ROMANIAN JOURNAL OF BIOLOGY

PLANT BIOLOGY

VOLUME 58, N^{o} 2 2013

CONTENTS

ROM. J. BIOL. – PLANT BIOL., VOLUME **58**, No. *2*, P. 83–122, BUCHAREST, 2013

IN VITRO STORAGE AT LOW TEMPERATURES OF THE ENDEMIC TAXON *PAPAVER ALPINUM* L. ssp. *CORONA-SANCTI-STEPHANI – PRELIMINARY RESULTS*

RODICA CATANĂ, IRINA HOLOBIUC[1]

Storage of dehydrated somatic embryos at low temperatures (4° and -20°C) may be used as a cheap method for *ex situ* conservation purpose. In the case of endemic *Papaver alpinum* L. ssp. *corona-sancti-stephani (*Zapal.) Borza, the plant material was represented by somatic embryos obtained through a direct embryogenesis process. Embryos having sizes varying between 0.2-0.5 cm were dehydrated under sterile air flow until they reached 45% from their initial weight. The low temperature tolerance of somatic embryos was evaluated based on the survival rate (viability) after storage. Around 60% of dehydrated embryos stored up to 3 weeks at 4°C and -20°C were viable and able to re-grow**.**

Key words: *P. corona-sancti-stephani*, somatic embryo, low temperatures, *in vitro* techniques.

INTRODUCTION

The genus *Papaver* is represented in Romanian alpine vegetation only by one taxon – *Papaver alpinum* L. *corona-sancti-stephani* (Zapal.) Borza (Fig. 1), considered as an endemic (Ciocârlan, 2009) and a rare plant (Oltean *et al*., 1994) for SE Charpathian Mountains. This taxon is important because it is a pioneer plant in the rocky areas by fixing the detritus (Dihoru & Pârvu, 1987).

In situ conservation of the habitats represents the first strategy to conserve a plant species (Maunder *et al.*, 2004). The target taxa of plants for *ex situ* complementary conservation measures (including *in vitro* methods) is represented by the rare and threatened taxa (Harris *et al*., 2009). Endemic plant species are claiming also conservation actions (Işik, 2011).

P. corona-sancti-stephani is protected in *situ* in four Natura 2000 sites (the Rodnei, Retezat, Piatra Craiului and the Bucegi Mountains) and also *ex situ* in three Botanical Gardens collections (Sharrock & Jones, 2009). Being a rare and an endemic taxon, *in vitro* methods can complement *ex situ* preservation efforts (Benson, 1999).

[1] Plant and Animal Cytobiology Department, Institute of Biology Bucharest, Romanian Academy, 296 Splaiul Independenței St., 060031 Bucharest, P.O. Box 56-53.

Figure 1. *Papaver alpinum* L. *corona-sancti-stephani* (Zapal.) Borza in the natural habitat (Piatra Craiului Massif).

In vitro conservation was first extensively used to preserve the genetic resources of the economic important plant species. During the last decades, *in vitro* techniques brought an increased contribution in the case of the taxa with conservative importance, mentioned in Red Lists and books.

In vitro preservation can ensure the production of a large number of plants starting from a single individual in a relatively short time and in reduced space without affecting the natural populations owing to collecting methods.

Among *in vitro* developmental ways of regeneration, somatic embryogenesis allows the production of embryos originated from somatic cells, which develop similarly to zygotic embryos, rooting easily. They are more genetically stable compared to other *in vitro* generated propagules (buds, shoots).

Concerning the medium or long-term *ex situ* conservation based on *in vitro* techniques, there are no papers regarding this taxon. Some results were reported concerning the cryoconservation of transformed *Papaver somniferum* cells (Gazeau *et al.*, 1998, Elleuch *et al*., 1998).

The slow growth methods as medium-term conservation procedure can maintain the plant material for a few years (Kaviani, 2011). Slow growth is routinely used for few cultivated species conservation (banana, potato and cassava) in regional and international Germplasm Conservation Centers such as National Bureau of Plant Genetic Resources (NBPGR), Centro Internacional de la Papa (CIP), Instituto de Investigaciones Fundamentales en Agricultura Tropical (INIFAT) or International Institute for Tropical Agriculture (IITA).

The *ex situ* conservation protocols based on the maintenance at low temperatures are cheaper and safer, also suitable for conservation of plant material (Reed, 2002).

Comparing with cryoconservation, the mainterining of the plant material at low temperatures determines a slow metabolism without the total inhibition of biological activities. For this reason, plant material has a better capacity to recover after the cold treatment. The storage of plant material, shoots or embryos, obtained through *in vitro* methods at 4°C or -20°C, may be used as an alternative to field gene banks (Skene *et al.*, 1988).

The aim of our study was to develop a cheap and reproducible method for *in vitro* preservation of *P. corona-sancti-stephani* using the storage of dehydrated somatic embryos under low temperatures.

MATERIALS AND METHODS

Plant material is represented by somatic embryos previously obtained by us (unpublished results). The direct embryogenesis was induced on medium based on MS salts formula (Murashige & Skoog, 1962) added with Gamborg complex B vitamins (Gamborg *et al.*, 1968) and mannitol 3%, without plant growth factors, using leaf and root explants. The highest number of regenerants/explant was registered after 90 days (Fig. 2).

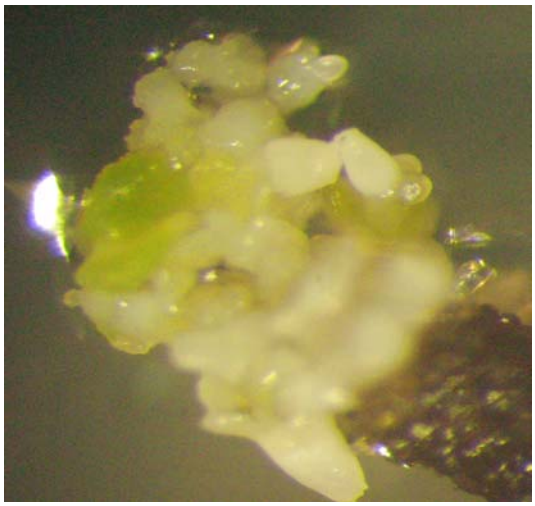


Figure 2. Aggregate of somatic embryos used as source for the experiment of dehydration and low temperatures storage.

Because the somatic embryogenesis is an asynchronous process, the somatic embryos in different stages were developed.

The isolated somatic embryos varying from 0.2-0.5 cm size, in different developmental stages (from globular to cotyledonary), were placed in a Petri dish in one layer on sterile filter paper and exposed to air drying in a laminar flow hood and dehydrated during 1 to 5 hours.

The relative water content (RWC) was determined by weighting samples consisting in 1g of somatic embryos (of different sizes) before and after dehydration after the formula: RWC = Initial weight – Final weight/ Initial weight *100.

After dehydration, the embryos were collected from the filter paper and placed in empty sterile Petri dishes and stored at 4° (in a normal refrigerator), -20°C (in freezer) and -75°C in darkness for 3 weeks.

For viability evaluation, the embryos were transferred from the storage dishes on recovery medium MS supplemented with 3% sucrose, 3% mannitol, solidified with 8% Phyto Agar, without any plant growth regulators. The cultures were maintained at 25°C and 1500 lux and 16/8 hours illumination regime. The viability evaluation was calculated after the formula: Embryo viability = number of viable embryos / total number of embryos * 100.

The viability of the somatic embryos maintained at low temperatures was evaluated after dehydration, at every hour in the first 3 hours of exposure at 4 and -20ºC, after 1 day and weekly during 3 weeks. The evaluation concerning the somatic embryos viability was recorded after 28 days.

An embryo was considered viable if, after 28 days, has the color green and was able to re-grow.

For each treatment were cultured 3 replicates consisting in 5 somatic embryos/Petri dish. The data were statistically analyzed based on Daniel's XL Toolbox program version 6.52 (http://xltoolbox.sourceforge.net). One-way analysis of variance (ANOVA) was used to calculate the statistical significance. The significant differences among the means were assessed by Tukey's test at 5% probability level to compare the variants means.

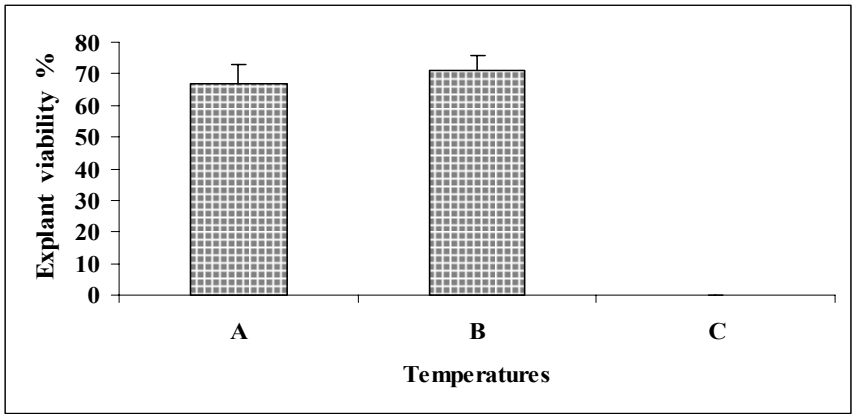
RESULTS

The dehydration process of the somatic embryos was achieved after 1-5 hours, in a laminar flow hood, at 25°C.

When the embryos were dehydrated for 1 hour prior to storage at the tested temperatures (4°C, -20ºC and -75ºC), their viability rate was affected. After embryos dehydration during 3 hours, they reached 45% RWC (relative water content). This prolonged exposure induced an optimal loss of water which allowed the best survival of the embryos and, for this reason, this interval of time was chosen for further experiments with embryos storage at low temperatures.

In our case, the 45% RWC allows to avoid the irreversible damages of the cells exposed to low temperatures which can affect the re-growth capacity of the plant material. During dehydration protocol, the somatic embryos decreased in size and changed their color becoming darker.

In our experiment, the embryos with 45% RWC were stored for 24 hours at 4°C, -20°C and -75°C. The dehydrated embryos maintained at -75°C were not viable (Fig. 3). The embryos viability maintained at 4°C and -20°C was not significantly different ($p > 0.05$).



Legend: A: 4°C, B: -20°C, C: -75°C.

Figure 3. Dehydrated somatic embryos viability after low temperature storage for 24 hours.

The viability of the dehydrated somatic embryos maintained at low temperatures showed the same response for both temperatures tested (4°C and -20°C), no significant differences ($p > 0.05$) being registered. 90% of the dehydrated embryos stored at 4°C and 60% from those stored at -20°C were viable after one hour. In the second hour of exposure, a decreased rate of explants viability was observed. Starting with storage from 3 hours until 1 week of exposure at low temperatures, the embryos reacted better. After 3 weeks, the dehydrated embryos achieved a survival rate more than 65% in the case of both tested temperatures (Figs. 4, 5). The embryos maintained at low temperatures had the capacity to re-grow, being able to convert into plants.

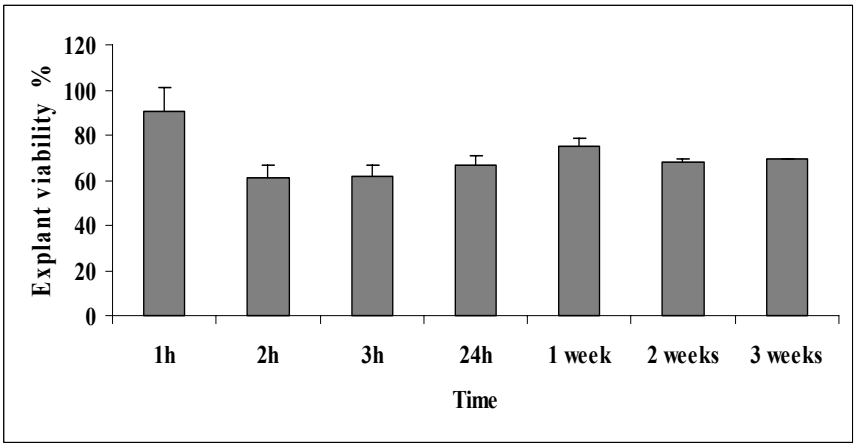


Figure 4. The viability of dehydrated embryos after 3 weeks of storage at 4°C.

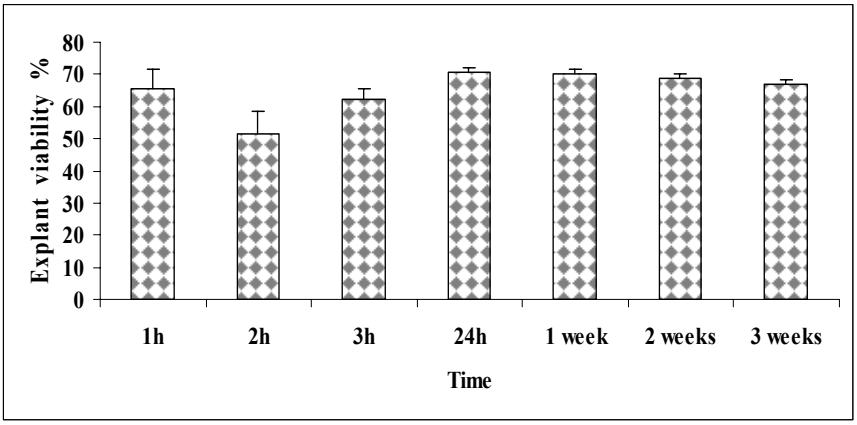


Figure 5. The viability of dehydrated embryos after 3 weeks of storage at -20°C.

DISCUSSION

Generally, the seeds are preferred plant propagules for medium or long-term conservation. Our previous researches regarding seed germination in *Papaver corona-sancti-stephani* showed that germination rate decreased in time. The seeds older than 5 years germinated neither *ex vitro* nor *in vitro* conditions. In the case of 2 years old seeds, only 10% germinated *in vitro* (Catană *et al*., 2013).

Owing to low seeds germination rate, *in vitro* methods are a reliable alternative to preserve this taxon on short and medium–term. In this frame, we tested the behavior of somatic embryos obtained by us at low temperatures.

Due to their similar structure with zygotic embryos, the somatic embryos may be used to mimic the seeds storage.

Somatic embryos of several species were preserved using low temperatures (Engelmann, 1997). Several studies concerning germination of dehydrated stored embryos in alfalfa (Senaratna *et al.*, 1990), orchardgrass (Gray *et al.*, 1987) and soybean (Parrot *et al.*, 1988), carrot (Lecouteux *et al*., 1992) suggested that this technique may be applied to other species as well. 90% of the dried somatic embryos stored at 4°C for a period of 42 months were converted into plants in the case of *Vitis vinifera* L cv. Chardonnay (Jayasankar *et al.*, 2005). *Ex situ* conservation protocols based on storage at low temperatures (1-4°C) were also described in apple (Orlikowska, 1992), mint (Reed, 1999), *Rubus* (Reed, 1993).

Generally, the pretreatments are used to allow the decrease of water content from the tissue in order to avoid the intracellular damages. The dehydration of the somatic embryos was considered not to affect the tissues (Moges *et al.*, 2003). The relative water content and time of dehydration are partially dependent on embryo

size. It was reported that the dehydration process had positive effects on the conversion of somatic embryos into plantlets in many species (Srinivas *et al*., 2006).

Similar results were obtained in *Cocos nucifera* L., embryos being dehydrated in a sterile air flow in a dehydration solution (containing glucose and glycerol). 12% of the dehydrated embryos maintained at -20°C for 3 weeks produced normal plants upon recovery (Sisunadar *et al.,* 2012).

In the nature, plants have several mechanisms to counteract the negative effects during freezing periods by decreasing the freezing point or increasing the degree of supercooling (Burke *et al*., 1976); increasing the concentration of solutes in the protoplasm (Li & Palta, 1978); increasing the permeability of the plasma membrane (Alden & Hermann, 1971; Levitt, 1980).

The viability of dehydrated somatic embryos exposed at -20°C, during 1 month may be explained by the ability to avoid the freeze-induced damages by normal strategy used by plants to resist to freezing at natural subzero temperatures (Meryman & Williams, 1985).

In the case of lower temperature of -75°C, probably, the embryos require a combination of pretreatments that could induce the tolerance. Only dehydration in laminar flow is not enough. Also, the thawing conditions may affect the plant material after the exposure at low temperatures. These results may be attributed to the formation or recrystallization of ice crystals which may lead to the destruction of cellular structures and death of the embryos.

CONCLUSIONS

Our preliminary results proved the possibility to preserve dehydrated embryos at low temperatures, in normal refrigerator or freezer, as a cheap alternative for *in vitro* conservation.

Comparing to the conservation at ultralow temperatures, more expensive and needing a lot of labor, the protocol of dehydrated somatic embryos at low temperatures may be useful in this taxon.

In normal conditions, the *in vitro* tissue cultures are stored at 25°C, with illumination regime of 16/8 hours in the growth chamber, being necessary periodical subcultures (1 month). Due to the necessity of labor connected to the transfers and because of hazards of contaminations, loss of entire cultures or genetic erosions can occur, methods based on slow-growth methods or cryoconservation can improve the duration and security of preservation.

Acknowledgements. This study was funded by Romanian Academy Research project RO1567-IBB06/2013.

REFERENCES

1. Alden J. and Hermann R.K., 1971, Aspects of the cold hardness mechanisms in plants, *Bot Rev*, **37**, pp. 37-142.
2. Burke M.J., George M.F., Gerber J.F., Janick J., Martsolf J.D., 1977, Does washing frost from plants reduce cold damage? *Hortscience*, **12**, pp**.** 101-103.
3. Catană R., Holobiuc I., Moldoveanu M., 2013, *In vitro* seed germination in three rare taxa from Romanian Carpathians flora, *Oltenia. Studii şi comunicări. Ştiinţele Naturii*. Muzeul Olteniei Craiova, **29**(1), pp. 85-92.
4. Ciocârlan V., 2009, *Flora ilustrată a României*. Edit. CERES, Bucureşti, 176 pp.
5. Dihoru G. & Pârvu C., 1987, *Plante endemice în Flora României*. Ed. Ceres, Bucureşti, 179 pp.
6. Engelmann F., 1997, In: Callow, Ford, Newbury (Eds.), Biotechnology and plant genetic resources *In vitro conservation methods*. CAB International, Oxford, 119-161.
7. Elleuch H., Gazeau C., David H., David A., 1998, Cryopreservation does not affect the expression of a foreign sam gene in transgenic *Papaver somniferum* cells, *Plant Cell Rep*, **18**, pp. 94-98.
8. Gamborg O.L., Miller R.A., Ojima K., 1968, Nutrient requirements of suspension cultures of soybean root cells, *Exp Cell Res*, **50**, pp. 151-158.
9. Gazeau C., Elleuch H., David A., Morisset C., 1998, Cryopreservation of transformed *Papaver somniferum* cells, *Cryo-Letters*, **19**, pp. 147-159.
10. Gray D.J., Conger B.V., Songstad D.D., 1987, Desiccated quiescent somatic embryos of orchard grass for use as synthetic seeds, *In Vitro Cell Dev B*, **23**, pp. 29-33.
11. Harris S., Shaw J., Crane N., 2009, Planning the integration of *ex situ* plant conservation in Tasmania, *Cunninghamia*, **11**(1), pp. 123–130.
12. Işik K., 2011, Rare and endemic species: why are they prone to extinction? *Turkish Journal of Botany*, **35**, pp. 411-417.
13. Jayasankar S., Van Aman M., Cordts J., Dhekney S., Li Z.T., Gray D.J., 2005, Low temperature storage of suspension culture-derived grapevine somatic embryos and regeneration of plants, *In Vitro Cell Dev Pl*, **41**(6), pp. 752-756.
14. Kaviani B., 2011, Conservation of plant genetic resources by cryopreservation, Review article, *Australian Journal of Crop Science*, **5**(6), pp. 778-800.
15. Lecouteux C., Tessereau H., Florin B., Courtois D., Petiard V., 1992, Preservation of carrot (*Daucus carota* L.) somatic embryos by dehydration after pretreatment with sucrose, Congrès Académie des sciences/Royal society, *Réunion conjointe*, **314**(9), pp. 1-94.
16. Levitt J., 1980, *Responses of Plants to Environmental Stresses*, vol. 1 (2nd ed.), New York NY: Academic Press, pp. 497.
17. Li P.H. and Palta J.P., 1978, Frost hardening and freezing stress in tuber-bearing solanum species, *in:* P.H. Li and A. Sakai (Eds). *Plant Cold Hardness and Freezing Stress.* Vol. I, New York, NY: Academic Press, pp. 49-71.
18. Maunder M., Guerrant E.O., Havens K., Dixon K.W., 2004, Realizing the full potential of *ex situ* contributions to global plant conservation. In: Guerrant EO, Havens K, Maunder M, (Eds.) *Ex Situ* Plant *Conservation: Supporting Species Survival in the Wild*. Washington (DC): Island Press, pp. 389–418.
19. Meryman H.T., Williams R.J., 1985, Basic principles of freezing injury to plant cells: Natural tolerance and approaches to cryopreservation. In: Kartha KK (Edit.) *Cryopreservation of Plant Cells and Organs*. CRC Press Inc., Boca Raton, Florida, pp. 14-47.
20. Moges A.D., Karam N.S., Shibli R.A., 2003, Slow growth *in vitro* preservation of African violet (*Saintpaulia ionatha* Wendl.) shoot tips, *Advanced HortScience*, **17**, pp. 1-8.
21. Murashige T. and Skoog F., 1962, Revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol Plant*, **15**(3), pp. 473-497.

22. Oltean M., Negrean G., Popescu A., Roman N., Dihoru G., Sanda V., Mihăilescu S., 1994, *Lista roşie a plantelor superioare din România*. Studii, Sinteze, Documentaţii de Ecologie, Academia Română, Bucureşti, pp. 26.
23. Orlikowska T., 1992, Effect of *in vitro* storage at 4°C on survival and proliferation of two apple rootstocks, *Plant Cell Tiss Org*, **31**, pp. 1-7.
24. Parrot W.A., Dryden G., Vogt S., Hildebrand D.F., Collins G.B., Williams E.G., 1988, Optimization of somatic embryogenesis and embryo germination in soybean, *In Vitro Cell Dev B*, **24**, pp. 817-820.
25. Reed B.M., 1993, Improved survival of *in vitro* stored *Rubus* germplasm, *Journal of the American Society for Horticultural Science*, **118**, pp. 890-895.
26. Reed B.M., 1999, *In vitro* storage conditions for mint germplasm, *HortScience*, **34**, pp. 350-352.
27. Reed B.M., 2002, Photoperiod improves long-term survival of *in vitro* stored strawberries, *HortScience*, **37** (5), pp. 811-814.
28. Srinivas L., Ganapathi T.R., Suprasanna P., Bapat V.A., 2006, Desiccation and ABA treatment improves conversion of somatic embryos to plantlets in banana (*Musa* spp.) cv. Rasthali (AAB), *I J Biotech*, **5**, pp. 521-526.
29. Senaratna T., Mckersie B.D., Bowley S.R., 1990, Artificial seeds of alfalfa *(Medicago)* spacer-length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences USA*, **81**, pp. 8014-8019.
30. Sharrock S. and Jones M., 2009, Conserving Europe's threatened plants: Progress towards Target 8 of the Global Strategy for Plant Conservation Botanic Gardens Conservation International, Richmond, UK.
31. Sisunadar S., Samosir P.A., Rival Y.M.A., Adkins S.W., 2012, Conservation of coconut (*Cocos nucifera* L.) germplasm at sub-zero temperature, *Cryo Letters*, **33**(6), pp. 465-75.
32. Skene K.G.M., Goodwins D.R., Barlass M., 1988, Ploidy stability in grapevines following long term storage *in vitro, Vitis*, **27**, pp. 41-44.
33. http://xltoolbox.sourceforge.net.

IN VITRO SYSTEMS, A FACILE TOOL FOR *CETRARIA ISLANDICA (L.)* (*LICHENOPHYTA*) ARTIFICIAL RESYNTHESIS AND BIOTECHNOLOGICAL POTENTIAL EXPLOIT – PRELIMINARY RESULTS

DIANA CRISTIAN[1], MONICA MITOI, AURELIA BREZEANU

This paper presents our findings regarding the possibility of elaborating an efficient methodology for lichens *in vitro* culture. The semiliquid consistency of the media contributed to obtaining of lichen biomass. Also, we mixed three types of media, namely MY medium (Ahmadjian, 1967a), BG 0 medium and basal Murashige – Skoog (1962) supplemented with BAP 0.4 mg/L and ANA 0.1 mg/L that allowed a better contact with the nutrients of the algae and fungal hyphae of investigated species – *Cetraria islandica* (L.) Ach. (Icelandic moss), used for bioconservation.

Key words: lichens, *in vitro* culture, symbiotrophic formations, biotechnology.

INTRODUCTION

Lichens are the expression of the weaving between the green partner (photobiont) and the fungal partner (mycobiont). The mycobiont uses the resources proved by the photobiont to construct and maintain a complex, "living habitat" that effectively addresses many of the environmental and nutritional requirements of the photobiont (Nash, 1996a; Palmqvist, 2000).

According to Ahmadjian (1993) and Alexopoulos (1996) classification, based on mycobiontic partner constancy, lichens can be enclosed in regnum Fungi, *Ascomycota*, respectively *Bazidiomycota*.

In the last decades the interest for study of lichens has increased, on manifold levels, generated especially by the capacity of many species to biosynthesize bioactive compounds of biotechnological interest; their use as model systems for production of secondary metabolites by "in vitro" culture is a special concern as well as artificial biosynthesis of lichens thalli for biomass production (Armitage and Hawe, 2007).

It is noteworthy in this regard that icelandic moss contains carbohydrates, proteins, fats, ascorbic and folic acid, B vitamins, iron, copper, manganese, chromium, as well as herbal antibiotics with high antimicrobial activity. *Cetraria islandica* (L.) Ach. (iceland moss) is well assimilated by the human body improving the functioning

[1] Institute of Biology, Romanian Academy, Splaiul Independenței, nr. 296.

of the internal organs and the immune system. Special attention was given to usnic acid, which is contained in the Icelandic moss and has a strong anti-bacterial effect against *Staphylococcus, Streptococcus*, and *Mycobacterium*. The mechanism of antibiotic action in usnic acid is associated with the rupture of oxidative phosphorylation process in the cells of microorganisms. Usnic acid selectively affects the infectious agents, without damaging the normal microflora. The dietary supplement Cetrazin is an example of product which demonstrates the action of Icelandic moss, besides other plant components (http://www.artlifedelhi.com/cetrazin-more.html). Comparing with higher plants, the richness of metabolites is surprisingly high (Manole and Banciu, 2015).

Apart from this, a considerable number of lichen species became endemic, a reason for their biodiversity conservation in repopulation strategies (Paunescu, 2009, Banciu *et al.* 2009, Manole *et al.* 2015). Also, their sensibility to environmental changes recommends them as indicators of quality for living environment.

In this context, our main objective was to test the optimal conditions for *in vitro* growth of *Cetraria islandica* (L.) Ach., to elaborate an alternative methodology of bioconservation, and biomass obtaining with a significant content of bioactive substances, many of them being unique for lichens, using "in vitro" systems benefits, with antimicrobian and/or antitumoral effects (usnic acid, for example).

Pharmacodynamic value of secondary metabolites, namely depsides (usnic acid), triterpenes and xanthones, the abundance of this species in Romania's lichenobiota, as well as non-relevant data regarding *in vitro* culture of this species in Romania are arguments for these studies.

MATERIAL AND METHODS

Plant material

The biological material investigated was represented by *Cetraria islandica* (L.) Ach., a terricolous lichen from the Retezat National Park, Lolaia Peak which was offered and determined by Ioana Vicol PhD (Institute of Biology).

In vitro culture

The inocula were represented by thallus fragments sampled from the edge of the lobes.

Superficial sterilization protocol included the following steps: thallus washing in running tap water (about three hours), the treatment with 70% ethanol (3 min), dichloroisocyanuric acid sodium salt 0.5 % (3 min) and sterile water.

Initially we tested the Bold's Basal Medium (BBM) (Deason & Bold, 1960) and Honegger **(**Honegger, 1993) media.

Further, we mixed, in equal proportions, three types of media, namely MY medium (Ahmadjian, 1967a), BG 0 medium (Rippka, R., Deruelles J., 1979) and basal Murashige – Skoog (1962) supplemented with BAP 0.4 mg/L and ANA 0.1 mg/L, solidified with agar.

The biological material was incubated in a growth chamber at a temperature of 19ºC and a photoperiod of 12/12 hour light/dark conditions.

Cytological analysis

The globular formations developed were cytologically investigated with the photonic microscope Scope A1, model Zeiss and scanning electron microscope too – JEOL-JSM-6610LV Scanning Electron Microscope.

The symbiotrophic formations were cytologically analyzed on squash preparations by phase contrast, in photonic microscopy after coloration with chlor-iodide of zinc solution (Kishnamurthy, 1999) or lactophenol/blue-cotton solution (Leck, 1999). For electron microscopy studies the samples were processed using Standard protocols of SEM laboratory techniques (Postek, 1980, Hall and Hawes, 1991, Fowke, 1995) and analyzed to the JEOL-JSM-6610LV Scanning Electron Microscope.

Determination of total phenolic compounds

For extraction of phenolic compounds, symbiotrophic formations obtained in *in vitro* cultures and native thalli were ground with mortar pestle. The extraction was performed in methanol or water for 24 hours at 200 rpm.

A reaction mixture consisting of 0.5 ml extracts 2.5 ml of Folin – Ciocalteu reagent (diluted 10 fold) and 2 ml Na_2CO_3 (75%) was used; the absorbance was measured at 765 nm (Mihailovic *et al*., 2013). The total phenol content was expressed in milligrams of gallic acid equivalent mg GAE /g extract.

Determination of DPPH free – radical scavenging activity

In order to determine scavenging DPPH radical activity of extracts, the method proposed by Marxen *et al.*, 2007 was used. 2.25 ml methanol, 0.1 ml extract and 0.15 ml DPPH were mixed in one measuring cuvette. After 30 min the absorbance to 550 nm was measured.

The results were calculated and expressed as µM Trolox equivalents per gram of fresh weight using calibration curve of Trolox. Linearity range of calibration curve was 50-150 µg/ml.

RESULTS AND DISCUSSION

The experiment started in November and after about four months some globular structures differentiated.

Initial attempts to culture lichen thallus *in vitro* were unsuccessful for more reasons, first of all because the contaminants could not be removed with weak sterilizing agents such as NaOCl 4% and ethanol; dichloroisocyanuric acid sodium salt was more efficient in concentrations of 0.5 %.

Also, the initially tested media Bazal Bold (BBM)(Deason & Bold, 1960); Honegger **(**Honegger, 1993) sustained the growth processes of the explants in a slow manner.

It is known that the mycobiont nutrient requirements refer to organic substances and differ from that of the alga which prefers the mineral ones (Stocker – Wörgötter and Elix, 2002). Literature and our previous experiments recommended MY medium (Ahmadjian, 1967a) for mycobiont development in *in vitro* culture (Cristian and Brezeanu A., 2013) and liquid BG 0 medium (Rippka and Deruelles 1979) for algae growth. During the experimental period many variants of these media were tested with modest results.

The best experimental variant was that which mixed the three types of media, namely MY medium (Ahmadjian, 1967a), BG 0 medium and basal Murashige – Skoog (1962) supplemented with BAP 0.4 mg/L and ANA 0.1 mg/L. After three months from the explants inoculation on culture medium, the first symbiotrophic formations (Fig. 1a) came out in response to *in vitro* condition.

The algae proliferate faster than the hyphae, coming out of the network hyphae.

The fact that both partners of the symbiosis are obvious on the microscopic slide sustained that the *in vitro* conditions tested by us were successful for the lichen fragments (Figs. 3-4) used as inoculum.

A decisive role in determining the favorable response was probably assigned by hormones that stimulated cells divisions. It is known that the development of the symbiotic structure depends on the culture medium. The two mixed culture media, namely BG0 (Rippka and Deruelles, 1979) and MY (Ahmadjian, 1967a) were suitable for both partners of the lichen symbiosis.

Thereby, the semiliquid consistency allowed a better contact with the nutrients of the algae and fungal hyphae.

In time the globular mass developed (Fig. 1b).

The scanning electron-microscope observations on green globular structures developed from *Cetraria islandica* thallus revealed a series of aspects which highlight a compact layer built by mycobiont hyphae.

Fig. 1a. Green symbiotrophic formations three months old.

Fig. 1b. Four months old *in vitro* developed structures.

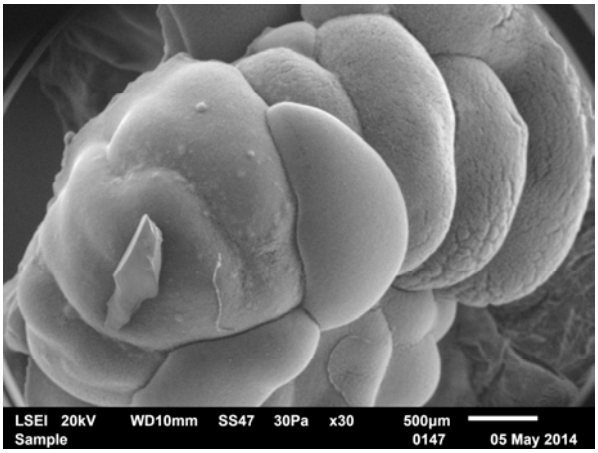


Fig. 2. Scanning - electron - micrograph (SEM) of the surface of symbiotrophic structure developed from *Cetraria islandica* thallus fragment.

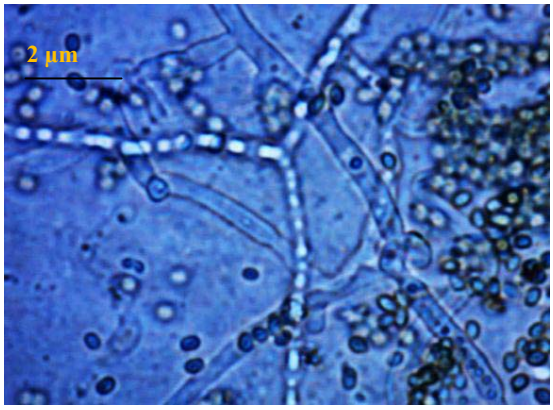


Fig. 3. Structural peculiarities of the *Cetraria islandica* symbiotrophic formations regenerated by " in vitro" culture from fragments of thallus. Numerous algae cells surrounded by fungal hyphae (see arrow) can be observed.

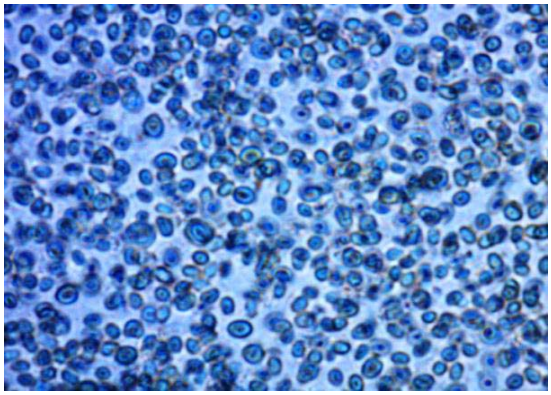


Fig. 4 Multitude of algae which explains green intense colour of the formations.

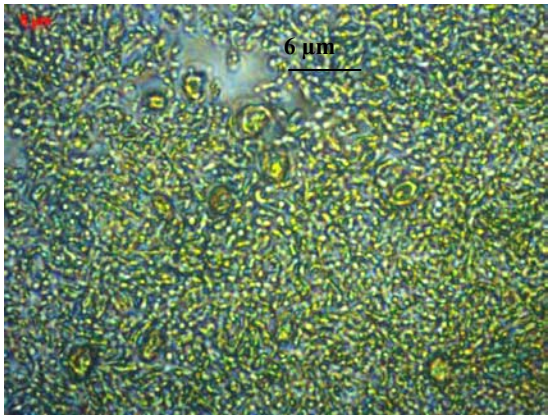


Fig. 5. Details of the lichen symbionts highlighted by staining with zinc chloride iodinated solution.

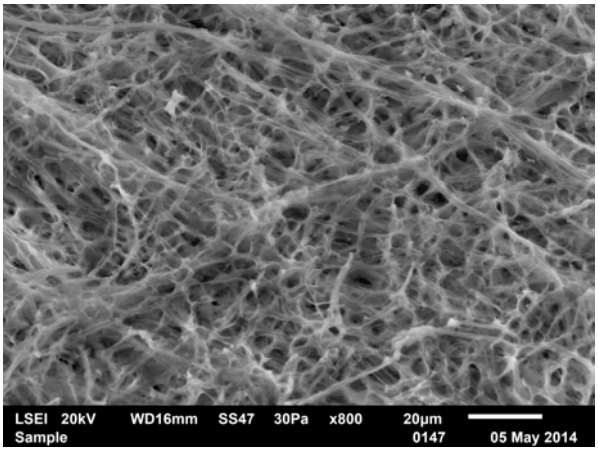


Fig. 6a. Surface scanning images of lichen formations.

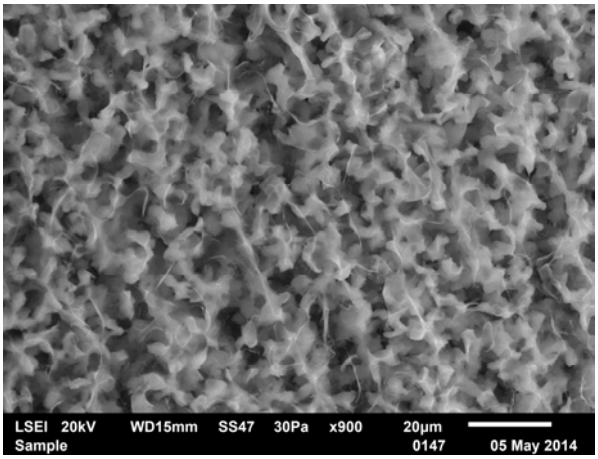


Fig. 6b. Details of the surface lichen mass.

The variant that was represented by mixing the three nominated media was the best. The data revealed that thallus fragments require a long time (about five months) until they grow and symbiotrophic formations differentiated. Temperature of about 19ºC has been found to represent optimal condition. Subculture procedures for lichens are longer than that of plant which requires shorter periods, this fact being an advantage for the first ones. Previous attempts tested the effectiveness of some media – BBM (1965) with kinetin (0.1 mg/L), (Honegger, 1993) listed in the literature for lichens (Voicu and Brezeanu, 2007). Choosing media specific for every partner of the symbiosis is to some extent a better way to obtain lichen biomass. The hormone that stimulates cells divisions in tissue culture seems to have a crucial role in determining the response.

Phenols content of the symbiotrophic formations

Phenols are very important plant constituents because of their scavenging ability to their hydroxyl groups (Hatano *et al*., 1989). In our aqueous extract of globular *in vitro* cultures of *C. islandica* 16.86 µg GAE/mg was detected. The phenolic compounds may contribute directly to antioxidant action (Duh *et al.*, 1999). It is suggested that polyphenol compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1 g daily ingested from a diet rich in fruits and vegetables (Takana *et al*., 1998).

In order to exploit the biotechnological potential of *in vitro* cultures of this lichen species we determined the total polyphenols content, flavonoids concentration and antioxidant activity. The preliminary results obtained in our experimental conditions did not show increased amounts of polyphenols content of about 21.023 µg GAE in methanol extracts and no higher antioxidant activity in *in vitro* culture than native thalli (*in vivo*).

Because in literature *C. islandica* is considered an antioxidant source, future studies will follow metabolites production in *in vitro* culture.

Table 1
Polyphenols concentration and antioxidant activity in methanol extracts

Sample	Polyphenols (µg GAE/mg fresh weight)	Antioxidant activity (µg Trolox equivalents/mg fresh weight)
In vitro culture	21.022	90. 529
Native thalli	99.296	194.758

CONCLUSIONS

1. *Cetraria islandica* (L.) Ach. is a reactive species to *in vitro* culture conditions and therefore is suitable for conservation and multiplication using this experimental system.

2. Because of their slow growth rates in nature (0.5-500 mm per year), the *in vitro* system represents a good alternative because their developmental condition can be easily controlled and modulated.

3. The results of this study show that the native extract of *C. islandica* has antioxidant activity and can be used as an easily accessible source of natural antioxidants and in pharmaceutical industry. Therefore, it is suggested that further work could be done on the identification of the some components with antioxidant activity in *C.islandica* (L.) Ach.

4. Our experimental data support the idea that this method can be applied to other lichen species and also the obtained material can be the objective of other investigations in the future.

5. Although our results indicate a lower concentration of polyphenols and a lower activity in *in vitro* culture in contrast to native thallus, *Cetraria islandica* is a rich source of antioxidants and our future experiments will focus on obtaining a higher biomass with a higher content of metabolites by modulating *in vitro* conditions.

Acknowledgements. The authors are grateful to Ioana Vicol, Department of Ecology, Institute of Biology, who offered us the biological material. Thanks are due to Anca Manole, for useful advice in microscopy analysis, Dr. Ioan Ardelean for technical help microscopic review, Brânzan Alexandru for technical support to scanning electron microscope investigations and laboratory technician Mariana Andrei for media preparations.

This paper was supported by project RO1567 – IBB06/2014 from the Institute of Biology Bucharest of the Romanian Academy.

REFERENCES

1. Ahmadjian V., 1967a, *The Lichen Symbiosis.* Blaisdell Publishing Company, Massachusetts, 152 pp.
2. Alexopoulos C.J., Mims C.W., Blackwell M., 1996, *Introductory Mycology*, John Wiley and Sons, New York, 868 pp.
3. Armitage M.H. and Hawe F.G., 2007, *The Ultrastructure of Lichens Cells*, Support Creation not Macroevolution, *CRSQ*, **44**, **1**, pp. 40-53.
4. Banciu C., Carasan M.E., Brezeanu A., 2009, *In vitro* propagation of the endangered species *Marsilea quadrifolia* L. – morphological and biochemical analysis of the regenerates, *Rom. Biotech. Lett.*, **14** (1): 4139-4145.
5. Brezeanu A., Voicu D., 2008, Ultrastructural peculiarities of *Usnea barbata (L.)Mott.* mycobiont and phycobiont cells "*in vivo*" and "*in vitro*" culture, *Rom. J. Biol. – Plant Biol.*, **53**(2), pp. 63-69.
6. Cristian D., Brezeanu A., 2013, *Xanthoria parietina* (L). Th. Fr. mycobiont isolation by ascospore discharge, germination and development in "in vitro" culture. Muzeul Olteniei Craiova. *Oltenia. Stud. Comunic. Şt. Nat*. **29** (2), pp.58-63.
7. Duh P.D., Tu Y.Y., Yen G.C., 1999. Antioxidant activity of the aqueous extract of Harng Jjyur (*Chrysanthemum morifolium* Ramat). *Lebensmittel-Wissenschaft and Technologie*, 32, 269-277.
8. Fowke LC. 1995. Transmission and scanning electron microscopy for plant protoplasts, cultured cells and tissues. In: Gamborg OL, Phillips GC, eds. Plant cell, tissue and organ culture. Fundamental methods. Berlin: Springer-Verlag, 229-238.
9. Hall J.L and Hawes C., 1991, *Electron microscopy of plant cells*. Academic Press, London, pp. 476.
10. Hatano T.R., Edamatsu, M. Hiramatsu, A. Mori and Y. Fujita *et al*., 1989. Effects of the interaction of tannins with co-existing substances. VI: Effects of tannins and related polyphenols on superoxide anion radical and on 1,1-diphenyl-2-picrylhydrazyl radical. *Chem. Pharmaceutical Bull*., 37: 2016-2021.
11. Honegger R., 1993, Developmental biology of lichens. *New Phytologist*, **125**, pp. 659-677.
12. Manole A., Banciu C., 2015, Optimization of shoot multiplication in *Ruscus aculeatus* L. from long term cultures, *Romanian Biotechnological Letters*, **20**(2), pp. 10200-10204.
13. Manole, A., Banciu, C., Indreica, A. 2015, Genetic diversity within a newly identified population of *Adenophora liliifolia* (L.) A.D.C. in Romania: implications for conservation. *Ann. For. Res.* **58**(2) DOI: 10.15287/afr.2015.389.
14. Mihailovic V., Matic S., Misic D., Solujic S., Stanic S., Katanic J., Mladenovic M., Stankovic N., 2013, Chemical composition, antioxidant and antigenotoxic activities of different fractions of *Gentiana asclepiadea* L. roots extract, *EXCLI Journal*, **12**, pp. 807-823.

15. Nash T. H., 1996a, Photosynthesis, respiration, productivity, and growth. In: T.H. Nash [ed.], *Lichen Biology*, Cambridge University Press, pp. 88-120.
16. Paunescu, A., 2009, Biotechnology for endangered plant conservation: a critical overview. *Rom. Biotech. Lett*., **14**(1), pp. 4095-4103.
17. Palmqvist K., 2000, Carbon economy in lichens. *New Phytologist*, **148**, pp. 11-36.
18. Postek M.T., Howard K.S., Johnson A.H., McMichael Kl., 1980, Scanning electron microscopy – a student's handbook. Ladd Research Industries, Burlington.
19. Rippka R., Deruelles J., Waterbury J.B., Herdman M., Stanier R.Y., 1979, Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.*, **111**, pp. 1-61.
20. Stocker – Wörgötter E. and Elix J.A., 2002, Secondary chemistry of cultured mycobionts: formation of a complete chemosyndrome by the lichen fungus of *Lobaria spathulata*. *Lichenologist*, **34**, pp. 351-359.
21. Tanaka K. *et al*., 1998. Inhibition of N - nitrosation of secondary amines in vitro by tea extracts and catechins. Mutation Research. 412 (1): 91-98.
22. Voicu D., Brezeanu A., 2008, Comportamentul in cultura *in vitro* a speciilor lichenice *Xanthoria parietina* Beltr. şi *Cetraria islandica* Ach. In: *Biotehnologii vegetale pentru secolul XXI*, Ed. Risoprint, Cluj-Napoca, pp. 101-112.
23. Voicu D., Brezeanu A., 2008, *In vitro* reactivity of *Usnea barbata (L.) Mott.*, *Rom. J. Biol. – Plant Biol.*, **53**(2), pp. 83-90.
24. http://www.artlifedelhi.com/cetrazin-more.html, accessed on 27.06.2014.

DISTRIBUTION OF THE *TELOSCHISTES CHRYSOPHTHALMUS* (L.) Th. Fr. IN ROMANIA

IOANA VICOL[1]

Investigations on the chorology of *Teloschistes chrysophthalmus* (L.) Th. Fr. were performed in the Ceahlău Mountain. The obtained results have pointed out the absence of this red listed lichen species from its cited site. Therefore it is of major importance to carry out the field activities because the extinction of a red listed lichen species has a negative impact on Romanian lichen diversity.

Key words: chorology, *Teloschistes chrysophthalmus*, Ceahlău Mountain, Romania.

INTRODUCTION

In Romania, *Teloschistes chrysophthalmus* (L.) Th. Fr. is a red-listed macrolichen considered as an European threatened species according to A (ii) sub-criterion used to Important Plant Areas (IPA) selection (Sârbu A. *et al*., 2007). In Italy, *T. chrysophthalmus* (L.) Th. Fr. is also included in A (ii) subcriterion of IPA category which was found within maquis, arborescent matorral and thermo-Mediterranean brushes habitats (Ravera S. *et al*., 2011).

In countries such as Czech Republic and Estonia *T. chrysophthalmus* is not a red-listed species (Liška J. *et al*., 2008; Randlane T. *et al*., 2008). Also for Alicante, Castellón and Valencia provinces from eastern Spain *T. chrysophthalmus* is not included in the Red List (Atienza V. et Segarra J. G., 2000).

T. chrysophthalmus (L.) Th. Fr. appears to be declining throughout many parts of North America because of habitat destruction and increased air pollution (Nelsen M.P., 2005).

From an otherwise point of view, this species has a practical importance within the pharmaceutical researches. Thus, it was used to obtain parietin acid as secondary metabolite with a virucidal effect against the arenaviruses Junín and Tacaribe (Fazio A.T. *et al*., 2007).

[1] Institute of Biology Bucharest, Romanian Academy, 296 Splaiul Independenței St., 060031 Bucharest, P.O. Box 56-53.

MATERIALS AND METHODS

The field activities were performed within Ceahlău National Park in November 2011 and June 2014. The chorological data and the nomenclature for *T. chrysophthalmus* is according to Ciurchea (2004).

RESULTS AND DISCUSSION

Within field activities performed in the Ceahlău National Park, *T. chrysophthalmus* has not been identifed on corticolous substrata. A great attention was attributed to Chernobyl accident (April 1986) when an important part of Europe was contaminated. Romania was one of the most radiopolluted countries due to its geographical position, climatic and environmental conditions (Bartók K., 1998). One of the causes responsible for the absence of *T. chrysophthalmus* from its cited habitat might be the impact of radiopollution caused by Chernobyl accident.

Taxonomy

Teloschites chrysophthalmus (L.) Th. Fr. syn. *Teloschistes chrysophthalmus* var. *hillmannii* (Ciurchea M., 2004).

Sociology

T. chrysophthalmus is present within *Xanthorion parietinae* Ochsner 1928 and is characteristic to *Buellion canescentis* Barkm. 1958, *Buellietum canescentis* Duv. 1941 (Ciurchea M., 2004).

Geographical element

The studied taxa is distributed in central-european, atlantic, subatlantic and mediterranean areas (Ciurchea M., 2004).

Ecology

T. chrysophthalmus is growing on corticolous substrata. Regarding the preferences towards substrata conditions this species is subneutrophilous, moderate xerophilous, and non-nitrophilous – moderate nitrophilous (Ciurchea M., 2004). It is found both on well illuminated and shaded substrata (Purvis O.W. *et al*., 1994).

Chorology

General distribution

T. chrysophthalmus is known in drier, sunny, warm temperate areas of both hemispheres (Purvis O.W., *et al*., 1994). Thus, in Africa it is distributed in areas

along the Mediterranean coast, especially from Morocco and Algeria, eastern and southern parts of Africa (Sudan, Ethiopia, Somalia, Kenya, Tanzania) and central and western parts of Africa (Angola, Uganda, Rwanda). It was identified in Madagascar Republic, Madeira Portuguese Archipelago and Canary Islands. In northern, central and southern America, *T. chrysophthalmus* is known from Winnipeg and Ontario (Canada), Texas, Minnesota, New England, California (United States), Mexico, Argentina, and Chile. Also it is widely distributed in Australia and New Zealand (Almborn O., 1989).

On European continent *T. chrysophthalmus* was cited from Great Britain as a very rare species, Romania, Italy, Czech Republic, Estonia, Spain, Germany (Purvis O.W. *et al*., 1994; Wirth V., 1994; Atienza V. et Segarra J.G., 2000; Sârbu A. *et al*., 2007; Liška J. *et al*., 2008; Randlane T. *et al*., 2008; Ravera S. *et al*., 2011). It is seems to be extinct in southern England, western France and southern Germany, whereas it is more frequent in Portugal, Spain and Italy (Almborn O., 1989).

National distribution

According to Moruzi C. *et al*. (1967) and Ciurchea M. (2004), in Romania *T. chrysophthalmus* was cited from the Ceahlău Mountain at Piatra Ciobanului by Moruzi C. et Cretzoiu P. (1944). The research on *T. chrysophthalmus* must be continued and its habitat yearly monitored.

CONCLUSIONS

As an important statement on chorology of *Teloschistes chrysophthalmus* in Romania is mentioned based on field activities, the absence of this species from its cited locality. The research on the presence of *T. chrysophthalmus* in Ceahlău Mountain will be continued especially to decide if it is extinct or extremly rare in its habitat.

REFERENCES

1. Almborn O. 1989. Revision of the lichen genus *Teloschistes* in central and southern Africa. *Nordic Journal of Botany*. **8**: 521-537.
2. Atienza V., Segarra J.G. 2000. Preliminary Red List of the lichens of the Valencian Community (eastern Spain). *Forest Snow and Landscape Research*. **75** (3): 391-400.
3. Bartók K., Mócsy I., Bolyós A., Dezsö Z. 1998. Studies on Cs137 content of lichens in mountain regions of Romania. *Sauteria*. **9**: 249-256.
4. Ciurchea M. 2004. *Determinatorul lichenilor din România*. Iaşi, Editura: Bit.
5. Fazio A.T., Adler M.T., Bertoni M.D., Sepúlveda C.S., Damonte E.B., Maier M.S. 2007. Lichen secondary metabolites from the cultured lichen mycobionts of *Teloschistes chrysophthalmus* and *Ramalina celastri* and their antiviral activities. *Verlag der Zeitschrift für Naturforschung*. **62c**: 543-549.

6. Liška J., Palice Z., Slaviková S. 2008. Checklist and Red List of lichens of the Czech Republic. *Preslia*. **80**: 151-182.
7. Moruzi C., Cretzoiu P. 1944. Ein felsbewohnendes *Teloschistes* aus Rumänien. *Bulletin de la section scientifique de l'Academie Roumaine*. **24** (10): 723-725.
8. Moruzi C., Petria E., Mantu E. 1967. Catalogul lichenilor din România. *Acta Botania Horti Bucurestiensis*: 309.
9. Nelsen M.P. 2005. Additions to the lichen flora of Wisconsin with new records of rare species. *The Michigan Botanist*. **44**: 188-191.
10. Purvis O.W., Coppins B.J., Hacksworth D.L., James P.W., Moore D.M. 1994. *The lichen flora of Great Britain and Ireland. London*. Natural History Museum Publications in association with The British Lichen Society.
11. Randlane T., Jüriado I., Suija A., Lõhmus P., Leppik E. 2008. Lichens in the new Red-list of Estonia. *Folia Cryptogamica Estonica*. **44**: 113-120.
12. Ravera S., Nimis P.L., Brunialti G., Frati L., Isocrone D., Martellos S., Munzi S., Nascimbene J., Potenza G., Tretiach M. 2011. The role of lichens in selecting important plant areas in Italy. *Fitosociologia*. **48** (2): 145-153.
13. Sârbu A., Sârbu I., Oprea A., Negrean G., Cristea V., Gheorghe C., Cristurean I., Popescu G., Oroian S., Tănase C., Bartók K., Gafta D., Anastasiu P., Crişan F., Costache I., Goia I., Maruşca T., Oţel V., Sămărghiţan M., Henţea S., Pascale G., Răduţoiu D., Baz A., Boruz V., Puşcaş M., Hiriţiu M., Stan I., Frink J. 2007. *Arii speciale pentru protecţia şi conservarea plantelor în România*. Bucureşti, Editura: Victor B Victor.
14. Wirth V. 1995. *Flechtenflora*. Stuttgart, Verlag Eugen Ulmer.

REVISION OF THE *ORCHIDACEAE* FAMILY FROM THE HERBARIUM OF "ALEXANDRU IOAN CUZA" UNIVERSITY OF IAŞI (3rd NOTE)

IRINA IRIMIA[1]

The paper presents the taxonomic and chorologic revision of two genera of *Orchidaceae* family. The study is based on herbarium materials that are in the Herbarium of "Alexandru Ioan Cuza" University of Iaşi [I]. There were revised 298 herbarium sheets with specimens collected from different parts of Romania during 1890-2005. Also, it was analyzed the dynamics of species entry into the herbarium collection.

Key words: *Cephalanthera*, *Epipactis*, flora.

INTRODUCTION

The *Orchidaceae* family is represented in Romania's flora through 26 genera and 59 species. Of these in the herbarium collection of "Al. I. Cuza" University of Iaşi there are 23 genera and 44 species.

Most of the orchid species from the Romanian flora are included in "The Red List" being rare or endangered (Oltean *et al*., 1994), especially due to the anthropic impact on their habitats (transforming the grassland into agricultural fields, deforestation, abusive and irrational pasturage).

The chorological aspects included in this paper provide to specialists important data regarding the distribution of these orchids in Romania.

METHODOLOGY AND MATERIALS

In this paper were included 2 genera of *Orchidaceae* family. There were revised 298 herbarium sheets with specimens collected from different parts of Romania during 1890-2005.

The species are: *Cephalanthera damasonium* (Mill.) Druce, *Cephalanthera longifolia* (L.) Fritsch, *Cephalanthera rubra* (L.) Rich., *Epipactis atrorubens* (Hoffm.) Besser, *Epipactis helleborine* (L.) Crantz, *Epipactis palustris* (L.) Crantz, *Epipactis purpurata* Sm.

[1] "Alexandru Ioan Cuza" University, Faculty of Biology, Bd. Carol I, no. 20A, Iaşi – 700506 Romania.

Every specimen was verified using the classical determination method. For the identification were used (Beldie, 1977; Ciocârlan, 2009; Moore, 1980; Paucă *et al*., 1972; Sârbu *et al*., 2001).

RESULTS AND DISCUSSION

The research results into herbarium are the following:

Cephalanthera L.C.M. Richard

1a. Ovary pubescent… *rubra*

1b. Ovary glabrous… 2

2a. Bracts much longer than ovary. Lower leaves ovate… *damasonium*

2b. Bracts much shorter than ovary. Lower leaves lanceolate… *longifolia*

C. damasonium (Mill.) Druce

BACĂU: Filipeşti, tufiş, 19.V.1968, leg. & det. A. Goagă (as *Cephalanthera alba*) [I 20853]; **Oneşti**, tufişuri, 7.VI.1968, leg. & det. A. Goagă (as *Cephalanthera alba* (Cr.) Simk.) [I 20852].

BOTOŞANI: Suharău, păd. Suharău, 16.VII.1966, leg. & det. G. Mihai (as *Cephalanthera alba* (L.) C. Rich.) [I 54626], *ibidem*, 8.VI.1968, leg. & det. I. Sârbu (as *Cephalanthera alba* (Cr.) Simk.) [I 41605].

GALAŢI: Balinteşti, păd. Rediu Grăpeni, în gorunet, 19.VI.1974, leg. & det. I. Sârbu [I 53525]; **Între Suceveni şi Onciu**, păd. Rediu Cerbului, în gorunet, 26.VI.1973, leg. & det. I. Sârbu [I 53524].

ILFOV: Comana, Valea Gurbanului, 15.V.1900, leg.?, det. A. Paucă (as *Cephalanthera alba* (Cr.) Simk.) [I 11134].

IAŞI: Bârnova, 16.VI.1930, leg. & det.? (as *Cephalanthera alba* Simk.) [I 11135], *ibidem*, 23.VI.1960, leg. & det. C. Dobrescu (as *Cephalanthera alba* (Cr.) Simk.) [I 71789], pădure, V.1896, leg. & det.? (as *Cephalanthera alba* (Cr.) Simk.) [I 11130], pădure 30.V.1951, leg. & det. C. Dobrescu (as *Cephalanthera alba* (Cr.) Simk.) [I 77081], păd. Bârnova, stânga gării, 30.V.1954, leg. & det. C. Dobrescu [I 71813], păd. Bârnova, 5.VI.1958, leg. & det. C. Dobrescu (as *Cephalanthera alba* (Cr.) Simk.) [I 71786]; **Dobrovăţ**, S, păd. Buda, 3.VIII.1970, leg. & det. C. Dobrescu (as *Cephalanthera alba* (Cr.) Simk.) [I 77058], spre Dobrovăţ, marginea drumului, 13.VI.1954, leg. & det. C. Dobrescu (as *Cephalanthera alba* Simk.) [I 71814]; **Dumbrava**, păd. Dumbrava-Ciurea, 10.V.1969, leg. & det. D. Solinschi Tinţu (as *Cephalanthera alba* Simkonkai) [I 20854]; **Glăvăneşti**, păd. Sohodol, 18.VI.1980, leg. & det. C. Dobrescu [I 72530]; **Grajduri**, V, păd. Scânteia, 18.VIII.1957, leg. & det. C. Dobrescu [I 77059], S, pădure, 2.VII.1969, leg. & det. C. Dobrescu [I 77060]; **Iaşi**, păd. Repedea, 26.VI.1939, leg. & det.? (as *Cephalanthera alba* (Cr.) Simk.) [I 11133], *ibidem*, 11.VI.1961, leg. & det. G. Viţalariu

[I 98718, 98721], Repedea, 11.V.1910, leg. & det.? (as *Cephalanthera alba* (Cr.) Simk.) [I 11125, 11127]; **Mârzeşti**, 15.V.1914, leg. & det. C. Oescu (as *Cephalanthera alba* (Cr.) Simk.) [I 11126], pădure (gorunet), 3.V.1966, leg. & det. C. Dobrescu (as *Cephalanthera alba* Simonkai) [I 50627]; **Piscu Rusului**, Dagâța, pădure, 6.VI.1963, leg. & det. C. Dobrescu (as *Cephalanthera alba* (Cr.) Simk.) [I 77083]; **Poieni**, păd. Poieni, 29.V.1968, leg. & det. I. Sârbu (as *Cephalanthera alba* (Cr.) Simk.) [I 41930]; **Voineşti**, V, 11.VI.1894, leg. & det.? (as *Cephalanthera alba* (Cr.) Simk.) [I 11132], "Catarg" pădure, 6.VI.1963, leg. & det. C. Dobrescu (as *Cephalanthera alba* (Cr.) Simk.) [I 77082].

NEAMȚ: Masivul Ceahlău, 20.V.1910, leg. & det.? (as *Cephalanthera alba* (Cr.) Simk.) [I 11131]; **Pângărați**, valea pârâului Pângărați, 28.VI.1961, leg. & det. T. Chifu (as *Cephalanthera alba* (Cr.) Simk.) [I 60844]; **Potoci**, dl. Bisericii, 5.VII.1980, leg. I. Pruteanu, det.? as *Cephalanthera rubra*, rev. I. Irimia, 10.X.2011 [I 74126]; **Rădeni**, Valea Răgoazelor, pădure, 12.VI.1970, leg. G. Mihai & I. Sârbu, det. I. Sârbu (as *Cephalanthera alba* (Cr.) Simk.) [I 42043].

SIBIU: In fagetis mixtis prope balneas **Bazna**, solo humoso. Alt. cca 400 m s. m., 30.VII.1940, leg. A. Borza [FRE nr. 2821 - I 48064, 48065, 49675, 63997].

VASLUI: Brăhăşoaia, păd. Hârboanca, 25.V.1971, leg. & det. C. Dobrescu [I 77069]; **Micleşti**, pădure, 13.V.2001, leg. & det. I. Blaj, rev. T. Chifu [I 120343]; **Răşcani**, 2.VI.1973, leg.?, det. I. Sârbu [I 62676]; **Vaslui**, NV Fabrica de Cărămidă, 14.V.1973, leg.?, det. I. Sârbu [I 62674].

Without a geographical placement: 19.V.1914, leg. & det.? (as *Cephalanthera alba* (Cr.) Simk.) [I 11128]; pădure, 19.V.1917 leg. & det.? [I 11129].

The most part of the herbarium material was collected in the period 1961-1980. The oldest herbarium sheet dates from 1894 and the newest from 2001 (Fig. 1a).

C. longifolia (L.) Fritsch

BACĂU: Ferăstrău, pădure, 13.V.1968, leg. & det. A. Goagă as *Cephalanthera rubra* (L.) Rich., rev. I. Irimia, 10.X.2011 [I 20857]; **Filipeşti**, livadă, 2.V.1968, leg. & det. A. Goagă as *Cephalanthera rubra* (L.) Rich., rev. I. Irimia, 10.X.2011 [I 20856]; **Mănăstirea Caşin**, făget, 14.VII.1998, leg. & det. M. Gurău, rev. A. Oprea [I 115064]; **Oneşti**, pădure, 29.V.1968, leg. & det. A. Goagă as *Cephalanthera rubra* (L.) Rich., rev. I. Irimia, 10.X.2011 [I 20858], Culmea Perchiului, 29.V.1968, leg. & det. A. Goagă as *Cephalanthera rubra* (L.) Rich., rev. I. Irimia, 10.X.2011 [I 20855].

BRAŞOV: Valea Teliului, E, fânaț 14.VI.1970, leg. & det.? [I 64561].

CARAŞ-SEVERIN: Ad margines Fageti prope stationem climaticam **Poiana Mărului**. Solo schistoso. Alt. cca 800 m s. m., 13.VI.1943, leg. A. Borza [FRE nr. 2509 – I 48067, 70423].

COVASNA: Floroaia Mică, N, plantație de molid, 22.VI.1970, leg. & det.? [I 64560].

DOLJ: Mânăstiricea, E, petic de coastă nordică, pădure, 23.V.1956, leg. & det.? [I 87585].

IAŞI: Bârnova, 16.VI.1930, leg. & det.? [I 11143, 11148], *ibidem*, V.1935, leg. & det.? [I 11140], pădure, 11.VI.1912, leg. & det. C. Papp [I 15502]; Bârnova-Repedea, 30.VI.1939, leg. & det.? [I 11154]; **Buda**, 15.V.1976, leg. & det. C. Burduja [I 95813]; **Dobrovăț**, pădure, 28.V.2005, leg. & det. I. Blaj [I 120719]; **Domnița**, Țibana, păd. Domnița, 9.V.1975, leg. & det.? [I 91664]; **Grajduri**, dl. Cocoarei, pășune 4.V.1950, leg. & det. C. Dobrescu [I 77063], păd. Scânteia, 18.VIII.1957, leg. & det. C. Dobrescu [I 77066], V, păd. Găunoasa, pădure, 18.VIII.1958, leg. & det. C. Dobrescu (as *Cephalanthera ensifolia* (Huds.) Fritsch. [I 77067]; **Iași**, fânațul Bucium (margine de pădure), 22.05.1941, leg. & det. C. Burduja [I 71191], Repedea, V.1895, leg.?, det. A. Paucă [I 11136, 11137, 11139], *ibidem*, 5.V.1910, leg. & det.? [I 11153], *ibidem*, 25.V.1929, leg. & det.? [I 11144], *ibidem*, 25.V.1930, leg. & det.? [I 11141, 11146, 11147], *ibidem*, 15.V.1931, leg. & det.? [I 11142], *ibidem*, 24.V.1935, leg. & det.? [I 11152], *ibidem*, 2.V.1954, leg. C. Dobrescu, det. I. Sârbu [I 77061], *ibidem*, 16.V.1958, leg. & det. G. Mihai [I 76410], *ibidem*, 15.V.1969, leg. A. Goagă, det. I. Irimia, 10.X.2011 [I 59305], păd. Repedea, 26.VI.1939, leg.?, det. A. Paucă [I 11138], *ibidem*, 20.V.1958, leg. & det. G. Vițalariu [I 97056]; **Piscu Rusului**, Dagâța, păd. Surda, 26.VIII.1968, leg. & det. C. Dobrescu [I 77062]; **Voinești**, 14.V.1975, leg. & det.? [I 91665], *ibidem*, 28.VI.1975, leg. & det.? [I 91666].

NEAMȚ: Mărgineni, pădure, 24.V.1971, leg. G. Mihai & I. Sârbu, det. I. Sârbu [I 37815]; **Mănăstirea Neamț**, 19.V.1967, leg. & det. T. Chifu & V. Slonovschi [I 91669]; **Poenari**, păd. Zimbru, 9.VIII.1968, leg. & det. C. Dobrescu [I 77065]; **Târgu Neamț**, dl. Brăilenei, 28.V.1968, leg. & det. T. Chifu [I 91667, 91670], *ibidem*, 11.VI.1968, leg. & det. T. Chifu & N. Ștefan [I 91668].

VASLUI: Băceşti, dl. Iezerul, 4.IX.1969, leg. C. Dobrescu, det. I. Sârbu [I 77064], păd. **Dumeşti**, 20.VI.1970, leg. & det. C. Dobrescu [I 77068]; **Laza**, păd. Scroafa, 7.V.1973, leg. C. Dobrescu, det. I. Sârbu [I 68743]; **Micleşti**, pădure, 13.V.2001, leg. & det. I. Blaj, rev. T. Chifu [I 120344].

Uncertain information about the county: Păd. Țiganca, 26.IV.1914, leg. & det.? [I 11149, 11151], *ibidem*, leg. & det. C. Oescu [I 11150]; **Buşaga**, 17.VIII.1917, leg.?, det. I. Irimia, 20.X.2011 [I 11428]; **Basarabi**, pădure, 12.V.1951, leg. & det. C. Burduja [I 72505]

The biggest part of the herbarium material comes from 1961 – 1980. The oldest herbarium sheet dates from 1895 and the newest from 2005 (Fig. 1a).

C. rubra (L.) Rich.

BOTOŞANI: Suharău, păd. Suharău, 8.VI.1968, leg. & det. I. Sârbu [I 41606]; **Dealul Verde**, 6.VII.1959, leg. & det. G. Mihai [I 70213].

BRAŞOV: Vama Buzăului, SV, pădure de fag, 24.VII.1970, leg. & det.? [I 64559].

GALAȚI: Roşcani, păd. Roşcani, pădure de *Quercus pubescens*, 25.VI.1973, leg. & det. I. Sârbu [I 53526]; **Slobozia Băneasa**, 18.VII.1995, leg. & det. A. Oprea [I 97355].

IAŞI: Bârnova, păd. Bârnova, 21.VI.1959, leg. & det. G. Mihai [I 70080]; **Iaşi**, Repedea, 21.VI.1951, leg. & det. A. Volcinschi [I 73623]; **Mogoşeşti**, 10.VII.1960, leg. & det. C. Dobrescu [I 71792]; **Piscu Rusului**, Dagâța, dl. Şanțuri, 13.VI.1950, leg.?, det. A. Paucă [I 59489]; **Voineşti**, pădure, 6.VII.1963, leg. & det. C. Dobrescu as *Cephalanthera alba* (Cr.) Simk., rev. I. Irimia, 10.X.2011 [I 77084].

NEAMȚ: Pângărați, dl. Botoşanul, 28.VI.1961, leg. & det. G. Vițalariu [I 98719]; **Țolici**, păd. Țolici, 27.VI.1968, leg. & det. I. Sârbu [I 42651], Valea Frasinului, pădure de fag, 2.VII.1969, leg. C. Burduja, G. Mihai & I. Sârbu, det. I. Sârbu [I 43761], în pădure pe Valea Drăganului, în fânaț, 27.VII.1969, leg. C. Burduja, G. Mihai & I. Sârbu, det. I. Sârbu [I 43617]; **Târgu Neamț**, dl. Brăilenei, 21.VI.1968, leg. & det. T. Chifu [I 91674]; **Valea Iapa**, Piciorul Gogii (pădure de fag), 3.VII.1938, leg. & det. C. Burduja [I 71228].

PRAHOVA: Sinaia, din pădure de molid-Stâncile Sf. Ana, 18.VII.1969, leg. & det. V. Iuncu, rev. C. Bârcă [I 28179].

SUCEAVA: Călineşti-Arşineasa, alt. 850-900 m, 12.VIII.1966, leg. & det. C. Dobrescu [I 73871].

Without dates on label: [I 11155].

The biggest part of the herbarium material comes from 1961-1980. The oldest herbarium sheet dates from 1938 and the newest from 1995 (Fig. 1a).

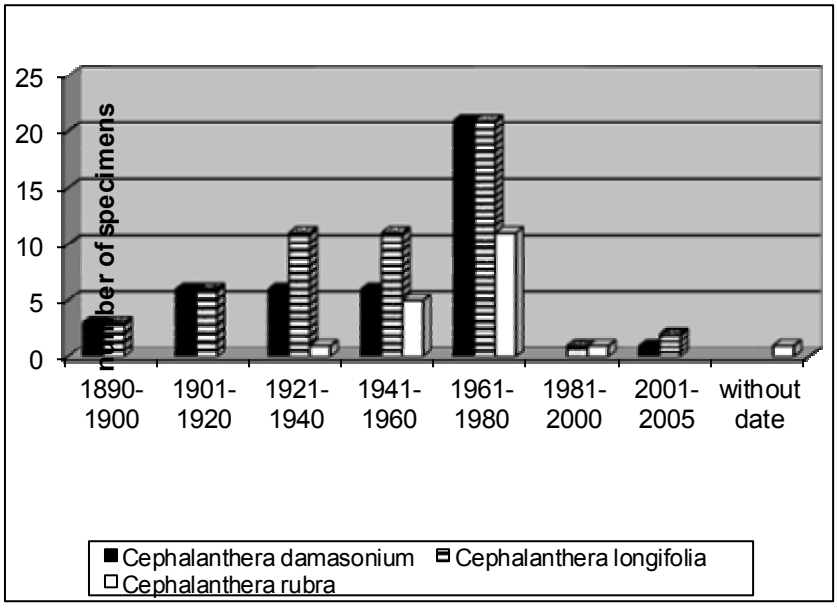


Fig. 1a. Dynamics of species entry in the herbarium collection.

Epipactis Zinn

1a. Epichile suborbicular, as long as wide… *palustris*
1b. Epichile ovate or cordate… 2
2a. Pedicels and ovary pubescent… *atrorubens*
2b. Pedicels and ovary glabrous… 3
3a. Leaves ovate to ovate-elliptical… *helleborine*
3b. Leaves ovate-lanceolate to lanceolate… *purpurata*

E. atrorubens (Hoffm.) Besser

BACĂU: Oituz, dl. Boişteanu, leg. & det. M. Gurău, rev. A. Oprea [I 115065]; **Grinduş**, margine de pădure, 28.VII.2001, leg. & det. L. Gorea, rev. D. Mititelu as *Cephalanthera damasonium* (Mill.) Druce, rev. I. Irimia, 12.X.2011 [I 100772]; **Tărhăuş**, pajişte, 22.VII.1998, leg. & det. L. Gorea, rev. D. Mititelu [I 100769].

GALAŢI: Munteni, 30.VII.1996, leg. & det. A. Oprea as *Epipactis helleborine* (L.) Crantz, rev. I. Irimia, 12.X.2011 [I 97352].

HARGHITA: Lacul Roşu (Cheile Bicazului), stânci (grohotiş cu sol), 11.VII.1950, leg. C. Burduja, det. A. Paucă (as *Epipactis rubiginosa* Gaud.) [I 69481, 69482], *ibidem*, loc înierbat, leg. & det. C. Dobrescu (as *Epipactis atropurpurea* Raf.) [I 88552], *ibidem*, 4.VII.1961, leg. & det. G. Viţalariu as *Epipactis helleborine* (L.) Crantz, rev. I. Irimia, 12.X.2011 [I 98722], *ibidem*, 1969, leg. & det. C. Dobrescu (as *Epipactis atropurpurea* Raf.) [I 88226].

ILFOV: In silvis circa pag. **Ciolpani.** Alt. cca 100 m s. m., 22.VII.1947, leg. & det. I. Morariu [FRE nr. 3097 - I 48073].

NEAMŢ: Cheile Bicazului, 22.VII.1959, leg. & det. G. Viţalariu as *Epipactis helleborine* (L.) Crantz, rev. I. Irimia, 12.X.2011 [I 97054]; **Masivul Surduc**, fânaţ, 11.VII.1950, leg. & det.? (as *Epipactis atropurpurea*) [I 61539].

SUCEAVA: Câmpulung Moldovenesc, 28.VIII.1949, leg. C. Burduja, det. A. Paucă (as *Epipactis rubiginosa* Gaud.) [I 69479, 69480]; **Masivul Rarău**, Pietrele Doamnei, VII.1896, leg. & det.? (as *Epipactis atropurpurea* Raf.) [I 11182, 11183], Stânca Şoimului, 15.VII.1963, leg. & det. G. Filipescu ca *Epipactis helleborine* (L.) Crantz, rev. I. Irimia, 12.X.2011 [I 83849]; **Schitul Rarău**, VII.1896, leg. & det.? (as *Epipactis atropurpurea* Raf.) [I 11181].

Most of the herbarium material comes from 1941-1960. The oldest herbarium sheet dates from 1896 and the newest from 2001 (Fig. 1b).

E. helleborine (L.) Crantz

ARGEŞ: Masivul Leaota, Colţii lui Dumitru, 13.VIII.1969, leg. & det. F. Diaconescu (as *Epipactis latifolia*) [I 76350].

ARAD: In silvis Ciala oppid. **Arad**, solo argill. Alt. cca 90 m s. m., 9.VII.1941, leg. C. Cosma [FRE nr. 2822 - I 48074, 48075, 49681, 63998].

BACĂU: Agăştin, pajişte, 2.VIII.2001, leg. & det. L. Gorea, rev. I. Sârbu [I 100783]; **Blăgeşti**, Lunca Ciubotei, 14.VIII.1951, leg. C. Burduja, det. A. Paucă as *Epipactis palustris*, rev. I. Irimia, 12.X.2011 [I 69640, 69458], valea lui Ioan-

Țârdeni, 9.IX.1951, leg. C. Burduja, det. A. Paucă (as *Epipactis latifolia* (L.) All.) [I 69489]; **Buda**, 21.VII.1948, leg. & det. C. Burduja (as *Epipactis latifolia* (L.) All. var. *platyphylla* (Irmisch) Mansf.) [I 69461]; **Curița**, Caşin, ariniş, 8.VII.1998, leg. & det. M. Gurău [I 115076]; **Runcu**, păd. Pârlitura, 21.VII.1951, leg. C. Burduja, det. A. Paucă (as *Epipactis latifolia* All. [I 69484], as *Epipactis latifolia* (L.) All. var. *viridiflora* (Rchb.) Irmisch [I 69486, 69488]).

BOTOŞANI: Bucecea, păd. Bucecea, 2.VIII.1961, leg. & det. T. Chifu as *Epipactis palustris* (L.) Cr., rev. I. Irimia, 12.X.2011 [I 60846]; **Horlăceni**, pădurea satului, 24.VIII.1967, leg. & det.? (as *Epipactis latifolia* (L.) All.) [I 20859]; **Hudeşti**, păd. Hudeşti, 9.VIII.1966, leg. & det. G. Mihai (as *Epipactis latifolia* (L.) All.) [I 54624]; **Lişna**, păd. Lişna, 29.VII.1964, leg. & det. G. Mihai as *Cephalanthera rubra* (L.) Rich., rev. I. Irimia, 10.X.2011 [I 55001]; **Sadoveni**, păd. Sadoveni, 28.VII.1966, leg. & det. G. Mihai (as *Epipactis latifolia* (L.) All.) [I 54622]; **Siminicea**, pădure, VII.1890, leg. & det.? (as *Epipactis latifolia*) [I 11189, 11191], *ibidem*, VII.1896, leg. & det.? (as *Epipactis latifolia*) [I 11187], *ibidem*, VIII.1897, leg. & det.? as *Epipactis atrorubens*, rev. I. Irimia, 12.X.2011 [I 11184]; **Suharău**, păd. Suharău, VII.1942, leg.?, det. I. Irimia, 20.X.2011 [I 11427]; *ibidem*, 16.VII.1966, leg. & det. G. Mihai (as *Epipactis latifolia* (L.) All.) [I 54623], *ibidem*, 17.VII.1966, leg. & det. G. Mihai (as *Epipactis latifolia* (L.) All.) [I 54625].

BUZĂU: Mănăstirea Ciolanu, 30.VIII.1950, leg. & det.? [I 77124]; **Sărata Monteoru**, 11.VII.1950, leg. & det. C. Burduja (as *Epipactis latifolia*) [I 59491].

GALAȚI: Iveşti, păd. Arhipoaia, 29.IX.1996, leg. & det. A. Oprea [I 97353]; **Munteni**, păd. Balta, 6.VII.1996, leg. & det. A. Oprea [I 97354]; **Torceşti**, pădure, locuri umbrite, 19.VII.1995, leg. & det. A. Oprea [I 97367].

HARGHITA: Cobăteşti, pădurea Rez, exp. N, alt. 900 m (*Fagetum*), 18.VII.1968, leg. & det. A. Kovacs (as *Epipactis latifolia* (L.) Druce) [I 21349].

ILFOV: Lipia Gruiu, S, păd. Căldărușani, pe malul bălții Vlăsia, 22.VII.1954, leg. & det. N. Roman (as *Epipactis latifolia*) [I 84090].

IAŞI: Bârnova, 23.VI.1960, leg. & det. C. Dobrescu (as *Epipactis latifolia*) [I 71791], spre Valea Călugărului, 28.VII.1957, leg. & det. C. Dobrescu [I 77086], păd. Bârnova, la sud de cabană, 28.VII.1957, leg.?, det. C. Dobrescu (as *Epipactis latifolia*) [I 71798]; **Dobrovăț**, N, Valea Pietrosu, pădure, 18.VIII.1951, leg. C. Dobrescu, det. I. Sârbu (as *Epipactis latifolia* (L.) All.) [I 77137], NE, pădure, 13.VIII.1965, leg. & det. C. Dobrescu as *Epipactis varians*, rev. I. Irimia, 12.X.2011 [I 77094], N, spre Bârnova, 18.VIII.1970, leg. C. Dobrescu, det. I. Sârbu [I 77138]; **Domnița,** Țibana, valea Ciurdea, 10.IX.1964, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77104], *ibidem*, pădure de fag, 10.IX.1969, leg. & det. C. Dobrescu [I 77107], *ibidem*, dl. Doamnei, pădure, 15.IX.1969, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77085], păd. Domnița, 31.VIII.1969, leg. & det. C. Dobrescu [I 77108]; **Gârbeşti**, Țibana, pădure, 3.VIII.1964, leg. C. Dobrescu, det. I. Sârbu [I 77105];

Grajduri, 22.VII.1970, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77093], V, păd. Scânteia, 18.VIII.1957, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77132], E, dl. Doamnei, pădure, 27.VII.1961, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77113]; **Hîrlău**, dl. Mare, rezervația forestieră Humosu, pădure de fag, 30.VII.1973, leg. C. Burduja, G. Mihai & I. Sârbu, det. I. Sârbu [I 37816]; **Iorcani**, Tătăruşi, Turbata, 28.VIII.1976, leg. & det. C. Dobrescu [I 75431]; **Mădârjac**, păd. Gheorghițoaia, VIII.1964, leg. C. Dobrescu, det. I. Sârbu [I 68337]; **Mirceşti**, zăvoi, 24.VII.1969, leg. & det. V. Slonovschi (as *Helleborine latifolia*) [I 28674]; **Miroslava**, 19.VI.1968, leg. & det.? (as *Epipactis latifolia* (L.) All.) [I 21348]; **Poiana cu Cetate**, pădure, 5.IX.2001, leg. & det. I. Blaj, rev. T. Chifu [I 120346]; **Slobozia**, la confluența văii Pietrosu cu Humăria, la capătul Poienii Perjului, 6.VII.1954 , leg. & det. C. Dobrescu (as. *Epipactis latifolia*) [I 71811]; **Țibana**, Valea Ciurdea, 31.IX.1969, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77131]; **Voineşti**, 15.VII.1975, leg. & det.? as *Cephalanthera rubra* (L.) L. C. Rich., rev. I. Irimia, 12.X.2011 [I 91673], trupul Ciurdea, 9.VI.1975, leg. & det.? ca *Cephalanthera rubra* (L.) Rich., rev. I. Irimia, 12.X.2011 [I 91671], păd. Voineşti, 17.VI.1975, leg. & det.? as *Cephalanthera rubra* (L.) L. C. Rich., rev. I. Irimia, 12.X.2011 [I 91672]; **Zagavia**, păd. Zagavia, 8.VII.1951, leg. & det. C. Burduja (as *Epipactis latifolia*) [I 69454, 72446].

NEAMȚ: Agapia, Mănăstirea Agapia, 11.IX.1967, leg. & det. T. Chifu & V. Slonovschi (as *Epipactis microphylla*), rev. I. Irimia, 12.X.2011 [I 91676]; **Bodeşti**, Precista, pădurea Ceuca-marginea de jos, 12.VIII.1952, leg. & det. C. Burduja (as *Epipactis latifolia* (L.) All.) [I 69485]; **Grumăzeşti**, punctul "La strâmturi", pădure de fag, 23.VII.1969, leg. C. Burduja, G. Mihai & I. Sârbu, det. I. Sârbu (as *Epipactis latifolia* (L.) All.) [I 43801]; **Masivul Surduc**, S, fânaț, 11.VII.1950, leg. & det.? (as *Epipactis latifolia*) [I 61538]; **Mănăstirea Neamț**, Dumbrava, 25.VII.1916, leg.?, det. I. Irimia, 20.X.2011 [I 11429]; **Poenari**, pădurea Zimbru, 9.VIII.1968, leg. & det. C. Dobrescu (as *Epipactis latifolia*) [I 77133], *ibidem*, 29.VIII.1968, leg. & det. C. Dobrescu (as *Epipactis latifolia*) [I 77129], pădurea Ariton, 10.VIII.1968, leg. & det. C. Dobrescu (as *Epipactis latifolia*) [I 77097]; **Potoci**, dl. Bisericii, 5.VII.1980, leg. I. Pruteanu, det.? as *Cephalanthera rubra*, rev. I. Irimia, 10.X.2011 [I 74126]; **Târgu Neamț**, dl. Cetății, 10.VII.1966, leg. & det. T. Chifu (as *Epipactis latifolia* (L.) All.) [I 91675].

PRAHOVA: Sinaia, de pe Stâncile Sf. Ana, 24.VII.1969, leg. & det. V. Iuncu, rev. C. Bârcă (as *Epipactis latifolia* (L.) All.) [I 28173].

SUCEAVA: Câmpulung Moldovenesc, 29.VI.1946, leg. C. Burduja, det. A. Paucă as *Epipactis varians*, rev. I. Irimia, 12.X.2011 [I 69465]; **Dolhasca**, păd. Vatra Ciungilor, 10.VII.1948, leg. C. Burduja, det. A. Paucă (as *Epipactis latifolia* (L.) All. var. *viridiflora* (Rchb.) Irm.) [I 69487]; **Izvorul Alb**, 26.VII.1936, leg. & det.? [I 11190]; **Solca**, pădure, 25.VI.1969, leg. & det.

A. Sfichi as *Epipactis atropurpurea* (L.) Cr., rev. I. Irimia, 12.X.2011 [I 21347]; **Tătăruşi**, pădure, 28.VII.1896, leg. & det.? (as *Epipactis latifolia*) [I 11186].

VASLUI: Băceşti, păd. Dumeşti, 26.VIII. 1969, leg. C. Dobrescu, det. I. Sârbu as *Epipactis helleborine* (L.) Cr. f. *gracilis* (Dageförde) Paucă et Morariu, rev. I. Irimia, 12.X.2011, *ibidem*, 31.VIII.1969, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77103], dl. Iezerul, 4.IX.1969, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77106]; **Bălteni**, S, păd. Bălteni, 25.VII.1953, leg. & det. C. Dobrescu [I 77112], N gara, păd. Bălteni, pădure, 10.VIII.1956, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77087], S gara, păd. Bălteni, 13.VIII.1967, leg. & det. C. Dobrescu [I 77096]; **Brăhăşoaia**, păd. Hârboanca, 3.IX.1967, leg. & det. C. Dobrescu (as *Epipactis latifolia*) [I 77134]; **Fâstâci**, Cozmeşti, pădure de fag, 27.VII.1953, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77114]; **Gârceni**, NE Schitul Mălineşti, 31.VII.1953, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77110]; **Mirceşti**, Tăcuta, păd. Larga, 4.VIII.1969, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77109]; **Rafaila**, Fundul Stemnicului, pădure, 6.VII.1963, leg. & det. C. Dobrescu (as *Epipactis latifolia* All.) [I 77128]; **Valea Mare**, Negreşti, pădure, 30.VII.1953, leg. & det. C. Dobrescu (as *Epipactis latifolia* (L.) All.) [I 77111]; **Zăpodeni**, păd. Academiei, 2.VIII.1970, leg. & det. C. Dobrescu as *Epipactis varians*, rev. I. Irimia, 12.X.2011 [I 77095].

The most part of the herbarium material comes from 1961-1980. The oldest herbarium sheet dates from 1890 and the newest from 2001 (Fig. 1b).

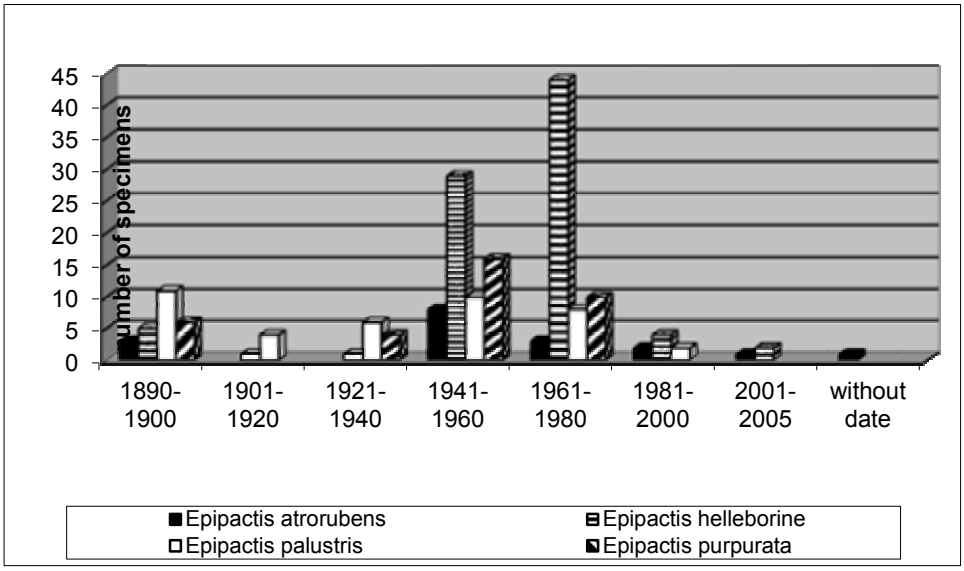


Fig. 1b. Dynamics of species entry in the herbarium collection.

E. palustris (L.) Crantz

BACĂU: Blăgeşti, 14.VIII.1951, leg. C. Burduja, det. A. Paucă [I 69458, 69460]; **Găureana**, Borzeşti, pădure de gorun, 2.VII.1998, leg. & det. M. Gurău, rev. A. Oprea [I 115072]; **Tărhăuş**, pajişte, 22.VII.1998, leg. I. Gorea, det. I. Irimia, 12.XII.2012 [I 181623].

HARGHITA: In pratis turfosis et inundatis prope pagum **Sâncrăieni**. Alt. cca 655 m. s. m., 15.07.1925, leg. E. I. Nyarady [FRE nr. 2824 - I 48076, 48077, 49680, 64000]; **Porumbenii Mari**, Lacul Racului, 12.VII.1969, leg. & det. A. Kovacs [I 21350].

IAŞI: Bârnova, 25.VI.1956, leg. & det. C. Dobrescu [I 77126], Poiana Schitului, 28.VII.1957, leg.?, det. C. Dobrescu [I 71808], Valea Săghiuții, 9.VII.1960, leg. & det. C. Dobrescu [I 77125, 71788], "În gropi", 9.VII.1960, leg. & det. C. Dobrescu [I 71787]; **Ciocârleşti**, Scânteia, 10.VI.1975, leg. & det. C. Dobrescu (as *Epipactis palustris f. ochroleuca*) [I 77089]; **Iaşi**, Repedea, 25.V.1929, leg. & det.? [I 11209]; **Iorcani**, Tătăruşi, dl. Pripon, 27.VI.1976, leg. & det. C. Dobrescu [I 75430].

NEAMȚ: Bicaz, dl. Fața Cârnului, 25.VI.1968, leg. & det. I. Sârbu [I 42273]; **Durău**, Schitul Durău, 3.VII.1914, leg. & det.? [I 11206], poiană de marginea pârâului, 3.VII.1914, leg. & det.? [I 11205]; **Masivul Ceahlău**, 4.VII.1897, leg. & det.? [I 11194, 11195, 11199], Cabana 7 Noiembrie, 1.VII.1961, leg. & det. G. Vițalariu as *Epipactis helleborine* (L.) Crantz, rev. I. Irimia, 12.X.2011 [I 98716], Ponor, 6.VII.1912, leg. & det.? [I 11207], Poiana Ponor, 3.VII.1914, leg. & det.? [I 11208]; **Negoeşti**, Dragomireşti, 13.VIII.1950, leg. & det. C. Burduja [I 69459].

SUCEAVA: Antoceni, fânaț Fălci, E pădurea Focşa, 11.VII.1948, leg. & det. C. Burduja [I 69462]; **Masivul Rarău**, 11.VII.1931, leg. & det.? [I 11204]; **Ponoare**, vale și coastă paralelă cu hotarul cosit, 19.VII.1960, leg.?, det. G. Mihai [I 68618]; **Tătăruşi**, VII.1896, leg. & det.? [I 11193, 11197]; **Schitul Rarău**, 2.VII.1896, leg.?, det. A. Paucă [I 11201], *ibidem*, 21.VII.1896, leg. & det.? [I 11196, 11202], *ibidem*, VIII.1896, leg. & det.? [I 11203], *ibidem*, prin păduri, 21.VII.1896, leg. & det.? as. *Epipactis latifolia* rev. C. Burduja [I 11198]; **Țoleşti**, 15.VII.1898, leg. & det.? [I 11200]; **Vicovu de Jos**, 16.VII.1971, leg. & det. T. Chifu, N. Ştefan & D. Florea [I 91677, 91678].

The most part of the herbarium material comes from 1896-1900 and 1941-1960. The oldest herbarium sheet dates from 1896 and the newest from 1998 (Fig. 1b).

E. purpurata Sm.

BACĂU: Blăgeşti, Lunca Ciubotei (aval cărpiniş), 14.VIII.1951, leg. C. Burduja, det. A. Paucă (as *Epipactis varians* Fleischm. et Rech.) [I 69467].

BOTOŞANI: Lişna, păd. Lişna, 26.VII.1964, leg. & det. G. Mihai (as *Epipactis varians* Fleischm. et Rech.) [I 55000]; **Siminicea**, VII.1896, leg.?, det. A. Paucă, 1954 (as *Epipactis varians* Fleischm. et Rech.) [I 181621, 181622].

IAŞI: Bârnova, Valea Călugărului, pădure, 18.VIII.1950, leg. & det. C. Dobrescu (as *Epipactis varians* Fleischm. et Rech.) [I 77136], păd. Bârnova (est de

cabană), 27.VII.1957, leg.?, det. C. Dobrescu (as *Epipactis varians*) [I 71803, 71804]; **Dobrovăț**, valea Dobrovăț, pădure, 19.VIII.1950, leg. & det. C. Dobrescu (as *Epipactis varians* Fleischm. et Rech.) [I 77135]; **Domnița**, Țibana, VII.1969, leg. & det. C. Dobrescu (as *Epipactis sessilifolia* Peterm.) [I 68338]; **Hîrlău**, Dl. Mare, rezervația forestieră Humosu, pădure de *Fagus sylvatica*, 30.VII.1973, leg. C. Burduja, G. Mihai & I. Sârbu, det. I. Sârbu (as *Epipactis sessilifolia* Peterm.) [I 37817]; **Mădârjac**, păd. Bojila, 29.VIII.1969, leg. & det. C. Dobrescu (as *Epipactis varians*) [I 77127]; **Piscu Rusului**, Dagâța, 5.VIII.1968, leg. & det. C. Dobrescu (as *Epipactis varians*) [I 77100], pădure, 26.VIII.1968, leg. & det. C. Dobrescu (as *Epipactis varians* Fleischm. et Rech.) [I 77099]; **Zagavia**, pădure, 8.VII.1951, leg. & det. C. Burduja (as *Epipactis varians* Fleischm. et Rech.) [I 72445].

NEAMȚ: Bodeşti, Precista, 19.VII.1951, leg. C. Burduja, det. A. Paucă (as *Epipactis varians* Fleischm. et Rech.) [I 69455], păd. Ceuca, 12.VIII.1952, leg. C. Burduja, det. A. Paucă (as *Epipactis varians*) [I 69456]; **Petricani**, Țolicea, dl. Păucișița, 16.IX.1951, leg. & det. C. Burduja (as *Epipactis varians* Fleischm. et Rech.) [I 69457, 69464]; **Poenari**, păd. Zimbru, 9.VIII.1968, leg. & det. C. Dobrescu (as *Epipactis varians*) [I 77101], păd. Ariton, 24.VIII.1968, leg. & det. C. Dobrescu (as *Epipactis varians*) [I 77098]; **Țolici**, păd. Țolici, 27.VI.1968, leg. & det. I. Sârbu as *Cephalanthera alba* (Cr.) Simk., rev. I. Irimia, 12.X.2011 [I 42652].

SIBIU: In fagetis et carpinetis *versus* N expositis prope balneas **Bazna**, solo humoso. Alt. cca 350 m s. m., 30.VII.1940, leg. A. Borza [FRE nr. 2823 - I 48078, 48079, 49679, 63999].

SUCEAVA: Câmpulung Moldovenesc, 29.VI.1946, leg. C. Burduja, det. A. Paucă (as *Epipactis varians* Fleischm. et Rech.) [I 69466]; **Cihoreni**, 1.VIII.1948, leg. & det. C. Burduja [I 69468]; **Dolhasca**, 10.VII.1948, leg. C. Burduja, det. A. Paucă (as *Epipactis varians* Fleischm. et Rech.) [I 69483]; **Tătăruşi**, 10.VII.1897, leg.?, det. A. Paucă (as *Epipactis varians* Fleischm. et Rech.) [I 11210], *ibidem*, VII.1897, leg.?, det. A. Paucă as *Epipactis latifolia*, rev. I. Irimia, 12.X.2011 [I 11188], *ibidem*, 19.VII.1897, leg. & det.? as *Epipactis palustris*, rev. I. Irimia, 12.X.2011 [I 11192]; **Ţoleşti**, VIII.1896, leg. & det.? as *Epipactis latifolia* (L.), rev. I. Irimia, 12.X.2011 [I 11185].

VASLUI: Băceşti, păd. Dumeşti, 31.VIII.1971, leg. & det. C. Dobrescu (as *Epipactis sessilifolia* Peterm.) [I 77088]; **Mirceşti**, Tăcuta, păd. Ciomag, 1969, leg. & det. C. Dobrescu (as *Epipactis sessilifolia* Peterm.) [I 77130].

The most part of the herbarium material comes from 1941-1960. The oldest herbarium sheet dates from 1896 and the newest from 1973 (Fig. 1b).

In Fig. 1c is represented the dynamics of species entry in the herbarium. It can be observed that the most part of the herbarium material was collected during 1961-1980 (39.6%) and 1941-1960 (28.52%). 10.40% from the herbarium material was collected during 1890-1900, 9.75% during 1921-1940, 5.70% during 1901-1920, 3.35% during 1981-2000 and 2.01% during 2001-2005. For 0.67% of the herbarium's material the collection date was not mentioned.

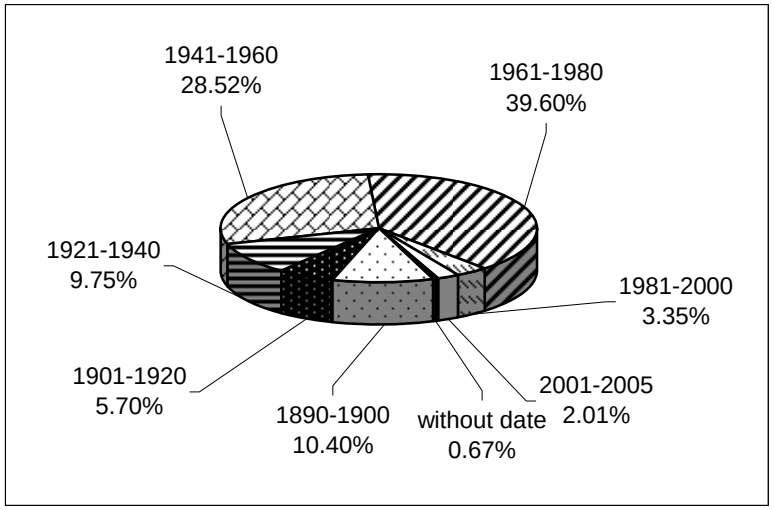


Fig. 1c. Dynamics of species entry in the herbarium collection.

CONCLUSIONS

In the collection of the herbarium [I] there are: 43 specimens of *Cephalanthera damasonium*, 55 specimens of *Cephalanthera longifolia*, 19 specimens of *Cephalanthera rubra*, 18 specimens of *Epipactis atrorubens*, 86 specimens of *Epipactis helleborine*, 41 specimens of *Epipactis palustris*, 36 specimens of *Epipactis purpurata*.

Some of the herbarium sheets could not be verified because the material is deteriorated (*Cephalanthera longifolia* [I 91664, 91666]).

The most part of the herbarium material was collected in 1961-1980.

From these 298 herbarium sheets, 5 were determined and 29 were revised.

REFERENCES

1. Beldie Al., 1977, *Flora României. Determinator ilustrat al plantelor vasculare*, **II**. Edit. Acad. Rom., pp. 375-389.
2. Ciocârlan V., 2009, *Flora ilustrată a României – Pteridophyta et Spermatophyta*, Edit. Ceres, Bucureşti, pp. 932-948.
3. Moore D.M., 1980, *Orchidaceae*, in Tutin T.G. (Ed.). *Flora Europaea*, **V**. Cambridge University Press, pp. 325-350.
4. Oltean M., Negrean G., Popescu A., Roman N., Dihoru G., Sanda V., Mihăilescu S., 1994, Lista roşie a plantelor superioare din România, Stud., Sint., Docum. Ecol., **I/1994**, Acad. Rom., Inst. de Biol., Bucureşti: pp. 52.
5. Paucă A., Morariu I, Beldie Al., 1972, Familia *Orchidaceae*, in: Săvulescu T. (Ed.). *Flora R.S.R.*, **XII**. Edit. Acad. R.P.R., Bucureşti, pp. 646-768.
6. Sârbu I., Ştefan N., Ivănescu L., Mânzu C., 2001, *Flora ilustrată a plantelor vasculare din estul României*, **II**. Edit. Univ. "Al. I. Cuza" Iaşi, pp. 284-297.

MARIUS-NICUŞOR GRIGORE, LĂCRĂMIOARA IVĂNESCU, CONSTANTIN TOMA, 2014, **Halophytes: An integrative anatomical study**, Springer International Publishing, Switzerland, 547 p., ISBN 978-3-319-05729-3 (e-Book)

Work of monographic character, the book ,"**Halophytes: An integrative anatomical study**", elaborated by Marius-Nicuşor Grigore, Lăcrămioara Ivănescu, Constantin Toma, represents an outstanding original contribution to the field of plant biology. This book, unique in it is own way, focuses on the study of halophytes, plants able to survive in highly saline and arid conditions. Characterized by a great diversity and widespread in the plant world, they have a great theoretical and practical significance.

The taxonomical diversity of halophytes is very high. They are heterogenously distributed in many plant families and this makes anatomical study very difficult. From this reason the studies on halophyte adaptations are extremely opportune. They represent numerous specific, interesting structural strategies that help plants to cope with harsh environments.

In this context, this book presents in its 547 pages a complex, extensive and very well documented analysis of morpho-histo-cytological peculiarities of this category of plants in close correlation with their specific environmental conditions. The work is structured in three parts.

In the first part, general considerations on halophytes are posed for discussion. In this frame, the authors presented, primarily, general considerations on halophytes definitions and classifications. General morphological and anatomical adaptations in halophytes as well as halophytes and salt stress are also analysed. Interesting is the classification system proposed by Grigore and Toma (2010) for halophytes based on integrative anatomy observations conducted in a large number of Romania salt-tolerant plants.

The most important and extensive part is represented by part two which represents an ample integrative anatomical study of halophytes from a large number of plant families. In a rigorous scientific manner numerous species, 62 from different habitats and climates belonging to numerous families, are presented (18).

The most important representatives of each taxonomic group including Cheniopodiaceae, Polygonaceae, Plumbaginaceae, Fabaceae, Apiaceae, Brassicaceae, Primulaceae, Plantaginaceae, Asteraceae, Iridaceae, Cyperaceae, Poaceae were analysed. Each species mentioned is accompanied by its morphohistological presentation of the structural features of vegetative organs (root, stem, leaves) supported by a rich iconography that impresses by the excellent quality of the execution and the important scientific information transmitted to the reader. This is also accompanied by an impresionant number of original drawings of an excellent quality. For a correct analysis each plant studied was generally sectioned at all organs level in three different parts, upper, middle and lower, which presumed extremely numerous microscopical observations. Each family ends with an extensive bibliography consisting of both old and new papers from Romania and abroad well integrated into the text.

The third part of the book established the conclusions which represent in fact an overall view on halophytes adaptation and their ecological significance.

A few aspects should be distinguished from this ample study. Additional cambia activity was a phenomenon highlighted in species from almost all families analysed. It may be assumed that it can contribute to salt retention, dilution and "delay" of salty water transport to more sensitive upper parts of the plant.

Intense lignification accompanying the successive cambia activity may be stimulated by salinity and can provide mechanical resistance of cells exposed to high osmotic pressure of soil water solution.

ROM. J. BIOL. – PLANT BIOL., VOLUME **58**, No. *2*, P. 121–122, BUCHAREST, 2013

Well developed endodermis as well as the sclereids, tracheodioblasts or salt glands presence represent only some of the adaptative features found in halophytes which are analysed and commented in detail, in this ample, original monograph.

Besides it is important original value for all scientists interested in plant science this book offers significant knowledge to those involved in plant biodiversity conservation area by analysing the plants morphofunctional modifications induced by the severe climatic conditions modification during the present day and of the mechanisms to get involved with. This could also open new perspectives for the identifications of salt-tolerant crop plants that can be used for bioremediations and revegetation. In this way it represents a work of reference, a valuable scientific information instrument in the field of theoretical and practical biology, generally.

AURELIA BREZEANU PhD.
Institute of Biology, Bucharest