and fatty acids; a flavone *chrysin*, cyanogenic glucoside sulphate *tetraphyllin* 8-4-sulphate and *epitetraphyllin* B-4-sulphate.

Analyzing plant materials from Hofigal experimental field, we obtained a first botanical evaluation, proving that our plants present the botanical characteristics of *P. caerulea* L. (PLATES IV-VI, Figs. 13-29), the oldest citation of *Passiflora* in the Flora

R.P.R. [GRINȚESCU, 1955]. In *Passiflora* taxonomy, it was a long confusion between *P. incarnata* and *P. edulis*, the two species being estimated as synonyms by J. HOOKER & al. (1843), in *Index Kewensis* (1895). After long time, DHAWAN & al. (2001) have focused on the correct identification of *P. incarnata* L. and *P. edulis* Sims. Comparing the recognized taxonomical references on *Passiflora* [GRINȚESCU, 1955; SCHULTZ, 1968; TUTIN & al. 1968; CERVI, 1997; DEGINANI, 2001; VANDERPLANK, 2000, 2003; BERNACCI & al. 2003, 2008; ULMAN & MacDOUGAL, 2004; VITTA & BERNACCI, 2004; SOUZA & LORENZI, 2005; REGINATTO & al. 2006; REJMÁNEK & REJMÁNEKOVÁ, 2009; BLANKESPOOR, 2012; HUTCHINSON, 2012; DE FARIAS, 2014; SAHELIAN, 2014; FRANCO, 2014 etc.] We have concluded that our material belongs to *P. caerulea* L. This species has given origin to more hybrids than any other species, because other species from sub-genus *Passiflora* accept its pollen easily [FRANCO, 2014] (Tab. 3). Different authors were focused on different taxonomical characters of *Passiflora* spp.: GARCIA & PÉREZ (2008) analyzed the presence of trichoms as scales on adaxiale epidermis and the presence of druses in the parenchyma cells in the main vein and in the parenchyma and colenchyma of petiole for *P. guazumaefolia* Juss., ornamentations of adaxiale cuticule on the leaf and petiole levels for *P.* aff. *tiliaefolia* L.; REJMÁNEK & REJMÁNEKOVA (2009) have been focused on the hypanthium (floral tube) to define *P. caerulea*. VIANA & al. (2010) performed morphological investigations on *Passiflora* spp. organs as: leaves (length, width, area), flowers (number), fruit (number, mass, diameter, length), seeds (number, mass, length). Their results have showed high inter- and intra-specific morphological variation for traits of interest in *Passiflora* plants; RAPD analyses indicated that there is polymorphism within and among accession of the studied species. The indication of the most divergent accessions was very important for the *Passiflora* breeding program, since the information will be used for selecting parents for interspecific crosses to produce ornamental hybrids [VIANA & al. 2010]. Other authors insisted on leaf constants number of vein-islets, number of vein endings, number of stomata/stomatal index [LIM & al. 2012], size of stomata 102.6 µm, stomatal index 38.2%, number of 22 stomata per cm^2, leaf size of 112 cm^2 [SREELAKHAMI & al. 2014] as taxonomical characters for *P. edulis* Sims. Recently, CHINNIAH & THIAGARAJAN (2015), WOSCH & al. (2015) have performed detailed studies on the leaf anatomy in order to characterize species of *Passiflora*. These data presume to continue the botanical analysis in the conditions of Romania with a higher number of samples in order to evaluate additional identification aspects as: plant phenotypic plasticity, hybridization, influence of environmental and technological factors [CARNEVALLI DIAS & al. 2010; INGALE & HIVRALE, 2010; IZAGUIRRE & al. 2013; OLIVEIRA & al. 2013; CHAGUT & al. 2014 a.o.].

Macroscopic aspects on the *Passiflora* cultivated in experimental field of Hofigal are presented in the PLATES IV – V and Figs. 13-29.

PLATE IV

***Passiflora caerulea* L. – TAXONOMICAL CHARACTERS**

Fig. 13

Fig. 14

Fig. 15

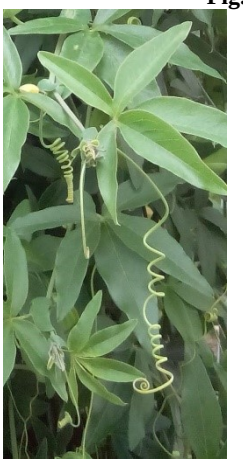


Fig. 16

Fig. 17

Fig. 19

Fig. 18

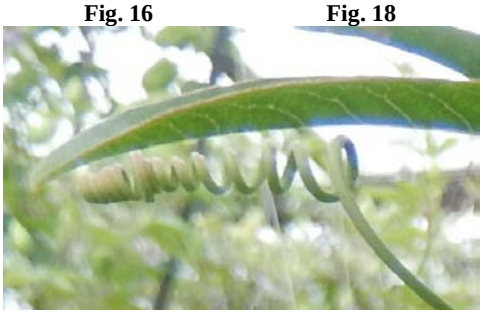


Fig. 20

PLATE V

***Passiflora caerulea* L. – TAXONOMICAL CHARACTERS**

Fig. 21

Fig. 22

Fig. 23

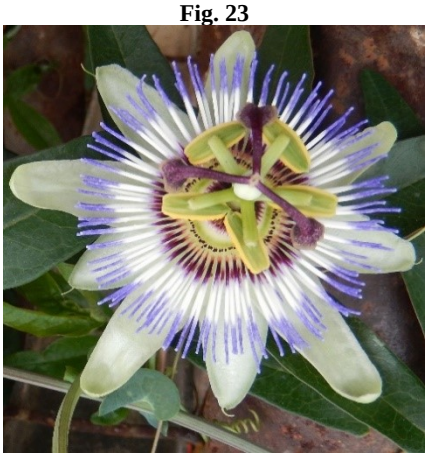


Fig. 24

Fig. 25

PLATE VI

***Passiflora caerulea* L. – TAXONOMICAL CHARACTERS**

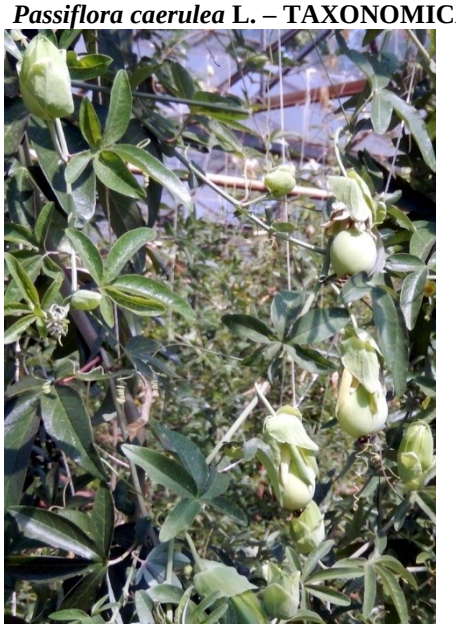


Fig. 26

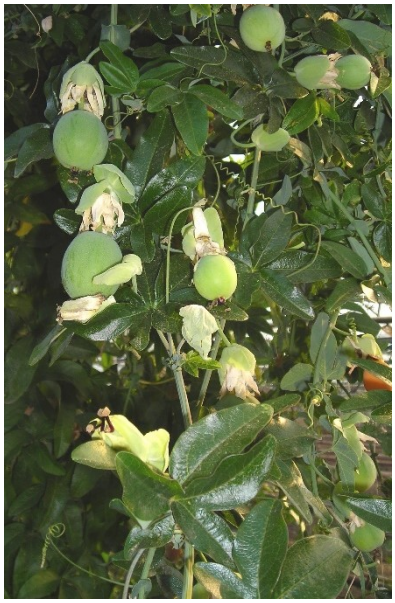


Fig. 27

Fig. 28

Fig. 29

Physiological parameters

In order to determine the morpho-anatomical (leaf length, leaf width, leaf area) and **physiological (leaf growing rate, photosynthesis intensity / chlorophyll fluorescence and stomatal conductance) parameters**, *Passiflora caerulea* plants from experimental plots have been monitorized during the whole time of this.

Classic method has been used for the estimation of leaf area for many plant species by different researchers [LAZAROV, 1965; BOLDOR & al. 1983; MONTGOMERY & al. 1985; CHANDA & SINGH, 2002; CRISTOFORI & al. 2008; DEMIROY & LANG, 2010; PANDAY & SINGH, 2011; AHMED & KHAN, 2011 a.o.]. For *Passiflora* spp., this method for estimation leaf area has been published by REIMBERG & al. (2009), MORGADO & al. (2013) a.o.

Leaf area. Using classic method of leaf area calculation in the formula **LA = L x l x k** (cm^2) appear the coefficient k. Average values calculated by us for the coefficient k have been 0.53, at 10.06.2015 and 0.47, after 20 days. Coefficient k, analyzed statistically by the test χ^2 (Hi/chi square) did not vary for the 3 factors (length, width and area), being the aspect which support and argue the specificity of this coefficient for each type of plant. These data confirm the literature data published by MOLGADO & al. (2013), which established the specificity of coefficient k for *Passiflora* spp., classifying the values calculated by us (k = 0.47-0.53) as moderate and sufficient for the characterization of *Passiflora* plants from the point of view of the leaf morphologic parameters length, width, leaf area.

Analyzing **leaf growing rate** during 14 days of experiment between the two determinations, at 7 and 20 days respectively (Tab. 4.) at *Passiflora caerulea*, the values were 4.1 cm^2 (calculated by classic method) and 6.1 cm^2 (calculated by computational method) in 14 days.

Tab. **4.** Morpho-physiological parameters of *Passiflora caerulea* leaves from the experimental field Hofigal: **Leaf area (cm^2), Leaf growing rate (cm^2), Chlorophyll fluorescence [Y(II)], Stomatal conductance** (nmol $m^{-2}s^{-1}$), 2015

Parameters	At 7 days	At 20 days	Diffe-rence	Test t (p)
Leaf length (cm)	6.7	7.8	1.1	0.00***
Leaf width (cm)	8.7	9.4	0.7	0.04*
Leaf area (L x l x k) (cm^2)*	30.0	34.1	4.1	0.05*
Coefficient k	0.53	0.47		
Leaf area (cm^2)**	30.0	36.1	6.1	
Leaf growing rate (cm^2)*		4.1		
Leaf growing rate (cm^2)**		6.1		
Chlorophylle fluorescence [Y(II)] (arbitrar unit)	3341	3046		
Stomatal conductance (nmol m^{-2} s^{-1})		554.2		
Yield green plant mass (kg/5 plants/replicate)		0.91667		

Legend: * - classical method; ** - computational method

Intensity of photosynthesis has been estimated by the parameter **chlorophyll fluorescence.** Through evaluation of chlorophyll fluorescence output it is possible to obtain information on the changes in photochemical efficiency and in the dissipation of heat [MAXWELL & JOHNSON, 2000; PASK & al. 2012]. Measurements on *Passiflora caerulea*

leaves have been showed the following values of chlorophyll fluorescence: 3341 arbitrary units, at 10.06.2015 and as 3046 arbitrary units, for the measurements at 22.06.2015. These values are quite similar at the both moments (10.06.2015 and 22.06.2015), they have not significantly varied.

Stomatal conductance. This physiological parameter [MONTEITH & al. 1988; DEWAR 2002; PASK & al. 2012] for *Passiflora* was determined through porometric method, with a porometer Delta-T Devices AP4, based on gas diffusion through pores, especially through leaf stomata. Plant transpiration being adjusted by the opening and closing of stomata, using of these parameters is essential in many plant research domains. This is a measure of plant resistance to the loss of vapors through stomata and it is an indicator of plant physiological status. The equipment is working through the evaluation of the necessary time for a leaf to release sufficient water vapors for changing the relative humidity in a certain chamber with a fixed/standard quantity. Our determinations (Tab. 4.) showed values of 554.2 $m^{-2}s^{-1}$, which have been compared with PASK & al. (2012) results obtained for irrigated wheat. After these data, the normal values for this parameter in the wheat irrigated crop, in the conditions of Mexico, are 300-700 nmol $m^{-2}s^{-1}$, and values between v 80-300 nmol $m^{-2}s^{-1}$ show a reduced stress action on the plants [PASK & al. 2012]. As a conclusion, our data, the value of 554.2 nmol $m^{-2}s^{-1}$ is normal, belonging to the value interval 300-700 nmol $m^{-2}s^{-1}$.

The parameter **green mass production/yield** (leaves, shoots, stems) determined at the end of our experiment has been estimated of 0.91667 kg/5plants replicate (Tab. 5).

As a conclusion for this research group, our investigations have given us data for characterization of some physiological parameters (Tab. 5), as leaf area, leaf growing rate, coefficient k, chlorophyll fluorescence, stomatal conductance. Comparing with the literature [MOLGADO & al. 2013], coefficient k = 0.47 – 0.53 can be evaluated as moderate and sufficient for *Passiflora* plants specific characterization.

It has been estimate the chlorophyll fluorescence for *Passiflora* leaves over 3000 au (3046 – 3341 au). Photosynthetic mechanisms respond very quickly to most of the stress that plants encounter [TOTH & al. 2007; GAMA & al. 2011; STRIBET & GOVINDJEE, 2011; IZAGUIRRE & al. 2013; CHAGUT & al. 2014 a.o.]. Characteristics of gas exchange photosynthetic pigment determination and chlorophyll α fluorescence have been widely tested in many plant species as a parameter for the plant developments and for possible use in breeding programs to improve plant stress tolerance [GAMA & al. 2013].

Our data on stomatal conductance (554.2 nmol $m^{-2}s^{-1}$), compared with the literature [PASK & al. 2012] have showed normal value, between 300 and 700 nmol $m^{-2}s^{-1}$, for this parameter in an irrigated crop, as *Passiflora* is under the greenhouse conditions in Hofigal experimental field. All the physiological data are newly for the botanical and horticultural Romanian literature.

Phytochemical investigations

***Qualitative analyses*.** After performing the qualitative methods we obtained the results shown in Tab. 5. Phytochemical screening showed that glycosides were detected in both studied plant extracts of *Passiflora caerulea*. In turn, tannins and alkaloids could not be detected. We observed that the alcoholic extracts contained more bioactive compounds than petroleum ether extracts, and saponins were detected only in ethanolic extracts. In the ethanolic extract of *Passiflora caerulea* leaves, saponins, glycosides and triterpenoids were detected, while the petroleum ether extract contained only glucosides and polysaccharides.

Tab. 5. Qualitative bioactive compounds from the extracts of *Passiflora caerulea* (PE – petroleum ether extract; Et-OH – ethanol extract)

Phytoconstituents	PE	Et-OH
Glycosides	+	+
Triterpenoids	-	+
Polysaccharides	+	-
Saponins	-	++
Tannins	-	-
Alkaloids	-	-

***Quantitative analyses*.** Quantitative data showed that high amount of total polyphenols (expressed in mg gallic acid/ g dw) and flavonoids (expressed as mg quercitine/ g dw) were found in ethanolic extracts of *Passiflora* leaves. The highest value determined in *Passiflora* leaves ethanolic extract was 15.46 mg GA/g dw. Similar results were also obtained for flavonoids content, the maxim value being 12.82 mg Q/g dw (Fig. 30).

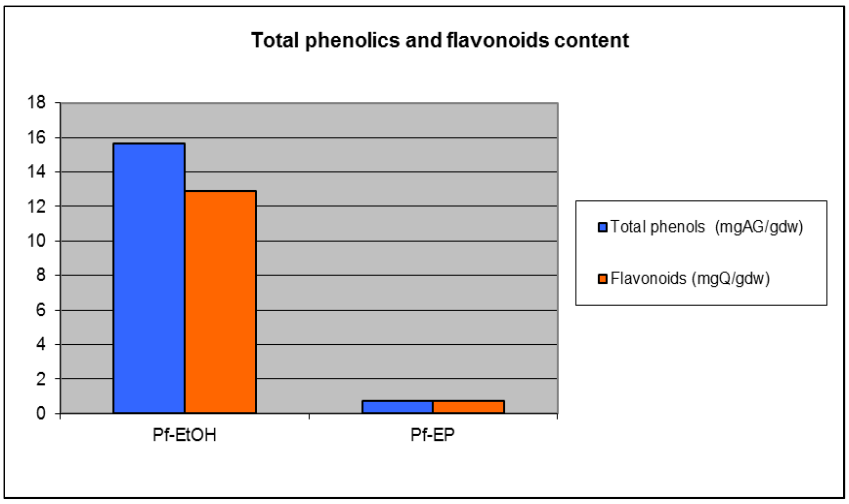


Fig. 30. The content of total phenols (determined by the Folin – Ciocâlteu) and flavonoids for ethanol (Pf - EtOH) and petroleum ether extracts of *Passiflora caerulea* leaves (Pf - EP)

Antioxidant capacity evaluation. Data obtained by the DPPH method shows that ethanol extract of *Passiflora caerulea* leaves had the largest content of free radical DPPH, with a concentration IC50 value of 54.01 µg/ml, similar to the standard BHT (57.16 µg/ml) (Fig. 31).

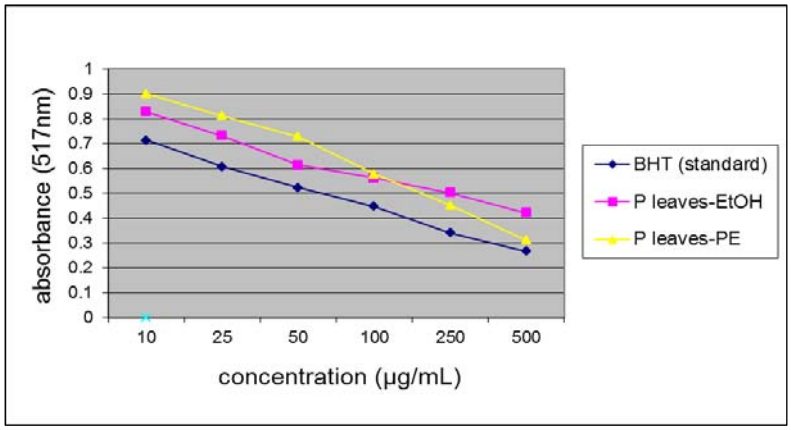


Fig. 31. Antiradical activity of *Passiflora caerulea* extracts measured by DPPH method

Similar results were obtained for the antioxidant capacity of plant extracts studied through inhibition of the radical ABTS assay. In this regard, *Passiflora* ethanol extract of leaves showed the highest antioxidant activity (79.77 ± 6.76 µmol TE / GMU) (Tab. 6).

Tab. 6. The antioxidant activity of the ethanolic and petroleum ether extracts of *Passiflora caerulea* leaves, determined by ABTS and DPPH methods

Analyzed extract	Trolox content (µmol/g dw)	DPPH test IC50 (µg/mL)
Passiflora caerulea leaves – ethanol 70%	79.77 ± 6.76	54.01
Passiflora caerulea leaves – petroleum ether	51.47 ± 4.69	289.16
BHT	-	57.16

Cell viability determination by Neutral Red method (NR).

Test results showed a higher values of cell viability for concentrations between 1-150 µg/ml *Passiflora caerulea* extracts, that did not inhibited cells growth at 24 hrs respectively 48 hrs, the cell viability being greater than 80% (98.3%, 97.86%, 94.26% and respectively 93.10%, compared to the control culture (considered 100%) (Fig. 32). After 48 hrs, we observed a slight decrease in the cell proliferation, but all values were maintained above 80% at the mentioned concentrations. Extract concentrations between 250 and 500 µg/ml affected the normal development of culture and had a toxic effect on individual cells. It was observed a significant inhibition on the cell viability, less than 70%, at 24 hrs and 48 hrs, respectively (Fig. 32).

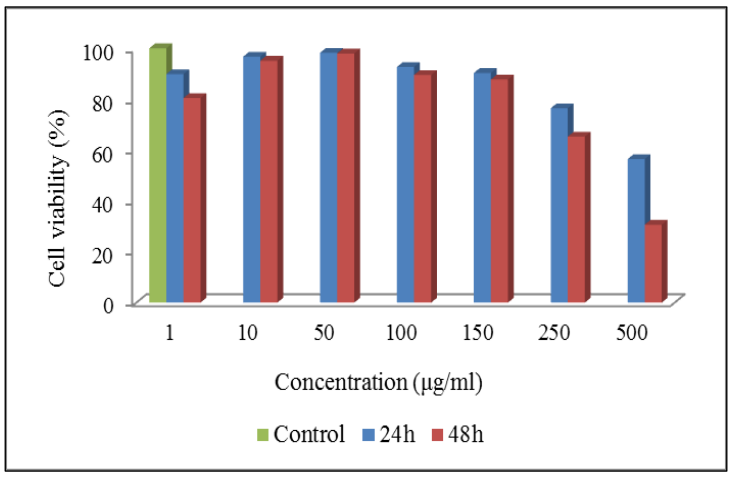


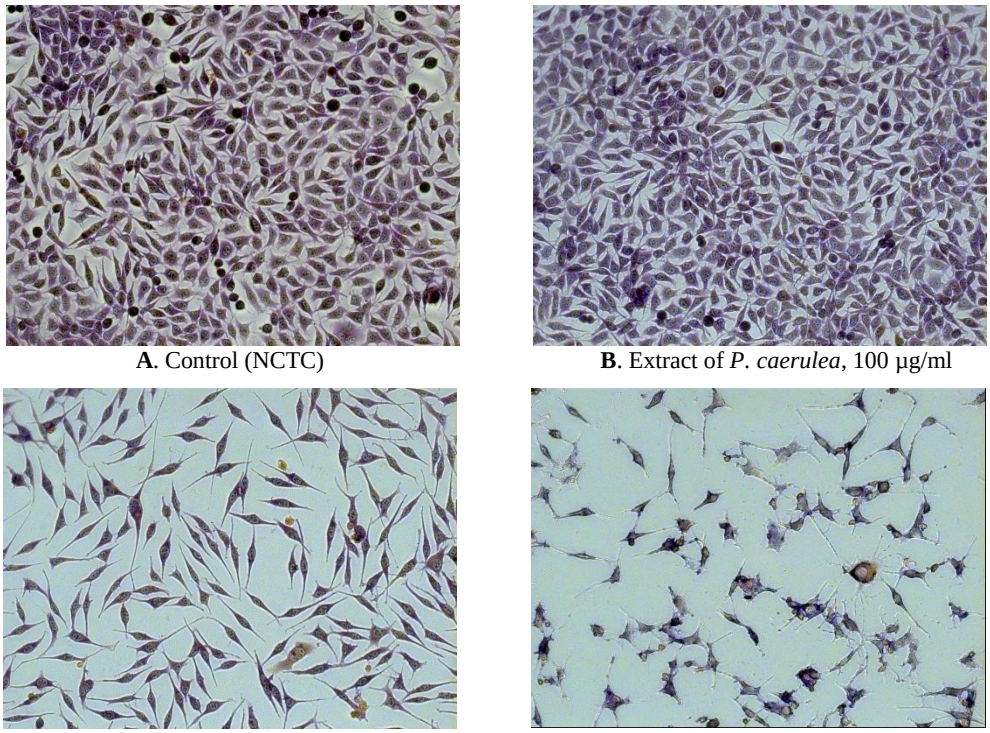
Fig. 32. The effect of *Passiflora caerulea* extracts on the culture of fibroblasts (NCTC clone 929), after 24 hrs respectively 48 hrs after cultivation, determined by Neutral Red method

The results obtained by Neutral Red method showed that *Passiflora caerulea* extract concentrations between 1 and 150 µg/ml did not inhibit cells growth at 24 hrs and 48 hrs, respectively, cells viability being greater than 80%. We also observed a slight decrease in cell viability at 48 hrs. At concentrations of 250 and 500 µg/ml, *P. caerulea* extract determined a decrease of cell viability after 24 hrs and 48 hrs, respectively.

Cell morphology analysis through light microscopy.

Our results indicated that the influence of different concentrations of *Passiflora* extracts on the NCTC cells morphology depend on the concentration. At extract concentrations between 1 and 150 µg/ml there are not an important changes in the cells morphology and density. Cells had a normal aspect, with 2-3 cytoplasmatic extensions, monochrome cytoplasm and very few rounded cells were observed (Fig. 33.B). At the concentration of 250 µg/ml it was observed an obvious decrease in cell density and the cells showed an altered morphology (Fig. 33.C). At the concentration of 500 µg/ml, *P. caerulea* extracts had a toxic effect, determined cells membranes integrity alteration and affecting the normal development of cell culture (Fig. 33.D).

In summary, our results have proved that: (i) The highest concentration in polyphenols and flavonoids was recovered in the ethanolic extracts of *P. caerulea*; (ii) A correlation between the polyphenols/flavonoids content of *P. caerulea* extracts and their antioxidant activity was observed. The highest values of antioxidant activity were calculated for the ethanolic plant extracts; (iii) The NR method showed a good biocompatibility of *P. caerulea* extracts in NCTC cell line, up to 10-150 µg/ml concentration, sustained by a normal cell morphology. At concentrations higher than 250 µg/ml, the plant extract become cytotoxic, altering the cell membrane structure, the cells viability and proliferation.



A. Control (NCTC) **B**. Extract of *P. caerulea*, 100 µg/ml

C. Extract of *P. caerulea*, 250 µg/ml **D.** Extract of *P. caerulea*, 500 µg/ml

Fig. 33. Effect of *P. caerulea* extracts on culture of fibroblasts NCTC at concentrations of 100, 250 and 500 µg/ml after 48 hrs of cultivation (Giemsa stain, 20X objective)

These data allowed us to select the optimal range of non-cytotoxic concentrations of the *Passiflora* extracts (less than 250 µg/ml) that will be use in further experiments.

Conclusions

Our complex investigation, morpho-anatomical, physiological, taxonomical and phytochemical on *Passiflora* nutraceutical plants from Hofigal experimental field, lead us to the following conclusions:

1. Structural aspects analyzed are important in characterization of *P. caerulea* samples: (i) Leaf lamina with a dorsi-ventral structure, hipostomatal and with lack of trichoms; (ii) Stomates of anomocitic and anizocitic types; (iii) Leaf petiole with an adaxial large kennel and a monosimetrical structure with vascular distinct fascicles; (iv) Irregular-ribbed stem which differentiates a secondary structures with concentric xylem and phloem rings, respectively; (v) Stem presenting collenchyma and sclerenchyma tissues; (vi) Leaves generating only collenchyma; (vii) Calcium oxalate crystals present in different cells from the leaf and stem structure of the druse type. In Romania, present histo-anatomical studies with taxonomic importance are the first ones with a significant highlighting to define more accurately the taxa of *Passiflora* genus. We will continue the botanical analysis, investigating a higher number of samples in order to evaluate additional identification aspects as: plant phenotypic plasticity, hybridization, influence of environmental and technological factors.

2. From **taxonomical point of view**, plant materials sampled for these studies from S.C. Hofigal Export-Import S.A. have been analyzed, obtaining a botanical evaluation that these plants belong taxonomical to *Passiflora caerulea* L., in concordance with the data of the monographers of *Passiflora* genus in the world: VANDERPLANK (2000) and ULMAN and MacDOUGAL (2004).

3. Physiological aspects determined in this approach have given us data for characterization of some parameters, as: (i) **Coefficient k** = 0.47-0.53, evaluated as moderate and sufficient for *Passiflora* plants specific characterization; (ii) **Leaf area** varied between 30 cm^2 and 34.1 (calculated by classic method), respectively 36.1 cm^2 (computational method); (iii) **Leaf growing rate** varied between 4.1 cm^2, approximately 0.29 cm^2/experimental day (classic method) and 6.1 cm^2, approximately 0.43 cm^2/day (computational method); (iv) **Chlorophyll fluorescence** was evaluated over 3000 arbitrary units (3046–3341); (v) **Stomatal conductance** (554.2 nmol $m^{-2}s^{-1}$), with a normal value (554.2 nmol $m^{-2}s^{-1}$), between 300 and 700 nmol $m^{-2}s^{-1}$, in an irrigated crop, as *Passiflora* is under the greenhouse conditions in Hofigal Company; (vi) **Yield green plant mass** (kg/5 plants/replicate) has reached in our experiment to an average value of 0.91667 kg/5 plants. All these results are new for the Romanian botanical literature.

4. Referring to the phytochemical approach, the influence of the extraction medium on the content of some active principles was analyzed and (i) the highest concentration in polyphenols and flavonoids was recovered in the ethanolic extracts of *Passiflora*; (ii) a correlation between the polyphenols/flavonoids content of *P. caerulea* extracts and their antioxidant activity was observed, the highest values of antioxidant activity being calculated for the ethanolic plant extracts; (iii) The NR method showed a good biocompatibility of *P. caerulea* extracts in NCTC cell line, up to 10-150 µg/ml concentration, sustained by a normal cell morphology. At concentrations higher than 250 µg/ml, the plant extract become cytotoxic, altering the cell membrane structure, the cells viability and proliferation. All these data are new for the Romanian phytochemical literature and allowed us to select the optimal range of non-cytotoxic concentrations of the *Passiflora* extracts (less than 250 µg/ml) that will be use in further experiments.

Authors contributions: T. E. ŞESAN coordinated and monitorized the project 160/2014, performed designed experiments, prepared the manuscript, analyzed and gave general interpretation of data (leaf morphology, physiological data, taxonomical evaluation, phytochemical data, photos), prepared periodical project reports, techno-manufactured the manuscript. A. SÂRBU and D. SMARANDACHE performed section of vegetal material, provided anatomical data and their interpretation for taxonomical evaluation, took microscopic photos; F. OANCEA prepared and supervised finishing of manuscript. A. OANCEA, S. SAVIN, A. TOMA and L. ŞTEFAN performed phytochemical analyses and their interpretation. G. NEGRU, A. F. BIRA and G. VLĂSCEANU applied in the experimental field of Hofigal the experiment designed by the coordinator of project, managed *P. caerulea* crop and provided samples for laboratory analyzes. M. GHIUREA, G. VASILESCU and L. JECU obtained and interpreted physiological experimental data. C. M. POMOHACI analyzed statistically experimental data.

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Explanation of plates and figures

Fig. 1. A. Working A4 squared sheet of paper/page used for classical method of leaf area estimation; 1.B. *Passiflora caerulea* simple, palmat-partite leaves (herborized and scanned) (Şesan, 2015)
Fig. 2. Main medicinal and nutraceutical importance of *Passiflora* spp.
PLATE I (photo: Anca Sârbu) – *Passiflora caerulea* – LAMINA
Cross section through median zone of the leaf segment, colorants Iodine Green and Carmine Alum (Fig. 3)
Cross section through the median zone of leaf segment, with evidence of epidermis and mesophyll, colorants iodine green and Carmine alum (Fig. 4)
Adaxiale epidermis (superior) in apical image (Fig. 5)
Abaxiale epidermis (inferior) in apical image (Fig. 6)
Abaxiale epidermis in apical image, with evidence of calcium oxalate crystals (polarized light) (Fig. 7)
Abaxiale epidermis in apical image, with evidence of calcium oxalate crystals (polarized light) (Fig. 8)
PLATE II (photo: Anca Sârbu) – *Passiflora caerulea* – PETIOLE
Cross section through petiole, colorants Iodine Green and Carmine Alum (Fig. 9)
Cross section through petiole, colorants Iodine Green and Carmine Alum (Fig. 10)
PLATE III (photo: Anca Sârbu) – *Passiflora caerulea* – STEM
Cross section through stem, colorants Iodine Green and Carmine Alum (Fig. 11)
Cross section through stem, with evidence of epidermis, cortex and of central cylinder elements, colorants Iodine Green and Carmine Alum (Fig. 12)
PLATE IV (photo: Tatiana Eugenia Şesan, 30.04.2015) – *Passiflora caerulea* – TAXONOMIC CHARACTERS
General aspect of the *Passiflora caerulea* vine in the experimental field of Hofigal (climbing vine, flowers, floral calyx, 5-diveded lobate leaves) (Fig. 13)
Passiflora stipules and tendrils (Fig. 14-15**)**
Passiflora stipule (Fig. 17)
Passiflora stipule and 2 nectary glands at the petiole base (Fig. 18)
Passiflora tendrils (Fig. 16, 19, 20)
PLATE V (photo: Tatiana Eugenia Şesan, 30.04.2015) – *Passiflora caerulea* – TAXONOMIC CHARACTERS
Passiflora caerulea flowering in the greenhouse at experimental field of Hofigal (climbing vine, flowers, floral calyx, 5-diveded lobate leaves) (Fig. 21)
Passiflora caerulea flower, 5-lobed leaf, tendrils (Fig. 22)
Passiflora caerulea white flowers, corolla white; sepals 5, white to pale, calyx tube cup-shaped, petals 5, similar as form and size with sepals; petals white inside and outside, corona (petals) disposed in 2 verticils, extern verticil consisting in many filiform appendices (radia), purple at the base, in the middle white and blue at the apex, corona filaments in 4 series, outer 2, purple at the base, then white and blue towards apex, gynophore well developed; stamens 5, unilocular ovary, styles 3, reddish (Fig. 23 – 25)
PLATE VI (photo: Tatiana Eugenia Şesan) – *Passiflora caerulea* – TAXONOMIC CHARACTERS
Passiflora caerulea floral buds in the experimental field of Hofigal (26.07.2016) (Fig. 26)
Passiflora caerulea at the phenofase of green fruits (16.10.2012) (Fig. 27)
Passiflora caerulea at the phenofase of orange fruits at the ripe status: 07.09.2016, left (Fig. 28); 26.07.2016, right (Fig. 29)
Fig. 30. The content of total phenols (determined by the Folin – Ciocâlteu method) and flavonoids for ethanol (Pf - EtOH) and petroleum ether extracts of *Passiflora caerulea* leaves (Pf -EP)
Fig. 31. Antiradical activity of extracts of *Passiflora caerulea* measured by DPPH method
Fig. 32. The effect of *Passiflora caerulea* extracts on the culture of fibroblasts (NCTC clone 929), after 24 hrs. respectively 48 hrs. after cultivation, determined by neutral red method
Fig. 33. Effect of *Passiflora caerulea* extracts on culture of fibroblasts NCTC at concentrations of 100, 250 and 500 mg/ml after 48 hrs. of cultivation (Giemsa stain, 20X objective)

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MORPHOLOGICAL AND MICROMORPHOLOGICAL INVESTIGATIONS REGARDING THE LEAVES OF SEVERAL *ROSA* L. SPECIES

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Abstract: Multicellular secretory glands are present on *Rosa* L. species, at least on the stipellae edge; some of them have a larger number and different particularities.
The researches have been made on leaves of the following species: *Rosa agrestis*, *R.* x *damascena*, *R. multibracteata*, *R. pimpinellifolia* and *R. rubiginosa*.
Micromorphological studies emphasized a large number of cells which forming both terminal secretory part and foot, even though their dimensions are small.
These researches highlight the micromorphological aspect of these glands, tector hairs and epicuticular wax. All of them were examined through scanning electron microscopy method.

Key words: epicuticular wax, micromorphology, *Rosa*, secretory glands, tector hairs.

Introduction

The classical literature concerning to morpho-anatomy of *Rosa* L. genus mentioned both names, either "secretory gland" (especially in plant morphology papers) [BELDIE & PRODAN, 1956; CIOCÂRLAN, 2009], or "glandular trichomes" (especially in plant anatomy papers without express reference to *Rosa* genus) [FAHN, 1987]. The multitude of writings about *Rosa* L. species places secondly after cereals [BELDER & MISSONE, 1994]. Furthermore, there still are things to clarify.

The present scientific literature present many electron-microscopy researches for many genera and, some of them, are about rings of few *Rosa* species or varieties. Likewise, epicuticular wax has been studied at a limited number of species [WERLENMARK & al. 1999; WISSEMANN, 2000]

Often, secretory emergencies from *Rosa* are treated as "glandular trichomes" [CAISSARD & al. 2006; HASHIDOKO & al. 2001; SULBORSKA & WERYSZKO-CHMIELEWSKA, 2014].

It can be observed that these emergencies are pediculous formations, which has a foot with variable length, branched or not, multiseriate (generally 12-13 series of cells), multicellular (each series being composed of 8-12 cells for short pediculous glands from the teeth of leaflets and for some stipellae, 30-40 cells in glands with branched foot from *R.* x *damascena*, *R. multibracteata*, *R. pimpinellifolia*). These pediculous formations shows, most often, terminal but sometimes from the side, on their pediculous ramifications, a secretory part more or less globulous or hairy-shaped; this character varies with species. The external

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layer of secretory part consists of at least 40 cells. For these reasons we agree with the term "secretory gland" instead of the term "glandular trichomes" [GOSTIN & ADUMITRESEI, 2010]. Secretory emergences found in *Rosa L* are structures more massive than the ones described as glandular trochomes from *Acer campestre* [ASCENSAO & PAIS, 1985], *Hibiscus permambucensis* [ROCHA & MACHADO, 2009], *Salvia officinalis* [VENCLATACHALAMK & al 1984].

Material and methods

The **investigated material** is from collection of The Botanical Garden "Anastasie Fătu" from Iaşi. We have studied the following species: *Rosa agrestis* Savi, *R.* x *damascena* Herrm., *R. multibracteata* Hemsl. & E. H. Wilson, *R. pimpinellifolia* L. (syn. *R. spinosissima* L.), *R. rubiginosa* L. Among them, *R. agrestis*, *R. pimpinellifolia*, *R. rubiginosa* are spontaneous species from Romanian Flora and *R.* x *damascena* and *R. multibracteata* are non-indigenous species. The investigated species belong to a polyploid series. Some of them are tetraploid species (for example *R.* x *damascena*, considered by many authors with hybrid origin), *R. multibracteata* and *R. pimpinellifolia*, or pentaploid species (for example *R. agrestis* and *R. rubiginosa*).

Microphotographs that refer to morphologic aspects of a leaf have been made at Karl Zeiss Jena eyeglass with an Olympus SP 500UZ camera.

Scanning electron microscopy (SEM) investigations – fragments of leaves, rachis and stipellae were fixed in FAA in ethanol 50% for 48 hours, stored in 70% ethanol (Johansen 1940). After dehydration in a graded ethanol series, the material was dried at critical point with CO_2, sputter-coated with a thin layer of gold (30 nm) and, finally, examined in a scanning electron microscopy (Tescan Vega II SBH) at an acceleration voltage of 27.88 kV.

Results and discussion

The leaf is odd-pinnate composed by 5-9 leaflets, several at R. *multibracteata*, *R. pimpinellifolia*, and *R. rubiginosa*.

The leaflets shape is different from oblong-elliptic to cuneate-obovate at *R. agrestis*, oblong-ovate at *R.* x *damascena*, wide-ovate at *R. multibracteata*, elliptical at *R. pimpinellifolia*, and ovate at *R. rubiginosa*.

The leaflets edge is either double-glandulous serrate (at *R. agrestis*, *R. pimpinellifolia* and *R. rubiginosa*) or simple serrate (at *R.* x *damascena* and *R. multibracteata*). Most often, on the teeth of leaflets are present glands or hydathodes.

The leaflets have different size: from 1.4/0.7 cm (at *R. pimpinellifolia*) and 2.2/3 cm (at *R. multibracteata*), which are the smallest leaflets, up to 6/3 cm (at *R.* x *damascena*), which are the largest leaflets.

The leaflets surface is glabrous on both sides (i.e. *R.* x *damascena* and *R. multibracteata*), short-hairy on the upper surface (i.e. *R. agrestis*, *R. pimpinellifolia* and *R. rubiginosa*), hairy and glandulous on the lower surface (at *R. agrestis*, *R. pimpinellifolia* – which have spherical glands with a foot slightly longer than in other species, or *R. rubiginosa* – which have glands located only on the nervure of all orders). On the median nervure, both hairs and glands are more numerous than in other segments of leaf, for all investigated species.

Usually, leaf rachis presents thorns typical for species on abaxial surface, very common at *R.* x *damascena* (it has thorns with a disposition in 2-3 parallel rows and some of them ending with a gland), and at *R. pimpinellifolia* (with acicular thorns and some glands have either a relatively short foot or an extremely long and lignified foot, seeming to be acicular-like with a gland on extremity). Generally, hairs and glands are more abundant on rachis than on leaflets surface, except the adaxial channel, where they are missing or they are rarely and shorter. Also, hairs and glands have an increased frequency on petiolules in the case of species where they are present.

The stipellae are fused with leaf petiole and they have a specific shape for each species. Thus, at *R. agrestis* stipellae are slightly rounded in the middle, with auricles oriented apart, with a rich-glandulous and hairy edge. The glands are situated side by side, which secretory spherical part and foot with different lengths (but not too long). The glands are reddish with a translucent secretion that smells like green apples (Pl. I, Fig. 1). In the same time, the stipellae of *R.* x *damascena*, are long and not too wide (2.1/0.6 – 0.7 cm), with parallel edges and auricles with a parallel disposition to leaf rachis; edges presents glands and tector hairs. At *R. multibracteata*, stipellae are very short (less than 1 cm length) and relatively narrow, with parallel edges, which have relatively rare glands, but more frequent on auricles. They have short foot, are approximately spherical and red colored at maturity. During the young stage, gland secretion is translucent (Pl. I, Fig. 2) and at *R. pimpinellifolia* stipellae are narrow, with widened auricles and with an external orientation, with glands both on their edges and lower side. Stipellae have glands on terminal side. They are almost spherical, slightly flattened on the top, and the foot has variable length (Pl. IV, Fig. 21). At *R. rubiginosa*, stipellae have parallel edges and rounded auricles, with outward disposition with medium size (1.5 - 0.5 cm), they have high-glandulous edges with spherical gland and foot with different length (but relatively short compared to that of other species and varieties). The secretion of these glands smells like green apples and it is translucent (Pl. V, Fig. 25, 26).

We have to mention that, glands begin to regress at some point, and in most cases it overlap with dropping flowers and, rarely, the glands remain active much later - *R. agrestis*, *R. pimpinellifolia*, *R. rubiginosa*. The regression only partially and somewhat later than flowering of glands from leaf can be explained by the fact that a significant part of these gland secretions are sesquiterpenes, which are not characteristic secretions of flower, leaf by going to its metabolism beyond the flower (flavones and volatile oils are characteristic for both leaves and petals) [ADUMITRESEI & al. 2009; CAISSARD & al. 2006; HASHIDOKO & al. 2001; ZAMFIRACHE & al. 2006].

Micromorphology of leaf appearance highlights the peculiarities of the investigated species. Thus, the species more closely related genetically are *R. agrestis* and *R. rubiginosa* and they have some similarities.

R. agrestis presents clavate secretory glands (stipitate) on leaflets underside, along some different orders of nervure, leaflets teeth, stipellae edges, petiole and rachis (Pl. I, Fig. 3-6).

On the lower surface of leaflets, on petiole and rachis glands alternate with tector hairs: on leaflets are more glands, while on rachis there are more hairs; only glands are present on stipellae edges.

At the young leaf, glands are very close to each other; as much as leaf grows and epidermal cells between nervures are dividing, these distances became larger.

Glands have pluriseriate and pluricellular feet; their terminal part are under-globulous and it has a small central concavity at the apical extremity. The glands are of two

types: some of them are big, with a foot length between 40-50 μm, and the diameter of secretory part is between 110-130 μm; the others have the diameter of secretory part between 80-100 μm. Glands situated on rachis have slightly longer foot, reaching up to 65 μm. On the stipellae edges a gland with a longer foot alternating with a small one with a shorter foot.

Glands situated on leaflets teeth have a very short foot (15-20 μm).

The number of cells from the external layer are more than 40.

The tector hairs are numerous on the upper surface of leaflets and rachis; they are missing on stipellae (Pl. I, Fig. 3-6) and they are very rare on the leaflets underside. The hairs from those two epidermis from leaflets are relatively long (200-250 μm) and flexuous. The hairs from rachis are of two types: some are long (250-500 μm) and rare, and the others are short (30-90 μm) and more frequently.

Epidermal cells from around the base of tector hairs form a slight protuberance around them.

At *R. rubiginosa*, clavate secretory glands are present in the same leaf segments with some peculiarities. Both on rachis and underside leaflets, and on stipellae edges, the glands alternate with tector hairs; on rachis, hairs are more frequent than glands, and on leaflets and stipellae proportions of these parts are balanced, tilting slightly for glands (Pl. V, Fig. 25, 26, 28, 30).

The glands of this species are somewhat smaller compared with *R. agrestis* having foot length of 40-50 μm, and diameter of secretory part is around 100 μm. At *R. agrestis*, glands from leaflets teeth have an extremely short foot (about 20 μm), and some glands from rachis have a foot much longer than the majority of them (about 180 μm), as the secretory part (170 μm).

Form of the secretory part is almost globulous (in the case of glands situated on leaflets and stipellae, but even some glands situated on rachis) with a slight central protuberance on apical pole and under-globulous protuberance on glands with a long foot on the rachis.

The number of cells from external layer of secretory part varies between 20 and 50, and this number is correlated with gland size.

On leaflets teeth hydathodes are present (Pl. V, Fig. 29).

Tector hairs are present on both sides of leaflets, on stipellae edges and rachis. Also, some of them are long (up to 200 μm) and flexuous, while others are shorter (40-100 μm) and straight (Pl. V, Fig. 27, 30).

Rosa pimpinellifolia also shows a large number of glands on all leaf segments, and it has a specific series of particular aspects.

Some glands have a relatively short foot, and others had extremely long and lignified foot (seeming to be a needle-like emergence with a gland at its extremity) (Pl. IV, Fig. 19, 20, 22, 23).

On the stipellae edges under-globulous glands are present, with a foot of different size, gland diameter varies between 120 and 150 μm.

Also, at this species the branching of foot glands can be noticed; the ramifications ends with the same type of glands. The foot which shows lateral branches is extremely long (up to 1600 μm), and it have stretched strong base (270-300 μm), but also its upper part is considerably widened (about 150 μm) in which the foot thickness is adjusted, this part is similar with gland foot for the most investigated species. Such foot shows 2-3 lateral branches (on both sides) with typical glands (Pl. IV, Fig. 21).

In addition, on stipellae edges can be observed globulous glands; they have a long foot (420-680 μm) and widened to near the top (140-100 μm), and also common globulous glands with short foot (90-100 μm) or average (250 μm) and with the same thickness throughout its length, gland diameter varying around 110-120 μm. The upper part of the gland is slightly concave.

R. x *damascena* and *R. multibracteata* species presents only few glands located on stipellae edges, and on rachis at *R.* x *damascena* species.

Thus, at *R.* x *damascena* on stipellae edge, both secretory glands and tector hairs are present, these last in a smaller number.

The glands are of two types: some are globulous (with short foot by 80 mm, and gland by 80-90 μm) and others are piriform (with foot long 300 μm, and 150 x gland axes 90-110 μm). The bottom part of the pyriform gland foot is stretched. The top of globulous gland is, in this case, as a perfect sphere (Pl. II, Fig. 9-12).

Tector hairs have a medium length (250-270 μm).

At *R. multibracteata*, in the adaxial channel of the petiole (the area where it accrete with stipellae) are present short and thick hairs, which are rarer on rachis.

The rachis presents glands (rare and only on lower surface) and tector hairs, especially on insertion points of leaflets; tector hairs are present in adaxial channel.

This specie presents on the stipellae edges both pyriform and globulous glands, both categories are active in young leaf, the pyriform glands ending his their activity first (Pl. III, Fig. 15-18).

The pyriform glands have a foot slightly widened at the bottom, with a medium length (450-600 μm) and glands with axes 250-260 x 100 μm.

Globulous glands have a shorter foot (150 μm) and the secretory part about 100 μm being strongly curved at the top.

Epicuticular wax is of granular type at *R. agrestis* (Pl. I, Fig. 6), *R.* x *damascena* (Pl. II, Fig. 12) and *R. multibracteata* (Pl. III, Fig. 18) species, and of triangular type at *R. pimpinellifolia* (Pl. IV, Fig. 24).

Stomata length varies between 15 μm at *R. agrestis* (among the lowest compared to other investigated species), 20 μm at *R.* x *damascena* and *R. multibracteata*, 25 μm at *R. pimpinellifolia*; in the case of *R. rubiginosa* stomata have variable lengths, between 15 and 20 μm.

Conclusions

Secretory glands are spread on leaves in all cases, the differences are concerning to number, shape and scattered thereof (only on the stipellae edges and/or leaflets teeth, rachis, median nervure, etc.). Presence, shape, size and disposition of glands (characteristic for some other species of Rosaceae family) are characters used for diagnosis and they are very interesting by their own great variability within the *Rosa* genus.

On the stipellae edge glands are present in all species. At some species (*R. agrestis*, *R. pimpinellifolia* and *R. rubiginosa*), both hairs and glands are present in all leaf segments, in different proportions. However, in most species, leaflets are hairless, tectors and secretory formations are located only on the stipellae edges, rachis, and sometimes on median nervure and leaflets teeth.

The shape of secretory glands is generally elliptical or under-globulous, with variations between them, sometimes encountered both forms at the same taxa (*R.* x *damascena* and *R pimpinellifolia*).

Secretory glands are present in many cases even on rachis at *R. agrestis*, *R.* x *damascena*, *R. pimpinellifolia* and *R. rubiginosa*, their frequency considerably increases at their insertion points of leaflets and on their petiolules.

On rachis, glands can alternate with hairs as at the following species: *R. agrestis*, *R.* x *damascena*, *R. multibracteata* (where glands are rare) and *R. rubiginosa*.

The leaflets teeth are ending with glands, as in the following species: *R. agrestis*, *R. pimpinellifolia*, *R. rubiginosa* (where glands alternating with hydathodes). The presence of hydathodes in large numbers on leaflets teeth was observed at *R. rubiginosa*.

Secretory glands at *Rosa* present a pluriseriate and multicellular foot, and its clavate terminal part is either globulous or under-globulous in some cases; either elliptical-oblongated or piriform in some other cases. Among the investigated taxa, some of them show only under-globulous to globulous glands (*R. agrestis*, *R. rubiginosa* and *R. pimpinellifolia*).

R. x *damascena* and *R. multibracteata* species present both type of glands: more or less globulous and elliptical to piriform.

The branching of foot in elliptical or pyriform gland is present at *R. multibracteata* and *R. pimpinellifolia*.

The length of the foot glands vary within some wide limits, from 20 μm in under-globulous glands from leaflets teeth of *R. agrestis*, to 1600 μm from stipellae edges glands from *R. pimpinellifolia*. Broadly, globulous and under-globulous glands (unless the case shown above) have shorter foot which ranging from 20 to 200 μm, and elliptical glands present a foot longer ranging from 300 to 600 μm.

The diameter of under-globulous to globulous secretory part varies between 80 μm (at *R. agrestis*) and 180 μm to those on the rachis (at *R. rubiginosa*).

The dimensions of secretory part of elliptical or piriform glands ranges from 90 to 110 μm for minor axis and from 150 and 260 μm for major axis.

In terms of number of cells which forming external layer of secretory glands part, it varies between 40 and 50, and it is correlated with glands size.

The number of cells (in cross-section) that forms a foot gland is approximatively 12-13, the foot external layer which consists of some series by at least 8 to 12 cells.

The tector hairs have different lengths ranging: from 30 to 90 μm for short hairs (*R. agrestis* and *R. rubiginosa*); from 250 to 270 μm for long hairs (*R.* x *damascena*); 200-500 μm for hairs present on rachis (*R. agrestis*). At the same species was observed both short hairs and long hairs.

Generally, epidermal cells forming a slight protuberance around tector hairs.

The stomata are either irregular dispersed among epidermal cells (*R. agrestis*, *R. pimpinellifolia* and *R. rubiginosa*), or with a grouped disposition on hybrids (*R.* x *damascena*).

In terms of cell stomata length, we distinguished species with smaller stomata (15-20 μm): *R. agrestis* (not exceeding 15 μm); *R.* x *damascena*, *R. multibracteata* and species with larger stomata (greater than 25 μm) as *R. pimpinellifolia*.

The epicuticular wax could be of granular type (*R. agrestis*, *R.* x *damascena*, *R. multibracteata* and *R. rubiginosa*), and by triangular type (*R. pimpinellifolia*).

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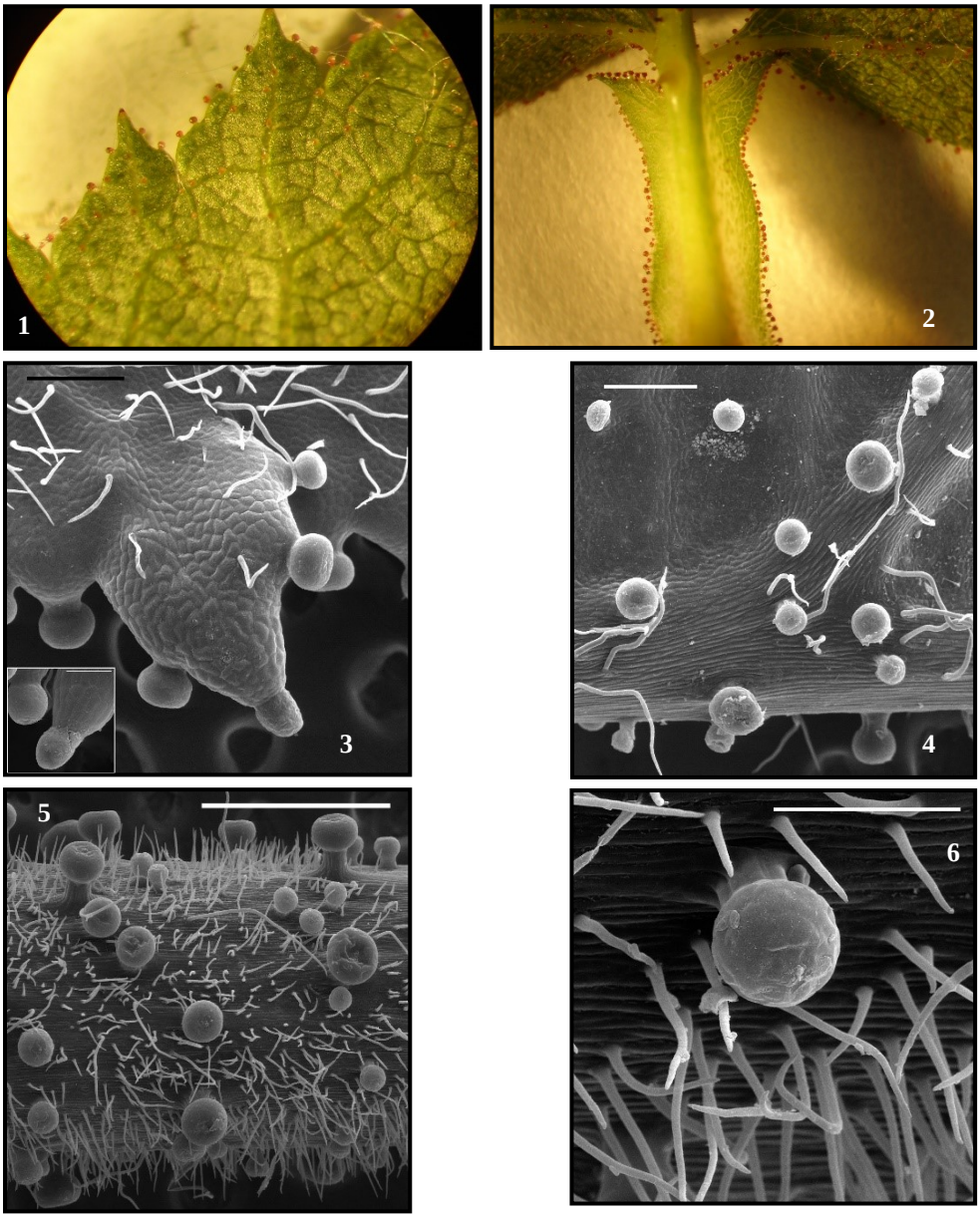
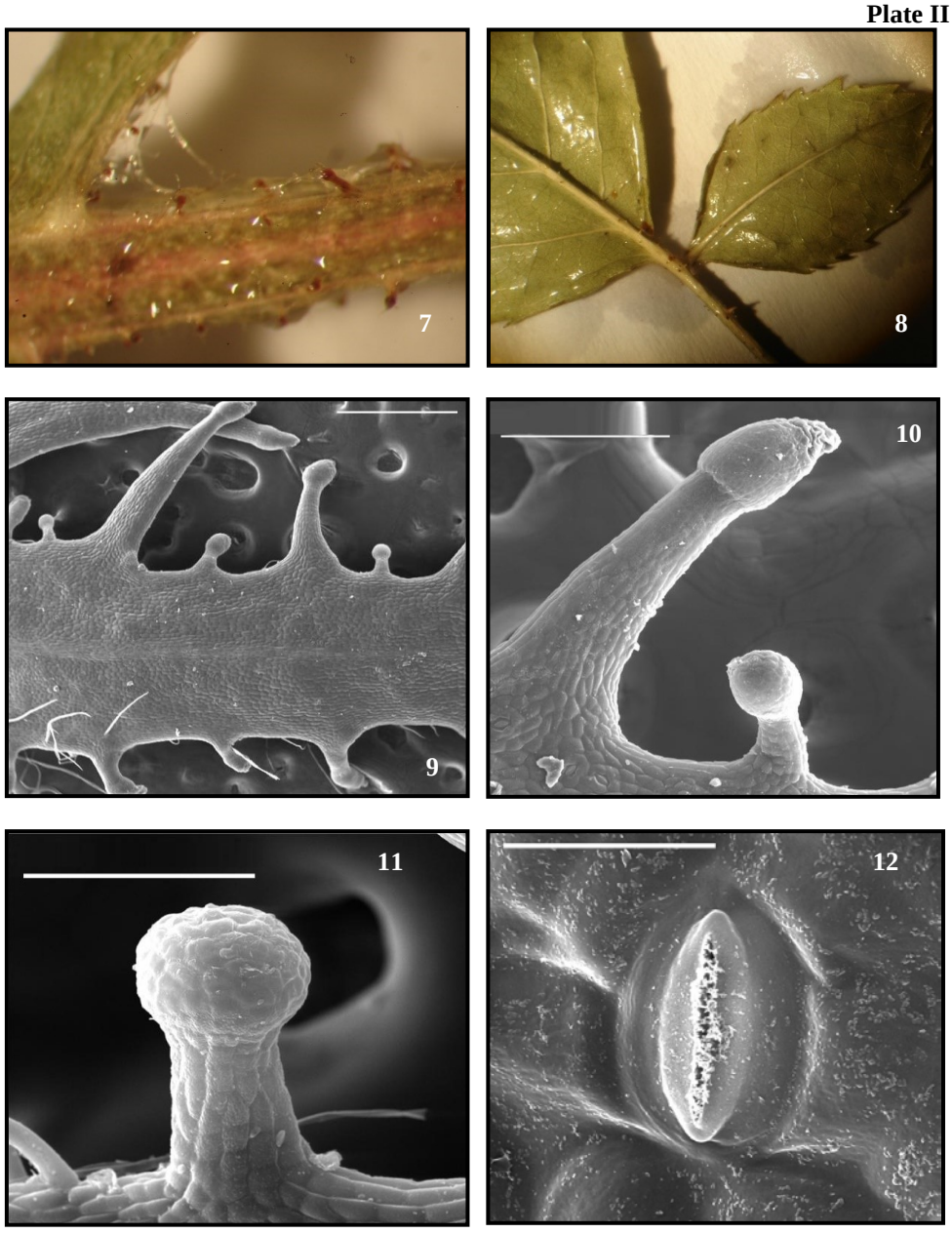
Plate I



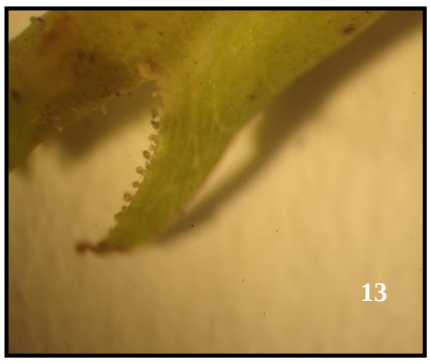
***Rosa agrestis*: 1)** leaflet, lower epidermis (scale bar 6.3 x 2.5); **2)** leaflet (6.3 x 1.6); **3)** margin of leaflet (200 µm, details = 100 µm) (ADUMITRESEI & al. 2009); **4)** leaflet (200 µm) (ADUMITRESEI & al. 2009); **5)** rachis (500 µm); **6** rachis (100 µm).

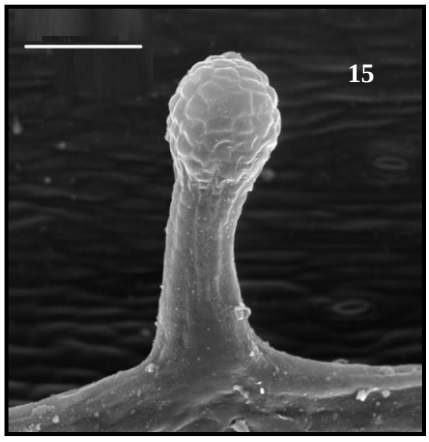
Plate II

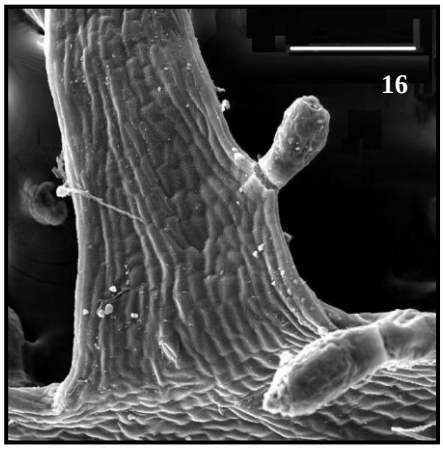


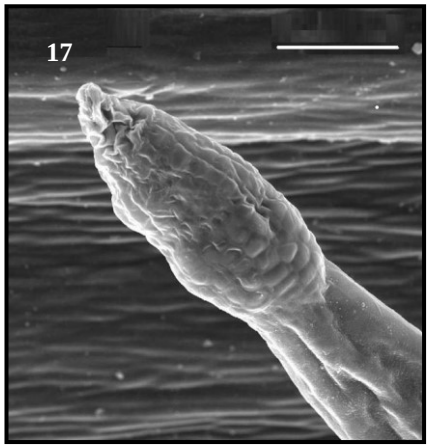
***Rosa* x *damascena*: 7)** stipellae and rachis (6.3 x 4); **8)** leaflets (6.3 x 1.6); **9)** stipellae (500 µm); **10)**, **11)** stipellae glands (100 µm); **12)** epicuticular wax and stomata (20 µm).

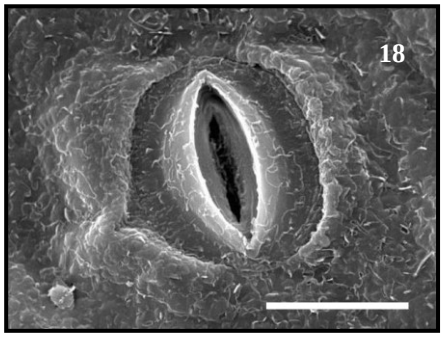
Plate III





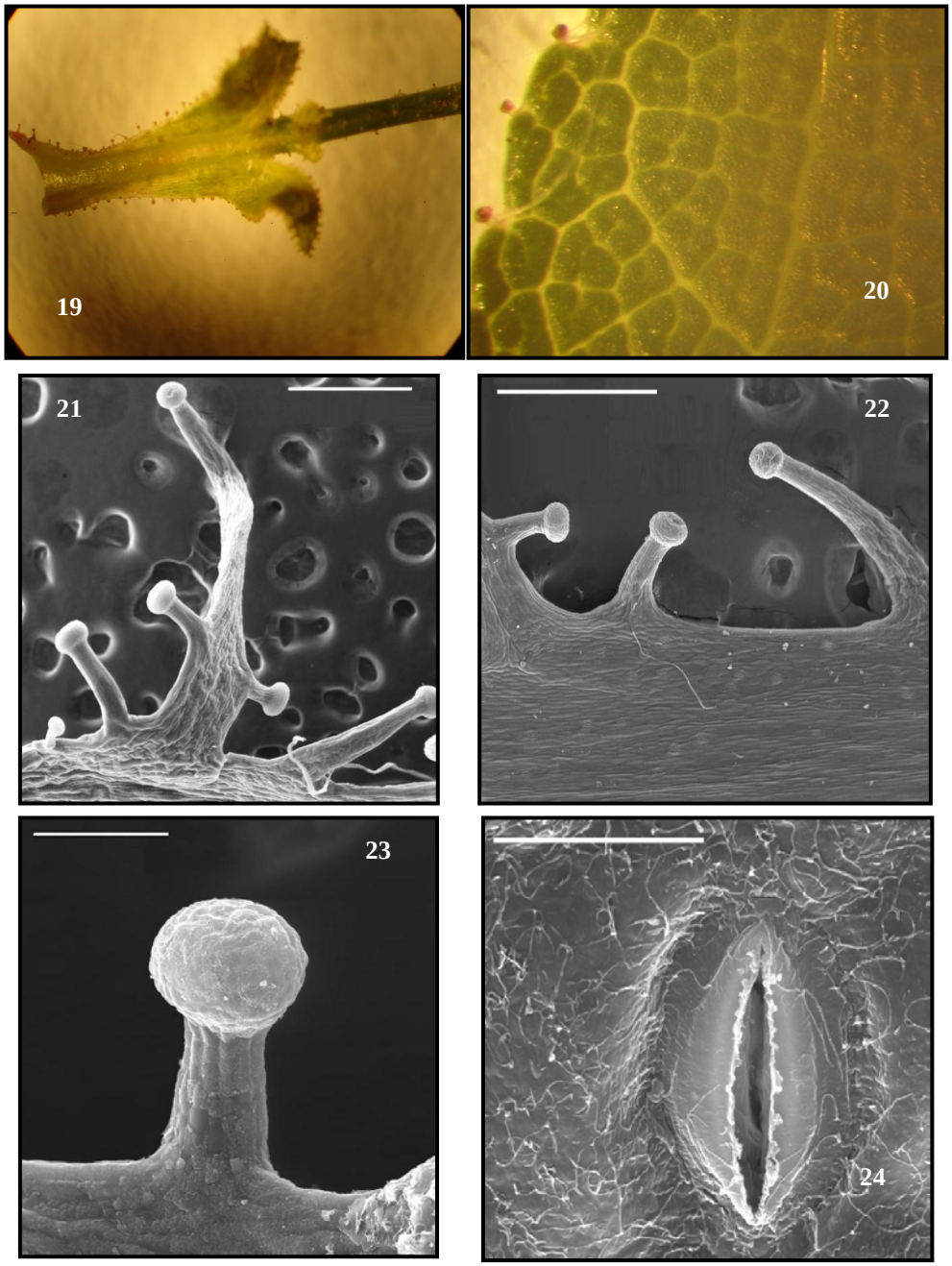






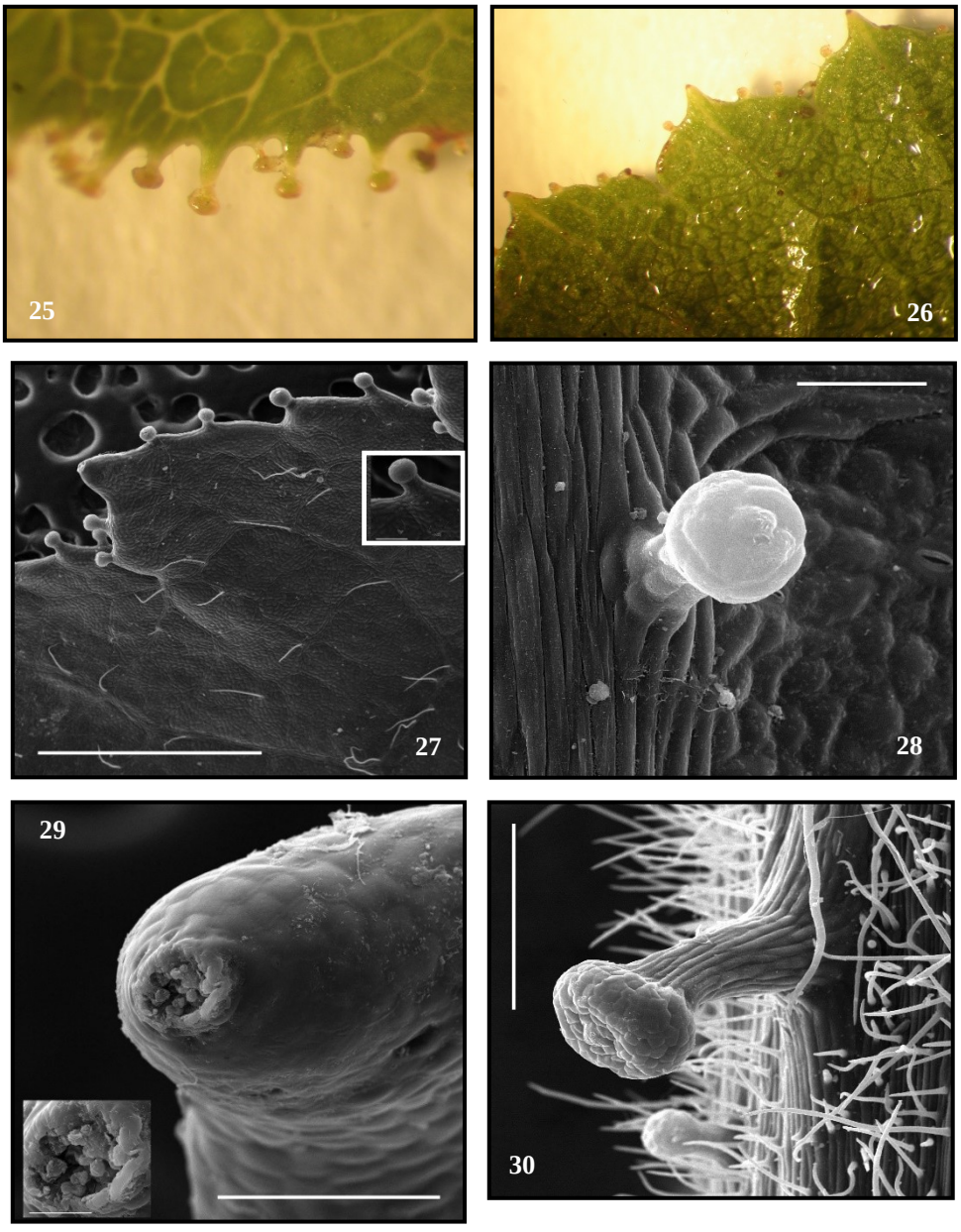
***Rosa multibracteata*: 13)** stipellae (6.3 x 4); **14)** leaflet (6.3 x 1.6); **15)** stipellae (200 μm); **16), 17)** stipellae glands (100 μm); **18)** epicuticular wax and stomata (20 μm)

Plate IV



***Rosa pimpinellifolia*: 19)** stipellae (6.3 x 1.6); 2**0)** leaflet (6.3 x 4); **21)** stipellae glands (50 µm); **22)** stipellae glands (500 µm); **23)** stipellae glands (100 µm); **24)** epicuticular wax and stomata (20 µm).

Plate V



***Rosa rubiginosa*: 25)** leaflet (500 μm); **26)** mature leaflet (50 μm); **27)** mature leaflet (1 mm and 100 μm) (ADUMITRESEI & al. 2009); **28)** leaflet gland (500 μm); **29)** leaflet hydathode (100 μm and 20 μm), **30)** rachis (500 μm).

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THE MACROMYCETES OF THE DOWNY OAK FORESTS FROM MOLDOVA

Ştefan MANIC[1]

Abstract: The article contains original data, accumulated over several years, about mushrooms from the forests of downy oak, inventoried during the growing seasons in 1976-2015. The paper includes a systematic list of all the species inventoried to date, with short eco-chorological descriptions. The rare and protected species are also listed.

Keywords: chorology, downy oak forests, macromycetes.

Introduction

The downy oak associations are spread, mainly, throughout the southern part of Moldova, where they can be found in separate small areas. Insignificant areas of downy oak forests are also found in the southern part of "Codri", alongside Dniester and Prut rivers, on southern slopes.

The first layer of stands consists, mainly, of downy oaks. The distribution of woody species is uneven; trees are arranged in groups, forming groves alternating with glades. Rarely, in the first layer, pedunculate oak, sessile oak, Tatarian maple, common pear and sweet cherry can be found [POSTOLACHE, 1995].

An important part in the distribution of mushrooms throughout the phytocoenoses of downy oaks is played by the placement of trees in groups. Based on that, the saprotrophic foliicolous fungi and the mycorrhizal fungi are met in groves, while in the glades, among groves, there are favourable conditions for the development of sporophores and species of the genera *Hygrocybe, Lepiota* and *Macrolepiota*, which prefer open areas [MANIC, 1980].

Materials and methods

The research on macromycetes from downy oak forests was systematic, taxonomic, chorological and ecological in nature. Each of the studied issues was scientifically documented by examining a wide range of specialized literature: [EYSSARTIER & ROUX, 2011; GALLI, 1996; KIRK & al. 2008; MOSER, 1993; SĂLĂGEANU & SĂLĂGEANU, 1985; SINGER, 1986].

The field research started with the delimitation of the territory to be investigated and the establishment of the itineraries to be covered, according to methodological recommendations [CONSTANTINESCU, 1974; VASILIEVA, 1965].

The sampling of biological material was carried out during the years 1976-2015, according to the methodical recommendations [COURTECUISSE, 2000], in downy oak forests from southern Moldova and not too large, separate stands of downy oak which are

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also found in the southern part of "Codri" and the along Dniester and Prut rivers, on southern slopes.

Macromycetes were collected from the appearance of the first mushrooms until late autumn, in different biotopes from the territory of the Republic, in various stages of development and in all seasons. This was preceded by the macroscopic analysis, on the spot, of sporophores with the registration of all phenotypic characters as follows: size, shape, colour and area of the cap, type of hymenophore and spore print, type of universal and partial veil, shape and colour of the stipe (stem), presence or absence of the ring, flesh and its consistency, colour and changes caused by air, taste, smell, presence/absence of latex etc. [MOSER, 1993].

The macro-chemical reactions of various structural-functional components of sporophores, especially the cuticle and the external part of the stipe, the hymenophore, the spores and the latex were studied with particular attention. The changes that took place as a result of the action of chemicals, which are incontestable taxonomic characters, have also been studied: [COURTECUISSE, 2000; MOSER, 1993].

The macroscopic analyses were supplemented by photonic-microscopic analyses that targeted the structure of the *hymenial layer*, with particular emphasis on the qualities of asci and ascospores as well as basidia and basidiospores, respectively, that have been focused on colour, size, ornamentation and amyloid reaction of spores, phenotypic characteristics of great taxonomic value. Special attention was paid to biometrics, which was very useful in determining various species.

The identification of taxa was accomplished by using the well-known methodology, that is, the study of specialised literature that contains keys to the determination and diagnosis of species [EYSSARTIER & ROUX, 2011; GALLI, 1996; KIRK & al. 2008; MOSER, 1993; SĂLĂGEANU & SĂLĂGEANU, 1985].

Results and discussion

In the associations of downy oak, 240 species have been inventoried. The inventoried macromycobiota, in terms of systematics, belong to 95 genera, 43 families, 12 orders and 4 classes, included in 2 phyla of the kingdom *Fungi* (Tab. 1)

The phylum *Ascomycota* is represented by 16 species of macromycetes, which belong to 14 genera, included in 8 families, 4 orders and 3 classes. The most prevalent order of the phylum *Ascomycota* is *Pezizales* – with 11 species of 10 genera, belonging to 6 families.

The phylum *Basidiomycota* is represented by 224 species of 78 genera, 18 families and 9 orders of the class *Agaricomycetes*. The most prevalent order of the phylum *Basidiomycota* is *Agaricales* with 144 species, followed by the order *Russulales* with 40 species and *Polyporales* with 16 species. The most common families are *Russulaceae* with 36 species, *Agaricaceae* with 25, followed by *Tricholomataceae* with 21 and *Boletaceae* with 12 species, respectively. A high diversity, in this phylum, is characteristic of the genera: *Russula* with 22 taxa, *Lactarius* with 14 taxa, *Boletus* and *Mycena* with 10 taxa each (Tab. 1).

An annotated list of species from downy oak forests is presented below. The nomenclature and synonyms used in this list are in full compliance with the decisions taken at the 18th International Botanical Congress (Melbourne, Australia), in July 2011, when the new International Code of Nomenclature for algae, fungi and plants was adopted.

The scientific name, chorological data, the phenophase when *sporophores* formed and some information on gastronomic importance are given for each species from the annotated list.

Tab. 1. Taxonomic diversity of macromycetes from downy oak forests

Family	Genus	Species	Trophic group	Pheno-phase	Abun-dance
Helotiaceae	*Bisporella*	*B. citrina*	Lg	IX-XI	Rare
	Chlorociboria	*Ch. aeruginascens*	Lg	IX-XI	Rare
Bulgariaceae	*Bulgaria*	*B. inquinans*	Lg	IX-XI	Rare
Helvellaceae	*Helvella*	*H. acetabulum*	Hum	III-V	Rare
Morchellaceae	*Morchella*	*M. deliciosa*	Hum	III-V	Rare
	Verpa	*V. digitaliformis*	Hum	III-V	Rare
Pezizaceae	*Peziza*	*P. echinospora*	Hum	III-V	Rare
		P. vesiculosa	Hum	IX-XI	Rare
	Terfezia	*T. arenaria*	M	V-XI	Rare
Pyronemataceae	*Aleuria*	*A. aurantia*	Hum	IX-XI	Rare
	Scutellinia	*S. scutellata*	Lg	V-XI	Rare
	Tarzetta	*T. catinus*	Hum	V-XI	Rare
Sarcoscyphaceae	*Sarcoscypha*	*S. austriaca*	Lg	IX-III	Rare
Tuberaceae	*Tuber*	*T. aestivum*	M	IX-XI	Rare
Xylariaceae	*Xylaria*	*X. digitata*	Lg	IX-ZI	Rare
		X. polymorpha	Lg	I-XII	Com.
Agaricaceae	*Agaricus*	*A. altipes*	Hum	IX-XI	Com.
		A. arvensis	Hum	IX-XI	Com.
		A. campestris	Hum	VI-XI	Com.
		A. koelerionis	Hum	IX-XI	Com.
		A. langei	Hum	VI-XI	Com.
		A. phaeolepidotus	Hum	VI-XI	Com.
		A. pseudopratensis	Hum	VI-XI	Com.
		A. silvicola	Hum	VI-XI	Com.
		A. subperonatus	Hum	III-XI	Com.
	Bovista	*B. tomentosa*	Hum	IX-XI	Com.
	Calvatia	*C. candida*	Hum	IX-XI	Freq.
	Chlorophyllum	*Ch. rachodes*	Hum	VI-XI	Freq.
	Cyathus	*C. striatus*	Lg	VI-XI	Freq.
	Echinoderma	*E. asperum*	Hum	IX-XI	Freq.
	Lepiota	*L. clypeolaria*	Hum	IX-XI	Com.
		L. forquignonii	Hum	IX-XI	Com.
		L. helveola	Hum	VI-XI	Com.
		L. oreadiformis	Hum	VI-XI	Com.
		L. severiana	Hum	VI-XI	Rare
	Leucoagaricus	*L. meleagris*	Hum	VI-XI	Rare
		L. pilatianus	Hum	VI-XI	Rare
	Lycoperdon	*L. pratense*	Hum	VI-XI	Freq.
	Macrolepiota	*M. excoriata*	Hum	IX-XI	Freq.
		M .konradii	Hum	VI-XI	Freq.
		M. procera	Hum	IX-XI	Freq.
Amanitaceae	*Amanita*	*A. battarrae*	M	VI-XI	Freq.

Family	Genus	Species	Trophic group	Pheno-phase	Abun-dance
		A. crocea	M	VI-XI	Freq.
		A. excelsa	M	VI-XI	Rare
		A. fulva	M	V-XI	Com.
		A. phalloides	M	VI-XI	Rare
		A. rubescens	M	VI-XI	Com.
		A. solitaria	M	VI-XI	Rare
		A. vaginata	M	VI-XI	Com.
Bolbitiaceae	*Conocybe*	*C. percincta*	Hum	VI-XI	Rare
	Panaeolus	*P. papilionaceus*	Hum	IX-XI	Rare
		P. semiovatus.	Hum	IX-XI	Com.
Cortinariaceae	*Cortinarius*	*C. boudieri*	M	IX-XI	Rare
		C. collinitus	M	IX-XI	Rare
		C. elatior	M	IX-XI	Rare
		C. galeobdolon	M	IX-XI	Rare
		C. infractus	M	IX-XI	Rare
		C. meinhardii	M	IX-XI	Rare
		C. torvus	M	IX-XI	Rare
Entolomataceae	*Entoloma*	*E. aprile*	M	III-IX	Com.
		E. farinolens	Hum	III-V	Com.
		E. politum	Hum	IX-XI	Rare
		E. prunuloides	M	III-XI	Com.
		E. rhodopolium	M	IX-XI	Com.
Hygrophoraceae	*Hygrocybe*	*H. acutoconica*	Hum	IX-XI	Com.
		H. conica	Hum	VI-XI	Com.
	Cuphophyllus	*C. pratensis*	Hum	VI-XI	Com.
	Hygrophorus	*H. chrysodon*	M	IX-XI	Com.
		H. cossus	M	IX-XI	Com.
		H. eburneus	M	IX-XI	Com.
		H. penarius	M	VI-XI	Com.
	Lichenomphalia	*L. umbellifera*	Fol	IX-XI	V. rar.
Inocybaceae	*Crepidotus*	*C. mollis*	Lg	VI-XI	Com.
	Inocybe	*I. asterospora*	Hum	VI-XI	Freq.
		I .cookei.	Hum	VI-XI	Freq.
		I. geophylla	Hum	IX-XI	Freq.
		I. godeyi	Hum	VI-XI	Freq.
		I. margaritispora	Hum	VI-XI	Freq.
		I. rimosa	Hum	VI-XI	Freq.
	Phaeomarasmius	*Ph. rimulincola*	Lg	VI-XI	Rare
	Tubaria	*T. furfuracea*	Lg	VI-XI	Com.
Lyophyllaceae	*Calocybe*	*C. gambosa*	M	III-V	Com.
	Lyophyllum	*L. rhopalopodium*	Gs Hum	IX-XI	Vul.
Marasmiaceae	*Marasmius*	*M. bulliardii*	Fol	IX-XI	Rare
		M. cohaerens	Fol	VI-XI	Rare
		M. epiphyllus	Fol	IX-XI	Com.
	Megacollybia	*M. rotula*	Fol	VI-XI	Com.
		M. wynneae	Fol	IX-XI	Rare
		M. platyphylla	Lg	VI-XI	Com.

Family	Genus	Species	Trophic group	Pheno-phase	Abun-dance
Mycenaceae	*Mycena*	*M. aetites*	Gs	III-V	Rare
		M. corticola	Lg	IX-XI	Rare
		M. filopes	Lg	IX-XI	Rare
		M. galopus	Fol	III-V	Rare
		M. niveipes	Lg	VI-XI	Com.
		M. polygramma	Lg	VI-XI	Com.
		M. pura	Gs	IX-XI	Com.
		M. romagnesiana	Lg	VI-XI	Com.
		M. rosea	Fol	IX-XI	Com.
		M. vitilis	Lg	VI-XI	V. rar.
Omphalotaceae	*Gymnopus*	*G. brassicolens*	Fol	IX-XI	Com.
		G. dryophilus	Fol	IX-XI	Com.
		G. erythropus	Fol	IX-XI	Com.
		G. foetidus	Fol	IX-XI	Com.
		G. fusipes	Fol	IX-XI	Com.
		G. impudicus	Fol	IX-XI	Com.
		G. peronatus	Fol	VI-XI	Com.
		G. subpruinosus	Fol	IX-XI	Com.
Physalacriaceae	*Armillaria*	*A. cepistipes*	Lg	IX-XI	Com.
		A. mellea	Lg	IX-XI	Com.
	Hymenopellis	*H. radicata*	Hum	VI-XI	Com.
	Xerula	*X. pudens*	Hum	VI-XI	Com.
Pleurotaceae	*Pleurotus*	*P. cornucopiae*	Lg	VI-XI	Freq.
Pluteaceae	*Pluteus*	*P. cervinus*	Lg	V-XI	Com.
		P. leoninus	Lg	V-XI	Rare
		P. petasatus	Lg	IX-XI	Freq.
	Volvariella	*V. caesiotincta*	Lg	V-XI	Rare
		V. pusilla	Lg	V-XI	Rare
Psathyrellaceae	*Coprinellus*	*C. micaceus*	Lg	III-XI	Com.
		C. radians	Cop	III-XI	Com.
	Coprinopsis	*C. laanii*	Cop	V-XI	Com.
	Coprinus	*C. alopecia*	Cop	IX-XI	Rare
	Parasola	*P. plicatilis*	Cop	IX-XI	Freq.
	Psathyrella	*P. candolleana*	Lg	III-V	Freq.
		P. leucotephra	Hum	VI-XI	Rare
		P. melanthina	Hum	IX-XI	Rare
		P. piluliformis	Hum	VI-XI	Freq.
		P. spintrigera	Hum	VI-XI	Rare
Schizophyllaceae	*Schizophyllum*	*S. commune*	Lg	I-XII	Com.
Strophariaceae	*Agrocybe*	*A. dura*	Hum	III-XI	Freq.
	Hebeloma	*H. crustuliniforme*	M	IX-XI	Rare
		H. quercetorum	M	IX-XI	Rare
	Hypholoma	*H. sinapizans*	M	IX-XI	Rare
		H. fasciculare	Lg	III-XI	Com.
		H. lateritium	Lg	VI-XI	Com.
	Kuehneromyces	*K. mutabilis*	Lg	VI-XI	Freq.
	Pholiota	*P. lenta*	Hum	VI-XI	Rare

Family	Genus	Species	Trophic group	Pheno-phase	Abun-dance
		P. squarrosoides	Lg	VI-XI	Freq.
Tricholomataceae	*Arrhenia*	*A. rustica*	Fol	V-XI	Rare
	Clitocybe	*C. gibba*	Gs	V-XI	Com.
		C. houghtonii	Fol	IX-XI	Com.
		C. infundibuliformis	Hum	VI-XI	Com.
		C. metachroa	Fol	IX-XI	Com.
		C. odora	Fol	IX-XI	Com.
		C. phyllophila	Fol	IX-XI	Com.
	Delicatula	*D. integrella*	Lg	VI-XI	Com.
	Haasiella	*H. splendidissima*	Lg	IX-XI	Com.
	Infundibulicybe	*I. geotropa*	Gs	IX-XI	Com.
	Lepista	*L. flaccida*	Fol	VI-XI	Rare
		L. panaeolus	Fol	IX-XI	Rare
		L. sordida	Hum	IX-XI	Rare
	Melanoleuca	*M. arcuata*	Fol	IX-XI	Rare
		M. brevipes	Hum	IX-XI	Rare
		M. grammopodia	Hum	IX-XI	Rare
		M. stridula	Fol	VI-XI	Rare
	Resupinatus	*R. applicatus*	Lg	VI-XI	Rare
	Tricholoma	*T. basirubens*	M	IX-XI	Rare
		T. gausapatum	M	IX-XI	Rare
		T. orirubens	M	IX-XI	Rare
		T. saponaceum	M	IX-XI	Rare
Typhulaceae	*Macrotyphula*	*M. fistulosa*	M	IX-XI	V. rar.
Boletaceae	*Boletus*	*B. aereus*	M	VI-XI	Freq.
		B. badius	M	VI-XI	Freq.
		B. chrysenteron	M	VI-XI	Freq.
		B. edulis	M	III-XI	Freq.
		B. erythropus	M	V- XI	Freq.
		B. impolitus	M	VI-XI	Freq.
		B. luridus	M	III-XI	Freq.
		B. queletii	M	III-XI	Freq.
		B. reticulatus	M	V-XI	Freq.
		B. subtomentosus	M	V-XI	Freq.
	Xerocomellus	*X. armeniacus*	M	III-XI	Freq.
		X. pruinatus	M	III-XI	Freq.
Sclerodermataceae	*Scleroderma*	*S. citrinum*	Hum	IX-XI	Freq.
		S. verrucosum	Hum	IX-XI	Freq.
Corticiaceae	*Vuilleminia*	*V. comedens*	Lg	I-XII	Rare
Geastraceae	*Geastrum*	*G. fimbriatum*	Hum	IX-XI	Freq.
		G. melanocephalum	Hum	IX-XI	Freq.
		G. pectinatum	Hum	IX-XI	Freq.
		G. triplex	Hum	IX-XI	Freq.
Gomphaceae	*Ramaria*	*R. stricta*	Lg	VI-XI	Rare
Hymenochaetaceae	*Inonotus*	*I. cuticularis*	Lg	IX-XI	Freq.
		I. hispidus	Lg	VI-XI	Freq.
		I. obliquus	Lg	I-XII	Freq.

Family	Genus	Species	Trophic group	Pheno-phase	Abun-dance
Schizoporaceae	*Basidioradulum*	*B. radula*	Lg	IX-XI	Freq.
Fomitopsidaceae	*Daedalea*	*D. quercina*	Lg	I-XII	Freq.
	Phaeolus	*Ph. schweinitzii*	Lg	VI-XI	Freq.
	Postia	*P. stiptica*	Lg	VI-XI	Freq.
Ganodermataceae	*Ganoderma*	*G. applanatum*	Lg	I-XII	Freq.
		G. lucidum	Lg	V-XI	Freq.
Meripilaceae	*Junghuhnia*	*J. nitida*	Lg	IX-XI	Freq.
Meruliaceae	*Abortiporus*	*A. biennis*	Lg	IX-XI	Freq.
	Merulius	*M. tremellosus*	Lg	Ix-XI	Freq.
Phanerochaetaceae	*Byssomerulius*	*B. corium*	Lg	IX-XI	Freq.
Polyporaceae	*Lenzites*	*L. betulina*	Lg	VI-XI	Freq.
	Polyporus	*P. arcularius*	Lg	III-V	Freq.
		P. leptocephalus	Lg	VI-XI	Freq.
		P. squamosus	Lg	VI-XI	Freq.
	Trametes	*T. gibbosa*	Lg	VI-XI	Freq.
		T. suaveolens	Lg	IX-XI	Freq.
		T. versicolor	Lg	I-XII	Freq.
Auriscalpiaceae	*Artomyces*	*A. pyxidatus*	Lg	IX-XI	Freq.
Russulaceae	*Lactarius*	*L. acerrimus*	M	IX-XI	Freq.
		L. azonites	M	IX-XI	Freq.
		L. citriolens	M	IX-XI	Freq.
		L. decipiens	M	VI-XI	Freq.
		L. evosmus	M	VI-XI	Freq.
		L. flexuosus	M	IX-XI	Freq.
		L. illyricus	M	V-XI	Freq.
		L. mairei	M	VI-XI	Freq.
		L. pergamenus	M	VI-XI	Freq.
		L. piperatus	M	VI-XI	Freq.
		L. quietus	M	VI-XI	Freq.
		L. vellereus	M	VI-XI	Freq.
		L. volemus	M	IX-XI	Freq.
		L. zonarius	M	VI-XI	Freq.
	Russula	*R. adusta*	M	VI-XI	V. rar.
		R. amarissima	M	V-XI	Freq.
		R. atropurpurea	M	VI-XI	Rare
		R. cyanoxantha	M	VI-XI	Com.
		R. decipiens	M	V-XI	Rare
		R. delica	M	VI-XI	Com.
		R. farinipes	M	VI-XI	Com.
		R. foetens	M	VI-XI	Com.
		R. furcata	M	VI-XI	Com.
		R. heterophylla	M	V-XI	Com.
		R. integra	M	V-XI	Rare
		R. luteotacta	M	V- XI	Rare
		R. minutula	M	V- XI	Rare
		R. nana	M	V- XI	Rare
		R. nuragica	M	V- XI	Rare

Family	Genus	Species	Trophic group	Pheno-phase	Abun-dance
		R. pectinata	M	V- XI	Rare
		R. risigallina	M	V- XI	Rare
		R. subfoetens	M	VI-XI	Rare
		R. subterfurcata	M	VI-XI	Com.
		R. vinosa	M	VI-XI	Rare
		R. virescens	M	VI-XI	Com.
		R. zvarae	M	V-XI	Freq.
Stereaceae	*Stereum*	*S. hirsutum*	Lg	I-XII	Freq.
Sebacinaceae	*Sebacina*	*S. incrustans*	Lg	VI-XI	Freq.
Thelephoraceae	*Thelephora*	*T. penicillata*	Hum	V-XI	Freq.

Abbreviations: M – mycorrhizal; Hum – humicolous; Lg **–** lignicolous; Fol – foliicolous; Cop – coprophilous; Com. – common; Freq. – frequent; Rare – rare; V. rar. – very rare; vul. – vulnerable.

The ecotrophic spectrum of macromycetes from associations of downy oak is dominated, as in the other associations that include plants of the genus *Quercus*, by mycorrhizal fungi with 79 (32.92%) species. The following categories of saprotrophic macromycetes have been identified: lignicolous – 68 (28.33%); humicolous – 65 (27.08%); foliicolous – 24 (10%) and coprophilous – 4 (1.67%) species (Fig. 1).

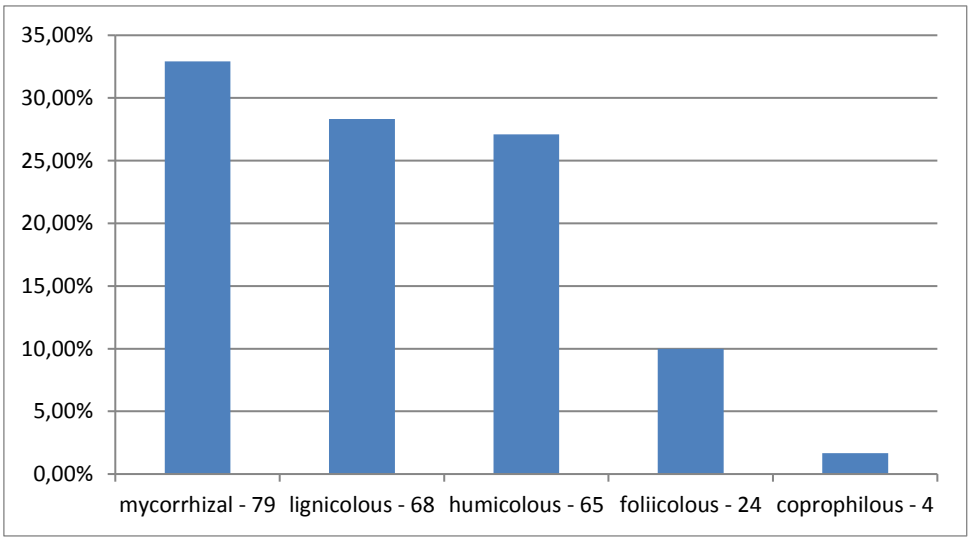

Fig. 1. Ecotrophic categories of macromycetes in downy oak forests

In the specific composition of macromycetes from downy oak stands, the species of the genera *Macrolepiota* and *Lepiota* play an important role. They form very often the so-called "fairy rings". Among mycorrhizal fungi, *Calocybe gambosa*, *Entoloma aprile* and *Entoloma prunuloides* often form spore-producing structures. These species are found only during spring and summer and are usually associated with trees and shrubs of the family Rosaceae. Besides the mycorrhizal fungi associated with woody species of the family

Rosaceae, such species as *Amanita solitaria*, *Amanita vaginata* and *Xerocomellus chrysenteron* associated with downy oak are quite common.

The most common saprotrophic foliicolous fungi, in the years with high humidity, is *Clitocybe geotropa*, and from lignicolous fungi – the species of the genus *Polyporus*.

Conclusions

The research on macromycetes was conducted in phytocoenoses of downy oak forest, and as a result, 240 species were identified.

Considering the specific habitats of phytocoenoses with downy oak, the spectrum of ecotrophic categories denotes a high diversity of mycorrhizal macromycetes.

Symbiotrophic macromycetes are important elements, characteristic of forest stands, where their spatial distribution is closely related to the composition of the stand, that's why the fungus-plant relationship is largely specific.

The saprotrophic species from glades and the mycorrhizal species that are associated with trees and shrubs of the family *Rosaceae* are characteristic of downy oak forests from the Republic of Moldova.

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ASTRAGALUS PSEUDOPURPUREUS GUŞUL. IN ROMANIA'S VEGETATION

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Abstract: *Astragalus pseudopurpureus* was described as a new species for science by the botanist Mihail Guşuleac, from Bicaz and Şugău Gorges. It is a very rare and endangered plant species in Romania, being endemic for Eastern Carpathians (it is a Dacian floristic element).
From phytosociological point of view, this species has been previously attributed, by various authors, to class *Elyno-Seslerietea* or to alliance *Seslerion bielzii*.
It is made a proposal of a new syntaxon in this paper, namely: subassociation *astragaletosum pseudopurpurei* subass. nova, subordinate to the association *Thymetum comosi* Pop et Hodişan 1963. This new subassociation is described from Şugău Gorges (Neamţ county, Romania). This new syntaxon is framed, as follow:
Class *Thlaspietea rotundifolii* Br.-Bl. 1926
Order *Thlaspietalia rotundifolii* Br.-Bl. 1926
Alliance *Achnatherion calamagrostis* Br.-Bl. 1918
Asociation *Thymetum comosi* Pop et Hodişan 1963
– subassociation *astragaletosum pseudopurpurei* subass. nova
The differential species for this subassociation, *Astragalus pseudopurpureus*, together with all the other species, edify phytocoenoses with soil coverages between 30% and 50%, having a stabilizing role of limestone screes.

Keywords: *Astragalus pseudopurpureus*, Eastern Carpathians, endemic, new syntaxon, Şugău Gorges, Romania.

Introduction

Astragalus pseudopurpureus is an endemic plant species in Romanian flora, distributed only in the Eastern Carpathians Mountains. The vernacular name is that of "cosaci bicăjean".

It was described by the romanian botanist Mihail Guşuleac, as a new species for science, in 1932. It was cited from Bicaz Gorges, being identified on "Piatra Panţârului", at 840 m a.s.l. and "Dealul Glodului", between 800 m a.s.l. and 1300 m a.s.l. [GUŞULEAC, 1933].

Materials and methods

Individuals of *Astragalus pseudopurpureus* have been examined right on the field in Şugău Gorges, Eastern Carpathians, Romania (Fig. 1-2). It has also been looked after in Nemira Mountains, Hăşmaş Mountains, and Bicaz Gorges (Eastern Carpathians), without any trace of existence, at least, for the time being.

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Fig. 1. *Astragalus pseudopurpureus* (habitus) (Adrian Oprea)

Fig. 2. *Astragalus pseudopurpureus* (flowers) (Adrian Oprea)

Herbarium sheets were also examined for distribution, in IAGB (Fig. 3) and I (abbreviations follow Thiers) [THIERS, 2009].

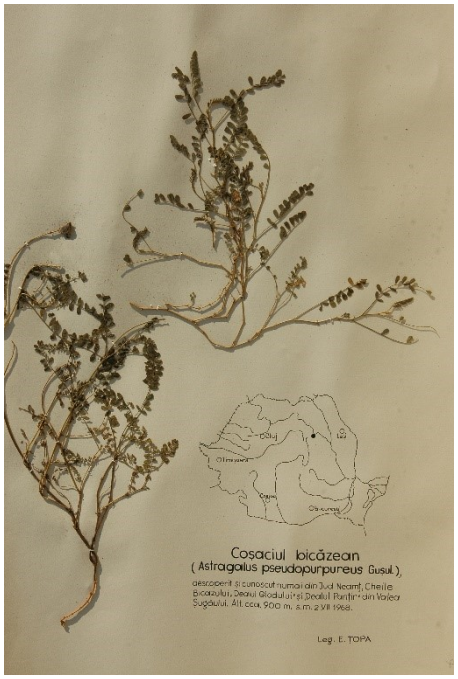

Fig. 3. A herbarium sheet with *Astragalus pseudopurpureus* (in IAGB) (Adrian Oprea)

The plant populations were followed between the years of 2007 and 2016, for their dynamics. The phytosociological surveys were made on the field in Şugău Gorges, Eastern Carpathians. The methodology for phytosociological surveys follow that one of Central European School of Phytosociology [BRAUN-BLANQUET, 1964].

Results and discussion

Plant species nomenclature

The nomenclature of *Astragalus pseudopurpureus* follow that existed in "Flora României", vol. 5 [GUŞULEAC, 1957], "Flora Europaea", vol. 2 [CHATER, 1968], "Plante endemice în România" [DIHORU & PÂRVU, 1987], "Cartea Roşie a Plantelor Vasculare din România" [DIHORU & NEGREAN, 2009], and in two monographs special dedicated to genus *Astragalus* [PODLECH, 1987, 2008]. The name and author(s) of all the plant species in this paper follow the most recent field identification book for plants in Romania [SÂRBU & al. 2013].

Distribution

Astragalus pseudopurpureus is distributed in the middle part of Eastern Carpathian Mountains only. The next distribution points for this species, are given as they are:

● Şugău Gorges: "Piatra Glodului", between 800 m a.s.l. and 1300 m a.s.l. (meters above sea level) [GUŞULEAC, 1932; SOÓ, 1940; ŢOPA, 1953 (in herbarium BUCA), 1960, 1968 (in herbarium IAGB), 1969; HOREANU, 1979 a, b; NEGREAN, 2009 (in herbarium BUCM];

● Bicaz Gorges: "Piatra Panţârului", at 840 m a.s.l. [GUŞULEAC, 1932];

● Bicaz Gorges: "Dealul Glodului" and "Dealul Panţa", "in pineto", at cca 900 m a.s.l., leg. (collected, in latin language), at 28.V.1934 [BORZA, 1935];

● Munticelu [SOÓ, 1940];

● Surduc, at 1267 m a.s.l. [GUŞULEAC, 1932; NYÁRÁDY, 1937; LÖRINCZI & GERGELY, 1977];

● **?** Suhardul Mic, between 900 m a.s.l. and 1300 m a.s.l. [GUŞULEAC, 1932], Suhardul Mic above the chalet called "Suhard" [LÖRINCZI & GERGELY, 1977];

● **?** Poliţele Bardosului, at 1132 m a.s.l. [GUŞULEAC, 1932; SOÓ, 1940];

● **?** Suhardul Mare [SOÓ, 1940; BUCA];

● **?** Nemira Mountains, at Dărmăneşti [MITITELU & al. 1971, 1993; CHIFU & al. 1987];

● **?** "… Limestone rocks. E. Carpathians (near Târgu Mureş and Bacău)" [CHATER, 1968].

As a matter of fact, the locations of *Astragalus pseudopurpureus* known for sure till now, are the Şugău Gorges only, on different local points, as they are "Piatra Glodului" /known also as "Dealul Glodului"/ and "Piatra Panţârului" /known also as "Dealul Panţa"/ [GUŞULEAC, 1932]. This species was identified on the entire length of the ridge Munticelu in the recent years, having as a maximum distribuiton area (in terms of individual numbers and populations) in Şugău Gorges (which are the southernmost part of the Nature Reserve Munticelu-Şugău Gorges, Neamţ county) (Fig. 4 a, b).

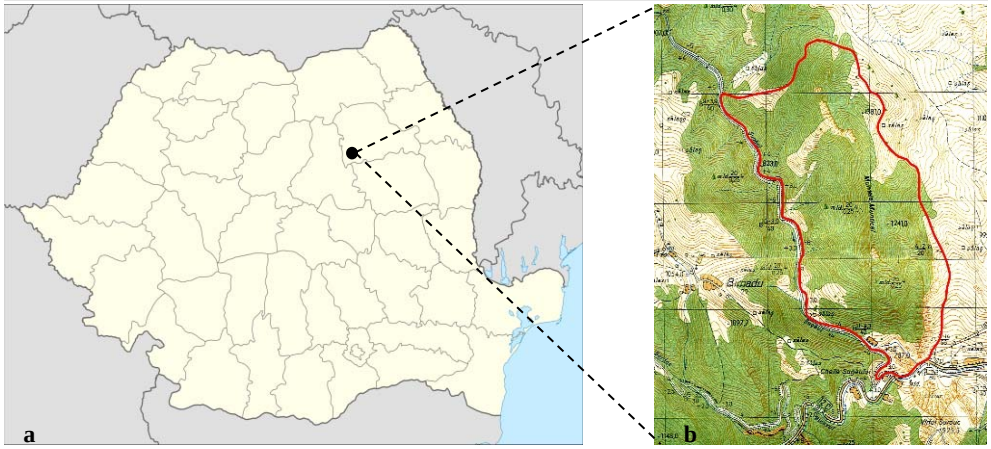

Fig. 4 a, b. Geographical location of the Nature Reserve Munticelu-Şugău Gorges (copyright: a) https://www.google.ro/search?q=harta+Romania&rlz; b) Romanian Mountain Club)

Plant ecology

a. *Geology*

Astragalus pseudopurpureus grows on limestone screes, moderately moisted, on medium inclined slopes, with various aspects, in lower mountain belt of vegetation (beech trees belt of vegetation), being in blossom in May-June, fruits reaching their maturity in July (sometimes, at the end of June).

The limestone screes are mobile or semi-mobile, with diameters of stones most often between 3 cm and 10-12 cm, underlying at the base of limestone walls of the Şugău Gorges (debris coming from the degradation of rocks or boulders detached walls).

The rocks are of upper age Jurassic limestones (Neocomian and Urgonian divisions), rich in fossils [CIOBANU & al. 1972; GRASU & al. 2010]. The peak called Munticelu is of 1387 m a.s.l.; the length of Şugău Gorges is about 350 m, with openings between the walls of 3-4 m only. There are also travertine rock formations situated at the northern part of the Şugău Gorges [OPREA & al. 2007].

b. *Pedology*

Lithomorphyc soils, of proto-rendzines and rendzines types, associated with outcrops of limestones and dolomites [BARBU & al. 1977].

c. *Climate*

Temperate-continental, with maximum absolute amplitudes, annual and diurnal quite large and winters over 4 months long. Annual average temperatures = cca 6 °C, the average annual precipipitations = cca 760 mm [HOREANU, 1979a].

The synthetic ecological factors that characterize this species, are the next ones: $L_9T_4U_3R_8N_x$, meaning that *Astragalus pseudopurpureus* is a plant growing in full sunlight (=heliophylous), in mountain and high mountain belts of vegetation (=mesothermal), on relatively dry soils at surfaces (=xero-mesophylous), usually indicative of limestone rocks (=neutral-basophylous) and euri-nitrophylous [SÂRBU & al. 2013].

Astragalus pseudopurpureus is accompanied by other plant species, normally growing among the limestone rock crevices, as: *Campanula carpatica*, *Hieracium pojoritense*, *Helictotrichon decorum*, *Androsace villosa* subsp. *arachnoidea*, *Silene zawadskii* and so on [LÖRINCZI & GERGELY, 1977].

Plant description

Astragalus pseudopurpureus is a perennial, hemicriptophyte, heliophylous, saxicolous, and calciphyluous plant species, 10-40 cm tall, with procumbent-ascending stems (Obs.: in "Flora României", vol. **V**, it is said that the stems are erected **!** [GUŞULEAC, 1957]); the stipules are connate around the stem at least on 1/3 of their length; the colour of corolla is bluish to violet, with standard weakly emarginate, of 17-18 mm long, keel connate, with a mucron at the apex on adaxial side; the fruit (legume) is of 10-15 mm long, villous, inflated at the rippen time (Fig. 1-2).

A black and white ink-drawing accompanies the description of the species in "Flora României", vol. **V**, page 283, fig. 1 a-e [GUŞULEAC, 1957].

The chromosoms number (2n) is unknown [DIHORU & NEGREAN, 2009].

Pathways of reproduction

It is an amphymictic and apomictic species, being reproduced by seeds and stolons, exhibiting also the phenomenon of polycormy; it is an allogamous, anemochorous, endozoochorous or barochorous species [DIHORU & NEGREAN, 2009]. This species does not produce viable seeds each year on the field, so the vegetative reproduction by stolons is more presumable.

Sozology

Astragalus pseudopurpureus is one of the very rare plant species in Romanian flora, being endemic for Eastern Carpathians (it is a Dacian floristic element), distributed in the mountain inferior belt of vegetation [BELDIE, 1967; ŢOPA, 1960]. It is an endangered (EN) plant species [DIHORU & NEGREAN, 2009].

Aspects on this topics are added in other papers [MANOLIU & al. 1979; HOREANU, 1979b; DIHORU & PÂRVU, 1987; DIHORU & NEGREAN, 2009], but with no any new or valuable information.

It is parasited by the fungus *Ovularia tuberculiformis* Höhnel 1987, matrix nova Negrean 2009 (in BUCM) [DIHORU & NEGREAN, 2009].

Specimens examined

Astragalus pseudopurpureus was collected for FRE, no. 1277, by the romanian botanist Emilian Ţopa, from Bicaz Gorges, on "Dealul Glodului" and "Dealul Panţa, in pineto", at cca 900 m a.s.l., in 1934. In herbarium IAGB (abbreviations according to Thiers [THIERS, 2009]) there is a single herbarium sheet only, under no. 4267, collected also by Emilian Ţopa, on July, the 2nd, 1968, from Şugău Gorges, on "Dealul Glodului" and "Dealul Panţir", at cca 900 m a.s.l. Another herbarium sheet (no. 10834) is in herbarium IASI, a specimen collected in Bicaz Gorges, on „Dealul Glodului and Dealul Panţa", on May, 28th, 1934, by the same Emilian Ţopa, for FRE [BORZA, 1935].

Phytosociology

From phytosociological point of view, *Astragalus pseudopurpureus*, has been previously attributed by various authors, either to Class *Elyno-Seslerietea* Br.-Bl. 1949 [CHIFU & al. 2006; SÂRBU & al. 2013) or to the Alliance *Seslerion bielzii* Br.-Bl. 1949 [SANDA & al. 1983], an alliance formerly named *Seslerion bielzii* (Pawł. 1935 em. A. Nyár. 1967) *calcophilum* I. Pop 1968 (also from Class *Elyno-Seslerietea* Br.-Bl. 1949) [SANDA & al. 1980].

Due to the fact that *Astragalus pseudopurpureus* is a strictly, local, and endemic species, located in Eastern Carpathians only, being accompanied by other endemic or near-

endemic plants, there is proposed a new syntaxon, namely subassociation ***astragaletosum pseudopurpurei* subass. nova** (ICPN, app. 1-11) [WEBER & al. 2000], subordinated to the association *Thymetum comosi* Pop et Hodişan 1963.

This plant association, *Thymetum comosi*, was originally named as "Asoc. *Thymus comosus*", by I. Pop and I. Hodişan [POP & HODIŞAN, 1963]. Later on, this association has received in synonymy [COLDEA, 1991, 1997], based on the Art. 25 of ICPN, the association *Galietum erecti*, initially described by I. Pop and collaborators, from Runc Gorges [POP & al. 1964b], and the association *Teucrietum montani*, described by I. Csűrös, from the Nature Reserve Scăriţa-Belioara [CSŰRÖS, 1958], under two names: "*Teucrietum montani*" in text of the paper, and "As. de *Teucrium montanum*" in table no. 5 attached to the same paper, based on the Art. 8, of ICPN [COLDEA, 1991, 1997].

The association *Thymetum comosi* Pop et Hodişan 1963, was described from Bulzeşti Gorges (Bihor-Vlădeasa Mountains) [POP & HODIŞAN, 1963]; it has also been identified in Runc Gorges (Gilău-Muntele Mare Mountains) [POP & al. 1964b], located on brown soils, rich in sand and gravel, of different sizes.

So far, within the plant association *Thymetum comosi* Pop et Hodişan 1963, there were described some infrataxa, as they are:

– subassociation *typicum* Coldea 1991 [COLDEA, 1991, 1997], described from Bulzeşti Gorges [POP & HODIŞAN, 1963]; it was cited also from Grohot Massif [ŞUTEU & FAUR, 1977], Runc Gorges [POP & al. 1964], Iadu valley [RAŢIU & al. 1984], Leaota Massif [DIACONESCU, 1973], and Tălmaciu-Podu Olt [SCHNEIDER-BINDER, 1970];

– subassociation *galietosum albi* (Pop et al., 1964b) Coldea 1991 [COLDEA, 1991, 1997], described from Runc Gorges [POP & al. 1964]; it was also identified in Vadu Crişului [BOŞCAIU & al. 1966], Râmeţ Gorges [ŞUTEU, 1968], Grohot Massif [ŞUTEU & FAUR, 1977], Iadu valley [RAŢIU & al. 1984], Sighiştel valley [POP & HODIŞAN, 1969], Ascunsa valley [POP, 1971], and Leaota Massif [DIACONESCU, 1973];

– subassociation *teucrietosum montani* (Csűrös 1958) Coldea 1991 [COLDEA, 1991, 1997] (syntaxon synonym association *Galio albi-Teucrietum montani* Ştefan et al. 2007 [ŞTEFAN & al. 2006-2007]), described from the Nature Reserve Scăriţa-Belioara [CSŰRÖS, 1958]; it was also cited from Crăciuneşti valley [POP & HODIŞAN, 1964a], Leaota Massif [DIACONESCU, 1973], and Bicaz Gorges/Eastern Carpathians [ŞTEFAN & al. 2006-2007].

There is a relatively high similarity of phytocoenoses with *Astragalus pseudopurpureus* from the Nature Reserve Munticelu-Şugău Gorges (Eastern Carpathians), with the phytocoenoses of the other subassociations (i. e. *typicum*, *galietosum albi*, *teucrietosum montani*), assigned to ass. *Thymetum comosi* Pop et Hodişan 1963 [POP & HODIŞAN, 1963]. Thus, we assumed that this new subassociation, *astragaletosum pseudopurpurei* subass. nova, is a vicariant one of the previous three syntaxa.

Coenotaxonomical framing of the newly proposed syntaxon is as follow:

Class *Thlaspietea rotundifolii* Br.-Bl. 1926

Order *Thlaspietalia rotundifolii* Br.-Bl. 1926

Alliance *Achnatherion calamagrostis* Br.-Bl. 1918

Association *Thymetum comosi* Pop et Hodişan 1963

– **subassociation** *astragaletosum pseudopurpurei* subass. nova

Holotype relevé: Tab. 1, relevé no. 5 (*****)

From phytosociologic point of view, *Astragalus pseudopurpureus*, together with all the other taxa belonging to association and alliance (e.g. *Teucrium montanum*, *Galium album*

subsp. *album*, *Geranium macrorrhizum* etc.), order (e.g. *Acinos alpinus* subsp. *alpinus*, *Gymnocarpium robertianum*), and class (e.g. *Carduus defloratus* subsp. *glaucus*, *Sedum telephium* subsp. *maximum* etc.), made a strong core of this new subassociation. Other taxa belong to alliance *Bromo-Festucion pallentis* (e.g. *Alyssum saxatile* subsp. *saxatile*, *Biscutella laevigata* subsp. *laevigata*, *Seseli libanotis* subsp. *libanotis* etc.) and to class *Festuco-Brometea* (e.g. *Anthemis tinctoria* subsp. *tinctoria*, *Centaurea stoebe* subsp. *australis*, *Teucrium chamaedrys*, *Campanula sibirica* subsp. *sibirica* etc.), indicating thus some xeric features of this habitat type. Other taxa are belonging to class *Asplenietea trichomanis* (e.g. *Asplenium ruta-muraria*, *Jovibarba heuffelii*, *Silene nutans* subsp. *dubia*, *Gypsophila petraea* etc.), thus indicating a series of species who entered here from the limestone walls surrounding the screes substratum of this new syntaxon. Other taxa are coming into the phytocoenoses of this coenotaxa from nearby, as: either from the meadows (many species belong to *Arrhenatheretalia* et *Molinio-Arrhenatheretea*: *Leucanthemum vulgare*, *Linum catharcticum* subsp. *catharcticum*, *Anthyllis vulneraria* subsp. *polyphylla* etc.), or forests (species belonging to *Querco-Fagetea*, *Vaccinio-Piceetea* and *Franguletea*: *Epipactis atrorubens* subsp. *atrorubens*, *Polygonatum verticillatum*, *Frangula alnus* juv., *Melica nutans* etc.), or from the weed communities (belonging to *Trifolio-Geranietea*, *Galio-Urticetea*, *Epilobietea*, or *Dauco-Melilotion*: *Geranium phaeum*, *Pteridium aquilinum*, *Cirsium erisithales*, *Epilobium montanum*, *Melilotus albus* etc.) (Tab. 1).

Tab. 1. The phytosociological table of association *Thymetum comosi* Pop et Hodişan 1963 subassociation *astragaletosum pseudopurpurei* subass. nova

***** = holotype relevé

Live's form	Geoelement	Relevé area (sq. m)	9	9	9	9	9
		Altitude (m a.s.l.)	1310	738	696	686	711
		Aspect	S	W	S	S-W	W
		Slope (degrees)	25°	30°	30°	20°	35°
		Herb layer coverage (%)	35	30	30	50	40
		Relevé no.	1	2	3	4	**5***
Difer. subass.							
H	End.	*Astragalus pseudopurpureus*	1	1	1	1	1
Achnatherion calamagrostis							
H	Centr. eur.-submedit.	*Teucrium montanum*	2	1	1	2	1
H	Eur.	*Galium album* subsp. *album*	+	+	+	+	+
H	Centr. eur.-medit.	*Melica ciliata* subsp. *ciliata*	-	+	+	+	+
T-Ht	Cosm.	*Geranium robertianum*	-	-	-	-	+
H	Euras.	*Origanum vulgare* subsp. *vulgare*	-	-	-	+	+
H	Pont.-medit.	*Scutellaria altissima*	-	-	-	-	+
H	Euras. cont.	*Vincetoxicum hirundinaria* subsp. *hirundinaria*	-	+	+	+	+
H	Alp.-carp.-balc.	*Geranium macrorrhizum*	-	+	-	-	-
Thlaspietalia rotundifolii							
H	Alp.-carp.	*Acinos alpinus* subsp. *alpinus*	-	-	-	+	+
G	Circ.	*Gymnocarpium robertianum*	-	-	-	-	+
Thlaspietea rotundifolii							
T-H	End. carp.	*Campanula carpatica*	-	+	+	-	+

H	E alp.-carp.	*Carduus defloratus* subsp. *glaucus*	+	-	-	+	+
Ch	Euras. arct.-alp.-eur.	*Saxifraga paniculata*	-	-	+	-	+
H	Eur.	*Sedum telephium* subsp. *maximum*	-	-	-	+	+
Ch	End. Romanian Carp.	*Thymus comosus*	-	-	-	+	+
Bromo-Festucion pallentis							
H	Grecia, Romania (E & S Carp.)	*Gentiana phlogifolia*	-	+	+	-	-
Ch	Cont. eur.	*Alyssum saxatile* subsp. *saxatile*	-	+	+	+	+
T-Ht	S-E Eur. (naturalized also in N Eur.)	*Sedum hispanicum*	-	+	+	+	-
H	Balc.-pan.	*Silene flavescens*	-	+	+	-	-
Ht	End. E Carp.	*Erysimum witmannii* subsp. *witmannii*	-	-	-	-	+
H (Ch)	Near-end. carp.(Romania, Ukraine)	*Dianthus spiculifolius*	-	+	+	-	-
H	Near-end. (Carp.)	*Dianthus tenuifolius*	-	-	+	+	+
H	Centr. eur. (mont.)	*Biscutella laevigata* subsp. *laevigata*	+	-	-	-	+
H	End. Romanian Carp.	*Helictotrichon decorum*	1	+	+	+	+
H	End. E Carp.	*Hieracium pojoritense* subsp. *pojoritense*	-	+	+	+	+
Ph	Eur. mont. - V As.	*Juniperus sabina*	-	+	+	+	+
H	Dacian / End. E Carp.	*Poa rehmannii*	-	-	+	+	+
H	Medit.	*Primula veris* subsp. *columnae*	+	-	-	-	-
H	Carp.-balc.	*Scrophularia heterophylla* subsp. *laciniata*	-	-	-	+	+
Ht-H	Euras. cont.	*Seseli libanotis* subsp. *libanotis*	-	+	+	+	+
H	Carp.-balc.	*Sesleria bielzii*	-	-	-	-	+
H	Centr. - S eur. (mont.)	*Pedicularis comosa* subsp. *comosa*	-	-	-	+	+
Stipo-Festucetalia pallentis							
H	Euras.	*Aconitum anthora*	-	-	-	+	+
G	Centr. eur.-submedit. (mont.)	*Allium lusitanicum*	+	-	+	-	-
H	Euras. cont.	*Carex humilis*	-	-	-	+	-

H	Centr. eur. (mont.)	*Festuca pallens* subsp. *pallens*	-	+	+	+	-
H	Alp.-eur.	*Kernera saxatilis* subsp. *saxatilis*	-	-	-	-	+
Ch	Centr. eur.-balc.-pont	*Minuartia setacea* subsp. *setacea*	-	-	-	+	+
H	Euras. cont.	*Thalictrum foetidum*	-	-	-	+	+
Festuco-Brometea							
H	Euras.	*Agrimonia eupatoria* subsp. *eupatoria*	-	-	-	+	-
H	Euras.	*Ajuga genevensis*	-	-	-	+	-
H	Euras. cont.	*Anthemis tinctoria* subsp. *tinctoria*	-	-	-	+	-
H	Centr. eur.-medit	*Asperula cynanchica*	-	-	-	+	-
Ht-H	Pont.-pan.-balc.	*Centaurea stoebe* subsp. *australis*	-	-	-	+	-
Ch	Eur.	*Helianthemum nummularium* subsp. *nummularium*	+	+	+	+	-
H	Pont.-pan.-balc.	*Inula ensifolia*	-	+	+	+	-
Ht-H	Euras. cont.	*Isatis tinctoria* subsp. *tinctoria*	-	-	-	-	+
H	Euras. (submedit.)	*Pimpinella saxifraga* subsp. *saxifraga*	-	-	-	+	-
H	Eur. cont.	*Potentilla heptaphylla*	+	-	-	+	-
H	Centr. eur.-medit.	*Salvia verticillata*	-	+	+	+	+
H	Pont.-medit.-centr. eur.	*Stachys recta* subsp. *recta*	-	-	-	+	+
Ch	Centr. eur. (submedit.)	*Teucrium chamaedrys*	-	+	+	+	
H	Euras. cont.	*Veronica teucrium* subsp. *teucrium*	-	+	+	-	+
Ht	Euras.	*Echium vulgare*	-	-	+	+	-
H	Carp.-balc.-cauc.-anat.	*Phleum montanum*	-	-	-	+	-
Ht	Euras. cont.	*Campanula sibirica* subsp. *sibirica*	-	+	+	+	+
Ht-H	Circ.	*Erigeron acris* subsp. *acris*	-	-	-	+	+
H	Pont.-pan.-balc.	*Linum flavum*	-	+	+	-	-
Asplenietea trichomanis							
H	Euras.	*Asplenium ruta-muraria*	-	+	+	-	+
H	Cosm.	*Asplenium trichomanes* subsp. *trichomanes*	-	-	-	-	+
Ch	Carp.-balc. (Dacian)	*Jovibarba heuffelii*	-	+	+	+	+
G	Circ.	*Polypodium vulgare*	-	-	+	-	+
H	End. Romanian Carp.	*Silene nutans* subsp. *dubia*	-	+	+	+	+

Ch	End. E Carp.	*Silene zawadzkii*	+	-	-	+	+
Ch	End. E & S Carp.	*Gypsophila petraea*	+	-	-	-	+
H	Euras.	*Sedum acre*	-	+	-	-	-
Arrhenatheretalia et Molinio-Arrhenatheretea							
H	Euras.	*Leucanthemum vulgare* subsp. *vulgare*	-	-	-	+	-
T-H	Eur.	*Linum catharcticum* subsp. *catharcticum*	-	+	+	-	-
H	Euras.	*Lotus corniculatus*	-	-	-	+	-
H	Euras.	*Potentilla erecta*	-	+	+	+	+
T	Eur.	*Trifolium campestre*	-	-	-	+	+
H	Euras. (submedit.)	*Valeriana officinalis*	-	-	-	+	+
H	Carp.-balc.	*Ranunculus montanus* subsp. *pseudomontanus*	+	-	-	+	+
H	Eur.	*Scabiosa columbaria* subsp. *columbaria*	-	-	+	+	+
G	Eur.	*Gymnadenia conopsea* subsp. *conopsea*	-	-	-	+	+
H	Eur. cont.	*Anthyllis vulneraria* subsp. *polyphylla*	-	-	-	-	+
Vaccinio-Piceetea							
H	Cosm.	*Athyrium filix-femina*	-	+	-	-	-
G	Euras.	*Epipactis atrorubens* subsp. *atrorubens*	-	-	-	-	+
Ph	Circ.	*Juniperus communis* subsp. *communis* juv.	-	-	-	-	+
Querco-Fagetea							
H	Eur.	*Carex muricata*	-	-	-	-	+
H	Euras	*Hieracium murorum* s. lato	-	-	-	-	-
H (G)	Euras.	*Melica nutans*	-	-	-	+	+
G	Euras.	*Platanthera bifolia*	-	+	-	-	-
G	Euras.	*Polygonatum verticillatum*	-	+	-	-	-
Ph	Eur.	*Ribes uva-crispa* subsp. *grossularia*	-	-	+	-	-
G	Euras.	*Polygonatum odoratum* subsp. *odoratum*	-	-	-	-	+
Franguletea							
Ph	Euras.	*Frangula alnus*	-	+	+	-	-
Trifolio-Geranietea							
H	Alp.-eur.	*Bupleurum falcatum* subsp. *falcatum*	-	+	+	+	+
H	Centr. eur.	*Geranium phaeum*	-	-	-	-	+
H	Eur. (mont.)	*Laserpitium latifolium*	-	-	-	+	+
G	Cosm.	*Pteridium aquilinum*	-	+	-	-	-
H	Circ.	*Solidago virgaurea* subsp. *virgaurea*	-	-	-	+	
Ht	Eur.	*Verbascum lychnitis* subsp. *lychnitis*	-	-	-	+	+

H	Euras	*Cruciata glabra* subsp. *glabra*	+	-	+	+	-
H	Centr. eur.-submedit.	*Coronilla varia*	-	+	+	+	+
H	Alp. eur.	*Geum urbanum*	-	+	-	-	-
Galio-Urticetea							
H	Centr. eur. (mont.)	*Cirsium erisithales*	-	-	-	-	+
H	Eur. cont.	*Sisymbrium strictissimum*	+	-	-	-	-
Epilobietea							
H	Euras.	*Epilobium montanum*	-	+	-	-	-
Rhamno-Prunetea							
Ph	Euras.	*Rhamnus catharcticus* juv.	-	-	-	-	+
Dauco-Melilotion							
Ht	Euras.	*Melilotus albus*	-	-	-	+	
T-H	Euras.	*Medicago lupulina*	-	-	-	+	+
Aliae							
H	Euras.	*Euphorbia cyparissias*	-	-	-	+	+

Date and place of relevés: May, 23rd, 2015, the Nature Reserve Munticelu - Şugău Gorges (Neamţ county)

i. The **differential species** for this newly proposed subassociation is *Astragalus pseudopurpureus*, which, together with the other species, edify pioneer phytocoenoses, covering the soil between 30% and 50%, sometimes even more.

Previously, *Astragalus pseudopurpureus* has been recorded in a single phytosociological relevé only, within the association *Calamintho baumgarteni-Galietum anisophylli* Beldie 1967, an association identified in Bicaz Gorges, as a characteristic species attributed to class *Elyno-Seslerietea* [ŞTEFAN & al. 2006-2007].

In a monography dedicated to flora and vegetation from Bicaz Gorges, Red Lake and Hăşmaş Mountains [NECHITA, 2003], *Astragalus pseudopurpureus* was not attributed to any coenotaxa.

ii. The **ecology** of these phytocoenoses is: slopes of various aspects (South, West, or South-West), with different slope degrees (from 20° to 35°), on surfaces of 10-15 sq. m, achieving coverages up to 50% of soil. All the species fits into a thin soil layer, mostly of them being heliophilous or helio-sciadophilous, xero-mesophilous, and calciphilous.

a) The live's forms spectrum of the new syntaxon shows the next value (both in terms of absolute figures and percentage): **H=62 (60,19%); Ch=10 (9,70%); G=9 (8,73%);** T-H=3 (2,91%); Ht=5 (4,85%); Ht-H=4 (3,88%); H(Ch)=1 (0,97%); T-Ht=2 (1,94%); Ph=5 (4,85%); H(G)=1 (0,97%); T=1 (0,97%) (in bold are given the prevailing categories) (Fig. 5).

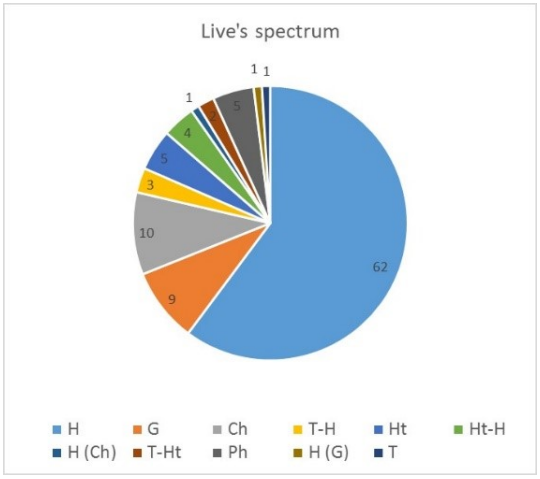

Fig. 5. The live's forms spectrum of subassociation *astragaletosum pseudopurpurei* subass. nova

b) The floristic elements (geoelements) spectrum of the new syntaxon shows the next value: **Euras.=34 (33%)**; **Eur.** (incl. eur. mont.)=**25 (24.27%)**; **Endemic=10 (9.70%)**; **Near-endemic=3 (2.91%)**; Centr. eur.-medit.-submedit.=11 (10.67%); Alp.-carp.-balc.=6 (5.82%); Pont.-pan.-balc.=5 (4.85%); Circ.=5 (4.85%); Cosm.=4 (3.88%) (in bold are given the prevailing categories) (Fig. 6). Beside the *Astragalus pseudopurpureus* itself, there are other several endemic or near-endemic species into the Romanian flora, (e.g. *Thymus comosus*, *Gentiana phlogifolia*, *Dianthus tenuifolius*, *Helictotrichon decorum*, *Poa rehmannii*, *Campanula carpatica* etc.), which increase the value of this new proposed subassociation.

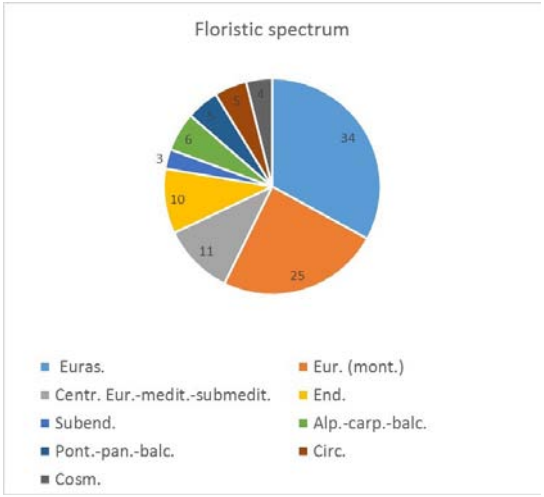

Fig. 6. The floristic elements spectrum of subassociation *astragaletosum pseudopurpurei* subass. nova

c) The soil moisture/humidity spectrum of the new syntaxon shows the next value: U_1=1 (0.97%); U_2=8 (7.76%); **U_3=31 (30.09%)**; **U_4=34 (33%)**; U_5=18 (14.47%); U_6=2

(1.94%); U_7=3 (2.91%); U_8=0; U_9=0; U_x=7 (6.79%) (in bold are given the prevailing categories) (Fig. 7).

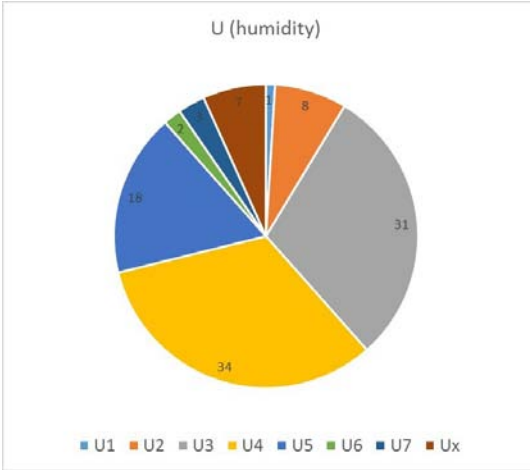

Fig. 7. The soil moisture spectrum of subassociation *astragaletosum pseudopurpurei* subass. nova

d) The temperature spectrum of the new syntaxon shows the next value: U_1=1 (0.97%); U_2=8 (7.76%); **U_3=31 (30.09%)**; **U_4=34 (33%)**; U_5=18 (14.47%); U_6=2 (1.94%); U_7=3 (2.91%); U_8=0; U_9=0; U_x=7 (6.79%) (in bold are given the prevailing categories) (Fig. 8).

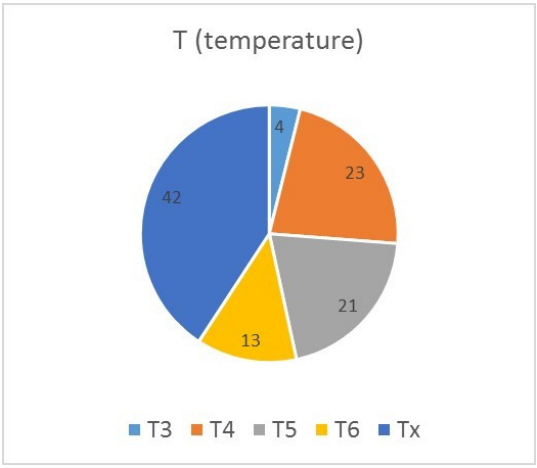

Fig. 8. The temperature spectrum of subassociation *astragaletosum pseudopurpurei* subass. nova

e) The *p*H soil spectrum of the new syntaxon shows the next value: R_1=0; R_2=1 (0.97%); R_3=1 (0.97%); R_4=4 (3.88%); R_5=8 (7.76%); R_6=13 (12.62%); **R_7=24 (23.30%)**; R_8=16 (15.53%); R_9=0; **R_x=36 (34.95%)** (in bold are given the prevailing categories) (Fig. 9).

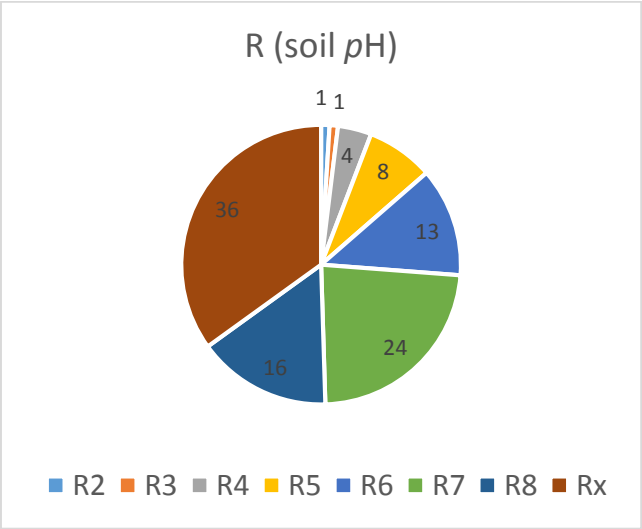

Fig. 9. The soil reaction (*p*H) spectrum of subassociation *astragaletosum pseudopurpurei* subass. nova

f) The light preferences spectrum of the of the plant species of the new syntaxon shows the next value: L_1=0; L_2=0; L_3=0; L_4=9 (8.73%); L_5=2 (1.94%); L_6=11 (10.67%); **L_7=35 (33.98%)**; **L_8=35 (33.98%)**; L_9=11 (10.67%) (in bold are given the prevailing categories) (Fig. 10).

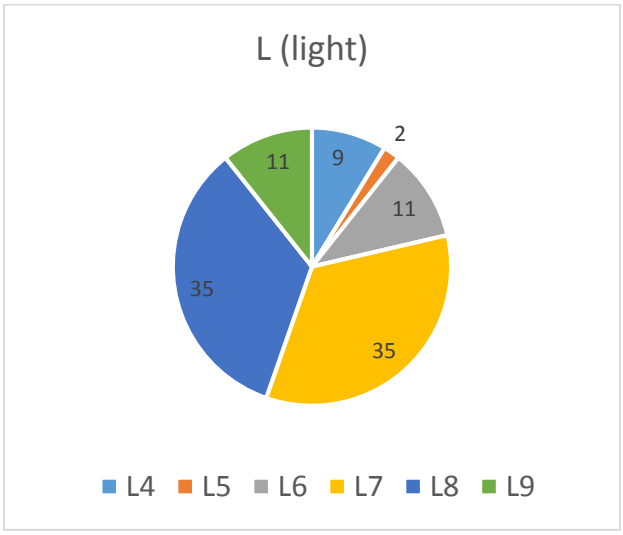

Fig. 10. The light spectrum of subassociation *astragaletosum pseudopurpurei* subass. nova

iii. The **distribution area** of this newly proposed infrataxa, subass. *astragaletosum pseudopurpurei*, is the Nature Reseve "Munticelu-Șugău Gorges" (Eastern Carpathians, Romania), solely.

Concerning the natural habitat of this new subassociation, it seems to be the best alloted to **8120** Calcareous and calcashist screes of the montane to alpine levels (*Thlaspietea rotundifolii*) [Habitat Directive 92/43/EEC, 1992; GAFTA & al. 2008; European Commission, 2013] (Fig. 11-12).

Fig. 11. Calcashist stones habitat of subassociation *astragaletosum pseudopurpurei* subass. nova (Adrian Oprea)

Fig. 12. Calcareous screes habitat of subassociation *astragaletosum pseudopurpurei* subass. nova (Adrian Oprea)

From 2007, this nature reserve is a *Natura 2000* site, namely ROSCI0033 Cheile Şugăului-Munticelu [Ord. MESD, 2008], thus being protected the natural habitats, the most precious plant and animal species, as well as the natural landscapes, inside those 318 hectaries.

Conclusions

Astragalus pseudopurpureus is a very rare and endangered (EN) plant species, being endemic for Eastern Carpathians (Romania).

From phytosociological point of view, this species has been formerly attributed by various authors, to Class *Elyno-Seslerietea*, or to Alliance *Seslerion bielzii*.

It was made a proposal for a new coenotaxa in this paper, namely subassociation *astragaletosum pseudopurpurei* subass. nova, subordinate to the ass. *Thymetum comosi* Pop

et Hodişan 1963, alliance *Achnatherion calamagrostis*, order *Thlaspietalia rotundifolii*, and class *Thlaspietea rotundifolii*.

The differential species for this new subassociation is *Astragalus pseudopurpureus*.

The phytocoenoses are installed on limestone screes, on various aspects (South, West, or South-West), with different slope degrees (from 20° to 35°).

All the plant species fits into a thin soil layer, mostly of them being heliophilous or helio-sciadophilous, xero-mesophilous, and calciphilous.

Phytocoenoses of this new subassociation are distributed on nature reserve Munticelu-Şugău Gorges (Neamţ county, Romania) only.

The natural habitat of this subassociation is **8120** Calcareous and calcashist screes of the mountain to alpine levels (*Thlaspietea rotundifolii*).

Abbreviations used in this paper:

BUCA – herbarium of the Institute of Biology Bucharest, Romanian Academy
BUCM – mycological herbarium of the Institute of Biology Bucharest, Romanian Academy
FRE – Flora Romaniae Exsiccata
I – herbarium of the University "Alexandru Ioan Cuza", Iaşi, Romania
IAGB – herbarium of the Botanic Garden "Anastasie Fătu" - University "Alexandru Ioan Cuza", Iaşi, Romania
ICPN – International Code of Phytosociological Nomenclature
NGO – Non-Guvernamental Organisation
Ord. MESD – Order of the Minister of Environment and Sustainable Development

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PHYTOSOCIOLOGICAL STUDY OF THE POPULATION OF *PAEONIA PEREGRINA* MILL. (*PAEONIACEAE*) IN THE REPUBLIC OF MOLDOVA

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Abstract: The article contains information on the phytosociological study of the population of *Paeonia peregrina* Mill. in the Central Moldavian Plateau, in the Republic of Moldova. Three releveés (plots), with a total area of 3 ha, are described. These phytocoenoses are proposed to be grouped in the association *Paeonio peregrinae-Quercetum pubescentis* (Sârbu 1982) Popescu et Sanda 1999.

Keywords: *Paeonia peregrina*, phytosociological study, Republic of Moldova.

Introduction

The species *Paeonia peregrina* Mill. (*Paeoniaceae*) is a Balkan geophyte, xeromesophile, common in the thermoxerophilous forests from Italy (Basilicata, Calabria), Albania, Greece, Macedonia, Serbia, Bulgaria, Romania (Dobrogea Plateau) and Turkey (Anatolia) [AKMAN & al. 1979; AKSOY & GEMICI, 2010; CHIFU & POPA, 2006; CONTI & al. 2005; DONIŢĂ & al. 1992, 2005; DUMAN, 1988; EKIM & AKMAN, 1991; PETRESCU, 2006-2007; SANDA & al. 2008; SÂRBU, 1982; SÂRBU & al. 1995; ŢUPU & CHIFU, 2009; CHIFU (ed.), 2014]. In the Republic of Moldova, this species of peony has been found in Boltun village, Nisporeni district, it has been protected by the State since 1975 and included in the Red Book of the Republic of Moldova (1978, 2001, 2015, category: Critically Endangered [CR]). Populations of *Paeonia peregrina*, in Moldova, are found at the eastern part of the studied area. Uprooting of plants, grazing and mowing in glades and forest edges are limiting factors for this species [GHEJDEMAN & al. 1982; PÎNZARU & al. 2016]. No phytosociological research on the populations of this species in our country has been done so far, therefore the phytosociological study has been the main objective of this work.

Materials and methods

Phytosociological studies were carried out in May and August 2016, in the Central Moldavian Plateau, using the methods of the European Central school [BRAUN-BLANQUET, 1964]. Three relevés (plots), of 10.000m² each, have been described; the area of the releveés – according to [IVAN & SPIRIDON, 1983]. The floristic nomenclature according to [PÎNZARU & SÎRBU, 2014].

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Results and discussion

Peony (*Paeonia peregrina*), in the Republic of Moldova, is found in thermoxerophile forests of downy oak (*Quercus pubescens* Willd.) with European smoketree (*Cotinus coggygria* Scop.), on hillsides with south-western and western aspect and an angle of slope degree of 10-25º, at the altitude of 180-240 m.a.s.l., in levigated chernozems.

In the vertical structure of phytocoenoses, 3 layers are observed:

1. The degree of canopy – 60-75%. The height of trees – about 7-8 m, the dominant species is downy oak (*Q. pubescens*), with the diameter at breast height 20-24 cm, secondary species: *Tilia tomentosa* Moench, *T. cordata* Mill., *Acer campestre* L., *Cerasus avium* (L.) Moench, *Fraxinus excelsior* L., less frequently – *Acer platanoides* L.

2. The degree of canopy – 30-80%. The height of the layer is about 1.50-3.0 m, it consists of the species: *Acer tataricum* L., *Cotinus coggygria*, *Crataegus monogyna* Jacq., *Rhamnus catartica* L., *Cornus sanguinea* L., *Rosa canina* L., *Euonymus verrucosus* Scop., *E. europaeus* L., *Ligustrum vulgare* L. Young trees of several species are also part of this layer.

3. Herbaceous cover of (5-) 50-80%, in glades – up to 100%, consists, mostly, of herbaceous plants, small shrubs, branches of young shrubs and trees. The height of this layer varies between 25-30 cm (if it consists of medium-sized herbaceous plants) and 80-90 cm (in areas where the abundance+dominance is up to 3-5 of the species *Alliaria petiolata* (M.Bieb.) Cavara et Grande, in thickets of *Cotinus coggygria* or in glades with tall herbaceous plants). In the thickets of European smoketree (*Cotinus coggygria*), there are few herbaceous plants, their abundance varies between ″r″ and 1. In the described phytocoenoses, only the species: *Convallaria majalis* L., *Buglossoides purpurocaerulea* (L.) I. M. Johnst., *Fragaria viridis* Duchesne, *Vinca herbacea* Waldst. & Kit., *Anemone sylvestris* L. have an abundance+dominance of 2-3; the other herbaceous species are represented by an insignificant number of individuals. The lower layer also consists of small groups of *Amygdalus nana* L., *Rosa pimpinellifolia* L. small shoots and plantlets grown from seeds of woody species. Peony (*Paeonia peregrina*) is also found in this layer, in groups or sporadically. Other rare species that have been found are: *Clematis integrifolia* L., *Pulsatilla montana* (Hoppe) Rchb., *Adonis vernalis* L., *Iris graminea* L., *I. variegata* L. Altogether, the floristic composition of the three relevés, in summer, comprises about 140 species; this information has been completed by additional data obtained as a result of the research carried out in spring.

In Romania, the phytocoenoses containing *P. peregrina* have been grouped in several associations: *Paeonio peregrinae-Quercetum pubescentis* (Sârbu 1982) Popescu et Sanda 1999, *Cotino-Quercetum pubescentis* Soó 1932, *Paeonio peregrinae-Carpinetum orientalis* Doniţă 1970, *Galio dasypodi-Quercetum pubescentis* Doniţă 1970; the last two associations, are considered by some authors synonymous with *Cotino-Quercetum pubescentis* Soó 1932 [SANDA & al. 2008].

We consider questionable the inclusion of the associations with *P. peregrina*, found on Dobrogea Plateau, in different alliances: *Paeonio perigrinae–Quercetum pubescentis* (Sârbu 1982) Popescu et Sanda 1999 in the alliance *Quercion petraeae* Zólyomi et Jakucs in Soó 1963 and *Cotino-Quercetum pubescentis* Soó 1932 in the alliance *Fraxino orni-Cotinion* Soó 1960 [SANDA & al. 2008], because both associations are thermoxerophilous, found on levigated chernozems, at the same altitude, differing only in the absence or the presence of the species *Cotinus coggygria*. Analysing the list of species from the associations of *Quercus pubescens* with *Paeonia peregrina* (Tab. 1) and taking into consideration the characteristics

of the sites described by the authors who determined these associations, we propose to combine all the phytocoenoses of *Quercus pubescens* with *Paeonia peregrina* into a single association: *Paeonio peregrinae-Quercetum pubescentis* (Sârbu 1982) Popescu et Sanda 1999.

Tab. 1. The associations of the alliance *Quercion pubescenti-sessiliflorae*

Number of the column	1			2	3	4	5	6	7
Number of the relevés	3			10	65	12	10	20	10
	1	2	3						
Abundance (AD) or constancy (K)	AD	AD	AD	K	K	K	K	K	K
Characteristic species:									
Paeonia peregrina	r	+	+	III	IV	V	III	IV	V
Quercus pubescens	4	5	5	V	V	IV	IV	V	V
Diff. sous-ass.									
Cotinus coggygria	4	2	2	V	III	IV	-	V	-
Differentiated species:									
Carpinus orientalis	-	-	-	-	IV	V	V	-	-
Galium dasypodum	-	-	-	-	I	-	-	IV	-
Tree layer [A = 60-75%]									
Quercus dalechampii	-	-	-	-	-	-	II	-	III
Or. Quercus pedunculiflora	-	-	-	-	-	I	-	-	-
Or. Quercus virgiliana	-	-	-	-	-	I	-	-	-
Cl. Acer campestre	+	1	+	I	II	-	IV	-	I
Acer platanoides	+	-	-	I	-	-	I	-	-
Al. Carpinus orientalis	-	-	-	-	IV	V	-	-	-
Cl. Cerasus avium	-	+	+	-	-	-	-	-	III
Fraxinus coriariifolia	-	-	-	-	-	-	-	-	I
Cl. Fraxinus excelsior	+	+	+	I	-	V	II	-	I
Or. Fraxinus ornus	-	-	-	I	V	II	III	-	I
Cl. Malus sylvestris	-	-	-	I	-	I	-	-	I
Morus alba	-	-	-	-	-	-	-	-	I
Pyrus elaeagrifolia	-	-	-	II	-	-	-	-	II
Pyrus pyraster	-	-	-	III	II	I	II	II	IV
Robinia pseudacacia	-	-	-	-	-	-	-	-	I
Sorbus domestica	-	-	-	-	I	-	-	I	-
Sorbus torminalis	-	-	-	-	II	-	II	-	-
Tilia cordata	+	+	+	-	-	-	-	-	-
Tilia tomentosa	1	+	+	-	III	-	II	-	II
Ulmus procera	-	-	-	II	-	-	I	-	II
Cl. Ulmus minor	-	-	-	-	-	I	-	-	-
Shrub layer [B = 30-80 %]:									
Acer tataricum	1	2	1	III	-	I	-	-	IV
Berberis vulgaris	-	-	-	-	-	I	-	-	-
Clematis vitalba	-	-	-	-	I	I	-	-	I
Cornus mas	1	+	-	III	V	V	V	III	I
Cornus sanguinea	+	+	+	-	-	-	-	-	-

Crataegus monogyna	+	+	+	V	III	V	-	V	V
Euonymus europaeus	1	+	+	I	-	I	-	-	2
Euonymus verrucosus	2	2	1	II	II	V	-	I	III
Ligustrum vulgare	-	1	1	III	II	V	II	II	V
Prunus spinosa s.l.	-	+	+	V	-	-	-	IV	V
Rhamnus cathartica	-	+	+	IV	-	-	-	-	III
Rosa canina	+	+	+	V	-	I	-	-	V
Rosa glauca	-	-	-	-	-	-	-	-	I
Viburnum lantana	+	+	+	II	II	V	-	-	I
Brushwood:									
Quercus pubescens	+	1	+	-	-	-	-	-	-
Cerasus avium	-	+	+	-	-	-	-	-	-
Acer campestre	-	+	+	-	-	-	-	-	-
Acer platanoides	+	-	-	-	-	-	-	-	-
Fraxinus excelsior	+	+	+	-	-	-	-	-	-
Tilia cordata	+	+	+	-	-	-	-	-	-
Tilia tomentosa	+	+	+	-	-	-	-	-	-
Herbaceous layer [C = 5-60(100)%]:									
Achillea pannonica	-	+	+	I	-	-	-	-	-
Achillea setacea	-	+	+	I	-	-	II	-	IV
Adonis vernalis	-	+	+	I	-	-	-	-	I
Agrimonia eupatoria	-	+	+	III	-	-	-	I	IV
Agropyron cristatum s.l.	-	-	-	-	-	-	I	-	-
Ajuga genevensis	-	+	+	-	-	-	-	-	-
Ajuga laxmannii	+	+	-	-	I	-	-	I	I
Ajuga reptans	-	-	-	-	-	-	-	-	I
Alliaria petiolata	5	2	1	II	-	-	I	-	III
Allium oleraceum	+	-	-	-	-	-	-	-	-
Allium paniculatum	-	-	-	-	I	-	-	I	-
Allium rotundum	-	-	-	-	I	-	-	-	-
Althaea cannabina	-	-	-	I	-	-	-	II	I
Althaea officinalis	-	-	-	-	-	-	I	-	-
Anemone sylvestris	-	2	1	-	-	-	-	-	-
Anemonoides ranunculoides [=*Anemone ranunculoides*]	-	-	-	-	-	I	I	-	-
Anthericum ramosum	1	-	-	-	-	-	-	-	-
Anthriscus longirostris [= A. cerefolium ssp. trichosperma]	+	1	+	-	-	-	I	-	-
Anthriscus sylvestris	-	-	-	-	-	-	-	-	I
Arabis turrita	-	-	-	-	I	-	-	-	-
Arctium lappa	-	-	+	-	-	-	-	-	-
Arenaria serpyllifolia	-	-	-	I	-	-	-	-	-
Artemisia austriaca	-	-	-	I	-	-	I	-	II
Arum orientale	-	-	-	-	I	-	IV	-	-
Asparagus officinalis	r	r	-	I	-	-	-	-	III
Asparagus tenuifolius	r	+	+	IV	III	-	III	IV	III

Asparagus verticillatus	-	-	-	-	III	IV	II	V	I
Asperula cynanchica	-	-	-	-	-	-	I	-	-
Asperula tenella	-	-	-	I	-	-	-	-	I
Astragalus glycyphyllos	-	-	-	II	I	-	II	II	II
Ballota nigra	-	-	-	-	-	-	-	-	I
Bothriochloa ischaemum	-	-	-	I	-	-	-	-	II
Brachypodium pinnatum	-	-	-	I	II	-	-	-	II
Brachypodium sylvaticum	-	1	+	II	III	III	-	-	II
Bromus benekenii	-	1	-	-	-	-	-	-	-
Bromus inermis	-	-	-	-	I	-	-	V	-
Bromus sterilis	-	-	-	-	-	-	-	-	I
Buglossoides arvensis	-	+	+	-	-	-	-	-	-
Buglossoides purpurocaerulea	1	2	2	IV	III	IV	-	V	V
Bupleurum affine	-	-	-	-	I	-	-	I	-
Calamagrostis epigejos	-	-	-	I	-	-	-	-	I
Campanula bononiensis	+	+	+	-	-	-	-	-	-
Campanula glomerata var. cervicarioides	-	r	r	-	-	-	-	-	-
Campanula grossekii	-	-	-	-	-	IV	-	-	-
Campanula persicifolia	-	+	+	III	-	-	-	-	II
Campanula rapunculoides	+	+	+	I	-	-	-	-	I
Campanula rapunculus	-	-	-	I	-	-	-	-	I
Capsella bursa-pastoris	-	-	-	-	-	-	I	-	-
Carduus crispus	-	-	-	-	-	-	I	-	-
Carex contigua (= *Carex spicata*)	-	+	-	III	-	-	-	-	II
Carex divulsa	-	-	-	III	-	-	-	-	II
Carex halleriana	-	-	-	-	II	I	-	-	-
Carex michelii	-	-	-	-	III	-	-	IV	-
Carex pilosa	-	-	-	-	-	I	-	-	-
Carex praecox	-	-	-	I	II	-	II	-	I
Carex tomentosa	-	-	-	-	I	-	-	II	-
Centaurea orientalis	-	+	-	-	-	-	-	-	-
Centaurea stenolepis	+	+	+	-	-	-	-	-	-
Chenopodium album	-	-	-	I	-	-	II	-	I
Chaerophyllum bulbosum	-	+	-	-	-	-	I	-	-
Chaerophyllum nodosum [= *Myrroides nodosa*]	-	-	-	-	I	-	II	I	-
Chaerophyllum temulum	-	-	-	I	-	-	II	-	I
Chamaecytisus austriacus	-	-	-	-	-	-	-	-	I
Chamaecytisus lindemanii	-	-	-	-	-	-	-	II	-
Chondrilla juncea	-	-	-	-	-	-	I	-	-
Chrysopogon gryllus	-	-	-	I	-	-	-	-	I
Cichorium intybus	-	-	-	-	-	-	I	-	-
Clematis integrifolia	-	+	+	-	-	-	-	-	-
Clinopodium vulgare	-	+	+	V	II	-	-	-	V
Convallaria majalis	2	-	-	-	-	-	-	-	-
Convolvulus arvensis	-	-	-	-	-	-	II	-	-
Corydalis cava	-	-	-	-	-	-	II	-	-

Corydalis solida	-	-	-	-	-	-	II	-	-
Crepis foetida s.l.	-	-	-	-	-	-	I	-	-
Cruciata glabra	-	-	-	-	-	-	I	-	-
Cruciata laevipes	-	-	-	-	I	-	-	I	-
Cynanchum acutum	-	-	-	-	-	-	IV	-	-
Dactylis glomerata	+	+	+	III	-	-	II	-	III
Dactylis polygama	-	-	-	-	III	III	-	-	-
Dianthus andrzejowskianus	-	+	+	I	-	-	-	-	-
Dictamnus albus	-	-	-	V	-	-	-	-	II
Digitalis lanata	-	-	-	I	-	-	II	-	I
Echium maculatum	-	-	-	I	-	-	-	-	-
Eryngium campestre	-	-	+	-	-	-	I	-	I
Elytrigia intermedia	-	2	1	-	-	-	-	I	-
Elytrigia repens (= *Elymus repens*)	-	2	-	I	-	-	-	II	I
Epipactis helleborine [=*E. latifolia*]	-	-	-	-	-	III	-	-	-
Erysimum canescens	-	+	-	-	-	-	-	-	-
Euphorbia agraria	-	-	-	-	-	-	-	I	-
Euphorbia glareosa	-	-	-	II	-	-	-	-	II
Euphorbia illyrica	-	+	-	-	-	-	-	-	-
Euphorbia stepposa	-	+	+	-	-	-	-	-	-
Falcaria vulgaris	-	+	+	-	-	-	-	-	I
Fallopia convolvulus (= *Polygonum convolvulus)*	+	+	+	III	-	-	-	-	III
Ferulago sylvatica	-	-	-	-	-	-	-	I	-
Festuca rupicola	-	-	-	-	II	-	-	I	-
Festuca valesiaca	+	+	+	IV	I	-	II	I	II
Ficaria verna s.l.[= *R. ficaria* subsp. *calthifolius*]	-	-	-	-	-	-	I	-	-
Filipendula vulgaris	-	+	+	V	I	-	-	III	V
Fragaria vesca	-	-	-	-	-	-	II	-	-
Fragaria viridis	1	2	1	IV	III	I	III	II	V
Fumaria schleicheri	-	-	-	I	-	-	-	-	I
Galanthus graecus	-	-	-	-	-	V	-	-	-
Galanthus plicatus	-	-	-	-	-	-	I	-	-
Galatella lynosiris [= *Lynosiris vulgaris*]	-	-	-	-	-	-	-	I	-
Galium aparine	2	1	+	III	-	-	I	-	II
Galium dasypodum	-	-	-	-	I	-	-	IV	-
Galium humifusum	-	-	-	I	-	-	-	-	I
Galium mollugo	-	+	-	I	-	-	-	I	I
Galium octonarum	-	-	-	I	-	-	-	-	I
Galium odoratum	-	-	-	-	-	III	-	-	-
Galium schultesii	-	-	-	-	-	-	I	-	-
Galium verum	-	+	-	V	-	-	I	II	IV
Geranium robertianum	-	+	+	I	-	-	-	-	-
Geranium sanguineum	-	1	+	-	-	-	II	I	-
Geum urbanum	+	+	+	IV	III	II	V	-	V
Glechoma hirsuta	1	1	1	III	-	-	II	-	III

Hedera helix	-	-	-	-	-	I	I	-	-
Helichrysum arenarium	-	-	-	-	-	-	-	-	I
Hesperis tristis	-	-	+	-	-	-	-	-	-
Hieracium robustum	+	+	+	-	-	-	-	-	-
Himantoglossum hyrcinum	-	-	-	-	-	I	-	-	-
Hyacinthella leucophaea	-	-	-	-	-	-	I	-	-
Hypericum hirsutum	-	-	-	-	-	-	-	-	I
Hypericum perforatum	-	-	-	III	I	-	II	-	I
Inula germanica	-	-	-	I	-	-	-	I	I
Inula hirta	-	-	-	IV	-	-	-	-	III
Inula salicina	-	+	-	-	I	-	-	II	-
Iris graminea	-	+	-	-	-	-	-	-	-
Iris variegata	+	+	+	-	-	-	-	-	-
Koeleria macrantha	-	-	-	I	-	-	-	-	I
Knautia arvensis	-	+	+	II	-	-	-	-	I
Lactuca muralis (= *Mycelis muralis*)	-	-	-	-	-	-	-	-	I
Lamium amplexicaule	-	-	-	-	-	-	I	-	-
Lamium maculatum	-	+	-	-	-	-	I	-	-
Lamium purpureum	-	-	-	-	-	-	III	-	II
Lapsana communis	-	-	-	II	-	-	-	-	III
Lappula squarosa	-	-	-	I	-	-	-	-	I
Laser trilobum	-	-	-	-	I	I	-	III	-
Lathyrus lacteus	-	-	-	V	-	-	-	-	III
Lathyrus niger	-	-	-	III	-	-	-	III	III
Lathyrus pallescens	-	+	+	-	-	-	-	-	-
Lathyrus pannonicus	-	1	+	-	-	-	-	-	-
Leonurus cardiaca	-	-	-	I	-	-	I	-	I
Lepidium draba [= *Cardaria draba*]	-	-	-	-	-	-	I	-	-
Lepidium ruderale	-	-	-	-	-	-	I	-	-
Leopoldia comosa	-	+	+	-	-	-	-	-	-
Linaria genistifolia	-	-	-	I	-	-	-	-	I
Malva thuringiaca (= *Lavathera thuringiaca*)	-	-	-	I	-	-	-	-	I
Marrubium peregrinum	-	-	-	-	-	-	III	-	I
Medicago falcata	-	+	-	II	-	-	-	-	I
Medicago lupulina	-	-	-	I	-	-	-	-	I
Melampyrum arvense	-	1	1	-	-	-	-	-	-
Melica ciliata	-	-	-	III	-	-	I	-	III
Melica nutans	-	1	1	-	-	-	-	-	-
Melica transsilvanica	+	+	+	-	-	-	-	-	-
Melica uniflora	-	-	-	-	III	-	V	-	-
Mercurialis ovata	-	-	-	-	III	II	-	-	-
Milium effusum	-	-	-	-	-	-	I	-	-
Muscari racemosum	-	-	-	-	-	-	I	-	-
Myosotis arvensis	-	+	-	-	-	-	I	-	-
Nepeta nuda	-	r	r	I	-	-	-	-	I
Orchis purpurea	-	-	-	-	-	I	I	-	-
Origanum vulgare	-	+	-	II	-	-	-	-	I

Orlaya grandiflora	-	-	-	-	-	-	I	-	-
Ornithogalum fimbriatum	-	-	-	-	I	-	I	-	-
Peucedanum alsaticum	-	r	-	-	-	-	-	-	-
Phleum phleoides	-	1	1	I	-	-	-	-	I
Phlomis tuberosa	-	+	-	-	-	-	-	-	-
Physospermum cornubiensis[=*Danaa cornubiensis*]	-	-	-	-	-	IV	-	-	-
Pilosella bauhinii (=*Hieracium bauhinii*)	-	+	-	I	-	-	-	-	I
Pilosella cymosa	-	+	+	-	-	-	-	-	-
Piptantherum virescens	-	-	-	I	III	-	II	III	II
Plantago media	-	-	-	I	-	-	-	-	I
Plantago lanceolata	-	+	-	I	-	-	-	-	I
Plantago urvillei	.	+	+	-	-	-	-	-	-
Poa angustifolia	-	1	-	V	II	I	I	I	III
Poa bulbosa	-	-	-	-	-	-	I	-	-
Poa nemoralis	-	1	1	II	II	I	-	-	II
Polygonatum odoratum	1	-	-	III	-	-	-	II	III
Polygonatum latifolium	-	1	1	II	II	IV	-	II	II
Potentilla alba	-	-	-	-	-	-	-	-	I
Potentilla argentea	-	-	-	III	-	-	-	-	III
Potentilla patula	-	+	+	-	-	-	-	-	-
Potentilla recta	r	+	r	II	-	-	-	-	I
Primula veris	-	+	-	-	-	-	-	-	IV
Pulmonaria mollis	-	-	-	-	-	-	III	-	III
Pulsatilla montana	-	r	-	-	-	-	-	-	-
Ranunculus auricomus	-	+	+	-	-	-	-	-	-
Rubus caesius	-	-	-	I	-	-	-	-	I
Rubus tomentosus	-	-	-	-	-	-	-	I	-
Rumex acetosa L.	-	+	-	-	-	-	-	-	-
Salvia austriaca	-	+	-	-	-	-	-	-	I
Salvia verticillata	-	-	-	I	-	-	-	-	I
Scilla bifolia	-	-	-	-	I	-	II	-	-
Scutellaria altissima	+	+	+	-	-	I	II	-	-
Securigera varia (=*Coronilla varia*)	+	+	+	I	-	-	-	-	II
Sedum maximum	+	+	+	II	-	-	I	-	III
Silene alba	-	-	-	-	-	-	-	-	I
Silene coronaria (= *Lycnis coronaria*)	-	-	-	II	I	-	-	-	I
Silene nutans	-	+	+	-	-	-	-	-	-
Silene otites	-	-	-	II	-	-	-	-	-
Silene vulgaris	-	+	-	-	-	-	-	-	-
Solanum dulcamara	-	-	-	I	-	-	-	-	I
Stachys germanica	-	-	-	I	-	-	-	-	II
Stachys officinalis	-	+	+	III	-	-	-	-	I
Stachys recta	-	+	+	-	-	-	-	-	I
Stachys sylvatica	-	-	-	-	-	-	I	-	-
Stellaria graminea	-	+	-	-	-	-	-	-	-
Stellaria media	-	+	-	-	-	-	I	-	I

Stipa capillata	-	-	-	-	-	-	-	-	I
Stipa pennata	-	-	1	-	-	-	-	-	-
Tanacetum corymbosum [=*Pyretrum corymbosum*	-	+	+	I	III	IV	-	V	IV
Taraxacum camyloides (= *T. officinalis*)	-	-	-	I	-	-	III	-	II
Taraxacum serotinum	-	-	-	-	-	-	II		
Teucrium chamaedrys	1	1	1	III	II	I	II	I	III
Thalictrum aquilegiifolium	-	-	-	II	-	-	-	-	I
Thalictrum minus	+	+	+	III	II	I	I	III	II
Thlaspi perfoliatum	+	+	+	-	II	-	-	-	-
Thymus pannonicus s.l. [= T. pannonicus subsp. auctus]	-	+	+	-	-	-	I	-	-
Torilis arvensis	+	+	+	-	-	-	-	-	I
Tragopogon orientalis	-	+	+	-	-	-	-	-	-
Trifolium alpestre	-	+	+	III	-	-	-	II	IV
Trifolium campestre	-	-	-	-	-	-	-	-	I
Trifolium medium	-	-	-	-	-	-	-	-	III
Trifolium ochroleucon	-	-	-	-	-	-	-	-	I
Trinia kitaibelii	-	-	+	-	-	-	-	-	-
Turritis glabra	-	+	+	I	-	-	-	-	I
Urtica dioica	-	-	-	I	-	-	I	-	I
Valeriana collina	-	+	+	-	-	-	-	-	-
Valerianella locusta	-	-	-	-	-	-	I	-	-
Veratrum nigrum	-	-	-	-	II	-		IV	-
Verbascum chaixii	-	-	-	-	I	-	-	I	-
Verbascum nigrum	-	-	-	-	-	-	-	I	-
Verbascum phlomoides	-	-	-	II	-	-	I	-	I
Verbascum phoeniceum	-	-	-	-	I	-	-	II	-
Verbascum speciosum	-	-	-	-	-	-	-	-	I
Veronica arvensis	-	+	+	-	-	-	-	-	-
Veronica austriaca	-	-	-	II	-	-	-	-	I
Veronica chamaedrys	-	+	-	III	-	-	-	I	III
Veronica hederifolia	-	-	-	-	-	-	I	-	-
Veronica jacquinii	-	+	+	-	-	-	-	-	-
Veronica orchidea	-	-	-	-	-	-	-	-	I
Vicia angustifolia	-	-	-	-	-	-	-	I	-
Vicia cassubica	-	+	+	-	-	-	-	-	II
Vicia cracca	-	-	-	II	-	-	-	-	-
Vicia grandiflora	-	-	-	-	-	-	-	I	-
Vicia hirsuta	-	-	-	-	-	-	I	-	-
Vicia pisiformis	+	-	-	-	-	-	-	-	-
Vicia tenuifolia	-	-	-	-	-	-	-	II	-
Vicia tetrasperma	-	-	-	-	-	-	-	-	I
Vinca herbacea	-	2	2	III	II	-	II	II	III
Vincetoxicum hirundinaria	+	+	+	III	-	-	-	-	III
Viola arvensis	-	+	-	-	-	-	-	-	-
Viola hirta	-	+	-	V	-	-	II	-	V

Viola jordanii	-	-	-	-	I	-	-	II	-
Viola mirabilis	-	-	-	I	-	I	-	-	I
Viola odorata	+	1	1	-	-	-	II	-	I
Viola reichenbachiana	-	-	-	-	-	-	IV	-	-
Viola suavis	-	-	1	I	I	-	III	-	III
Amygdalus nana	+	2	1	-	-	-	-	-	II
R. pimpinellifolia	-	1	-	-	-	-	-	-	-
Brushwood:									
Cotinus coggygria	3	4	4	-	-	-	-	-	-
Quercus pubescens	1	1	+	-	-	-	-	-	-
Acer campestre	+	+	+	-	-	-	-	-	-
Cerasus avium	-	+	+	-	-	-	-	-	-
Euonymus verrucosus	+	+	+	-	-	-	-	-	-

Content of the columns: **1** (3 relevés) from R. Moldova; **2** -as. *Cotino-Quercetum pubescentis* Soó 1932: CHIFU & POPA, 2006; **3** - **4** - as. *Paeonio peregrinae-Carpinetum orientalis* Doniţă 1970: DONIŢĂ & al. 1992, tab. 32; **5** - as. *Paeonio perigrinae-Carpinetum orientalis* Doniţă 1970: ŢUPU & CHIFU, 2009; **6** - As. *Galio dasypodi-Quercetum pubescentis* Doniţă 1970: DONIŢĂ & al. 1992, tab. 32; **7** - as. *Paeonio peregrinae-Quercetum pubescentis* (Sârbu 1982) Popescu et Sanda 1999: CHIFU & POPA, 2006.

Conclusions

The studied plant communities with *Paeonia peregrina* from the forests of *Quercus pubescens* and *Cotinus coggygria*, from the Republic of Moldova, fit in the association *Paeonio peregrinae-Quercetum pubescentis* (Sârbu 1982) Popescu et Sanda 1999.

The association *Paeonio peregrinae-Quercetum pubescentis* (Sârbu 1982) Popescu et Sanda 1999 is proposed to be included in the alliance *Quercion pubescenti-petraeae* Br.-Bl. 1932, ord. *Quercetalia pubescenti-petraeae* Klika 1933, cl. *QUERCO-FAGETEA* Br.-Bl. et Vlieger in Vlieger 1937.

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THE DIVERSITY OF MEDICINAL AND AROMATIC PLANTS ENCOUNTRED IN NATURA 2000 6520 HABITAT FROM GURGHIU MOUNTAINS

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Abstract: Researches on medicinal plants used in various human disorders are particularly important and timely, as an alternative to medication. The studied semi-natural mountainous grasslands occur in the Gurghiu mountains. Special interest today worldwide for herbal medicine has led us to study the Gurghiu Mountains medicinal plants used in various diseases. In order to identify the taxa we used classical methods, described in the literature and statistical analyze was also carried out. In the study area 2 plant associations rich in medicinal plants were identified. They belong to 6520 Mountain hay meadows habitat.

Keywords: grasslands, habitats, medicinal plants, Mureş county.

Introduction

The study area stretches on the part of Gurghiu Mountains that belong to Mureş County. In terms of vegetation this area is characterized by the predominance of forest ecosystems, along with semi-natural mountainous meadows with much lower share. Special interest today, worldwide, for herbal medicine has led us to study especially medicinal plants used in phytotherapy, and the risk of their irrational collection. Also, we plan an assessment of habitat conservation.

Material and methods

The study of medicinal plants was carried out on the basis of own research in the field and of bibliography data [OROIAN, 1998; SĂMĂRGHIȚAN & OROIAN, 1999; OROIAN & SĂMĂRGHIȚAN, 2000; SĂMĂRGHIȚAN, 2005] by using classic techniques and procedures promoted by the literature [CRISTEA & al. 2004], to which was added the statistical analysis. The name of the species was given in accordance to CIOCÂRLAN (2009) and SÂRBU & al. (2013).

The type of habitat has been coded in accordance with Manual interpretation of habitats in Romania [GAFTA & MOUNTFORD, 2008] and Habitats from Romania [DONIȚĂ & al. 2005, 2006]. Habitat structure characterization was done using phytosociological surveys. The inventory of the medicinal species was based on the active principles contained therein, and data obtained from bibliographic information [RÁCZ & al.

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1970; CIULEI, 1993; ISTUDOR, 1998, 2001; KATHE & al. 2003; STĂNESCU & al. 2004; OROIAN, 2011; YBERRT & al. 2013; EŞIANU & al. 2016].

Results and discussions

The field studies were carried out in 2014 and data analysis was made in 2015.

Two plant associations were identified: *Poo – Trisetetum flavescentis* Knapp ex Oberdorfer 1957 and *Festuco – Agrostetum capillaris* Horv. 1951. They were classified according to COLDEA (2012) and SANDA & al. (2001, 2008) as follows:

Cls. Molinio-Arrhenatheretea
Ord. Arrhenatheretalia
All. Arrhenatherion Koch 1926
Ass. *Poo – Trisetetum flavescentis* Knapp 1951 em. Oberdorfer 1983
All. Cynosurion R.Tx.1947
Ass. *Festuco – Agrostetum capillaris* Horv. 1951

The phytocoenosis of these two associations belong to **6520** – Mountain hay meadows habitat of community interest listed in Annex I of Habitats Directive [Council Directive 92/43/E, 1992].

These meadows have a good and very good conservation status. The field observations found that the trend of global conservation status of the habitat type 6520 is unfavourable-inadequate.

The most important risk factors/threats observed in the area are: overgrazing, land abandonment and the intrusion of invasive species. A major risk is also the massive collection of medicinal plants, which results in the future severe drop in the number of medicinal species which entails changes in the structure and composition of vegetation due to the proliferation of invasive species.

The flora of these associations includes many medicinal and fodder varieties, species having indicators of high natural value grasslands. This kind of meadows are considered to be among the best pastures in the country, with a very high productivity and very good nutritional value [SÂRBU & al. 2004; VEEN & al. 2009].

The diversity of medicinal and aromatics plants in the identified plant associations

Ass. *Poo-Trisetetum flavescentis* Knapp 1951 em. Oberdorfer 1983

The coenosis edified by *Trisetum flavescens* (Photo 1), were described in the low montane level on altitudes between 504 and 1634 m a.s.l. They occur on less moist and acid soils, rich in nutrients. Eight phytosociological surveys were taken in following localities of Mureş County: Sălard, Neagra, Ciobotani, Răstolița and Androneasa.

In the floristic composition of the coenosis edified by *Trisetum flavescens* and *Poa pratensis* 141 species were recorded. In mesophilous to xerophilous phytocoenosis prevail the species characteristic to Arrhenatherion alliance: *Arrhenatherum elatius* ssp. *elatius*, *Campanula patula*, *Centaurea nigrescens*, *Centaurea phrygia* ssp. *phrygia*, *Crepis biennis*, *Geranium pratense*, as well as those of Cynosurion alliance: *Cynosurus cristatus*, *Trifolium repens* ssp. *repens*, *Leontodon autumnalis*, *Phleum pratense* and Polygono-Trisetion alliance: *Centaurea pseudophrygia*, *Polygonum bistorta.*

In these coenosis can be found also a significant number of species of Arrhenatheretalia order: *Achillea millefolium* ssp. *millefolium*, *Briza media*, *Campanula glomerata* ssp. *glomerata*, *Carum carvi*, *Dactylis glomerata* ssp. *glomerata*, *Galium mollugo*, *Knautia arvensis* ssp. *arvensis*, *Leontodon hispidus* ssp. *hispidus*, *Leucanthemum vulgare* ssp. *vulgare*, *Plantago media*, *Rhinanthus rumelicus*, *Tragopogon pratensis* ssp. *orientalis*, *Veronica chamaedrys* etc., and of Molinio-Arrhenatheretea class: *Agrostis capillaris*, *Anthoxanthum odoratum*, *Cerastium holosteoides*, *Euphrasia rostkoviana*, *Holcus lanatus*, *Plantago lanceolata*, *Prunella vulgaris*, *Stellaria graminea*, *Trifolium pratense* ssp. *pratense*, *Vicia cracca*, *Viola tricolor* ssp. *tricolor* etc.

Photo 1. 6520 habitat, mountain hay meadows with *Trisetum flavescens* and *Poa pratensis* (foto M. Sămărghițan)

Ass. *Festuco-Agrostetum capillaris* Horv. 1951

Mesophilic phytocoenosis of the association (Photo 2) were identified in the grasslands that stretch on cleared forests, planes or on slopes with various inclinations and aspects and also on mountain plateaux. 21 phytosociologic surveys were conducted in the following locations of Mureş County: Chiheru de Sus; Câmpul Cetății – Pârâul Cald; Câmpul Cetății – Nirajul Mic Valley; Chiheru de Jos – Nirajul Mic Valley; Chiheru de Jos – Pârâul Diceau; Gurghiu – Orsova; Lăpușna; Sovata – Platoul Repaș; Ilieși – Sig; Sălard –Belciu Valley; Gurghiu – Toaca, Poiana Căpităneasa; Lăpușna – Poiana Borta; Lăpușna – Gitea; Răstolița – Peșcoasa Mare; Răstolița – Listeș; Răstolița – Podirei; Răstolița – Borzia; Meștera; Neagra – Jinga Valley; Gudea. The surveys were taken on various elevations between 518 and 1255 m a.s.l.

The coenosis occur on low acid soils, moderately moist, rich in humus, developed on crystalline schists. They are cohesive, have very good vegetation coverage (97-100%)

with a continuous and dense plant canopy. The grasslands edified by *Festuca rubra* and *Agrostis capillaris* have a remarkable floristic diversity proved by the 186 taxa recorded in 21 surveys. Along with the association`s diagnose species *Festuca rubra* and *Agrostis capillaris*, there are many species belonging to the superior syntaxonomic units, which the association was reported to, as well a series of transgressive species of the xeromesophilous grasslands. Thus were recorded species from Arrhenatherion alliance such as: *Arrhenatherum elatius* ssp. *elatius*, *Campanula patula*, *Centaurea nigrescens* ssp. *nigrescens*, *Centaurea phrygia* ssp. *phrygia*, *Crepis biennis*, *Geranium pratense*, *Orchis coriophora* ssp. *coriophora*, *Trisetum flavescens* and from Cynosurion alliance: *Carum carvi*, *Centaurea pseudophrygia*, *Cynosurus cristatus*, *Leontodon autumnalis*, *Phleum pratense*, *Trifolium repens* ssp. *repens*, *Veronica serpyllifolia* ssp. *serpyllifolia*.

Also numerous species of Arrhenatheretalia order were identified: *Achillea millefolium* ssp. *millefolium*, *Ajuga reptans*, *Briza media*, *Dactylis glomerata* ssp. *glomerata*, *Daucus carota*, *Galium mollugo*, *Heracleum spondylium* ssp. *spondylium*, *Knautia arvensis* ssp. *arvensis*, *Leontodon hispidus* ssp. *hispidus*, *Leucanthemum vulgare* ssp. *vulgare*, *Lotus corniculatus*, *Plantago media*, *Rhinanthus rumelicus*, *Senecio jacobea*, *Stellaria graminea*, *Tragopogon pratensis* ssp. *orientalis*, *Trifolium hybridum*, *Veronica chamaedrys* etc., and species belonging to Molinio-Arrhenatheretea class such as: *Alopecurus pratensis*, *Anthoxanthum odoratum*, *Cerastium holosteoides*, *Euphrasia rostkoviana*, *Festuca pratensis*, *Holcus lanatus*, *Ononis arvensis*, *Plantago lanceolata*, *Primula veris*, *Prunella vulgaris*, *Rumex acetosa*, *Poa pratensis*, *Trifolium pratense* ssp. *pratense*, *Viola tricolor* ssp. *tricolor*, *Myosotis arvensis*, *Medicago lupulina* etc.

Photo 2. 6520 habitat, *Festuco – Agrostetum capillaris* association (foto Mihaela Sămărghițan)

These mountain hay meadows assigned to 6520 habitat, are rich in medicinal species. As they are a vegetable treasure of inestimable value and therefore their inventory enables us their quantitative knowledge in order to rational exploitation without jeopardizing

their existence. A comparative analysis of the floristic composition of the two associations included in 6520 habitat (Tab. 1), shows that of the 148 species present in the phytosociologic surveys of *Festuco – Agrostetum capillaris*, 74 species have certain content in therapeutic chemical compounds. These species are classified as medicinal and aromatic herbs and are used in traditional medicine and phytotherapy. In the second association *Poo – Trisetetum flavescentis*, recording 141 species, 57 species are considered medicinal and aromatic.

The most common herb, whose presence in phytosociologic surveys is very high (**81-100%**) are: *Achillea millefolium* ssp. *millefolium*, *Plantago lanceolata*, *Prunella vulgaris* and *Trifolium pratense* ssp. *pratense*, followed by those with high frequency (**61-80** %): *Alchemilla xanthochlora*, *Carum carvi*, *Equisetum arvense*, *Euphrasia rostkoviana*, *Pimpinella saxifraga*, *Plantago media*, *Rumex acetosella*, *Thymus pulegioides*, *Veronica chamaedrys*, *Viola tricolor* ssp. *tricolor* etc. The following species have an average frequency between **41-60**%: *Daucus carota*, *Fragaria vesca*, *Galium mollugo*, *Galium verum*, *Mentha longifolia*, *Polygala vulgaris*, *Potentilla erecta* etc.

Tab. 1. Presence of medicinal and aromatic species in 6520 habitat

SPECIES	Ass. *Festuco rubrae – Agrostietum capillaris*	Ass. *Poo – Trisetetum flavescentis*	SPECIES	Ass. *Festuco – Agrostetum capillaris*	Ass. *Poo – Trisetetum flavescentis*
	K	**K**		**K**	**K**
Achillea millefolium	V	IV	*Mentha longifolia*	III	II
Agrimonia eupatoria	I	-	*Ononis arvensis*	I	-
Ajuga reptans	II	II	*Origanum vulgare*	II	-
Alchemilla xanthochlora	III	IV	*Pastinaca sativa*	I	-
Anchusa officinalis	I	-	*Petasites hybridus*	-	I
Anthyllis vulneraria	I	II	*Picea abies*	II	-
Artemisia vulgaris	II	I	*Pilosella officinarum*	II	I
Bellis perennis	I	-	*Pimpinella saxifraga*	IV	IV
Carum carvi	IV	III	*Plantago lanceolata*	IV	V
Centaurium erythraea	II	-	*Plantago major*	II	-
Cichorium intybus	I	II	*Plantago media*	III	IV
Clematis vitalba	I	-	*Polygala comosa*	-	I
Colchicum autumnale	I	I	*Polygala vulgaris*	III	III
Convolvulus arvensis	II	II	*Polygonum bistorta*	-	II
Crataegus monogyna	I	-	*Populus tremula*	I	-
Cruciata glabra	-	II	*Potentilla argentea*	I	-
Cruciata laevipes	II	II	*Potentilla erecta*	III	III
Daucus carota	II	III	*Potentilla recta*	I	-
Digitalis grandiflora	I	-	*Potentilla reptans*	II	I
Echium vulgare	II	-	*Primula veris*	II	II
Equisetum arvense	I	IV	*Prunella vulgaris*	IV	V
Eupatorium cannabinum	I	-	*Rosa canina*	I	-
Euphrasia rostkoviana	IV	IV	*Rubus idaeus*	I	-
Filipendula ulmaria	I	I	*Rumex acetosa*	II	III
Fragaria vesca	II	III	*Rumex acetosella*	III	IV
Fragaria viridis	I	-	*Rumex crispus*	I	I
Galium mollugo	III	III	*Salix alba*	-	I
Galium verum	III	II	*Senecio jacobaea*	II	II

Genista tinctoria	I	-	*Stachys germanica*	II	-
Gentiana asclepiadea	I	-	*Stachys officinalis*	II	II
Geranium robertianum	I	I	*Symphytum officinale*	I	I
Geum urbanum	-	I	*Taraxacum officinale*	-	II
Glechoma hederacea	I	I	*Thymus glabrescens*	II	1
Heracleum sphondylium	II	II	*Thymus pulcherrimus*	-	I
Hypericum maculatum	II	II	*Thymus pulegioides*	III	IV
Hypericum perforatum	II	-	*Trifolium pratense*	IV	V
Juniperus communis	I	-	*Tussilago farfara*	I	-
Linaria vulgaris	I	I	*Urtica dioica*	I	I
Lysimachia nummularia	I	II	*Veratrum album*	I	-
Lythrum salicaria	I	I	*Verbascum lychnitis*	I	I
Malus sylvestris	I	-	*Veronica chamaedrys*	II	IV
Medicago falcata	I	II	*Veronica officinalis*	II	I
Medicago lupulina	II	I	*Vincetoxicum hirundinaria*	I	-
Melilotus officinalis	I	-	*Viola tricolor*	IV	III
Mentha arvensis	I	-			

These species were gathered according to the dominant active principles for which they are used in traditional medicine or phytotherapy [Farmacopeea Română, 1993; European Pharmacopoeia, 2015]

The most numerous species contain: tannins (16.66%), essential oils (12.22%), coumarins (11.11%), flavonoids (10%), saponins (8.88%), alkaloids and mucilage (6.66% each), iridoids, bitter compounds and organic acids, vitamins and provitamins (4.44% each) etc. (Fig. 1)

Conclusions

The purpose of this study was a better knowledge of taxa of medicinal plants with high content of active compounds in Gurghiu Mountains.

There were identified semi-natural mountainous meadows rich in medicinal plants. The most numerous species contain: tannins (16.66%), essential oils (12.22%), coumarins (11.11%), flavonoids (10%) etc.

These meadows have a good and very good conservation status.

The most important risk factors/threats observed in the area are: overgrazing, land abandonment and the intrusion of invasive species.

A major risk is also the massive collection of medicinal plants, which results in the future severe drop in the number of medicinal species which entails changes in the structure and composition of vegetation due to the proliferation of invasive species.

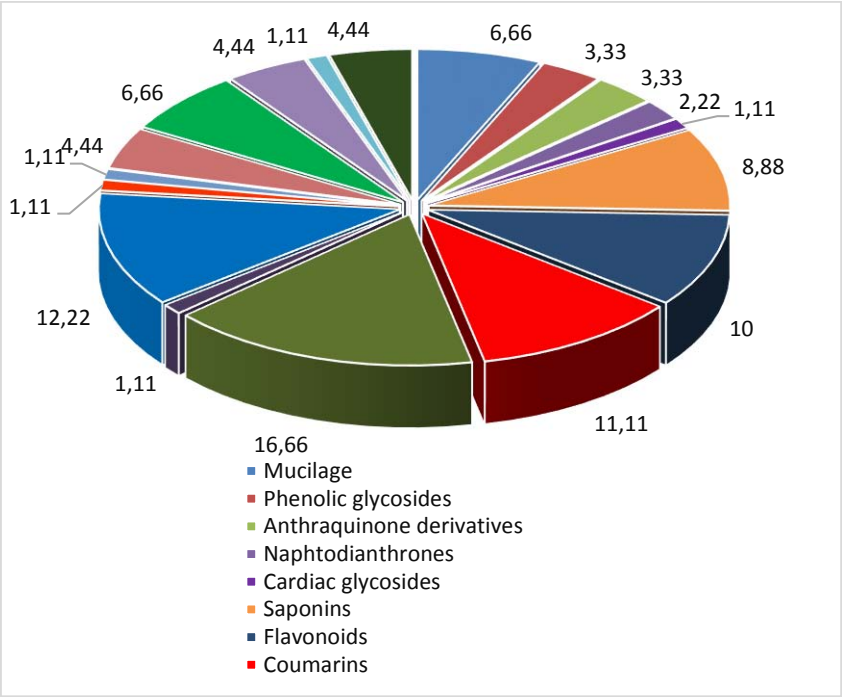

Fig. 1. The spectrum of active principles contained in the identified medicinal plants

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QUERCUS ROBUR, Q. CERRIS AND *Q. PETRAEA* AS HOT SPOTS OF BIODIVERSITY

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Abstract: Three different bipartite networks (pathogenic, ectomycorrhizal and galling insects) established by *Quercus robur* L., *Q. cerris* L. and *Q. petraea* (Matt.) Liebl. were merged in order to investigate the topological properties of the complex network, shading light on how biodiversity was organized through complex interactions. The complex network contains 290 species – 137 are pathogens (parasitic interaction), 72 are mycorrhizal fungi (mutualists) and 81 species of galling insects (herbivores). Most relevant network descriptors, connectivity, nestedness and modularity were analyzed. The main network and subnetworks displayed different behaviors in terms of topological properties, three of four networks showing significant modularity (galling insects network was marginally significant in what regards modularity). High connectivity and different degrees of nestedness characterized all networks. Clustering and Non Metric Multidimensional scaling refined the information provided by network analysis showing that networks occupy distant positions in ordination space and there are differences in terms of resemblance patterns.

Key words: biodiversity, community assembly, composite bipartite network, modularity, nestedness, subnetworks.

Introduction

One of the recurrent questions in ecology, why are there so many species, an inspiring interrogation since the seminal paper of G. E. HUTCHINSON (1959) is still in the quest of a conclusive answer. The answer is not simple and there are only partial explanations for the phenomenon of biodiversity. However, a common ground for investigation lies in the assembly rules which govern the structure of communities and ecosystems [GÖTZENBERGER & al. 2011] and in the structure itself. There is an obvious link between biodiversity as system property [HOLLAND, 1995] and the functioning of the system [LEVIN, 1998].

Network theory provides tools for the analysis of complex networks, ecological networks included [STROGATZ, 2001]. These tools permit the analysis of links simultaneously across the whole network. In a larger context, ecological networks incorporate interaction networks (established by antagonistic and mutualistic interactions, facilitation included) and spatial networks (such as habitat structure at landscape and meta-ecosystem levels), being complex real world networks with a specific small world and scale free topology [BARABÁSI & REKA, 1999; DUNNE & al. 2003; WATTS & STROGATZ, 1998]. Network approach facilitates the description of complex interactions summarized by large matrices, providing insight into ecological processes such as species co-existence, biogeographic frameworks, vulnerability to invasion or to extinction, population genetics and also, mechanisms of biodiversity patterns [ECONOMO & KEITT, 2008; THÉBAULT &

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FONTAINE, 2010; SIMARD & al. 2012; BAHRAM & al. 2014]. From a different perspective, species interaction diversity is analogous to species diversity in its simplest form, as interaction richness with characteristic network properties added [TYLIANAKIS & al. 2010].

Ecological networks depicting species interactions display non-random structure that allows them to persist despite their complexity [BASCOMPTE, 2010] however, the assembly of species within webs is modeled by deterministic and stochastic events which must be considered in the assessment of community structure [GÖTZENBERGER & al. 2011]. Network architecture is described by four main properties: number of nodes, connectance which describes the relative number of interactions, nestedness which describes the level of sharing of interaction patterns among species and modularity showing the degree of compartmentalization of the network [THÉBAULT & FONTAINE, 2010].

Species interactions are generally depicted by unipartite (trophic) and bipartite graphs (depicting mutualistic or antagonistic interactions). Bipartite networks are graphs with constrained wiring, with links between two groups of species, but not within each group. The two distinct sets of species are linked through coevolution and, the bipartite graph captures the main properties of the coevolved interactions such as asymmetry and modularity [JORDANO, 2010]. Intimate interactions established by symbiotic organisms (mutualistic or antagonistic) build networks of different pattern and structure as compared to free living organisms such as consumers and their prey [THOMPSON, 2005]. Being more specialized, networks integrating symbiotic organisms and their hosts display low modularity [PIRES & GUIMARÃES, 2013] and high nestedness [BASCOMPTE & al. 2003; FORTUNA & al. 2010]. It is generally accepted that low intimacy interactions which define consumers and their prey (consumption of plant tissue included), generate networks of different architecture, with high modularity and low nestedness. Connectance, as traditional network topology measure is dependent on species richness (higher in small networks) but has particular signature in trophic and mutualistic networks, with consumers' network being less connected than mutualistic network [THÉBAULT & FONTAINE, 2010].

Network analysis does not exclude multivariate analysis as traditional class of analytic tools but offers a new perspective and complements it [MELLO & al. 2011], with multivariate analysis focusing on species and network analysis on interactions. Compared to multivariate analysis, networks integrate different aspects including specificity and association breadth [CHAGNON & al. 2012].

Trees are complex organisms in terms of architecture, size, diversity of microhabitats, types of resources and longevity [LAWTON, 1983]. By consequence, biodiversity of associated species from higher trophic levels is vast [TEWS & al. 2004]. Organizing in a meaningful way this type of data becomes an important scientific goal in order to understand why indeed so many species are connected to trees and if trees are biodiversity generators in ecosystems, what consequences will have the unprecedented tree species decline that we witness [BUTCHART & al. 2010].

From higher trophic level perspective, trees are complex resources providing habitat and food for many categories of consumers and mutualists. Galling insects are typical consumers but with an elaborate strategy of reprograming the host-plant development, resulting in new structures beneficial to insects at the expense of the plant, being considered the most sophisticated herbivores [SHORTHOUSE & al. 2005]. These new structures are the galls which are composed of plant tissue generated as response to stimuli produced by gall inducers [STONE & SCHÖNROGGE, 2003]. The intimate level of interaction involving

molecular recognition mechanisms and genetic matching makes this category of highly specialized consumers superficially similar to pathogens. Also they are ideal models for the study of ecological diversity due to their abundance, specificity and richness [PORTUGAL-SANTANA & DOS SANTOS-ISAIAS, 2014].

Trees harbor a vast community of pathogens, the interaction with this category of organisms being antagonistic and the result of the co-evolution process, also intimate, modulated at molecular and genetic level. The impact of herbivores and pathogens on tree diversity was first hypothesized by JANZEN (1970) and CONNELL (1971). This was confirmed experimentally by BAGCHI & al. (2014) who showed that specialized consumers harmed more when their host were abundant, consequently leaving way to the establishment of less competitive tree species and therefore leading to higher biodiversity, at least in tropical areas. On the other hand, these groups of co-evolving organisms include rare parasitic species, mainly wood degrading fungi which are considered reliable indicators of ecosystem's biodiversity [CHRISTENSEN & al. 2004; ADAMČÍK & al. 2007].

Ectomycorrhizal fungi (ECM) establish beneficial mutualistic relationships with trees, essentially trophic interactions established through co-evolution, with different levels of specificity. There are ECM fungi with broad host range while others colonize few host species or closely related species within one genus [MOLINA & al. 1992; VAN DEN HEIJDEN & al. 2015]. Mycorrhizal interactions only recently were approached in the frame of mutualistic network studies focused more on pollinator and seed dispersal webs [BAHRAM & al. 2014].

The present study addresses the bipartite network properties of three types of communities (pathogens, ectomycorrhizal fungi and galling insects) linked to three important late successional tree species within same genus, dominant in mixed broadleaved forests of North-Western Transylvania, *Quercus robur* L., *Q. cerris* L. and *Q. petraea* (Matt.) Liebl. The summary networks for the three communities were merged in one complex network in order to investigate the topology and properties of the emerged network, assessing the contribution of each subnetwork. The assembly of the three communities was devised to highlight interaction diversity on taxonomically resolved networks. Combined with the information provided by the alternate multivariate analysis, it permits the analysis of possible assembly rule governing the association of different tree dependent communities and is an indirect indication of the importance of tree species as biodiversity key species.

Materials and methods

Compilation of species lists

We constructed complex bipartite network summarizing species of galling insects, pathogens and ectomycorrhizal fungi and three, phylogenetically close tree host species – *Q. robur, Q. cerris* and *Q. petraea*. Each of the corresponding subnetworks resulted from long term field observations. The number of species is considered to be a first order property of the network [DORMANN & al. 2009] and one of the fundamental network properties [THÉBAULT & FONTAINE, 2010]. The cumulative nature of the networks excludes strict spatial and temporal synchrony but is useful in the search of non-randomness in community assemblies. The interactions are resource mediated in the sense of host providing food and shelter for the associated species. Species inventory was assembled in one complex presence-absence matrix, used to generate the complex network and corresponding subnetworks (of galling insects, pathogens and ectomycorrhizal fungi).

The bipartite network approach in the case of galling insects which are linked to trees as consumers (traditionally included in unipartite trophic webs) was preferred due to the particular intimate nature of interaction resembling host-pathogen association.

Q. cerris, Q. robur and *Q. petraea* co-occur in forests located in hilly regions of North Western Transylvania, but our observations are restricted to areas in the proximity of cities as Cluj-Napoca and Oradea, in pure or mixed stands. As dominant, late stage species. *Q robur* and *Q. petraea* are closely related species, hybridizing frequently [SAMUEL & al. 1995; JENSEN & al. 2009], sharing many common features and being sympatric in most of their areal [ELLENBERG, 2009]. *Q. cerris* is more tolerant to drought, a feature correlated with its' predominantly southern distribution in Europe and Asia minor, more restricted than *Q. robur* and *Q. petraea* and expected to extend toward North due to the climate change [HLÁSNY & al. 2011].

ECM fungi inventory was based almost exclusively on aboveground observations on EC fungi fructifications over the last 20 years in mixed broadleaved forests in hilly areas of North Western Transylvania, on *Q. robur, Q. cerris* and *Q. petraea*, with the exception of *Coenococcum geophilum* observed on detached metabolically active roots of the investigated host species. The link to a specific host was assessed by the attachment of the sporophore to the root system. The long period of field observations suggests that at least most important and frequent active mycobionts were assessed but molecular data are needed to cover completely the diversity of ectomycorrhizal fungi [BAHRAM & al. 2014].

Observations on pathogenic, sapro-parasitic and parasitic fungi were gathered over the same extended period, within same types of ecosystems. Galling insects were inventoried beginning with 2006, within same locations.

The nomenclature of ectomycorrhizal fungi and pathogens follows Index Fungorum [www.indexfungorum.org], Global Biodiversity Information and Mycobank [www.mycobank.org]. For galling insects, nomenclature follows Fauna Europaea [www.faunaeur.org] and Melika (2006).

Network analysis

The complex network and separately, galling insects, pathogens and ECM fungi subnetworks were generated in Pajek ver.4.09 [BATAGELJ & MRVAR, 1998] using Kamada-Kawai layout. Unipartite versions of bipartite networks were also generated in Pajek.

Network metrics were calculated using several software packages for better significance testing, since different software authors provided different significance testing methods. Small networks (below 1000 nodes) are characterized by unstable metrics, a problem that arises when using iterative algorithms as in the case of the calculation of modularity and nestedness indices. In fact, many indices are affected by network size [OLESEN & al. 2007] and in the search of most stable results, using different software is helpful.

Network size is the product of total number of interacting species, more precisely the product between the number of hosts and number of their interaction partners, consumers or/and mutualists.

Connectance is a second order property of the network being related to specialization [DORMANN & al. 2009]. Is the proportion of realized interactions from all possible interactions in the network [MAY, 1972]. In bipartite webs it is measured as:

$$C = \frac{L}{I \cdot J}$$

Where: J stands for lower trophic level species and I for higher trophic level species.

Web asymmetry is a simple measure of the balance between the lower (J) and higher trophic (I) level in bipartite networks [DORMANN & al. 2009].

$$W = \frac{J - I}{J + I}$$

Positive numbers indicate higher trophic level species while negative numbers indicate lower trophic level species prevalence. The index scales within the interval [-1; 1]. When there are more species in the higher trophic level set, the value approaches -1.

Modularity is a network property measuring the tendency of groups of nodes (species) to interact more strongly among themselves than with other nodes in the network, compartmentalization defining the real world networks as opposed to random networks lacking this property [BARABÁSI, 2016; BASCOMPTE, 2010]. Detection of communities in networks is considered to reveal links between topologies and functional traits of biological systems and is considered to be more obvious in antagonistic than in mutualistic networks [OLESEN & al. 2007]. There are many algorithms proposed to search and measure modularity. We chose Louvain algorithm [BLONDEL & al. 2008] (provided by the software Pajek) which estimated the modularity index (Q) using a greedy optimization algorithm. It is the same Newman - Girvan (2004) metric which incorporates hierarchy in successive community search iterations. The optimization is performed in two steps: first it optimizes the modularity locally by clustering the neighboring nodes and during the second phase clusters are aggregated until modularity ceases to increase.

$$Q = \sum_{i-1}^{N_M} \left[\frac{E_i}{E} - \left(\frac{k_i C * k_j R}{E^2} \right) \right]$$

Where: k_iC represents the sum of degrees of nodes within module *i* that belong to set *C* and k_jR represents the sum of degrees of nodes within module *i* that belong to set *R;* E_i represents the number of links in module *i; E* the number of links in the complete network.

The partition of nodes that gives rise to the maximum Q value is considered as community structure of a graph or network. Modularity index quantifies the degree of modules' clear delimitation [OLESEN & al. 2007].

The results are considered to be robust and comparable to simulated annealing results [POISOT & al. 2013]. We used the default settings of the algorithm: resolution parameter set to 1, number of random restarts was 5, maximum number levels at each iteration - 20 and the number of repetitions at each level set to 50. The algorithm was repeated 50 times until a stable number of modules and of modularity value was obtained.

For comparisons, NETCARTO software [GUIMERÀ & AMARAL, 2005] was employed. The metric used for the present study was the most popular with modularity analysis, Q metric of Girvan and Newman (2002). Guimerà and Amaral (2005) developed an algorithm for modularity optimization over possible partitions by using simulated annealing. Significance testing is performed according to null model III, number of links in random models is the same with number of links in observed network reproducing in this way the real data [GOTELLI, 2000].

MODULAR software [MARQUITTI & al. 2014] was employed for comparing the possibility of significance testing using two null models: ER null model and null model II, (number of links is kept constant during randomizations but marginal matrix distributions vary). The alternative hypothesis states that observed modularity should be greater than the average modularity calculated on 100 simulated matrices. An alternative significance testing uses z

scores, which use the empirical modularity, value Q_{calc}, mean and standard deviation of simulated matrices' modularity [GUIMERÀ & al. 2004]. Significant modularity implies $z \geq 1$.

$$Z = (Q_{calc} - \mu)/\sigma$$

The roles and degree of connectivity of each node can be assessed in parameter z-P space (P standing for participation coefficient which measures how well are distributed the links of node *i* among modules and z for within module degree, which measures how well is connected node *i* to other nodes in module). Nodes are classified in this space as non-hub and hub nodes while non-hub nodes can play different roles as: ultra-peripheral, peripheral, connector and kinless nodes [GUIMERÀ & AMARAL, 2005].

Nestedness is a presence-absence matrix property displayed also by the corresponding matrix quantified by an index which is the measure of how much of the matrix elements can be packed without holes [ARAUJO & al. 2010]. In nested matrices, specialist species interact with generalists hosts. The concept was first used in biogeographic context to explain the diversity pattern of species from poor sites as being subsets of species rich sites [ATMAR & PATTERSON, 1993] and became a key tool to characterize network structure [ARAUJO & al. 2010]. To calculate matrix temperature, the authors used an analogy from thermodynamics, characterizing matrix order as 0°C temperature and complete disorder as 100°C. At zero degrees temperature, a thermodynamic system presents particles in the state of minimal energy analogous to a completely nested structure while, in the state of complete disorder particles present high energy level analogous to a non-nested matrix. Nestedness is calculated as N=(100-T)/100, [BASCOMPTE & al. 2003], N being defined in the range [0,1] where 1 corresponds to a perfectly nested network and 0 corresponds to systems where interactions occur completely at random. Matrix temperature was calculated using BINMATNEST software [RODRÍGUEZ-GIRONÉS & SANTAMARÍA, 2006] using 100 randomizations for significance testing. We employed also results yielded by the package *bipartite* for R [DORMANN & al. 2008].

Different software use different null models to test significance of the results: the actual network against a randomly assembled network under more or less liberal restrictions. BINMATNEST provides the results of testing null models I, II and III but authors recommend to use as benchmark, null model III (FE-fixed row totals and equiprobable column totals) [ULRICH & GOTELLI, 2007]. R bipartite package employs null model r00 or null model I, the most liberal of null models assuming that column and row total vary freely. The alternative hypothesis states that observed statistic is greater or less than simulated statistic (1000 simulations). Alternative significance testing is provided by SES (Standardized Effect Size) which measures the number of standard deviations that the observed statistic is above or below the mean statistic of simulated null matrices. SES above 2 and below -2 indicates significant result at error level of 5% [ULRICH & GOTELLI, 2007].

C score, the index devised for community analysis for the search of possible assembly mechanism [STONE & ROBERTS, 1990] is also included in network analysis as characteristic metric for qualitative bipartite networks [DORMANN & al. 2009] originally being used as biogeographic index. C-score is the average number of checkerboard units that are for a pair of species [STONE & ROBERTS, 1990; GOTELLI, 2000]. In order to quantify the direction of deviation from the null model, standardized effect size (SES) is calculated. The calculations were performed in EcoSim ver. 7.0 [GOTELLI & ENTSMINGER, 2001].

Classical community analysis approach

To test the level of pattern detection provided by network analysis we employed classical community analysis methods such as cluster analysis and Non Metric Multidimensional Scaling (NMDS).

Clustering is an exploratory method of grouping entities (species) according to a resemblance measure. Clustering was performed using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm and as dissimilarity measure, Euclidean distance. The graphical representation, the dendrogram records the sequences of merges of entities, for instance species, and splits which generate groups. The species are compared pairwise.

Clustering depends highly on the similarity measure used: one robust measure is Euclidean distance [HAMMER & al. 2001] which we employed in our cluster analysis.

We tested for correlation between similarity matrices of the three categories of organisms and also between the complex matrix and each of the categories using Mantel test [MANTEL, 1967]. Mantel test analyzes the correlation between 2 distance matrices being a non-parametric significance test. It computes the significance of correlation through permutation of rows and columns of the input matrices. The test statistic is Pearson product moment, R coefficient taking values in the [-1:1] range.

Non-metric Multidimensional Scaling ranks distances among objects and uses the ranks to map the objects onto two-dimensional ordination space preserving the ranked differences [SHEPARD, 1966] and operating in most parsimonious way. The essence of the method consists in finding the configuration of points compatible with a given dissimilarity relation among them permitting the visualization of a structure hidden in the original data [KENKEL & ORLÓCI, 1986; BORG & GROENEN, 2005]. The method is iterative and several iterations are performed until the lowest stress value (the best goodness of fit) is obtained. The proximity among objects in the ordination space corresponds to their similarity. We used Euclidean distance as similarity measure.

Clustering and NMDS were performed using software PAST [HAMMER & al. 2001].

Results

Interaction matrix

The assembled interaction matrix contains 290 species of which, higher trophic level incorporates galling insects (81 species), tree pathogens (137 species) and ECM fungi (72 species). The lower trophic level is represented by *Q. robur*, *Q. cerris* and *Q. petraea* (Tab. 1). The constructed matrix and networks are taxonomically resolved at species level. Closely related tree species are expected to share their higher level partners, a fact that should be reflected in the topology of the complex network incorporating three different types of interactions. The repartition on categories and hosts of the species is depicted in Fig. 1.

Tab 1. Matrix of pathogenic, ectomycorrhizal and galling insects' species associated to host trees

Nr.1[2]	Nr.2[3]	Species	*Q. robur*	*Q. cerris*	*Q. petraea*
Pathogens					
1.	1.	*Abortiporus biennis* (Bull.) Singer	1	1	1
2.	2.	*Aleurodiscus disciformis* (DC.) Pat.	1	1	1
3.	3.	*Aleurodiscus oakesii* (Berk. & M. A. Curtis) Cooke	1	0	1
4.	4.	*Alternaria alternata* (Fr.) Keissl.	1	1	1
5.	5.	*Amphiporthe leiphaemia* (Fr.) Butin	1	1	1
6.	6.	*Anthostoma decipiens* (DC.) Nitschke	1	1	0
7.	7.	*Antrodia albida* (Fr.) Donk	1	1	1
8.	8.	*Antrodiella semisupina* (Berk. & M. A. Curtis) Ryvarden	1	1	0
9.	9.	*Apiocarpella quercicola* Tak. Kobay. & K. Sasaki	1	0	0
10.	10.	*Apiognomonia errabunda* (Roberge ex Desm.) Höhn	1	1	1
11.	11.	*Armillaria gallica* Marxm. & Romagn.	1	1	0
12.	12.	*Armillaria mellea* (Vahl) P. Kumm.	1	1	1
13.	13.	*Armillaria tabescens* (Scop.) Emel	1	1	1
14.	14.	*Ascochyta quercus* Sacc. & Speg.,	1	1	1
15.	15.	*Aureobasidium apocryptum* (Ellis & Everh.) Herm.-Nijh	1	0	0
16.	16.	*Bjerkandera adusta* (Willd.) P. Karst.	1	0	0
17.	17.	*Botryodiplodia* sp.	1	0	0
18.	18.	*Botryosphaeria stevensii* Shoemaker	1	1	0
19.	19.	*Botryotinia fuckeliana* (de Bary) Whetzel	1	1	1
20.	20.	*Buglossoporus pulvinus* (Pers.) Donk	1	1	1
21.	21.	*Cerrena unicolor* (Bull.: Fr.) Murrill	1	1	1
22.	22.	*Chondrostereum purpureum* (Pers.) Pouzar	1	0	1
23.	23.	*Ciboria batschiana* (Zopf) N. F. Buchw	1	0	1
24.	24.	*Colpoma quercina* (Pers.) Wahll.	1	1	0
25.	25.	*Cryphonectria parasitica* (Murrill) M. E. Barr	1	0	1
26.	26.	*Cryptocline cinerascens* (Bubák) Arx	1	1	1
27.	27.	*Daedalea quercina* (L.) Pers.	1	1	1
28.	28.	*Daldinia childiae* J. D. Rogers & Y. M. Ju	1	0	0
29.	29.	*Diaporthe insularis* Nitschke	1	0	1
30.	30.	*Diatrypella quercina* (Pers.) Cooke	1	1	1
31.	31.	*Dicarpella dryina* Belisario & M. E. Barr	1	1	0
32.	32.	*Diplodia corticola* A. J. L. Phillips, A. Alves & J. Luque	0	1	1
33.	33.	*Diplogelasinospora grovesii* Udagawa & Y. Horie	0	0	1
34.	34.	*Elsinoe quercicola* Bitanc. & Jenkins	1	0	0
35.	35.	*Entonaema cinnabarinum* (Cooke & Massee) Lloyd	1	0	0
36.	36.	*Erwinia herbicola* (Lohnis 1911) Dye	0	1	0
37.	37.	*Erysiphe alphitoides* (Griffon & Maubl.) U. Braun & S. Takam.	1	1	1
38.	38.	*Erysiphe hypophylla* (Nevod.) U. Braun & Cunningt.	1	0	1
39.	39.	*Erysiphe quercicola* S. Takam. & U. Braun	1	1	1
40.	40.	*Eutypa quercicola* Berk.	1	1	1
41.	41.	*Fistulina hepatica* (Schaeff.) With.	1	1	1
42.	42.	*Fomes fomentarius* (L.) Fr.	0	0	1
43.	43.	*Fuscoporia contigua* (Pers.) G. Cunn	1	1	0
44.	44.	*Fuscoporia ferruginos*a (Schrad.) Murrill	1	0	0
45.	45.	*Fuscoporia torulosa* (Pers.) T. Wagner & M. Fisch.	1	0	0
46.	46.	*Fusicoccum quercus* Oudem.	1	0	0

[2] Complex matrix

[3] Separate matrices

Nr.1[2]	Nr.2[3]	Species	*Q. robur*	*Q. cerris*	*Q. petraea*
47.	47.	*Ganoderma applanatum* (Pers.) Pat.	1	1	1
48.	48.	*Ganoderma lucidum* (Curtis) P. Karst.	1	1	1
49.	49.	*Gibberella baccata* (Wallr.) Sacc.	1	0	0
50.	50.	*Gibberella pulicaris* (Kunze) Sacc.	0	0	1
51.	51.	*Gibberella zeae* (Schwein.) Petch	1	0	0
52.	52.	*Globisporangium spiculum* (B. Paul) Uzuhashi, Tojo & Kakish	1	0	0
53.	53.	*Globisporangium ultimum* (Trow) Uzuhashi, Tojo & Kakish.	1	0	0
54.	54.	*Gnomoniopsis clavulata* (Ellis) Sogonov	1	1	1
55.	55.	*Grifola frondosa* (Dicks.) Gray	1	0	1
56.	56.	*Gymnopus fusipes* (Bull.) Gray	1	1	1
57.	57.	*Hapalopilus croceus* (Pers.) Bondartsev et Singer	1	1	1
58.	58.	*Hapalopilus nidulans* (Fr.) P. Karst.	1	1	1
59.	59.	*Helicomyces roseus* Link	0	0	1
60.	60.	*Hericium cirrhatum* (Pers.) Nikol	1	1	1
61.	61.	*Hericium erinaceus* (Bull.) Pers.	1	1	1
62.	62.	*Hymenochaete rubiginosa* (Dicks.) Lév.	1	1	1
63.	63.	*Hypospilina pustula* (Pers.) M. Monod,	1	1	1
64.	64.	*Inonotus andersonii* (Ellis & Everh.) Nikol	1	1	0
65.	65.	*Inonotus hispidus* (Bull.) P. Karst	1	1	1
66.	66.	*Inonotus nidus-pici* Pilat ex Pilat	0	1	0
67.	67.	*Inonotus obliquus* (Ach. ex Pers.) Pilát	1	1	0
68.	68.	*Inonotus rheades* (Pers.) Fiasson & Niemelä	1	1	1
69.	69.	*Inonotus rickii* (Pat.) Reid	0	1	0
70.	70.	*Irpex mollis* Berk. & M.A. Curtis	1	1	1
71.	71.	*Laetiporus sulphureus* (Bull.) Murrill	0	0	1
72.	72.	*Lentinus arcularius* (Batsch) Zmitr	0	1	0
73.	73.	*Lenzites betulina* (L.) Fr.	1	1	1
74.	74.	*Loranthus europaeus* Jacq.	1	1	1
75.	75.	*Meripilus giganteus* (Pers.) P. Karst.	1	0	0
76.	76.	*Microsphaera silvatica* Vlasov	1	0	0
77.	77.	*Microstroma album* (Desm.) Sacc.	1	1	1
78.	78.	*Monochaetia monochaeta* (Desmaz.) Allesch.	1	1	0
79.	79.	*Mycelium radicis-atrovirens* Melin	1	0	0
80.	80.	*Nemania serpens* (Pers.: Fr.) S. F. Gray.	1	0	0
81.	81.	*Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A. J. L. Phillips	1	0	0
82.	82.	*Nodulisporium corticioides*(Ferraris & Sacc.) S. Hughes	1	0	0
83.	83.	*Peniophora quercina* (Pers.) Cooke	0	1	0
84.	84.	*Pesotum piceae* J. L. Crane & Schokn	1	1	0
85.	85.	*Pestalotiopsis monochaeta* Maharachch. K. D. Hyde & Crous	1	0	0
86.	86.	*Pestalotiopsis neglecta* (Thüm.) Steyaert	1	1	1
87.	87.	*Pezicula cinnamomea* (DC.) Sacc	1	0	1
88.	88.	*Pezicula melanigena* (T. Kowalski & Halmschl.) P. R. Johnst.	1	0	1
89.	89.	*Phellinopsis conchata* (Pers.) Y. C. Dai	1	0	0
90.	90.	*Phialocephala dimorphospora* W. B. Kendr	1	1	0
91.	91.	*Phlebia radiata* Fr.	1	0	0
92.	92.	*Pholiota squarrosa* (Vahl) P. Kumm	1	1	0
93.	93.	*Phomopsis glandicola* (Lév.) Grove	1	0	1
94.	94.	*Phomopsis quercina* (Sacc.) Höhn. ex Died.	1	0	0
95.	95.	*Phyllactinia guttata* (Wallr.) Lév.	1	0	1

Nr.1[2]	Nr.2[3]	Species	*Q. robur*	*Q. cerris*	*Q. petraea*
96.	96.	*Phytophthora plurivora* T. Jung & T. I. Burgess	1	0	0
97.	97.	*Phytophthora cactorum* (Lebert & Cohn) J. Schröt.	1	0	0
98.	98.	*Phytophthora citricola* Sawada	1	1	1
99.	99.	*Phytophthora cryptogea* Pethybr. & Laff.	0	1	0
100.	100.	*Piptoporus quercinus*(Schrad.) P. Karst.	1	1	1
101.	101.	*Podoscypha multizonata* (Berk. & Broome) Pat.	1	0	0
102.	102.	*Polyporus squamosus* (Huds.) Fr.	1	0	0
103.	103.	*Porostereum spadiceum* (Pers.) Hjortstam & Ryvarden	1	0	0
104.	104.	*Postia subcaesia* (A. David) Jülich	1	0	1
105.	105.	*Pseudoinonotus dryadeus* (Pers.) T. Wagner & M. Fisch.	1	1	1
106.	106.	*Pseudomonas quercus* Schern	1	0	0
107.	107.	*Pythium inflatum* V. D. Matthews	1	0	1
108.	108.	*Pythium sterilum* Belbahri & Lefort	1	1	1
109.	109.	*Ramularia endophylla* Verkley & U. Braun	1	0	0
110.	110.	*Rigidoporus lineatus* (Pers.) Ryvarden	1	0	0
111.	111.	*Rosellinia necatrix* Berl. ex Prill.	1	1	1
112.	112.	*Schizophyllum commune* Fr.	1	1	1
113.	113.	*Schizopora paradoxa* (Schrad.) Donk	0	1	1
114.	114.	*Septoria quercicola* Sacc.	1	0	0
115.	115.	*Spongipellis litschaueri* Lohwag	1	1	1
116.	116.	*Spongipellis spumeus* (Sowerby) Pat	1	1	1
117.	117.	*Steccherinum ochraceum* (Pers.) Gray	1	0	1
118.	118.	*Stereum gausapatum* (Fr.) Fr.	1	1	1
119.	119.	*Stereum hirsutum* (Willd.) Pers.	1	1	1
120.	120.	*Stereum ochraceoflavum* (Schwein.) Sacc.	1	1	1
121.	121.	*Stereum rameale* (Schwein.) Burt	1	0	1
122.	122.	*Stereum rugosum* Fr.	1	0	0
123.	123.	*Stereum subtomentosum* Pouzar	1	0	1
124.	124.	*Taphrina coerulescens* (Desm. & Mont.) Tul.	0	1	0
125.	125.	*Trametes hirsuta* (Wulfen) Lloyd	1	1	1
126.	126.	*Trametes trogii* Berk	1	0	1
127.	127.	*Trametes versicolor* (L.) Lloyd	1	1	1
128.	128.	*Tyromyces fissilis* (Berk. & Curt.) Donk	1	1	1
129.	129.	*Valsa intermedia* Nitschke	1	0	1
130.	130.	*Verticillium dahliae* Kleb.	0	1	1
131.	131.	*Viscum album* L.	1	1	1
132.	132.	*Vuilleminia comedens* (Nees.) Maire	1	1	1
133.	133.	*Vuilleminia cystidiata* Parmasto	1	1	1
134.	134.	*Vuilleminia megalospora* Bres	1	1	1
135.	135.	*Xanthomonas campestris* (Pammel, 1895) Dowson, 1939	1	1	0
136.	136.	*Xylebolus frustulatus* (Berk & M. A. Curtis) Boidin	1	1	1
137.	137.	*Xylobolus subpileatus* (Berk. & M. A. Curtis) Boidin	1	1	0
Ectomycorrhizal fungi					
138.	1.	*Coenococcum geophilum* Fr.	1	1	1
139.	2.	*Lactarius circellatus* (Battara) Fr.	1	1	1
140.	3.	*Amanita rubescens* (Pers.:Fr.) Gray	1	1	1
141.	4.	*Amanita phalloides* (Vaill. ex Fr.) Link	0	0	1
142.	5.	*Russula foetens* (Pers.) Pers.	1	1	1
143.	6.	*Russula cyanoxantha* (Schaeff.) Fr.	1	1	1
144.	7.	*Lactarius quietus* (Fr.) Fr.	0	0	1
145.	8.	*Russula fragilis* sensu Cooke	0	0	1
146.	9.	*Lactarius vellereus* (Fr.) Fr.	1	0	1
147.	10.	*Russula delica* Fr.	1	0	1

Nr.1[2]	Nr.2[3]	Species	*Q. robur*	*Q. cerris*	*Q. petraea*
148.	11.	*Xerocomellus chrysenteron* (Bull.) Šutara	1	1	1
149.	12.	*Butyriboletus appendiculatus* (Schaeff.) O. A. Arora & J. J. Frank	1	1	1
150.	13.	*Xerocomellus porosporus* (Imler ex. Bon) Šutara	1	0	1
151.	14.	*Russula nobilis* Velen.	0	0	1
152.	15.	*Russula consobrina* (Fr.) Fr.	0	0	1
153.	16.	*Boletus reticulatus* Schaeff.	1	1	1
154.	17.	*Suillellusqueletii* (Schulzer) Vizzini, Simonini & Gelardi	0	0	1
155.	18.	*Suillellus luridus* (Schaeff.) Murrill	1	1	1
156.	19.	*Gyroporus castaneus* (Bull.) Quél.	0	0	1
157.	20.	*Lactarius piperatus* (L.) Pers.	1	1	1
158.	21.	*Lactarius volemus* (Fr.) Fr.	0	0	1
159.	22.	*Craterellus cornucopioides* (L.) Pers.	1	1	1
160.	23.	*Inocybe rimosa* (Bull.) P. Kumm.	1	1	1
161.	24.	*Scleroderma citrinum* Pers.	1	1	1
162.	25.	*Russula cessans* A. Pearson	0	0	1
163.	26.	*Boletus subtomentosus* L.	0	0	1
164.	27.	*Amanita vaginata* (Bull.) Lam.	1	1	1
165.	28.	*Russula virescens* (Schaeff.) Fr.	1	0	1
166.	29.	*Tricholoma saponaceum* (Fr.) P. Kumm.	1	0	1
167.	30.	*Russula claroflava* Grove	1	0	1
168.	31.	*Russula fellea* (Fr.) Fr.	1	0	0
169.	32.	*Hygrophorus eburneus* (Bull.) Fr.	0	0	1
170.	33.	*Russula vesca* Fr.	1	0	1
171.	34.	*Laccaria laccata* (Scop.) Cooke	1	1	1
172.	35.	*Laccaria amethystina* (Huds.) Cooke	1	0	1
173.	36.	*Hebeloma crustuliniforme* (Bull.) Quél.	1	1	1
174.	37.	*Russula pectinatoides* Peck	0	0	1
175.	38.	*Suillellus satanas* (Lenz) Blanco-Dios	1	1	0
176.	39.	*Cortinarius* sp.	0	0	1
177.	40.	*Amanita lividopallescens* (Secr. ex Boud.) Kühner & Romagn	0	0	1
178.	41.	*Russula rosea* Pers.	0	0	1
179.	42.	*Russula parazurea* Jul. Schäff.	1	0	1
180.	43.	*Paxillus involutus* (Batsch) Fr.	1	0	1
181.	44.	*Inocybe* sp.	0	1	1
182.	45.	*Tricholoma virgatum* (Fr.) P. Kumm.	1	0	1
183.	46.	*Leccinum aurantiacum* (Bull.) Gray	0	0	1
184.	47.	*Russula nigricans* Fr.	1	1	1
185.	48.	*Lactarius chrysorrhaeus* Fr.	0	0	1
186.	49.	*Lactarius camphoratus* (Bull.) Fr.	0	0	1
187.	50.	*Amanita echinocephala* (Vittad.) Quél.	0	0	1
188.	51.	*Russula romellii* Maire	0	1	1
189.	52.	*Russula ionochlora* Romagn.	0	1	1
190.	53.	*Russula atropurpurea* (Krombh.) Britzelm.	1	0	0
191.	54.	*Amanita virosa* Bertill.	1	0	1
192.	55.	*Amanita ceciliae* (Berk. & Broome) Bas.	0	0	1
193.	56.	*Amanita pantherina* sensu Gonnermann & Rabenhorst	1	1	1
194.	57.	*Lactarius fulvissimus* Romagn.	0	0	1
195.	58.	*Russula brunneoviolacea* Crawshay	0	0	1
196.	59.	*Scleroderma areolatum* Pers.	1	0	1
197.	60.	*Hemileccinum impolitum* (Fr.) Šutara	0	0	1
198.	61.	*Russula viridicans* Carteret & Reumaux	0	0	1
199.	62.	*Hydnum repandum* L.	1	1	1

Nr.1[2]	Nr.2[3]	Species	*Q. robur*	*Q. cerris*	*Q. petraea*
200.	63.	*Ramaria stricta* (Pers.) Quél.	1	1	1
201.	64.	*Lactarius blennius* (Fr.) Fr.	0	0	1
202.	65.	*Russula aurea* Pers.	0	0	1
203.	66.	*Cyanoboletus pulverulentus* (Opat.) Gelardi, Vizzini & Simonini	0	0	1
204.	67.	*Russula decolorans* (Fr.) Fr.	0	0	1
205.	68.	*Lactarius scrobiculatus* (Scop.) Fr.	0	0	1
206.	69.	*Suillus granulatus* (L.) Roussel	1	0	1
207.	70.	*Russula grata* Britzelm.	1	1	1
208.	71.	*Boletus edulis* Bull.	1	1	0
209.	72.	*Entoloma sinuatum* (Bull.) P. Kumm.	1	1	1
Galling insects					
210.	1.	*Andricus lucidus* (= *A. aestivalis* Giraud, 1859 ♀♂)	0	1	0
211.	2.	*Andricus amblycerus* Giraud, 1859 ♀	1	0	1
212.	3.	*Andricus aries* Giraud, 1859 ♀	1	0	0
213.	4.	*Andricus autumnalis* Mayr, 1882 ♀♀	0	0	1
214.	5.	*Andricus caliciformis* Giraud, 1859 ♀	1	0	1
215.	6.	*Andricus callidoma* (= *A. cirratus* Adler, 1881 ♀♂)	1	0	0
216.	7.	*Andricus callidoma* Hartig, 1841 ♀♀	0	0	1
217.	8.	*Andricus collaris* Hartig, 1840 ♀♀	1	0	1
218.	9.	*Andricus collaris* (= *A. curvator* Hartig, 1840 ♀♂)	1	0	1
219.	10.	*Andricus conglomeratus* Giraud, 1859 ♀	0	0	1
220.	11.	*Andricus coriarius* Hartig, 1843 ♀	1	0	1
221.	12.	*Andricus corruptrix* Schlechtendal, 1870 ♀♀	1	0	1
222.	13.	*Andricus crispator* Tschek, 1871 ♀♀	0	1	0
223.	14.	*Andricus cydoniae* Giraud, 1859 ♀♂	0	1	0
224.	15.	*Andricus foecundatrix* Hartig, 1840 ♀♀	1	0	0
225.	16.	*Andricus galeatus* Giraud, 1859 ♀	0	0	1
226.	17.	*Andricus gallaetinctoriae* Olivier, 1791 ♀♀	1	0	1
227.	18.	*Andricus gemmeus* Giraud, 1859 ♀♀	1	0	1
228.	19.	*Andricus gemmeus* Giraud, 1859 ♀♂	0	1	0
229.	20.	*Andricus glandulae* Hartig, 1840 ♀	0	0	1
230.	21.	*Andricus glutinosus* Giraud, 1859 ♀	1	0	1
231.	22.	*Andricus grossulariae* (= *A. mayri* Mayr, 1882 ♀♀)	1	0	0
232.	23.	*Andricus grossulariae* (= *A. grossulariae* Giraud, 1859 ♀♂)	1	0	0
233.	24.	*Andricus inflator* Hartig, 1840 ♀♀	1	0	1
234.	25.	*Andricus inflator* (= *Cynips globuli* Hartig, 1840 ♀♂)	1	0	0
235.	26.	*Andricus kollari* Hartig, 1843 ♀♀	1	0	1
236.	27.	*Andricus lignicolus* Hartig, 1840 ♀♀	1	0	1
237.	28.	*Andricus lucidus* Hartig, 1843 ♀♀	1	0	1
238.	29.	*Andricus malpighii* Adler, 1881 ♀♀	0	0	1
239.	30.	*Andricus mitratus* Mayr, 1870 ♀	0	0	1
240.	31.	*Andricus multiplicatus* Giraud, 1859 ♀♂	0	1	0
241.	32.	*Andricus paradoxus* Radoszkowski, 1866 ♀♀	1	0	1
242.	33.	*Andricus polycerus* Giraud, 1859 ♀	0	0	1
243.	34.	*Andricus quadrilineatus* Hartig, 1840 ♀♀	1	0	0
244.	35.	*Andricus quercuscalicis* Burgsdorff, 1783 ♀♀	1	0	0
245.	36.	*Andricus quercuscorticis* Linnaeus, 1761 ♀♀	0	0	1
246.	37.	*Andricus quercusradicis* Fabricius, 1798 ♀♀	1	0	1
247.	38.	*Andricus quercusradicis* (*A. trilineatus* Hartig, 1840 ♀♂)	1	0	1
248.	39.	*Andricus schroeckingeri* Wachtl, 1876 ♀	1	0	1
249.	40.	*Andricus seminationis* Giraud, 1859 ♀	1	0	0
250.	41.	*Andricus serotinus* Giraud, 1859 ♀	0	0	1
251.	42.	*Andricus solitarius* Fonscolombe, 1832 ♀♀	1	0	1

Nr.1[2]	Nr.2[3]	Species	*Q. robur*	*Q. cerris*	*Q. petraea*
252.	43.	*Andricus seckendorffi* Wachtl, 1879	0	0	1
253.	44.	*Andricus gallaeurnaeformis* (= *A. sufflator* Mayr, 1882 ♀♂)	1	0	0
254.	45.	*Andricus superfetationis* Giraud, 1859 ♀	1	0	0
255.	46.	*Andricus testaceipes* Hartig, 1840 ♀♀	1	0	0
256.	47.	*Andricus testaceipes* Hartig, 1840 ♀♂	1	0	0
257.	48.	*Andricus truncicola* Giraud, 1859 ♀	1	0	1
258.	49.	*Andricus vindobonensis* Müllner, 1901 ♀♂	0	1	0
259.	50.	*Aphelonyx cerricola* Giraud, 1859 ♀	0	1	0
260.	51.	*Biorhiza pallida* Olivier, 1791 ♀♂	1	0	1
261.	52.	*Chilaspis nitida* (= *C. lowi* Wachtl, 1882 ♀♂)	0	1	1
262.	53.	*Chilaspis nitida* Giraud, 1859 ♀♀	0	1	0
263.	54.	*Contarinia quercicola* Rubsaamen, 1899	0	1	0
264.	55.	*Cynips agama* Hartig, 1840 ♀♀	1	0	1
265.	56.	*Cynips disticha* Hartig, 1840 ♀♀	1	0	1
266.	57.	*Cynips divisa* Hartig, 1840♀♀	1	0	1
267.	58.	*Cynips longiventris* Hartig, 1840 ♀♀	1	0	0
268.	59.	*Cynips quercusfolii* (= *C. taschenbergi* Schlechtendal, 1870 ♀♂)	0	0	1
269.	60.	*Cynips quercusfolii* Linnaeus, 1758 ♀♀	1	0	1
270.	61.	*Trigonaspis megaptera* (= *Cynips renum* Hartig, 1840 ♀♂)	0	0	1
271.	62.	*Dryomyia circinans* Giraud, 1861	0	1	0
272.	63.	*Epinotia festivana* Hübner, 1799	0	0	1
273.	64.	*Heliozela sericella* Haworth, 1828	1	0	1
274.	65.	*Janetia nervicola* Kieffer, 1909	0	1	0
275.	66.	*Macrodiplosis pustularis* Bremi, 1847	1	0	1
276.	67.	*Macrodiplosis roboris* Hardy, 1854	1	0	1
277.	68.	*Neuroterus anthracinus* Curtis, 1838 ♀♀	1	0	1
278.	69.	*Neuroterus lanuginosus* Giraud, 1859 ♀	0	1	0
279.	70.	*Neuroterus quercusbaccarum* (= *Diplolepis lenticularis* Olivier, 1791 ♀♂)	1	0	1
280.	71.	*Neuroterus minutulus* Giraud, 1859 ♀	0	1	0
281.	72.	*Neuroterus numismalis* Fourcroy, 1785 ♀♀	1	0	0
282.	73.	*Neuroterus numismalis* (= *N. vesicatrix* Schlechtendal, 1870 ♀♂)	1	0	1
283.	74.	*Neuroterus politus* Hartig, 1840 ♀♂	1	0	1
284.	75.	*Neuroterus quercusbaccarum* Linnaeus, 1758 ♀♀	1	0	1
285.	76.	*Neuroterus saliens* Kollar, 1857 ♀♀	0	1	0
286.	77.	*Polystepha malpighi* Kieffer, 1909	0	0	1
287.	78.	*Pseudoneuroterus macropterus* Hartig, 1843 ♀	0	1	0
288.	79.	*Synophrus pilulae* Houard, 1911	0	1	0
289.	80.	*Synophrus politus* Hartig, 1843	0	1	0
290.	81.	*Trioza remota* Foerster, 1848	1	0	0

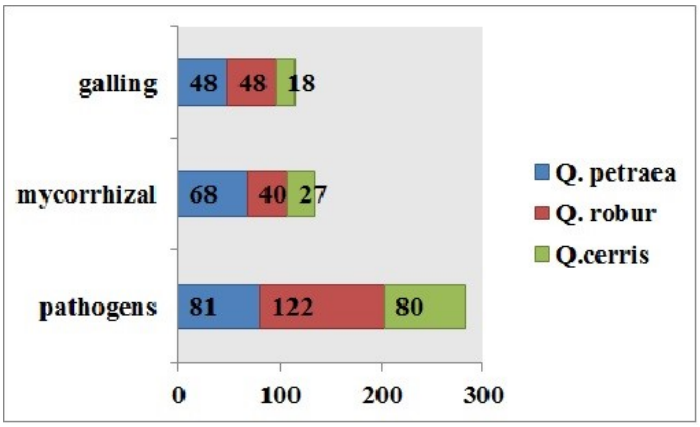

Fig. 1. The repartition of galling insects, tree pathogens and ECM fungi according to the hosts.

Network analysis

Networks are characterized by high asymmetry caused by high number of associated higher trophic level species (network size = 870). The complex network (Fig. 2) shows the highest level of asymmetry, followed by tree pathogens network, and galling insects together with ECM fungi with very close asymmetry values (Tab. 2).

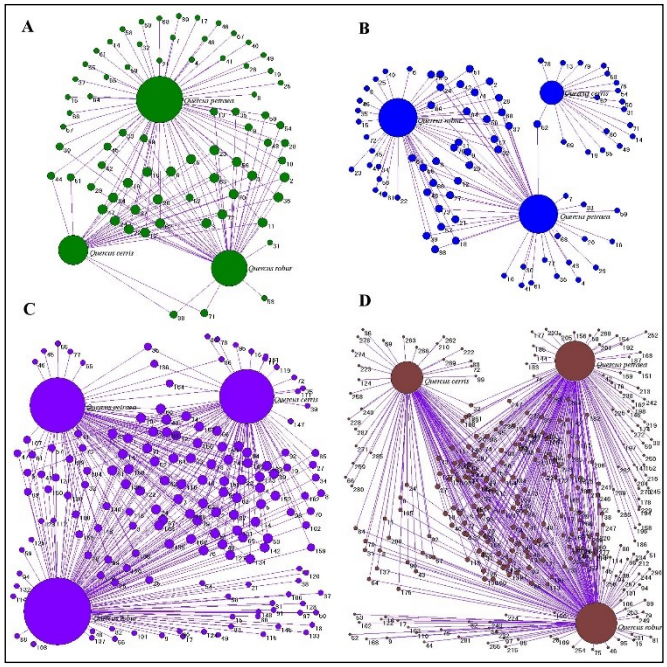

Fig. 2. Bipartite complex network and subnetworks: the radius of nodes is proportional to weighted node degree and the numbers of complex network correspond to species as they are listed in the adjacency matrix, first column. The node numbers in subnetworks correspond to second column in the adjacency matrix (A – ECM network, B – galling insects' network, C – pathogenic network, D – complex network)

Tab. 2. Nestedness results provided by package bipartite in R. Modularity results produced by software MODULAR with significance expressed in z score values (z scores ≥1, not significant result). C score estimated in EcoSim.

Descriptor	Composite		Galling insects		Tree pathogens		Mycorrhizal fungi	
Web asymmetry	-0.979		-0.928		-0.957		-0.92	
C score	0.288	SES= -27.18	0.592	SES= -5.60	0.152	SES= -69.94	0.102	SES= -20.64
Connectance	0.611		0.469		0.688		0.625	
Modularity	0.229 Z=2.15 ($Q_{simul.}$=0.190±0.010)		0.333 Z=0.86 ($Q_{simul.}$=0.311±0.02)		0.162 Z=2.12 (Q_{simul}=0.190±0.07)		0.210 Z=1.05 (Q_{simul}=0.191±0.01)	
Nestedness	0.736 P=0.002**		0.483 P=0.0009***		0.828 P=0.0009***		0.915 P=0.0009***	
Number of nodes	293		84		140		75	
Number of links	532		114		283		135	

Note: *significant at p<0.05; **significant at p<0.01; ***significant at p<0.001

The complex network and subnetworks are highly connected, lowest connectance characterizing galling insects subnetwork, the most host specialized organisms included in this study and herbivorous consumers, a pattern observed also elsewhere [THÉBAULT & FONTAINE, 2010].

For each network (complex and subnetworks) we ran the modularity detecting algorithm SA (simulated annealing) in MODULAR and NETCARTO and Louvain method in Pajek. All employed software produced same modularity indices but with different significance levels. According to significance testing using z scores, all networks were significantly modular (z scores ≥1), except for galling insects network (Tab. 2 reports the NETCARTO results).

The unipartite versions of the networks (Fig. 3) illustrate de level of species sharing among hosts, this display suggesting niche overlap [MELLO & al. 2011]. Lowest overlap is observed in galling insects and the highest in complex and pathogenic networks. Host specialization is greater in galling insects than in other associated species, a feature that explains differences in sharing pattern.

A rule of a thumb indicates that modularity refers to the situation with more links within modules than outside the modules and this situation is depicted in P-z space [GUIMERÀ & AMARAL, 2005] by assigning to nodes different roles. The complex network contains 23% connector nodes, 30% peripheral nodes and 47.58% ultraperipheral nodes (linked to only one host). Pathogenic network displays a similar repartition of node roles: 33.57% are connectors, 29.19% are peripheral nodes and 40.14% are ultraperipheral nodes. Galling insects establish with their hosts a peculiar network, without connectors, 40.24% peripheral nodes and 57.75% ultraperipheral nodes. This repartition of node roles generates an apparently modular network but the lack of connector nodes is causing a defective structure which cannot be tracked as significant. ECM network displays 30.55% connector nodes, 20.83% peripheral nodes and 50% ultraperipheral. All networks contain three module hubs corresponding to tree hosts.

Nestedness analysis yielded interesting results: complex network was highly nested together with ECM fungi subnetwork while galling insects showed lower but significant nestedness. Pathogens displayed marginally significant high nestedness under conservative null model III (results produced by BINMATNEST software) while all networks showed significant

nestedness under the liberal null model I (r00) (results provided by *bipartite* package of R). Nestedness results are shown in Tab. 2, as provided by R, considering null model r00.

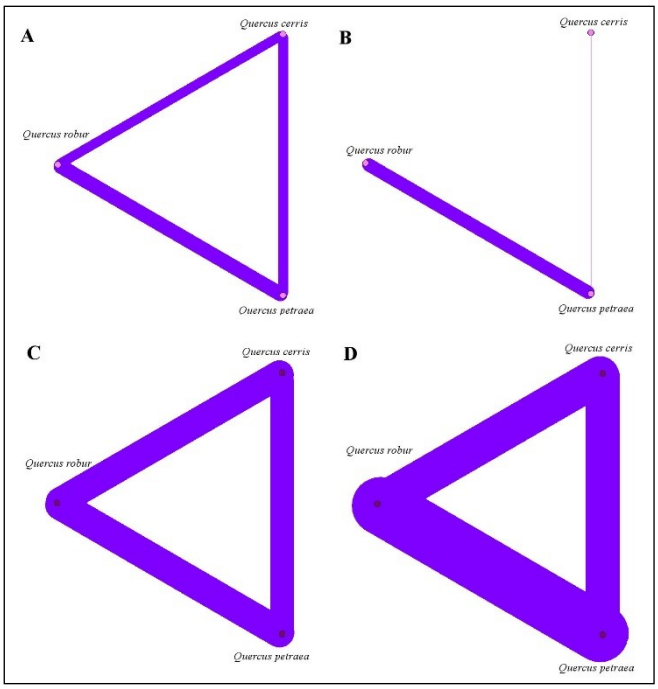

Fig. 3. Unipartite versions of the networks (A – ECM network, B – galling insects' network, C – pathogenic network, D – complex network)

The highest C score (Tab. 2) corresponds to galling insects community, apparently assembled by aggregation (indicated by negative SES scores). Same pattern at lower scores is encountered in complex network and the rest of subnetworks. C scores close to 0 however, indicate a pattern close to random suggesting that there is a degree of stochasticity in community assemblages.

Network topologies reflect the phylogenetic closeness among hosts, the number of shared species being high and suggesting large niche overlap in associated species sharing among hosts. The topology of the complex, with mixed interactions network is characterized by high connectance, low modularity and high nestedness, being closer to symbiotic networks pattern. Subnetworks follow the already observed pattern of low modularity for symbionts, higher for consumers, and high nestedness for symbionts, lower for consumers.

Community analysis

Clustering: Analyzing similarities among hosts in terms of shared associated species using the complex network and matrices summarizing galling insects, ECM fungi and tree pathogens, a general pattern emerged: ECM fungi and pathogens share same clustering pattern while galling insects and the complex network display a similar species share pattern (Fig. 4). Mantel test used for the search of correlations among resemblance matrices yielded non-significant correlations, showing that there are differences in sharing pattern among hosts with respect to different categories of associated organisms.

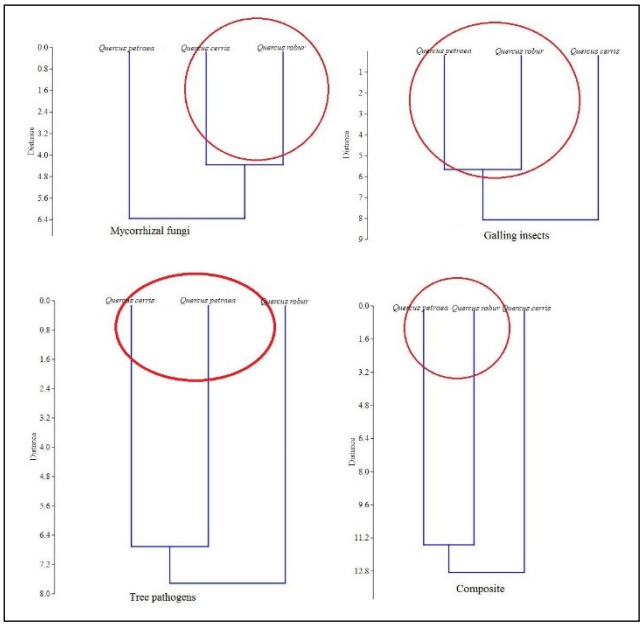

Fig. 4. Dendrograms of clustering pattern in ECM, galling insects', pathogenic, and complex communities (UPGMA clustering algorithm and Euclidean distance as resemblance measure)

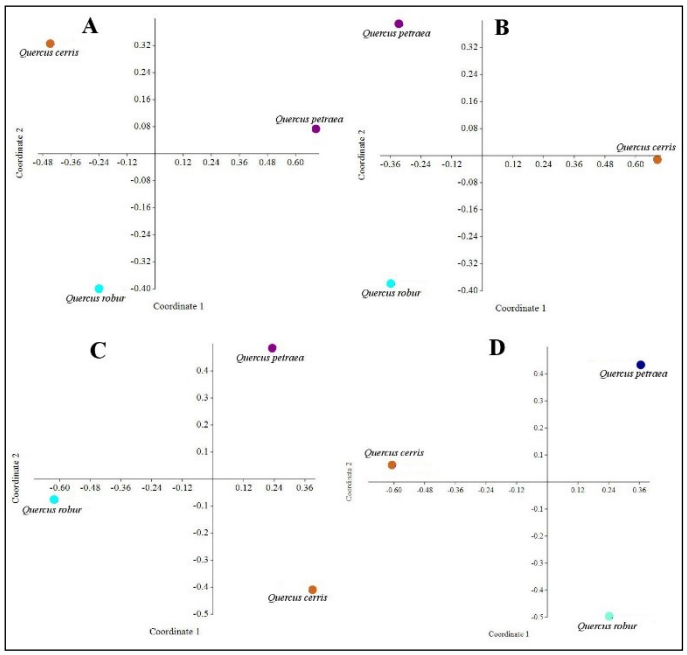

Fig 5. Mapping of ECM, galling insects, pathogenic and complex communities in NMDS ordination space (A – ectomycorrhizal network, B – galling insects' network, C – pathogenic network, D – complex network)

Modularity analysis suggests that there are three significant clusters meaning that a splitting line should be traced above the branching level in dendrograms.

Non Metric Multidimensional Scaling: The mapping of complex community and separate associated to tree hosts organisms' communities shows consistent differences between the three selected tree hosts in terms of shared communities, in this case the positioning being different for all investigated communities (Fig. 5). For the obtained configurations, minimum stress of 0 corresponds to best agreement between dissimilarity matrices and distances in ordination space (R^2 for complex network of 0.9903, for pathogens, 0.9921, for mycorrhizal fungi 0.9934 and for galling insects, 0.9997). The analysis of the resemblance matrix using NMDS highlighted more subtle differences among analyzed communities, a feature confirmed by Mantel tests (resemblance matrices were not significantly correlated).

Discussion

The evolution of plants in terrestrial environments triggered the evolution of various groups of associated organisms by species interactions, trophic webs and diversification of available niches. Closely related hosts tend to share many of their interaction partners, even more if they co-exist in the same geographic area or even location. All the interactions of the three categories of organisms presented in this study merged in one complex network are intimate, resource oriented.

The three functional groups of organisms affect tree hosts modifying the level of productivity and their fitness, hosts in turn present as functional trait susceptibility to herbivory and to pathogens/mutualists. The consumption/mutualistic interaction and susceptibility of hosts are community level traits affecting the ecosystem functions (productivity, nutrient cycling) and structure (habitat optimization via mutualistic interactions) [MATTHEWS & al. 2011]. The very same traits are responsible for the assemblage of associated communities.

The diversity of merged summary network revealed the fact that the number of species using trees is impressive. As networks are open structures, the attachment of new nodes and links covering other types of interactions (other categories of consumers, enemies of herbivorous insects, fungal endophytes, corticolous lichens, etc.) could increase consistently the size of the network.

The networks reflect the phylogenetic closeness of hosts and also the differences in their ecology. *Q cerris* is clearly separated from *Q. petraea* and *Q. robur* in terms of biogeographic range (confined more to Central and Southern Europe, also to Asia Minor) and fundamental niche (in terms of tolerance to drought and ability to vegetate on different types of soil, etc.) [ELLENBERG, 2009]. Dependent species assemblages (ECM, galling insects and pathogens) on *Q. cerris* are more dissimilar to the other two *Quercus* species (at greater extent observed for galling insects and the complex network).

The complex network displays interesting properties: subgraphs are smaller versions of the main graph in terms connectivity and modularity. High connectance and nestedness are reported for low intimacy antagonistic networks while significant nestedness and modularity are correlated with high and intermediate intimacy antagonistic interactions (as pathogenic, for instance) [PIRES & GUIMARAES, 2013]. High connectance is generally

linked to lower specialization [POISOT & GRAVEL, 2014], differences among hosts being small in terms of resource type use.

Connectance as network first order property is the result of co-evolution [PETANIDOU & POTTS, 2006; POISOT & GRAVEL, 2014] and the lower the connectance, the higher is the specialization, a structural feature observed in parasitic networks [BELLAY & al. 2013], applied also to pathogenic network under study. Higher diversity and connectance promotes stability in mutualistic networks and destabilize trophic networks (based on free living consumers) [THÉBAULT & FONTAINE, 2010]. However, small networks are characterized by default by high connectance [THÉBAULT & FONTAINE, 2010], a rule applying to our data as well.

Modularity is linked to ecosystem stability and co-evolution [OLESEN & al. 2007]. The striking result of low-modular structure of the investigated networks has an explanation found in the evolution of other biological systems such as genomes, showing that modular structures are usually less optimal than non-modular [KASHTAN & ALON, 2005]. Authors have shown that modularity evolves spontaneously in biological systems and causes emergent properties: stability, robustness under changing environment pressures because designs including modularity manifest higher survival rates. Modularity requires at least networks of 150 nodes to be significantly non-random [OLESEN & al. 2007]. Closely related hosts on the other hand share many of their associated species. As modularity evolves as a response to changes in environment, closely related hosts adapt in a correlative way. When several hosts from distantly related genera are used to construct the network (considering mycorrhizal species or pathogens), modularity is a significant structural feature [VACHER & al. 2008; CHAGNON & al. 2012; TOJU & al. 2014]. Using several software applications, same modularity index value was obtained and same number of modules, demonstrating that non-random modularity characterized three of the four analyzed networks.

Our analysis show that there is a pattern in the repartition of different categories of nodes according to their roles, similar in complex, mycorrhizal and pathogenic networks and different, lacking connector nodes in galling insects network. Under null model II (more conservative), galling insects network appear as marginally modular. As consumers, galling insects are expected to be organized in networks displaying modularity [PIRES & GUIMARÃES, 2013].

Trees are interconnected through mycorrhizae [KOTTKE & al. 2010], the level of share is recognized to be high among closely related and distant hosts, therefore modularity, if is expressed, must take only modest values. Many species are connectors among modules of which many are super-generalists. They are important for network topology because they link host species even when massive extinction affects other symbionts [GUIMARÃES & al. 2006] such is the case of the mutualist *Coenococcum geophilum*, a generalist mycorrhizal species with large ecological range. The conservation of ECM fungi biodiversity is an important issue recently highlighted by this category of fungi decline in various parts of Europe and North America [AMARANTHUS, 1998; SENN-IRLET & al. 2007; SUZ & al. 2015].

The galling insects selected as antagonistic consumers and free living organisms only during adult stage are presumed to reflect this mixed position between free living and intimately depending antagonistic organisms in network topology. Gall inducing insects and mining insects display an intermediate level of intimacy with their hosts but like pathogens they need specific host recognition systems and mechanisms to avoid defense reactions of the host [HESPENHEIDE, 1991; HARRIS & al. 2003]. Their species-specific interaction with hosts makes them highly predictive organisms of the level of diversity in various

ecosystems [DE ARAÚJO, 2011]. The network shows high nestedness, high connectance, aggregative mechanism in community assemblage (closer to random) and defective modularity (only marginally significant), characteristics of both antagonistic symbiotic and trophic networks.

Global climatic change will affect directly and through interspecific interactions the trees [HLÁSNY & al. 2011]; it is expected that emerging pathogens will put new threats to woody species, the case of *Phytophthora* species participation to trees' decline being largely documented [HANSEN, 2008; SANTINI & al. 2012; KEČA & al. 2016]. Modularity of pathogenic network enhances the pathogenic spread to other host species as for instance *Phytophthora plurivora* which could jump from the position of peripheral species to connector species, affecting all three host species in the future, in Romania. *Phytophthora citricola* is on the other hand a typical connector species. Also, pathogens are important density dependent population control factors. It might be hypothesized that in the *Q. petraea* and *Q. robur* range, the greater documented number of pathogens exerts a more significant control whereas for more xeric *Quercus* species (*Q. cerris*), abiotic factors play a more important role in population control. Also, contiguous host ranges imply that a pathogen parasitizing one host is more likely to parasitize another host placed in a close range [WARREN & al. 2010], a component of the local ecological network. Among pathogenic and parasitic fungi, however there are species important for biodiversity conservation, protected species such as *Piptoporus quercinus* [CROCKATT & al. 2010]. Wood decomposers attacking live and dead trees are hallmarks for forest ecosystems and important players of biodiversity.

Nestedness is ubiquitous in nature however, stochastic processes contribute greatly to the emergence of significant nestedness [HIGGINS & al. 2006] deriving from passive sampling, dynamics of extinction and colonization and use of inappropriate null models. As a consequence, caution must be taken in interpreting nestedness results; null model r00 employed by package bipartite in R more liberal than null model III provided by software BINMATNEST but significance analysis provided similar results confirming the fact that nestedness is a structural feature of the analyzed networks and not a methodological bias. For instance, it was stated that ECM networks display non-nested pattern of association which is unclear if this fact is determined by biased methodology or ecological pattern [BAHRAM & al. 2014]. Also, nestedness generating processes are determined by host abundance (sometimes equivalent to dominance in given ecosystems) and reciprocal specialization [JOPPA & al. 2010], more obvious with phylogenetically distant hosts. Our results show that ECM network is significantly nested, at least in interactions with closely related hosts. High nestedness is a common feature for all analyzed networks under the present study. However, larger networks of hosts and mycorrhizal partners analyzed elsewhere show no nestedness [TOJU & al. 2014] or low level of nestedness [BAHRAM & al. 2014].

As a proxy for niche overlap, the unipartite version of the investigated networks shows different patterns: lowest niche overlap is characterizing galling insects' network, other networks showing high overlap. High overlap suggests species redundancy [BLÜTHGEN & al. 2006] which in the case of mutualists is an insurance policy in the case of a series of extinctions.

Tree species were arranged in NMDS ordination space according to the variables represented by associated communities, symbiotic and herbivorous. The provided information adds dimensionality to species similarity pattern showing that hosts are

positioned distantly in ordination space. Clustering pattern suggest which hosts are closer in terms of shared species, the pattern being different for each network. Differences in similarity pattern lead to lack of correlation among similarity matrices (Mantel test results).

Modularity analysis revealed that the splitting line in dendrograms depicting the clustering pattern should separate three distinct branches corresponding to three tree hosts. As modularity splits networks in functional subsets, the separation is not always similar to classical clustering which is constructed on pairwise comparisons generating a resemblance matrix.

Network analysis of complex interactions and, at the smaller scale of one type interaction highlighted the differences in topologies which reflected differences in functional roles of species assembling communities, based on trees as food resources and habitats. At regional scale, it is presumed that community assemblage is incorporating stochastic events and mostly biotic drivers such as host affinity and predisposition, ecosystem type and interspecific interactions. As a conclusive remark, diversity of interactions is important from conservation perspective as much as the conservation of species or communities and new efforts must be made in the direction of the study of complex ecological networks, their structure and functioning to improve our understanding about biodiversity mechanisms.

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SPATIAL DISTRIBUTION OF SOME ECTOMYCORRHIZAL FUNGI (RUSSULACEAE, FUNGI, BASIDIOMYCOTA) IN FOREST HABITATS FROM THE NORTH-EAST REGION (ROMANIA)

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Abstract: Ectomycorrhizal macromycetes are, generally, an important ecological component for forest habitats, and a valuable resource in the context of sustainable development of rural communities in the North-East Region of Romania. The woody species distribution is an extremely important factor for the ECM macromycetes presence. The purpose of this study was to elaborate maps of potential distribution for some ECM edible macromycetes from *Russula* and *Lactarius* genera, based on chorological information, ICAS Forest Types Map, vegetation tables and bibliographical sources. These information allowed the elaboration of 15 potential maps of distribution for 15 edible species of *Russula* and *Lactarius*. The study was based entirely on the plant – fungal associations. The results highlighted that in the North-East Region of Romania there is a noteworthy potential for *Russulaceae* species. As expected, there is a large amplitude of species presence in the field depending on the fungal specificity for tree host and tree species distribution.

Keywords: ECM, *Lactarius*, *Russula*, North-East Region, potential distribution map.

Introduction

There are approximately 100000 known species of fungi on Earth [BOA, 2004] and many have economic value [BOA, 2004]. More than 3000 species possess some degree of edibility and are used in kitchens across world [GARIBAY-ORIJEL, 2009]. In temperate zones, many edible fungi are ectomycorrhizal (ECM). ECM fungi have a special relationship with plants, being in symbiosis with them [TĂNASE & MITITIUC, 2001]. Extensions of radicular system of plants, ECM fungi absorb nutrients and water from soil and give these resources to the plant [MARTINOVÁ & al. 2015; PEŠKOVA & al. 2012; WOLFE & PRINGLE, 2012]. On the other way, the plant supplies fungi the carbohydrates and other organic compounds necessary for development [TĂNASE & MITITIUC, 2001]. Plant diversity and composition is influenced by mycorrhizae [PEAY & al. 2009] and across forest ecosystems, fungi play a vital functional role [BUÉE & al. 2011]. On the other side, ECM fungi fruit bodies apparition is influenced especially by the host phenology [BUNTGEN, 2013]. Any change in the forest ecosystem can drive important phenology shifts, especially if the disturbance is powerful, like those induced by hazards (insect attacks [ȘTURSOVA & al. 2009], fires [KUTORGA & al. 2012], droughts [PEŠKOVA &

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al. 2012], strong storms [MILLER & LODGE, 1997]). In temperate zones, vegetation composition and structure influences fungal diversity and fungal distribution patterns [ANGELINI & al. 2014]. Important plant families with distribution in temperate areas are associated with ECM fungi: *Betulaceae, Fagaceae, Pinaceae, Tiliaceae* [MARTINOVÁ & al. 2015]. Numerous studies confirmed differences between different forest types, in relation to fungal diversity and composition, especially between broad-leaved and coniferous forests [ANGELINI & al. 2014; KUTSEGI & al. 2015]. Therefore, fungal geographical distribution is driven by the type of forests, as well as other related variables like soil upper layer's parameters [PEAY & al. 2009; SHI & al. 2013; MARTINOVÁ & al. 2015; DINCĂ & DINCĂ, 2015; KUTSEGI & al. 2015]. The forest type and its abiotic characteristics can be used as a powerful predictive variable for the macromycetes distribution, in our case from *Russula* and *Lactarius* genera.

Lactarius and *Russula* genera are included in *Russulaceae* family, *Agaricales* order, *Hymenomycetes* class and *Basidiomycotina* phylum. These genera are the main members of the *Russulaceae* family in Europe [ADAMČÍK & al. 2006]. The total number of species is unknown, but the family's diversity is high [ADAMČÍK & al. 2006].These macromycetes can form mycorrhizae with one tree species (e.g. *Lactarius deliciosus* with *Pinus*, *L. deterrimus* with *Picea*; COURTECUISSE & DUHEM, 2013) or with many woody hosts (e.g. *Russula delica* with *Carpinus*, *Fagus*, *Quercus*, *Tilia*; GERHARDT, 1999). There are two different ways of using edible fungi: personal subsistence or marketing [BOA, 2004], and in North-East Region, most of forest collected fungi are used for personal culinary use or for local marketing, *Russula* and *Lactarius* fungi having their part. Numerous studies have also highlighted the effect of some *Russulaceae* species to the good human health [SUN & al. 2010; NANDI & al. 2012; KOSANIĆ & al. 2016; RUTHES & al. 2013; SARIKURKCU & al. 2008]. On the other hand, *Russulaceae* family can prove as an important group for fungal diversity in North-East Region's forests, as PAVEL (2007) found that *Russulaceae* family and *Russula* genus was the most diverse genus in *Quercus* sp. edified forests. The value of *Russula* and *Lactarius* is therefore multiple, many species having commercial value, being important for human health or important from ecological perspective.

The importance of forest can be emphasized (among other important services and products) through their potential for wild edible fungi. The fact that a large part of these fungi are ECM [BOA, 2004] and the *Russulaceae* form an important group in forest's myco-diversity, made us to approach this group in the study. The aims of the study were: (i) to generate maps with common woody hosts across North-East Region; (ii) to generate maps of potential distribution for edible *Russula* and *Lactarius* species in North-East Region's forests; (iii) to prove that any literature source can be useful in primary assessment of potential distribution mapping; (iiii) to show, through mapping, that forests types have a powerful impact on fungi presence.

Materials and methods

In order to generate potential distribution maps, the first step was to determine the area in which those maps are set to be created. Forest ecosystems distribution across North-East Region of Romania was obtained from the ICAS forest types map (1997). After

clipping the interest region, a number of 76 forest types was obtained. Next, a correspondence between forest types and plant communities was realized using CHIFU & al. (2006). Thus, a distribution map of 12 plant communities in the North-East Region resulted. The *Pino–Quercetum* association, mapped in the south-western part of the region, was excluded because the study's purpose was to select only *Quercus* sp. forests, without coniferous species in their composition. Another reason for *Pino – Quercetum* exclusion was that it coveredless than 1% of the total area (Fig. 1).

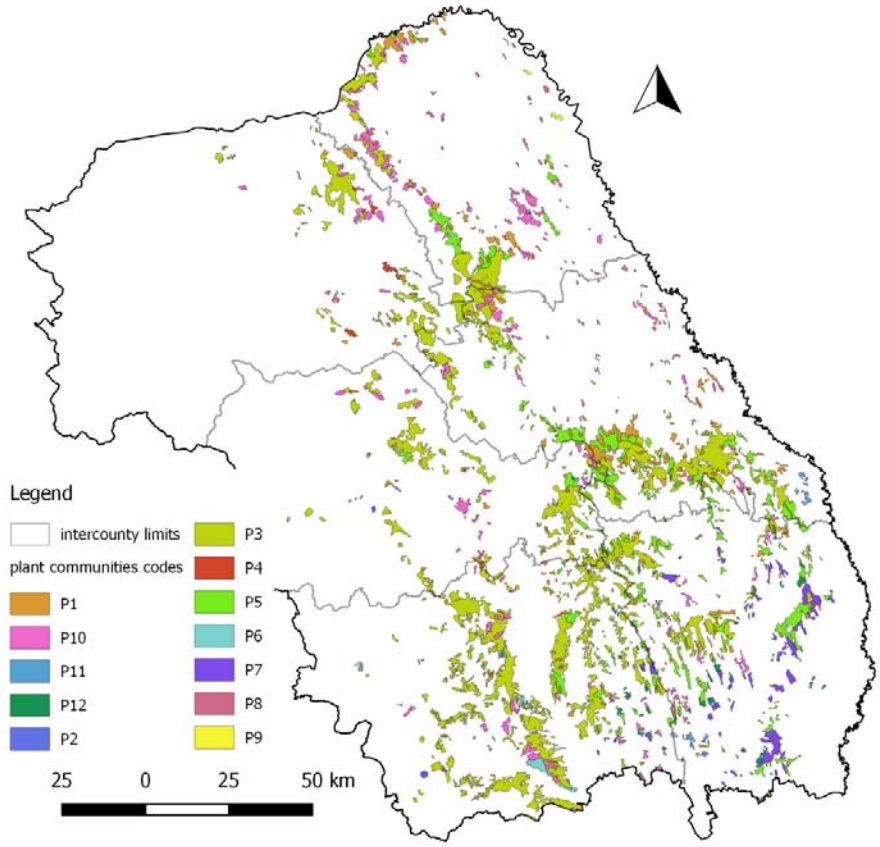

Fig. 1. Distribution mapof broad-leaved forests in North-East Region of Romania (adapted after ICAS Forest Types Map, 1997)

The investigated fungal species were selected as having a minimal edibility. Five *Lactarius* species and ten *Russula* species were chosen on this criterion, using literature [TĂNASE & al. 2009; GERHARDT, 1999; COURTECUISSE & DUHEM, 2013]. For these species, four tree genera were identified as hosts: *Carpinus*, *Fagus*, *Quercus* and *Tilia*.

Depending on the number of tree - hosts, probability numbers were assigned to fungi – plant mycorrhizae: 0 for no mycorrhizae, 100 for common mycorrhizae and 25 for rarely mycorrhizae (Tab. 2). For each studied tree genus all constancy classes were extracted from CHIFU & al. (2006). The maximum percentage value was chosen as the value for the tree genera in each plant community.

Tab. 1. Forest communities with *Quercus* sp. in the North-East Region of Romania

Code	Association	Class	Dominant tree genera (maximum constancy class >= 4)
P1	*Aro orientalis – Carpinetum *quercetosum roboris*	*Querco–Fagetea*	*Carpinus, Quercus, Tilia*
P2	*Aro orientalis – Carpinetum *quercetosum pedunculiflorae*	*Querco–Fagetea*	*Carpinus, Quercus, Tilia*
P3	*Lathyro venetus – Fagetum *fagetosum*	*Querco–Fagetea*	*Fagus , Carpinus*
P4	*Lathyro venetus – Fagetum *quercetosum dalechampii*	*Querco–Fagetea*	*Carpinus, Fagus, Quercus*
P5	*Dentario quinquefoliae – Carpinetum *typicum*	*Querco–Fagetea*	*Carpinus, Quercus*
P6	*Genisto tinctoriae – Quercetum petraeae*	*Quercetea robori–petraeae*	*Carpinus, Quercus*
P7	*Tilio tomentosae – Quercetum dalechampii *typicum*	*Quercetea pubescentis*	*Quercus, Tilia*
P8	*Aceri tatarico – Quercetum roboris*	*Quercetea pubescentis*	*Quercus, Tilia, Carpinus*
P9	Communities with *Acer campestre* and *Quercus robur*	*Quercetea pubescentis*	*Quercus*
P10	*Quercetum robori – petraeae*	*Quercetea pubescentis*	*Quercus, Carpinus*
P11	*Quercetum pedunculiflorae*	*Quercetea pubescentis*	*Quercus*
P12	*Cotino – Quercetum pubescentis*	*Quercetea pubescentis*	*Quercus*

The next step in map processing consisted in importing the final ecosystem map in R software and transforming map's data to a more appropriate type, the raster format. For each tree genera, the area (in ha) of each forest type per each constancy value was calculated. The total number of pixels, where the rasters resolution was 100 x 100 m (Fig. 2) were considered for this purpose.

After importing the potential distribution map of plant communities in R software, a data frame was generated for further analysis. There was a 30 columns with minimum and maximum probability of occurrence of fungi based on tree – fungi association and tree constancy values. In total, 360 cells. For computing the probability of fungi occurrence, a modified form of the law of total probability was used.

Tab. 2. Occurrence probability of selected *Lactarius* and *Russula* species and their tree genera hosts

code	mushroom species	*Carpinus*	*Fagus*	*Quercus*	*Tilia*
m1	*Lactarius pallidus*	0	100	25	0
m2	*Lactarius piperatus*	0	100	100	0
m3	*Lactarius quietus*	0	0	100	0
m4	*Lactarius subdulcis*	0	100	0	0
m5	*Lactarius vellereus*	0	100	25	0
m6	*Russula aurea*	0	100	25	25
m7	*Russula cyanoxantha*	0	100	100	0
m8	*Russula delica*	100	100	100	100
m9	*Russula graveolens*	25	25	100	0
m10	*Russula grisea*	100	25	100	0
m11	*Russula heterophylla*	25	100	100	25
m12	*Russula olivacea*	0	100	0	0
m13	*Russula vesca*	25	100	25	0
m14	*Russula violeipes*	0	100	25	0
m15	*Russula virescens*	0	100	100	0

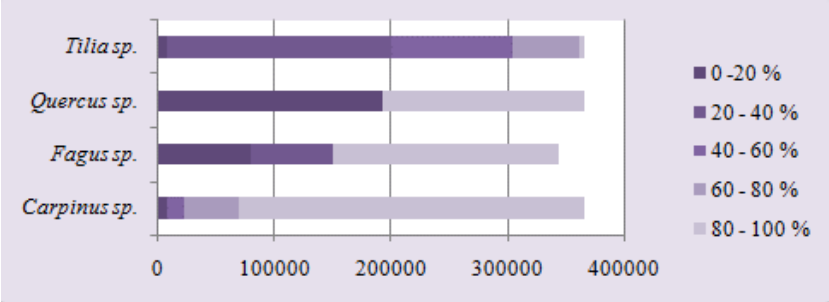

Fig. 2. Occurrence probability and occupied area (ha) of tree-hosts genera in the North-Eastern Romania

Suppose that B_1, B_2,…, B_n is a collection of exclusive events, each one with its specific probability space, for any event A, the probability of A's occurrence in the same total space is:

$$P(A) = \sum P(A \cap B_i) = \sum P(A|B_i)*P(B_i)$$ [ZWILLINGER & KOKOSKA, 2000].

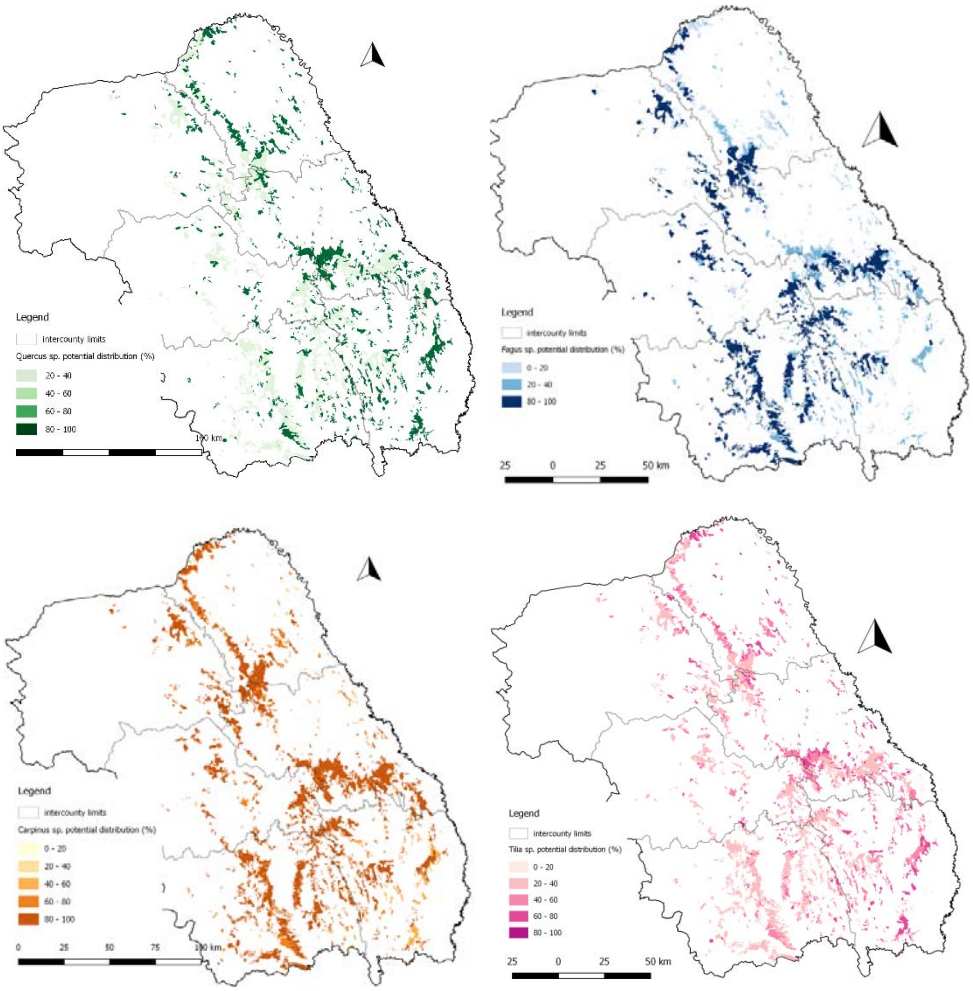

Fig. 3. *Quercus*, *Fagus*, *Carpinus* and *Tilia* genera potential distribution map
(the corresponding colors used are: green, blue, brown and pink)

Because the formula states that, given the total space, only one event A_i can happen, the formula was adapted so that each event can happen at the same time. In this way, the theorem's limitation that in a given forest type, a fungal species can occur only because of a single association, is eliminated. The fungi probability associations with tree genera were modified according to formula 1:

$$PFm_i(f_k t_j) = Pm_i(f_k t_j) * 100 / \sum Pm_i(f_k t_j).$$

The law of total probability was therefore generated in formula 2:

$$Pf_k(m_i) = \sum Pf_k(t_j) * PFm_i(f_k t_j) / 100,$$

where $Pf_k(m_i)$ it's the probability that the mushroom species m_i can be found in polygon f_k; $Pf_k(t_j)$ is the maximum probability that the woody plant genera t_j can be found in polygon f_k; $PFm_i(f_k t_j)$ is the maximum probability that the fungi species m_i can be found in polygon f_k only on available tree genera t_j while $PFm_i(f_k t_j)$ have the same interpretation without being transformed through formula 1; where *j* is from 1 to 4 and the number of tree genera; where *i* is from 1 to 15 and the number of fungi and *k* is from 1 to 12 and the number of plant communities. In this formula, were used: the total number of trees genera available in the polygon forest (*NTtF*), the total number of tree genera, 4 (*NTt4*) or the total number of trees available for the mushroom (*NTtM*). For an *in situ* fungi probability of occurrence, only the tree species growing on that site can be considered. Therefore, a fourth variable was taken, the total number of trees that grows on that site and have mycorrhizae with fungi (NTtMF). After incorporation of the final matrix in to the shapefile's data, 15 maps with the maximum distribution of the majority of edible *Russula* and *Lactarius* species in North-East Region's forests with *Quercus* sp. (Fig. 5 and Fig. 6a, 6b) were generated.

Another objective of this study was to observe if some groups of forest types could be differentiated by fungi probability occurrence. Thus, an attempt to group forest types depending on fungi probability of occurrence by K-means clustering was carried out. Silhouette index was calculated in order to identify the optimum number of clusters. This was obtained using *fviz_nbclust* function from *factoextra* package in R software (R Core Development Team, 2015). The data used was composed only from the mushrooms maximum probability occurrence values, in the 12 plant communities. In this case, the optimum number was three clusters (Fig. 4).

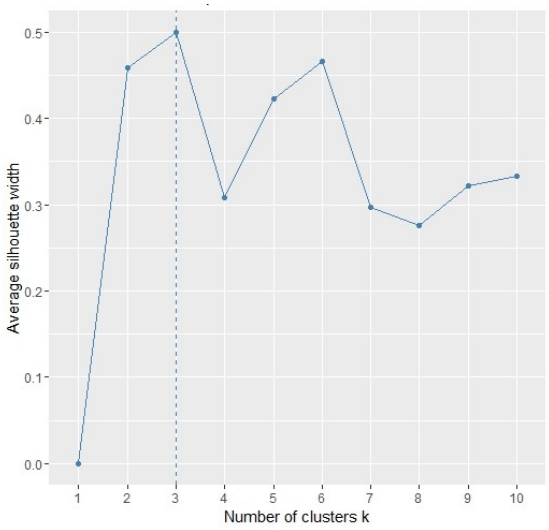

Fig. 4. Identification of optimum number of clusters using the *Silhouette* index

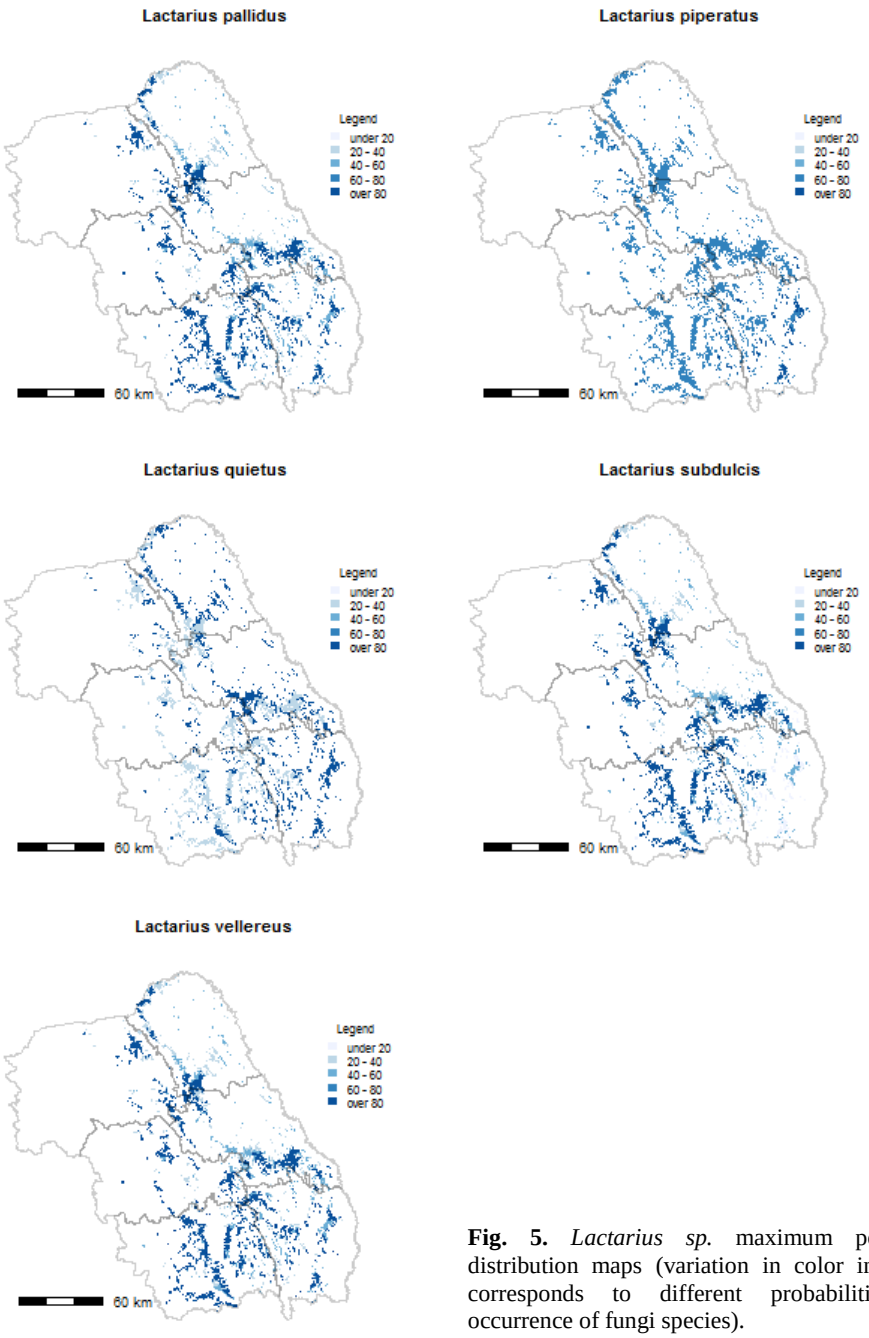

Fig. 5. *Lactarius sp.* maximum potential distribution maps (variation in color intensity corresponds to different probabilities of occurrence of fungi species).

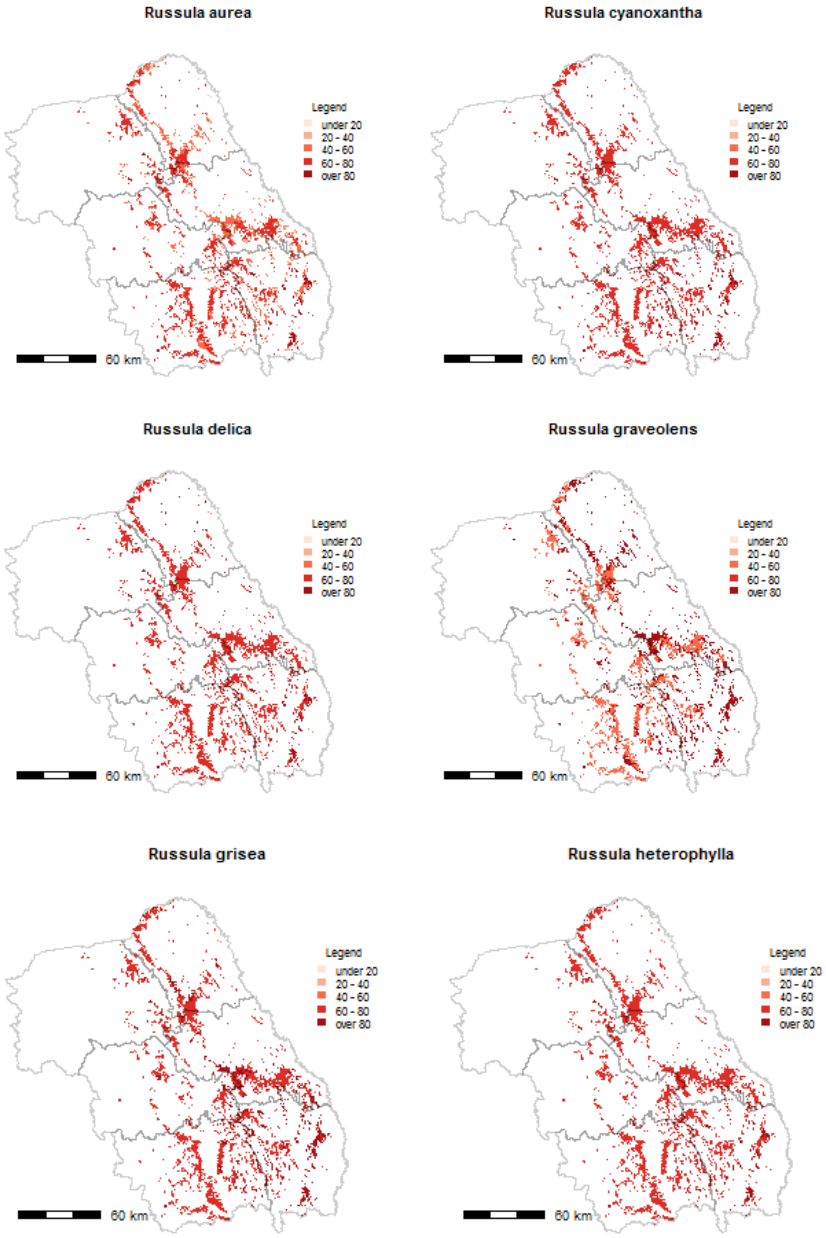

Fig. 6a. *Russula sp.* maximum potential distribution maps (variation in color intensity corresponds to different probabilities of occurrence of fungi species).

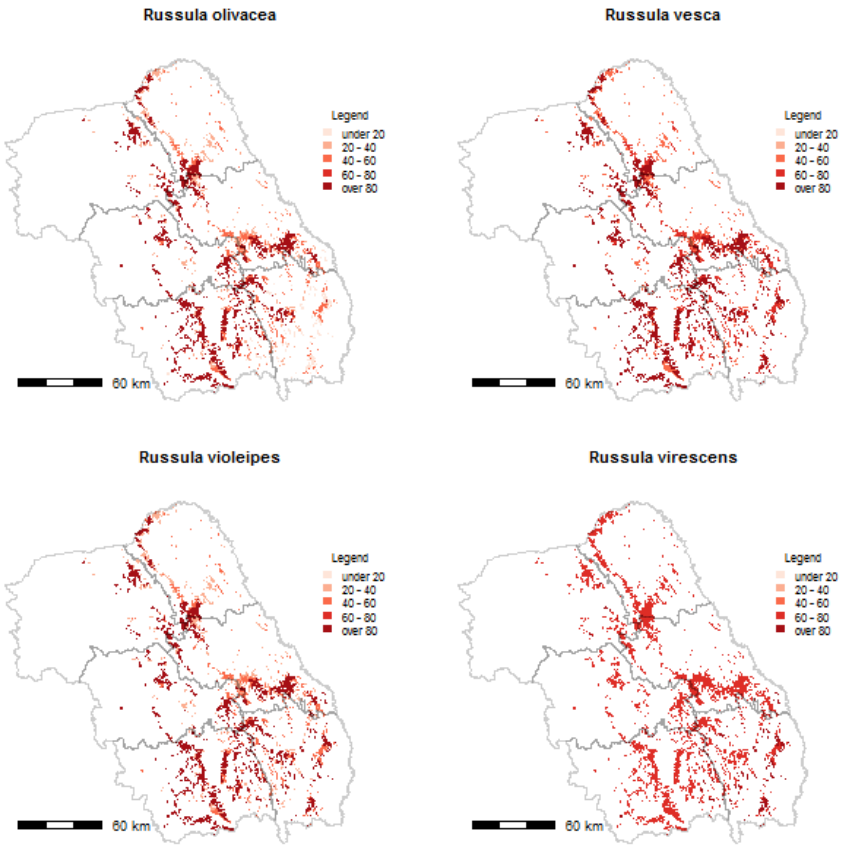

Fig. 6b. *Russula sp.* maximum potential distribution maps (variation in color intensity corresponds to different probabilities of occurrence of fungi species)

Results and discussion

The study methodology consisted in systematically use of the probabilities for fungi and tree species occurrence. This algorithm uses at maximum the plant constancy values and cartographic representations for generation of the potential distribution maps. These generated maps are a product of mathematics and one explanatory variable: fungi preference to particular plant tree species. The data base used was stretched from fungi to woody plant relations and from woody plant presence and constancy in certain plant communities.

A significant difference from a fungal species to another (in respect to probability of occurrence) was observed, due to different host preferences, different total number of hosts and different constancy values of trees in different plant communities. It was also found that there is a similarity of distribution probabilities between fungal species making mycorrhizae with identical or merely identical hosts. According to the maps of fungi

maximum probability of occurrence (Fig. 5 and Fig. 6a, Fig. 6b), the majority of fungi have a large distribution area. The Russulaceae species with lower probability of occurrence in different plant communities were *L. subdulcis* (code *m4*) and *R. olivacea* (code *m12*), because of their affinity only to *Fagus* species. On the other side, *L. quietus* (code *m3*), which form mycorrhizae only with *Quercus* genus, had a larger spectrum of occurrence.

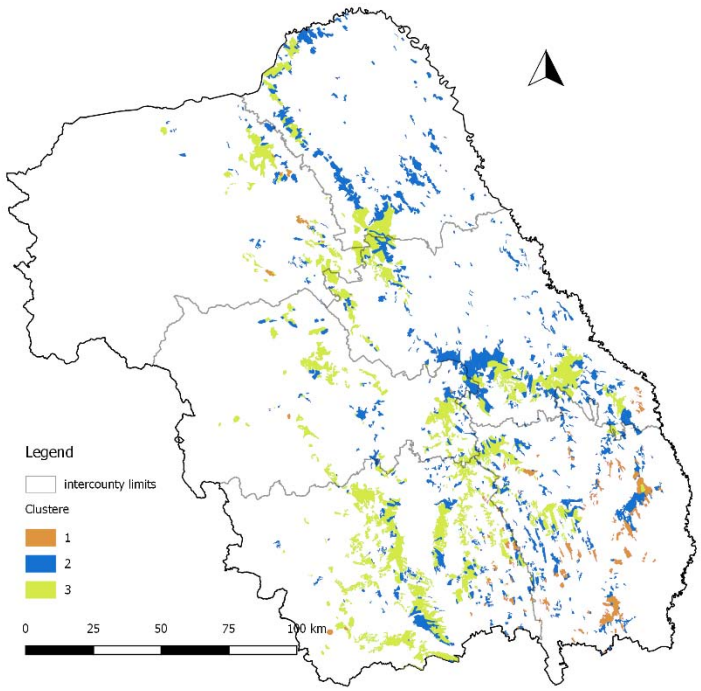

Fig. 7. Map of the three groups (resulted in k-means clustering) of forest types aggregated depending on fungi probability of occurrence in North-East Region of Romania.

Comparing the potential area of occurrence for each plant community and each fungal species, it was observed that, for the majority of fungi, association *Lathyro venetus – Fagetum *fagetosum* comprised the largest part of 100% probability of occurrence, about 136315 ha. The next three plant communities were *Dentario quinquefoliae – Carpinetum*, *Aro orientalis – Carpinetum *quercetosum roboris* and *Quercetum* robori – *petraeae*, with values between 20300 and 40300 ha. These four plant communities became the most important when considering edible *Russula* and *Lactarius* species in *Quercus* forests of North-East Region of Romania. Appreciated culinary species like *Russula cyanoxantha*, *R. delica* or *R. virescens* had a 100 % presence surface of approximately 235741 – 250097 ha, mostly in the former four plant communities. The species with the largest area of 100% probability occurrence were *R. vesca* and *R. grisea*, with over 260000 ha, both having 3 genera of trees as mycorrhizae partners: *Quercus*, *Fagus*, *Carpinus*.

The first group included *Tilio tomentosae – Quercetum dalechampii, Quercetum pedunculiflorae, Cotino – Quercetum pubescentis* and *Lathyro venetus – Fagetum* sub-association *quercetosum dalechampii* plant communities, all characterized by high frequency and dominance of *Quercus* species. Excepting the last plant community, all other were characterized by no *Fagus* sp. presence. Most of the studied mushrooms had a high probability of occurrence in this plant communities within this cluster. This shows that *Quercus* species was an important driver for the ECM fungi found in North-East Region forests. The last community was characterized by codominance of both *Fagus* and *Quercus* genera. It was a particular case in all clustered data. In the first 3 plant communities can be observed that the most frequent (and dominant) tree genera (*Quercus* and *Tilia*) were represented by xerophytic species (particularly *Q. pedunculiflora, Q. pubescens* and *T. tomentosa*) [SÂRBU & al. 2013]. Mean annual temperature was highlighted as an important driver for ECM distribution [SHI & al. 2013]. In this context, the above mentioned tree species, which are more thermophilous could explain the more restricted spatial distribution (only in the south-eastern part of the study area) of plant communities.

The second group comprised *Aro orientalis – Carpinetum, Dentario quinquefoliae – Carpinetum, Aceri tatarico – Quercetum roboris, Quercetum robori – petraeae, Genisto tinctoriae – Quercetum petraeae* and *Acer campestre* with *Quercus robur* communities. This group was characterized by increased frequencies and dominance of *Quercus* sp., lower values for *Fagus* sp., while the other two tree genera had maximum probabilities of occurrence ranging from 40% to 100%. The last cluster included *Lathyro venetus – Fagetum *fagetosum* and was characterized (distinctively from other plant communities) by *Fagus* sp. dominance and *Quercus* sp. low occurrence probability (maximum of 20%). Also, the cluster groups the only combination of low *Quercus* sp. probability occurrence with high *Carpinus* sp. occurrence probability. Both sub-associations of *Lathyro venetus – Fagetum* provides maximum probability of occurrence for mushrooms specialized with *Fagus* genera, as there were, in this study, *Lactarius subdulcis* and *Russula olivacea*. Regarding the differentiation between the second and the third clusters, the fungi that had large amplitude probability of occurrence were *Lactarius quietus, L. subdulcis* and *Russula olivacea*, all having only one tree genera host. This showed that, mushrooms affinity for particular tree genera can prove as an important driver for an entire fungal group. As others authors found, dominant tree species is one of the main drivers for shaping fungal communities in temperate forests [GOLDMANN & al. 2015; SHI & al. 2013]. Fungal species having multiple tree hosts, like *Russula delica, R. graveolens, R. grisea, R. heterophylla* and *R. vesca*, could be found in more forest types having at least one tree genera host in its composition. Because of that, at regional scales, some mushrooms can be found in many ecosystem types, from Mediterranean [ANGELINI & al. 2015; AZUL & al. 2011] to temperate forests [BUÉE & al. 2011], as shown for ECM species like *Russula cyanoxantha* or *R. delica*.

Regarding the distribution of these groups (Fig. 7), the clusters 2 and 3 had a large latitudinal and longitudinal amplitude, while the first cluster was preponderantly located in the southern and south-eastern part of the region. Clusters 1 and 2 comprised 7.3%, respectively 33.7% of the total forest area, while *Fagus* sp. dominated clustered had 59.0% of the total forest surface. Therefore, edible *Russula* and *Lactarius* species may prove a particular regional affinity, considering the forests dominant trees. Among the main factors separating the forests was the occurrence probability of *Quercus* sp. (especially clusters 1

and 2 from 3) and of *Fagus* sp. The fact that *Fagus* sp. is an important driver for edible *Lactarius* and *Russula* species could be explained by the fact that the number of these species associations with *Fagus* sp. is far larger (14 vs. 5 and 3) than other codominant species (*Carpinus* sp. and *Tilia* sp.) with *Quercus* sp. Dominant species in phytocenoses covering large areas stimulates diversity of other organism groups [FODOR & al. 2002].

The successful host genera *Quercus* (Tab. 2) in number of associative fungi - 50% from *Russulaceae* family was also demonstrated by RICHARD & al. (2004) who found that *Russula* and *Cortinarius* accounted for 34.4% of ECM fruitbodies and 50% of species diversity. As shown by TOJU & al. (2013), in a *Quercus* sp. temperate Japanese forest, roots colonized by both ECM fungi (including many species of *Lactarius* and *Russula* genera) and root endophytes may explain the complexity of fungal communities in oak dominated forests. Also, deep-rooted tree species like *Quercus robur* and *Q. petraea* [ȘOFLETEA & CURTU, 2007] might enhance survival of ECM fungi during prolonged drought, as shown with other *Quercus* species in California [SMITH & al. 2007]. PAVEL (2007) found more edible and non-edible *Russula* species in *Quercus* sp. edified forests than in *Picea abies* forests, both in Dofteana and Hemeiuș forests. O'HANLON (2011) found that Irish oak forests are the home for as many ECM species as the Irish Scots pine forests are. He found that oak forests hosts with 50% more *Lactarius* species than Scots pine, ash or Sitka spruce forests and has similar numbers in terms of *Russula* species as Scots pine forests. In total, *Russula* and *Lactarius* genera occupied the fourth and fifth places in a ranking fungal genera across all four forests types. Significant variation in composition of fungal communities was found also by GOLDMANN & al. (2015), who analyzed them in a comparative study between beech and coniferous forests. The most important driver which controls the fungal distribution, especially the ECM ones is the plant host [FODOR & al. 2002]. *Russula* and *Lactarius* genera are known to have a wider distribution than other ECM species [GOLDMANN & al. 2015].

Conclusions

In this study, 15 species with edibility potential included in *Russula* and *Lactarius* genera were investigated, in the forest ecosystems from the North-East Region of Romania, and maps of the potential distribution for each fungal species were generated. There are differences and similarities between distribution maps, determined by the fungal host specificity and host's probability of occurrence in the field. There are fungal species whose distribution maps are projections of the ecosystem edified by the dominant tree - in this case, the genus of tree-host. Significant differences between distribution maps of species preferring broad-leaved forests with *Quercus* sp. in North-East Region were observed.

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PROFESSOR PhD TOADER CHIFU AT THE 80TH ANNIVERSARY

On February 27, 2016, we celebrated the 80th anniversary of Professor PhD Toader CHIFU, a jubilee moment for the entire academic community as well as for his family. We celebrated a great scientific career, a Professor with high moral and professional standards.

He was born in Târnăuca, (Dorohoi County, nowadays in Ukraine Republic). In 1960 he graduated the Faculty of Natural Sciences-Geography of the "Alexandru Ioan Cuza" University in Iaşi. After graduation he become assistant professor at Botany Department (1960-1965). In next three years (1965-1968) worked as botanist in the Botanic Garden of Iaşi, where he organized the Systematic Section. Since 1968 he was Scientific Researcher in the Geobotany Laboratory of the Romanian Academy (Iași Filial) and since 1976 the Chief of the Terrestrial Ecology Collective in the Biological Research Center from Iaşi. In 1971 he was awarded with the PhD diploma in Phytosociology-Mycology at "Alexandru Ioan Cuza" University for the thesis: "*Floristic, ecologic-coenologic and economic research of the macromycetes from Neamțului Depression*". As a recognition of the value of his research he was invited to hold courses, talks, lectures and research stages in the National Agronomical Institute from Alger (1976-1980). In 1981 he returns to the Faculty of Biology and held courses of Systematic Botany, Tropical and Subtropical Flora, Vegetal taxonomy, Photosynthesis and productivity of ecosystems and Environment Protection. In 1995 he become full Professor at the Faculty of Biology and, currently, he is associate Professor in the same institution.

The research activity of Professor Chifu in domains as mycology, mycocoenology, botany, phytosociology, nature conservation is reflected by over than 180 articles and 10 monographies published. The most remarkable achievements are represented by over 30 coenotaxa new for science and numerous species of fungi and plants new for Romania.

His newest achievement, synthesizing his enormous experience in vegetation classification is the "*Phytosociological diversity of Romania's vegetation*", in four volumes, where there are described over 1200 coenotaxa. For this valuable work, he was distinguished with the "Emanoil Teodorescu" Prize of the Romanian Academy. Also, he participated at numerous scientific and didactic expeditions in Africa (Algeria, Morocco, Sahara Dessert etc.) and Europe (Switzerland, France, Andorra, Italy etc). He was responsible or coordinator of more than 50 research grants.

As a recognition of his experience, he is now a distinguished member in the scientific board of numerous prestigious journals and member in many scientific societies from Romania (*Romanian Phytosociological Society*) and abroad (*Amicale Internationale de Phytosociologie* (Bailleul – France) or *Association pour l'étude de la végétation* from Uppsala – Sweden). Also, he attended prestigious international scientific meetings and

conferences, presenting important aspects of Romanian vegetation: Excursion International de Phytosociologie en Suisse; International Excursion of Phytosociology in Apuseni Mountains, Colloque "Végétation et sols de montagnes", Grenoble – France, II-ème Congrès de la Fédération Internationale de Phytosociologie; XXVIII-ème Colloque Phytosociologique etc.

The passion for fungi, plants and plants communities, his tenacity, and perseverance made him a model and mentor for numerous PhD students, specialists, collaborators and friends. Therefore, on the behalf of all colleagues in the "Anastasie Fătu" Botanic Garden of Iași, we wish Professor Toader Chifu long years of health, energy to continue his prolific scientific career and all the best for many years to come.

Happy Anniversary!

Constantin MARDARI, Cătălin TĂNASE


Diversitatea fitosociologică
a vegetației României
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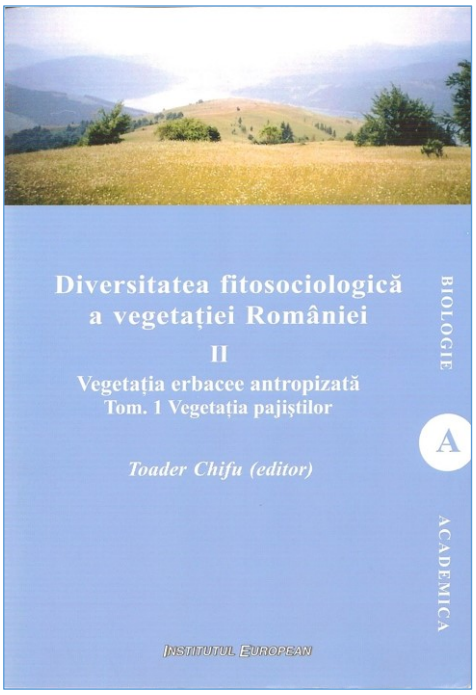
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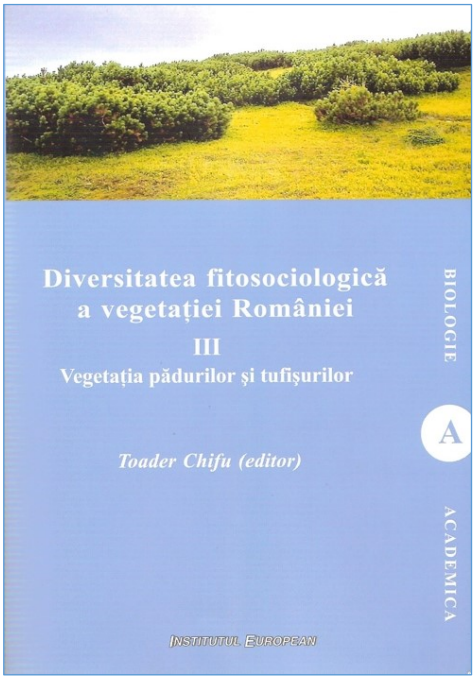
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Submission of a paper implies that it has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors, and that, if accepted, will not be published elsewhere in the same form, in English or in any other language, without the written consent of the publisher.

Authors are requested to submit their original paper and figures in digital format, to the Editor-in-Chief. The corresponding author should be indicated with an asterisk.

Manuscripts must be single-spaced, with wide margins. A font as Times New Roman, normal, is required.

The mirror of the page would be as follows: 13 x 20 cm (top 4.85 cm, bottom 4.85 cm, right 4 cm, left 4 cm).

The papers will be published only in a foreign language, structured as follows: title (the title would be also in the romanian language, if it is possible for the authors), authors, affiliation of the authors (including e-mails), abstract, keywords, introduction, material and method, results & discussions, conclusions, acknowledgements, references.

Titles would be written with bold, capital letters, 12 points, centered.

Names of the authors will be written with Times New Roman, 10 points, centered, capitals for surname (family name) and no capitals for first name (except initial letter). The names will not be abbreviated; each author name would be accompanied by a complete address, as a footnote on the first page. The affiliation should be provided in the following order: university (institution) name; faculty/department name; number and street name; city; country and email address.

Abstract: A concise and factual abstract is required (about 100-150 words). The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. References should therefore be avoided, but if essential, they must be cited in full, without reference to the reference list. Non-standard or uncommon abbreviations should be avoided but, if essential, they should be defined at their first mention in the abstract itself.

Key Words: five to seven words, the most important ones, in alphabetical order, after someone could discover your paper on the internet engines. Key words should not repeat the title of the manuscript.

Units: The SI system should be used for all scientific and laboratory data. In certain instances, it might be necessary to quote other units. These should be added in parentheses. Temperatures should be given in degrees Celsius.
The main text would be written at a single space, on A4 format page, Times New Roman, of 10 points.

The scientific names of taxa would be italicized.

Tables should be numbered consecutively in accordance with their appearance in the text and given suitable captions. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

Illustrations: photographs, charts and diagrams are all to be referred to as "Figure(s)", should be numbered consecutively in accordance with their appearance in the text. The mentions at the drawings, figures, pictures and tables will be placed inside the round brackets – for instance (Fig. 2); (Tab. 2); all illustrations should be clearly marked with the figure number and the author's name.

Obs.: all the schemes, drawings, etc. would be accompanied by a scale; the pictures must be very clear, being accompanied by the explanations. The diagrams should be made in Excel; pictures, ink drawings must be saved in JPG, JPEG, or BMP format, having a good resolution.

Other than the cover page, every page of the manuscript, including the title page, references, tables etc. should be numbered; however, no reference should be made in the text to page numbers.

All publications cited in the text should be presented in a list of references following the text of the manuscript. In the text, references are made using the author (s) name of a certain paper (e.g.: other authors [GÉHU, 2006] mentioned that…). The full reference should be given in a numerical list in the end of the paper. References should be given inside the square brackets.

Obs.: if there are two authors only, there must be written down both names (ex. [BOX & MANTHEY, 2006]); if there are more authors, there would be written the first author followed by "& al." (ex. [AMORFINI & al. 2006]).

References
For **scientific papers**: the name of the author (s) would be given in capital letters. The Christian name (s) would be abbreviated. Before the last but one and the last author you must insert the sign "&". In the reference list you must mention all the authors of a certain paper.
The year of a paper publication is put after the author (s).
Title: it should be fully written. The title of a book is written in italics. Between the year and the title we recommend to be inserted a dot sign. Next to it is the town and the publishing house of it (for books) or the periodical for papers. For periodicals, the abbreviations would be according to the international standards (BRIDSON & SMITH, 1991 or BROWN & STRATTON (eds), 1963-1965). Each periodical name is to be written in italics. A certain

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For **books**, after the title, is placed the name of the town, the publishing house and the number of pages.

The chapter in books: author (s), year, title, pages, a dot sign, followed by "In": author (s) of the book, city, publishing house, number of pages.
Serial papers or chapters in serial papers: like the previous, but with the specification of the serial volume.

Obs.: it is compulsory that all the papers from the reference list to be cited in the manuscript. The punctuation signs would not be in bold or italics. The references should be written in alphabetic order only. If a certain author has more papers with other contributors, the papers would be given also in alphabetic order (and not after the year of appearance). The number of a volume, the issue etc. would be given with Arabian numbers.

Examples of papers quotation:

References for papers in periodicals:

CIOCÂRLAN V. 2008. *Lathyrus linifolius* (Reichard) Bässler in the Romanian flora. *J. Plant Develop.* **15**: 3-6.
MEHREGAN I. & KADEREIT J. W. 2008. Taxonomic revision of *Cousinia* sect. *Cynaroideae* (*Asteraceae*, *Cardueae*). *Willdenowia*. **38**(2): 293-362.

References for books:

BOŞCAIU N. 1971. *Flora şi Vegetaţia Munţilor Ţarcu, Godeanu şi Cernei*. Bucureşti: Edit. Acad. Române, 494 pp.
HILLIER J. & COOMBES A. 2004. *The Hillier Manual of Trees & Shrubs*. Newton Abbot, Devon, England: David & Charles, 512 pp.

Serials:

JALAS J., SUOMINEN J., LAMPINEN R. & KURTTO A. (eds). 1999. *Atlas Florae Europaeae*. *Distribution of vascular plants in Europe*. Vol. **12**. *Resedaceae to Platanaceae*. Helsinki: Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo. Maps 2928-3270, 250 pp., ill (maps), ISBN 951-9108.
TUTIN T. G., BURGES N. A., CHATER A. O., EDMONDSON J. R., HEYWOOD V. H., MOORE D. M., VALENTINE D. H., WALTERS S. M. & WEBB D. A. (eds, assist. by J. R. AKEROYD & M. E. NEWTON; appendices ed. by R. R. MILL). 1996. *Flora Europaea*. 2nd ed., 1993, reprinted 1996. Vol. **1**. *Psilotaceae to Platanaceae.* Cambridge: Cambridge University Press, xlvi, 581 pp., illus. ISBN 0-521-41007-X (HB).
Chapters in books:
†TUTIN T. G. 1996. *Helleborus* L. Pp. 249-251. In: †T. G. TUTIN et al. (eds). *Flora Europaea*. 2nd ed., 1993, reprinted 1996. Vol. **1**. *Psilotaceae to Platanaceae*. Cambridge: Cambridge University Press, xlvi, 581 pp., illus. ISBN 0-521-41007-X (HB).

Short Communications: follow the same format as for the full papers, except that the Results and Discussion section (these should be combined). Manuscripts should not exceed 2000 words.

Special Issues: Proposals for Special Issues of full research papers that focus on a specific topic or theme will be considered.

Proofs will be sent to the corresponding author and should be returned within 48 hours of receipt. Corrections should be restricted to typesetting errors. All queries should be answered.

Manuscripts should be sent to:
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or
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