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REVIEW

Biotechnology for Endangered Plant Conservation: A Critical Overview

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Abstract

The loss of plant genetic resources has made necessary the development of new ex situ conservation methods. Advances in biotechnology provide new methods for plant germplasm conservation and evaluation. Biotechnological tools like in vitro culture, cryopreservation, and molecular markers offer a valuable alternative to plant diversity studies, management of genetic resources and ultimately conservation. This review summarizes the recent advances in plant conservation biotechnology with special emphasis on the preservation efforts of the Romanian flora. Strategies, as well as the plant species used for establishment and maintenance of germplasm collections are presented.

Keywords: *in vitro* conservation, endangered plants, recalcitrant seeds

Introduction

The high level of geographic and climatic diversity of Europe provides various habitats with over than 12,500 vascular plants (NATURA 2000 Newsletter [1]). Areas of particularly high plant diversity include the mountainous areas around the Mediterranean and Black Sea with the floras of Spain, Greece, Italy, Bulgaria and Romania enclosing the highest number of both endemic and endangered plant species. According to the latest release of World Conservation Union (http://cms.iucn.org/about/work/programmes/species/red_list) one in four mammals, one in eight birds, one third of all amphibians and 70% of the world's assessed plants on the 2007 Red List are in jeopardy. Plants are endangered by a combination of factors: over-collecting, unsuitable agriculture and forestry practices, urbanization, pollution, habitat destruction, fragmentation and degradation, spread of invasive alien species (non-indigenous species that heavily colonize a particular habitat) and climate change (PITMAN & al. [2]). Global concern about the loss of valuable genetic resources has stimulated many new programs for the conservation of plant genetic resources. Within past decade several conservation strategies were developed (ENGELMANN & al. [3], PAUNESCU [4]) mainly in the terms of *in situ* and *ex situ* conservation. Wild life conservation is based mainly on *in situ* conservation (conserving species on theirs natural habitats). *Ex situ* conservation involves preservation and maintenance of samples of living organisms outside their natural habitat, in the form of whole plants, seed, pollen, vegetative propagules, tissue or cell cultures. Ex *situ* techniques are generally used to complement *in situ* methods but in some cases are the only possible techniques to conserve certain species (RAMSAY & al. [5]). Among *ex situ* conservation methods the most common are cultivation in botanic gardens, seed storage, and *in vitro* cultivation. The world's 2204 botanic gardens cultivate more than one third of the world's flowering plants (BGCI Report [6]). Although cultivation in botanic gardens is an efficient way to conserve endangered species ex situ, it is limited in time and space and it has to overcome acclimatisation and accommodation problems. Among the various

ex situ conservation methods, seed storage seems to be one of the most convenient for long-term conservation. This involves desiccation and storage at low temperatures. However, there are a large number of threatened species, which produce immature, sterile or recalcitrant seeds that quickly lose viability and do not survive desiccation; hence conventional seed storage strategies are not suitable. Advances in biotechnology, especially *in vitro* culture techniques and molecular biology, provide some important tools for conservation and management of plant genetic resources. Several *in vitro* techniques have been developed, mostly for vegetatively propagated and recalcitrant seed producing species, with recent establishment of extensive germplasm collections. (ENGELMANN & al. [3], SARASAN & al.[7], PAUNESCU & al. [8], ZAPARTAN & al. [9]).

This review aims to summarize the biotechnological tools used for plant conservation. The progress in establishing in *vitro* germplasm collections of threatened Romanian phytotaxa is also presented.

In vitro conservation of plant germplasm

The term *in vitro* culture covers a wide range of techniques involving the growth under sterile conditions of plant germplasm (especially shoot tips, meristems, somatic embryos or embryogenic callus) on artificial culture media. Although each species require specific protocols, there are some common steps in establishing an *in vitro* collection: culture initiation, maintenance and multiplication, followed by long-term storage.

Culture initiation

The potential explant (the starting tissue originated from the donor plant) consist mostly of shoot (tip, nodal segments), leaf (lamina segments with ribs, petiole), flower pieces, immature embryos, hypocotyl fragments or cotyledons. Generally, younger, more rapidly growing tissue or tissue in early developmental stage are the most effective. Therefore, the initial quality of the explants will determine the success of the conservation procedure. The criteria for a good quality explants are: normal, true to type donor plant, vigorous and disease free (FAY [10]). Plant fragments are initiated into axenic culture from various sterilization procedures depending of the tissue used. As a common rule, fragile tissues (meristems, immature embryos, cotyledons, hypocotyls) requires less exposure to sterilizing agents than seeds or lignified organs. A successful sterilization is achieved when the explant is fully decontaminated and remains viable. An alternative for obtaining uncontaminated explants is to obtain explants from seedlings, which are aseptically grown from surface-sterilized seeds. An overview of successful decontamination for *in vitro* culture of some endangered Romanian endemic plants made in the Institute of Biology (Bucharest) is summarized in Table 1(PAUNESCU & al. [8, 11]).

Table 1. Explant sterilization strategies developed for some Romanian endemic plants

No.	Species	Explant type	Sterilizing agent	Concentration (%)	Time (min)
1	*Alyssum borzaeanum*	seeds	sodium dichloroisocyanurate	0.5	10
2	*Astragalus pseudopurpureus*	foliar fragments	sodium hypochlorite	5	10
		nodal segments	mercuric chloride	5	0.1
3	*Campanula romanica*	seeds	sodium dichloroisocyanurate	0.5	10

4	*Cerastium transsilvanicum*	foliar fragments	sodium hypochlorite	5	10
		nodal segments	mercuric chloride	5	0.1
		floral buds	calcium hypochlorite	9	5
5	*Dianthus callizonus*	seeds	mercuric chloride	0.1	10
		nodal segments	mercuric chloride	5	0.1
		floral buds	calcium hypochlorite	9	5
6	*Dianthus tenuifolius*	nodal segments	mercuric chloride	5	0.1
		floral buds	calcium hypochlorite	9	5
7	*Hieracium pojoritense*	foliar fragments	sodium hypochlorite	5	10
		stem fragments	mercuric chloride	5	0.1
		floral buds	calcium hypochlorite	9	5
8	*Primula leucophylla*	apical meristem	calcium hypochlorite	9	5

Following the sterilization step, the explant is placed on a sterile culture media containing mineral nutrients, vitamins, carbohydrates, and plant growth regulators (MURASHIGE & al. [12]). Plant growth regulators are the critical media components in determining the developmental pathway of the plant cells. Seven major types of growth regulators are generally recognized:

- auxins, usually involved in cell enlargement and differentiation
- cytokinins associated with cell division
- gibberellins stimulate cell elongation, seed germination
- abscisic acid involved in dormancy by inhibiting cell growth
- ethylene often associated with senescence
- brassinosteroids involved in gravitropism, stimulates cell elongation and division
- jasmonates associated with growth inhibition, senescence promotion, wound responses

Auxins and cytokinins are the most widely used growth regulators in plant tissue culture, usually being utilised together. The ratio between auxin and cytokinin is essential for the type of culture established. Generally, the indirect pathway (via callus) is avoided because of the increased risk of somaclonal variation occurrence. Although, somaclonal variation could provide some adaptive advantages, this is not a desired outcome for conservation in tissue culture.

Culture maintenance and multiplication

One major step in establishing *in vitro* germplasm collections is induction and multiplication of shoots. The media composition, especially the growth regulators, mineral salts and supplement factors are of paramount importance to successfully obtain viable tissue culture. Some successful multiplication strategies developed for a number of endangered plants in Romanian flora are summarized in Table 2 .

Storage of collections

In vitro storage techniques include the medium-term storage (for a determined period - a few months up to a few years) using slow growth strategy or artificial seed production, and long-term storage (tentatively for an indeterminate period of time) using cryopreservation. Generally, there are three recognized methods for reducing *in vitro* growth rates, including physical (reduced temperature and light conditions), chemical (using growth retardants), and a combination of the two (ENGELMAN & al. [3]). Temperature will vary upon the origin of stored species; temperate species may be stored at 4^0 C, whereas the tropical plants are requiring temperatures in the range of 15-20^0C. Light conditions may be darkness or a 12-16 h photoperiod, the light intensity varying upon the light requirement of the species stored. The humidity should be between 40-50%. These techniques enable extending the subculture periods from 12 month up to 4 years for many species (ASHMORE [13]). Slow growth storage strategy is used to preserve some endangered phytotaxa in the Institute of Biology (Bucharest) *in vitro* collections. The endangered species conserved by medium-term tissue culture are *Artemisia tschernieviana, Astragalus pseudopurpureus, Cerastium transsilvanicum, Dianthus callizonus, D. spiculifolius, D.tenuifolius, Erigeron nanus, Hieracium pojoritense,* and *Marsilea quadrifolia*.

Table 2. Multiplication strategies developed for some Romanian endangered plants

Species	Explant type	Multiplication media	Author
Alyssum borzaeanum	cuttings from *in vitro* germinated plantless	MS; Kin- 1 mgl^{-1},NOA - 0.1mgl^{-1}	Păunescu, 2007, [33]
Andryala levitomentosa	stalk cuttings	MS;Kin - 1 mgl^{-1}, 2,4D -1mgl^{-1}	Păunescu et al., 2002, [34]
Astragalus pseudopurpureus	leaf cuttings	MS; BAP - 1mgl^{-1},ANA- 0.1mgl^{-1}	Holobiuc et al., 2004, [35]
Astragalus péterfii	stem, leaf and floral cuttings	MS; BAP- 0.5mgl^{-1},ANA- 0.3mgl^{-1}	Şuteu et al., 1999, [36]
Arnica montana	nodal cuttings	MS; 2iP- 1mgl^{-1}, ANA- 1 mgl^{-1}	Butiuc-Keull et al., 2001[37]
Artemisia tschernieviana	stem and leaf cuttings	MS; BAP – 1 mgl^{-1}, Kin - 1 mgl^{-1} ANA- 0.25 mgl^{-1}	Holobiuc et al., 2006, [38]
Centaurea reichenbachii	nodal cuttings	MS; BAP - 1mgl^{-1},ANA- 0.1 mgl^{-1}	Cristea et al., 2004, [39]
Cerastium transilvanicum	nodal cuttings from *in vitro* germinated plantless	MS; BAP - 1mgl^{-1},ANA- 0.1 mgl^{-1}	Păunescu, 2005, [40]
Dianthus callizonus	nodal cuttings	MS; BAP- 1mgl^{-1}, ANA- 0.1 mgl^{-1}	Păunescu et al., 2003, [11]
Dianthus simonkaianus	floral buds and nodal segments	MS; BAP- 1mgl^{-1},ANA- 0.1 mgl^{-1}	Miclăuş et al., 2003, [41]

Dianthus spiculifolius	apical buds and nodal segments	MS; BAP-1mgl^{-1}, ANA- 0.1 mgl^{-1}	Cristea et al., 2002, [42]
Dianthus tenuifolius	nodal segments	MS; BAP - 1mgl^{-1}, ANA- 0.1mgl^{-1}	Păunescu et al., 2005, [8]
Drosera rotundifolia	leaf cuttings	MS FM - Mn:Zn:Fe/0.2:03:1	Corneanu et al., 1998, [43]
Ecbalium elaterium	cuttings from *in vitro* germinated plantless	MS; BAP-2.25mgl^{-1}, Kin- 0.2mgl^{-1} AIA- 0.18 mgl^{-1}	Voichiță et al., 2005, [44]
Ephedra distachya	stem cuttings	MS; Kin - 1 mgl^{-1}, 2,4D- 1mgl^{-}	Vântu, 2002, [45]
Erigeron nanus	leaf cuttings	MS; ANA - 1mgl^{-1},Ze- 1 mgl^{-1}	Blîndu, 2007, [46]
Gentiana punctata	cuttings from *in vitro* germinated plantless	MS;ANA- 1 mgl^{-1}, 2iP – 1 mgl^{-1} ME - 1 mgl^{-1}	Butiuc-Keull et al., 2005, [47]
Hieracium pojoritense	leaf cuttings	MS; BAP - 1mgl^{-1}, ANA- 0.1mgl^{-1}	Holobiuc et al., 2004, [48]
Leontopodium alpinum	floral buds	MS; BAP- 2mgl^{-1}, AIA- 2 mgl^{-1}	Zăpârțan, 1996, [49]
Marsilea quadrifolia	buds excised from rhizome	½ MS without growing regulators	Banciu et al., 2007, [50]
Pinguicula vulgaris	leaf cuttings	MS; AIA- 0.5-1mgl^{-1}, Kin-2-2.5mgl^{-1}	Popescu et al., 1997, [51]
Robinia preudacacia var.*oltenica*	apical and axilary buds	MS; AIA- 0.1 mgl^{-1} ,Kin – 1 mgl^{-1}	Corneanu et al., 2001, [52]

BAP -6 -benzylaminopurine **CM -** culture media, Côrte & Mendonça ,1985; **IAA –**Indole-3-acetic acid; **Kin –** Kinetin; **MS**- basal culture media, Murashige&Skoog , 1962; **½ MS** – basal media with half strenght minerals; **80% MS** – basal media with 80% mineral content; **NAA –**1-naphthylacetic acid; **NOA -** 2-naphthyloxyacetic acid; **SH** – culture media Schenk & Hildebrandt, 1972; **ZM –** culture media Zimmerman & Broome, 1980; **WPM-** culture media for woody plants;**ME** –maize extract; **FM –** magnetic fluids; **2iP –** 2 –isopentyl adenine; **2,4 D**- 2,4-dichlorophenoxyacetic acid

Some effective approaches for medium-term conservation techniques include reduction of the oxygen level available achieved by covering explants with a layer of liquid medium or mineral oil, or by placing them in controlled atmosphere (ENGELMAN & al.[3]).

Artificial seeds were first introduced in the 1970s as a novel analogue to the plant seeds, useful for propagation and medium term storage (REDENBAUGH & al. [14]). Artificial seeds are produced by encapsulating a plant propagule in a matrix, which will allow it to grow into a plant. Plant propagules may consist of shoot buds or somatic embryos that have been grown aseptically in tissue culture. In culture, these plant propagules can easily grow into individual plants by controlling the composition of culture media. The advantages of propagules encapsulation include multiplication of recalcitrant or non-seed producing species,

easier manipulation of fragile tissues, and direct protection during dehydration and thawing during the cryopreservation trial (SAIPRASAD [15]). This strategy was applied for conservation purposes to various plant species, like orchids from which encapsulated protocorms showed 70% viability after more than six months storage at 4^0C (Devi & al. [16]). One of the first Romanian researchers obtaining encouraging results in artificial seed production and cryopreservation is A. Halmagyi from the Institute of Biological Researches from Cluj-Napoca (HALMAGYI & al. [17, 18]).

To avoid the genetic alterations that may occur in long tissue cultures storage experimental protocols have been developed for the storage of germplasm at very low temperatures known as *cryopreservation* (MARTIN & al. [19]). The temperature used are those of liquid nitrogen (-196^{0}C) or its vapour phase (-150^{0}C). At these temperatures, all metabolic activity is suppressed minimizing the risk of genetic alterations and eliminating the requirement for refreshing the culture medium. Cryopreservation provides a safe and cost-effective method for the long-term storage of genetic resources. Cryopreservation as a conservation tool has been underlined by a number of authors (ENGELMAN [20], STACEY & al. [21]) and presently is recognized as the most effective technique for long-term storage of plant germplasm. The successful cryopreservation of plant cell suspension (QUATRANO [22]) and regeneration of somatic embryos from cryopreserved cells (NAG & al., [23]) have led to numerous studies on cryopreservation of plant system (FINKLE & al., [24], KARTA [25], PRITCHARD & al., [26]). Cryopreservation technique is based on the removal of all freezable water from tissues by physical or osmotic dehydration, followed by ultra-rapid freezing, resulting in vitrification of intracellular solutes. The main advantages are simplicity and the applicability to a wide range of genotypes. Basically, this can be achieved using different procedures, like encapsulation-dehydration, vitrification, encapsulation-vitrification, desiccation, pregrowth, pregrowth-desiccation and droplet-freezing (ENGELMANN [20]). The encapsulation-dehydration procedure is based on the technology developed for the production of artificial seeds. Vitrification consists of placing explants in the presence of highly concentrated cryoprotective solution followed by rapid freezing. Encapsulation–vitrification is a combination where explants are encapsulated in alginate beads and treated with vitrification solutions before freezing. Desiccation is the simplest procedure and consists of dehydrating explants and freezing them rapidly by direct immersion in liquid nitrogen and it may be used for freezing zygotic embryos or embryonic axes extracted from seeds (ENGELMANN [20], DUMET & al. [27]).

Genetic stability of preserved germplasm

To achieve the objective of maintaining the genetic integrity of the sample, the stored germplasm should be monitored visually every 1-3 month, depending on the species. Initial characterization of the accession is essential for comparison. In case that any abnormalities are found, the tissue culture plants must be grown for an entire cycle in greenhouse examining any morphological changes. Since the visual examination is not reliable, germplasm that has been kept in collection for more than a year should be assessed by biochemical and/or molecular methods.

Protein markers, usually named “biochemical markers” (seed storage proteins and isozymes) are generated through electrophoresis, taking advantage of the migrating proprieties of proteins, and subsequently revealed by specific histochemical stains. Genetic stability has been successfully studied using seven isozymes systems in the endangered Spanish plant *Centaurium rigualii*. (IRIONDO & al. [28]). A total of about 90 isozyme systems have been used for plants assessment, with isozyme loci being mapped in many cases. However, the major limitation of isozyme analysis is the reduced number of markers

analyzed. Another disadvantage of isozyme analysis consists in the phonotypical dependence of the markers. As such, the biochemical markers are limited due to their modifications by environment exposure and during the development.

Molecular (DNA) markers are derived from the initial DNA and considered to provide the best measure of genetic variation. DNA polymorphism can be detected in nuclear and organelle DNA and is not modified by environmental exposure. Further, the analysis can be carried out at any time during plant development and it may cover the entire genome. Many molecular markers have been developed for plant genetic diversity assessment (KARP & al. [29]). Commonly, DNA polymorphism assessment techniques based on polymerase chain reaction (PCR) and restriction fragment length polymorphism are applied to endangered species (CHASE & al., [30]). In particular, microsatellites have been effectively deployed to establish the genetic stability of long-term maintained germplasm. Microsatellite markers are favoured, since their preferential association with low copy regions of plant genomes (MORGANTE & al. [31]).

As a complementary method to molecular markers, flow cytometry is used to detect the possible changes in ploidy levels and DNA content (FUKAI & al. [32]).

Conclusions

The past two decades have witnessed significant advancement of biotechnology and currently new methods are available to conserve the threatened plant germplasm. The new developed techniques offer new options and facilitate conservation in the form of seeds, pollen, embryos, and *in vitro* cultures. *In vitro* techniques and storage methods are enabling the establishment of extensive collection using minimum space. Establishing *in vitro* plant stocks have an immediate benefit by reduction the collection pressure on the wild populations. These collections allow for continuous supply of valuable material for wild population recovery, molecular investigations, ecological studies, or economic uses.

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