METHODS OF CONSERVATION OF THE PLANT GERMPLASM. *IN VITRO* TECHNIQUES

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Abstract

In the last decade there has been an alarming increase in the number of disappearing species from the spontaneous flora due to the pressures put upon the environment (land clearing, drainage, pollution of the soil etc.) affecting their vitality, determining their number to fall under their biological possibilities of regeneration. In the present there are 60.000 vascular species on the verge of extinction on Earth and, from the European flora, evaluated at 12.000 of species, over 2000 are considered rare or extinct. The conservation of vulnerable species can be made either by traditional methods (see table 1) or by modern methods (see table 2). From the last, in vitro micropropagation presents a significant interest in the purpose of obtaining a large number of plants from the endangered plant. The plant biotechnologies laboratory of the University of Oradea, Faculty of Environmental Protection is designed to pursue the development of the modern methods of conservation. The paper presents some general aspects of the plant germoplasm conservation through the in vitro multiplication of some species of interest from the Romanian flora (Leontopodium alpinum, Drosera rotundifolia, Arnica montana, Dianthus spiculifolius, Syringa josikaea, Sequoia sempervirens) and also laboratory aspects of the in virto reaction of the above named species with the help of diverse methods: phenotypical, cytological (in situ hybridization), biochemical (elecrophoresis aspects etc.) and molecular (molecular markers).

Key words: germoplasm, biodiversity, micropropagation, in vitro, conservation.

INTRODUCTION

The partial destruction and the degradation of the natural habitat, the destabilization of the ecosystems due to the climatic modification, pollution, the increase of the number of invasive species and the implication of the human factor can be several of the causes of the biodiversities' decline. Because of this, the necessity of finding solutions for the disappearance of these species has determined the intensification of conservation actions of the plant germoplasm, the actualization of the lists and of the red book of plants for bringing to the attention of the experts in this field - researchers and those who are actively involved in the protection of the environment. The disappearance of species is from 100 to 1000 more alert since the invention of the anthropic factor in the environment, one in eight species in threatened by extinction. It is estimated that in the last 50 years, more than 300.000 species have become extinct[1]. The number of species depending of the level of endangerment is shown in picture 1. The situation presented

[1] http://.bbc.co.uk/nature/environment/conservationnow/global/biodiversity/page2.shtml

refers to 1996 – 1998 – 2006[2] (Blîndu & Holobiuc, 2008). Approximately 50% of the superior endemic European species have been threatened by extinction since 2001.

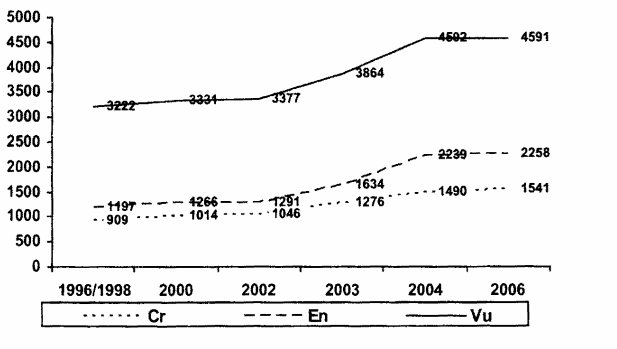


Fig. 1 The number of species endangered in relationship according to the endangerment degree. (Cr = critically threatened; En = endangered ; Vu = vulnerable)

The genetic erosion of natural populations is a growing phenomenon, due to the pressure of different factors on natural ecosystems. Conservation actions, according to Frankel and Hawkes (1975), represent a priority and measures must be taken for the conservation of the genetic variability of natural populations.

The danger of extinction of more and more species of flora in our country is acknowledged, which has determined the experts to manifest an interest in the preservation of rare and endangered elements. As a beneficial result, the forms of conservation, of whatever nature they may be, begin to be viewed and analyzed very closely. Romania is known for several lists and red books (Olteanu et al., 1994; Boşcaiu et al., 1994; Moldovan et al., 1994 and Dihoru – 1992; Gh Dihoru & Negrean - 2009), which took into account the classification of the species in the real category of endangerment, the loss rate, the expansion of protected areas and ex situ conservation projects, by developing rigorous research on the taxa considered missing and by pursuing the objectives imposed by the European institutions capable of managing the environment, etc..

Conservation programs regard species with economic value, wild species related with those cultivated, which represent the source of genes resistant to diseases and pests, medicinal, rare, endangered or vulnerable plants. There are two distinct methods of plant germoplasm conservation, *in situ* and *ex situ*. The first is based on the 1992 Convention on Biological Diversity, its role being to protect and monitor natural populations,

[2] http:/www.iucnredlist.org/info/2007RL_Stats_Table%202.pdf.

proposing to remove or maintain a certain level of causes that lead to the destruction of the species (Table 1).

Table 1

The method of species conservation *in situ*

The implications of the method	**The purpose of the method**	**Characteristics / objectives**
-preparation of the recovery plan of a single endangered species; -monitoring of the rare or vulnerable species; -protection plan of the habitats; -initiation of *in situ* conservation activities.	- to protect and monitor selected populations in their natural habitats; - to maintain the evolution processes for the adaptation to extreme conditions; - to reduce the fragmentation of the habitats.	- conservation of the ecosystems of natural habitats; - protection of viable populations in natural habitats; - elimination or reduction of the causes that affect the species.

The *ex situ* method is carried out outside of the natural habitats, with the objective of the accumulation of *ex situ* collections through the methods specified in Table 2. When the biological material (organs, seeds) cannot be stored in a classical manner, plant biotechnologies step in, which maintains it *in vitro* for different periods of time, providing its multiplication on the basis of *in vitro* micropropagation schemes. Studies were initiated for the endangered species and for the ones presenting a conservation interest in order to develop protocols for multiplication, rooting and maintenance of the culture *in vitro*. Issues raised by this mode of conservation are related to the reactivity of different species, the poor viability and the endogenous contamination (Cachiță, 2006). The success of micropropagation is an essential requirement of the method, especially for protected species, represented by a limited number of individuals, the use of related species in order to avoid further compromising the species being recommended.

The problem with conserving species through this method is a main preoccupation for many research institutions from Europe and from the entire globe Kew – Englad (Fay, 1992), Brussels – Belgium, Spain and other places from Europe, Australia, India, Brazil, Mexico, USA etc. In our country the conservation of the spontaneous flora through *in vitro* multiplication is of interest for almost all the biotechnology laboratories, for the Biological and Agricultural Research institutes, the institutions of higher education and the Botanical Gardens.

Table 2

Conservation method of plant species ex situ

The type of method	The place where the method is applied	Advantages and disadvantages
A. TRADITIONAL	- botanical gardens; - seed banks; - field gene banks	- disadvantages regarding the necessary space - the work performed, costs, exposure to risk factors.
B. MODERN	**Procedures/steps**	**Characteristics**
a. In vitro conservation	1. collection of the material; 2. sterilization; 3. initiation of in vitro culture 4. establishment of the multiplication and *in vitro* maintenance of the species	- ensures material for international trades. *Advantages*: rapid multiplication, high rate, short time, induces vigorousness, juvenilization, and obtainment of healthy plants, free of virus. *Disadvantages*: the necessity of qualified personnel, energy consumption for the standardization and automation of cultural conditions.
b. Reduction of growth	1. reduction of proliferation and growth 2. increase of the period between subcultures. For the maintenance of viability and of the regeneration capacity of the cultures are applied: a. Reduction of temperature and intensity of the light; b. Modification of the cultural environment by reducing the carbon or minerals sources; c. Reduction in the level of oxygen, by using the oil stratum or liquid environment;	- maintenance of cloned plant material by reducing proliferation and growth and by increasing the period between subcultures *Limitations of the method*: limited applicability; the existence of interactions between environmental factors (environment, retardants, temperature, light, photoperiod); limited information on the stability of the exposed material; not knowing the exact time period in which the method can be applied safely.
c. Cryoconservation	- involves basic steps: - cryoprotectant treatment, - pre-culture, - impregnation, freezing, rapid cooling, gradual and progressive thawing, resuming growth and multiplication	- involves maintaining plant material to liquid nitrogen temperature (-196^{o}C), without certain changes on periods of time. *Advantages*: limited space, protects plant material contamination, cost efficiency, unlimited maintenance in time, ensuring stability of biological material.
d. In vitro gene banks	1. Active banks; 2. Basic collections	- 1-3 years period with slow growth - contain duplicates, reserve, in case of loss of probes from active banks

Large collections of researchers are working in this field (Blîndu & Holobiuc, 2006, 2007 and 2008, Cristea et al. 2002 and 2004, Holobiuc & Blind, 2006, 2006-2007, Şuteu et al. 1999, Zăpârţan - 1994, Zăpârţan - 1995, 1996, 1997, 2000). Investigations are focusing on existing resources and the application of established technologies from them, so that our contury will properly respond to the regulations imposed by the European programs. Romania is a signatory of international conventions (Bern - 1979 -1970 Ramsar, Sofia - 1991 Rio - 1992, Aarhus - 2001, The Hague 2002)

that require special working methodology and environmental protection and legal institutional framework to ensure protection of flora (Cristea et al. - 1996).

Through the application of the *in vitro* multiplication method of vascular plants there were specific advantages to the conservation of the flora which were considered. Out of these advantages, we mention the following:

1. the possibility of obtaining a *large number of plant individuals*, identical or approximately identical to the mother plant; **2.** *relatively short time* for obtaining new plant with *low costs* (due to the replacement of hormones with natural extracts); **3**. this technique is the *only way of obtaining plants which cannot reproduce sexually* (via seeds) and *the only viable possibility for multiplying* unisexual plants; **4**. the method allows for the obtainment of *plants identical to the parent-plant*, the explant donor, this fact being possible either directly (via *in vitro* organogenesis) or indirectly (via somatic embryogenesis); **5.** the method can determine the generation of *genetic modifications*, useful in case one wants to obtain certain somatic variations (stress factor resistence, variations which lead to the production of useful compounds, etc.). For spontaneous plants, somaclonal variations are to be prevented, preferably, since they imply mutations in the biology of the regenerated plant and modification in its adaptive capabilities in its original habitat. The main disadvantage is the *danger of reducing the population's genetic baseline* since during the efforts to rebuild the population one starts from a small number of genotypes. The technique is also much *more expensive*, since it requires proper equipment and the training of specialists.

MATERIALS AND METHOD

Spontaneous species *in vitro* propagation technology aims not only to establish a species multiplication technique but also other aspects which have to do with the growth and development process of plants obtained *in vitro*, which is acchieveable by following the *organogenesis process* stages (caulogenesis, rizogenesis and philogenesis) and the correlation between these and the stages of evolution of new plants. *In vitro* organogenesis is dependent on species and aseptic growth environment composition. The type and nature of the explant, the physiological phase in which the tissue resides (the age of the explant donor plant)and the time of year in which the collection of vegetal material is performed are also factors with a determining role in the success of this method for species propagation and multiplication *in vitro*.

Biological material consists of *vegetal tissue fragments* (*explants* or *inocules*) which is a living unit, keeper of genetic information. The

metabolism and hereditary baggage of *in vitro* cultivated cells can be manipulated within the limits of unaltered conservation of cell **totipotence**. (Cachiță – 2006). The appropriate choice of explant is the essential condition of success for an *in vitro* culture, as the vegetal cells' ability to integrally reproduce the original plant is directly proportional to the youth of the donor plant. Theoretically any part of a plant can be an explant and can ensure its regeneration: *bits of stem, of leaf, of root, knot, interknot, buds, flower, flower organs, seeds, cells,* etc. This theory is backed by results we've obtained on a large number of studies species (M. Zăpârțan, 2000). We experimented with the following types of explants: seeds, natural vegetal material (plants taken from their original habitat), from which we separated different parts (*apex knots, meristema, leaf, bud, etc.*).

Culture environments are chosen either according to the nature of the speices, or according to the physiological stage of development of the plant material, having a broad or specific degree of utility, but always adequate to the desired goal. The composition of some of the more widely used culture media are linked to the years 1942, 1956, 1971, whereas in the present we're developing media with improved composition and meant to be specifically tailored for each plant group or family. *Fundamental growth media* used are media originally developed by Murashige-Skoog-1962 (MS) and Schenk-Hildebrandt-1972 (SH), to which one ads different hormonal balances, according to species and *in vitro* reaction of said species. For the germination of seeds *in vitro* use the MS1/2 medium (with improved micro and macro-elements). There are also species which behave very well *in vitro* on simple MS1/2 media when it comes to regeneration and multiplication. The addition of hormones make the technique more expensive from an economic stand point, so we have proceeded to replace hormones with natural extracts (for example 1 mg/l corn sprout extract can substitute zeatine – according to Butiuc-Keul & Zăpârțan, 1996).

RESULTS AND DISCUSSIONS

After inoculation, explants are kept in growth chamber conditions, 8 hours of darkness and 16 hours of light, light intensity varying accordg to species and desired result, temperature kept between 25ºC and 27ºC and air humidity between 50% and 100%, again, according to species. Cultures are supervized for determining their ability to regenerate and differenciate new plantules *in vitro* by taking note of the morphogenesis and rizogenesis procesees which occur in explants. The material obtained *in viro* must surpass an important but difficult threshold – the aclimatization by moving to greenhouses or cold or semi-cold hotbeds and then into the field. Survival in field condition and especially in the original areals constitutes the essential problem in the success of this method, thus acclimatization

requires certain steps in which optimal conditions need to be provided to each individual or species.

Table 3 shows the experiemental species we worked with, the culture medium for each of them, the type of explant and regeneration percentage (organogenesis, rhizogenesis, multiplication) and the acclimatization process.

Table 3

Studied plant species and obtained results

SPECIES /symbol	**MEDIUM**	**EXPLANT**	**% Multiplication**	**% Acclimatization**
Arnica montana L **(Am.)**	MS + g mg/l K + 1mg/l ANA; MS1/2+ 3 g/l Cv(coal)	seeds, meristema, apex	75-80%	46%
Dianthus spiculifolius Schur **(Ds.)**	MS+2mg/lBA+ 2mg/lAIB+40mg/l Ad.SO_4 ; MS1/2 + 3g/ Cv	Seeds, apex, knot	< 80%	50%
Drosera rotundifolia L **(Dr.)**	MS + 1mg/l ANA+ 0,1mg/l Z; MS1/2	Floral bud, Seeds	45%	32%
Leontopodium alpinum Cass **(La.)**	MS + 40mg/l Ad.SO_4 + 1mg/l corn extract; MS1/2	Floral bud, Meristema	> 73%	44%
Syringa josikaea Jack **(Sy.)**	SH+0,1mg/lBA + 0,1mg/lANA + 170 mg/l KH_2PO_4; MS1/2 + 3g/l Cv	seeds; saplings	54%	50%
Sequoia sempervirens D. Don. **(Sq.)**	SH + 4g/l Cv; SH + 1mg/lZ + 0,5mg/lAIB	apex	< 85%	74%

(MS = Murashige - Skoog - 1962: SH = Schenk – Hildebrand – 1972)

Analyzing figure 2 we can observe differences in the regeneration percentage according to the species being cultivated *in vitro* and different acclimatization percentages. According to the nature of the species, these percentages are higher for woody species.

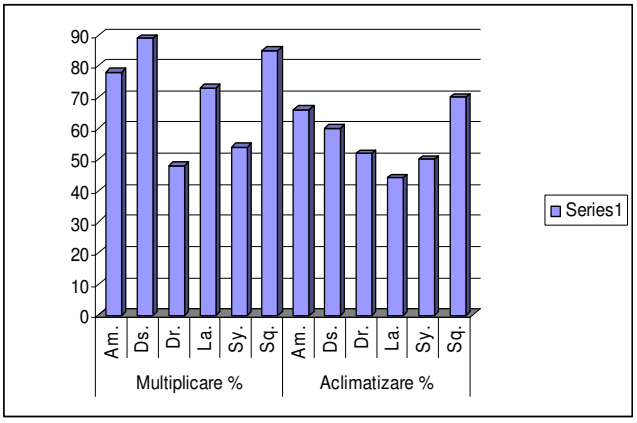
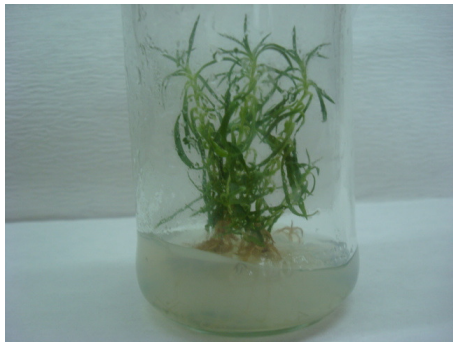


Fig. 2 Multiplication and acclimatization percentages for experimental species

CONCLUSIONS

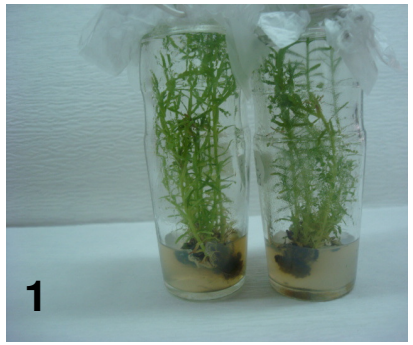
We recommend this method of *in vitro* multiplication of spontaneous flora species in order to conserve and repopulate their natural areals, as the technique ensures the obtainment of a large number of individuals in a relatively short time-span, identical from a phenotypical and genotypical stand point with the parent-plant from which the tissue is harvested originally. The essential advantage of this method lies in the fact that, in order to initiate a culture, *one can use a single parent-plant, a single seed, leaf, one apex, on meristema*, etc, in other words, a single explant, without compromising already endangered plants in their natural habitat. Research in this field have proven that spontaneous species are tolerant of *in vitro* multiplication in order to conserve and extend their presence in original areals, as well as in landscaping spaces, according to their natural and ornamental value.

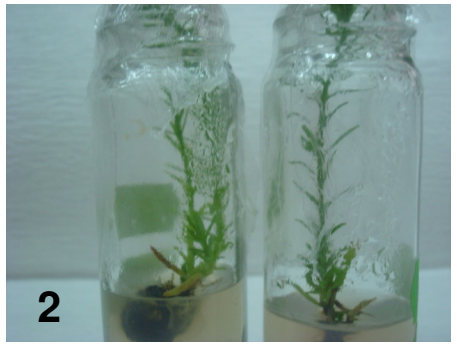
Drosera rotundifolia L



Dianthus spiculifolius Schur

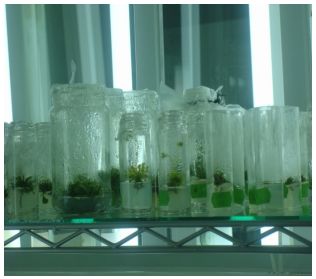
Leontopodium alpinum L.



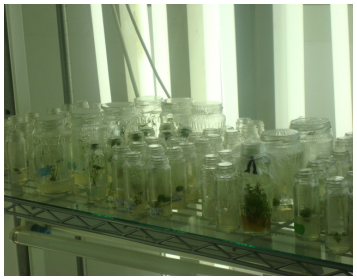


Sequoia sempervirens D. Don

(**1** - pe MS + BA; **2** – MS1/2)



a



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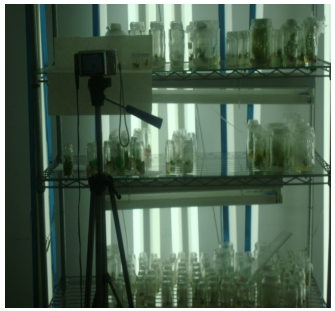

d

Photos a, b, c, şi d – images from the biotechnology laboratory

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