Acta Societatis Botanicorum Poloniae

Article ID: 9202
DOI: 10.5586/asbp.9202

Publication History
Received: 2022-03-23
Accepted: 2022-07-16
Published: 2023-02-09

Handling Editor
Robert Konieczny; Jagiellonian University, Poland; https://orcid.org/0000-0002-4205-2656

Authors' Contributions
BP & AP: writing – review and editing

Funding
This work did not involve any funding.

Competing Interests
No competing interests have been declared.

REVIEW in POLISH BOTANY CENTENNIAL

Application of *in vitro* culture and biotechnology for the protection of endangered national plant species in Poland

Bożena Pawłowska [1], Agata Ptak [2*]

[1] Department of Ornamental Plants and Garden Art, University of Agriculture in Kraków, 29 Listopada 54, 31-425 Kraków, Poland
[2] Department of Plant Breeding, Physiology and Seed Science, University of Agriculture in Kraków, Łobzowska 24, 31-140 Kraków, Poland

[*] To whom correspondence should be addressed. Email: agata.ptak@urk.edu.pl

Abstract

In Poland, research applying *in vitro* techniques to protect endangered national plant species began 30 years ago. Scientists from approximately 10 research centers comprising universities, research institutes, and botanical gardens, have conducted the most significant studies to date. In this paper, we review the knowledge and experience accumulated over the last few decades regarding the micropropagation of more than 40 wild Polish plant species. This research covered all groups of plants: ferns, monocotyledons, dicotyledons, and woody species. Most studies have focused on dicotyledon classes and species belonging to eight botanical families. Among them the first research was on the use of *in vitro* cultures for the protection of endangered plants of the Droseraceae family. Several micropropagation protocols have been established, in which organogenesis is a more frequently used morphogenetic pathway for plant propagation than somatic embryogenesis. The major aspects investigated included the selection of an appropriate initial explant and optimization of the medium composition. An embryogenic cell suspension culture was established, and a technology for obtaining artificial seeds was developed. Some studies have extended the acclimatization of plants to *ex vitro* conditions. Recent studies, apart from *in vitro* techniques, have also used molecular biology and genetic techniques, which may support the implementation of plant regeneration systems developed in Poland to protect endangered species.

Keywords

conservation; endangered plants; micropropagation; Polish wild species; tissue culture

1. Introduction

In Poland, the first six plant species were placed under strict protection in 1919, and in 1946, the first species protection ordinance listing 110 plants was issued. The Ministry of the Environment's regulation issued on October 9, 2014 (Journal of Laws, 2014), is currently in effect. A total of 415 species (including 270 seed plants) are strictly protected and 301 species (including 121 seed plants) are partially protected.

There are two basic strategies for preserving plant genomic reserves: *in situ* and *ex situ* conservation. Plants can be protected outside of their native habitats with a gene bank through traditional methods such as field collection, seed banks, and botanical gardens, as well as *in vitro* techniques using micropropagation and storage cultures. Many centers have tissue culture laboratories for the multiplication of plants that are difficult to propagate by conventional horticultural techniques.

Tissue culture permits mass production of cloned plants. Plants obtained *in vitro* can be of great value for research, living collections, and plant reintroduction programs if considered appropriate. Different morphogenetic pathways such as organogenesis, that is, the multiplication of shoots from axillary buds, the formation of adventitious organs, and somatic embryogenesis are used for *in vitro* production of native, protected, partly protected, endangered, critically endangered, and extinct species.

Australia is an example of a country that has successfully used *in vitro* cultures to protect endangered plant species for years (Ashmore et al., 2011). This country is currently implementing the Threatened Species Strategy 2021–2031 (Australian Government, 2021).

In vitro techniques were suggested for *ex situ* propagation of native endangered plant species in Poland for the first time by Professor Krystyna Kukułczanka from the Botanical Garden of the University of Wrocław. His paper, entitled "The role of *in vitro* cultures in the conservation of rare and threatened plant species" (Kukułczanka, 1987), referenced a seminar that took place ten years earlier in the Royal Botanic Gardens, Kew, England. At that time, the important role of tissue cultures in the protection and preservation of plant species that are difficult to reproduce using traditional methods was indicated. The author emphasized that plants could be multiplied *in vitro* by initiating a culture from a small number of seeds, organs, or tissues, and the resulting cultures could be maintained under optimal conditions for a long time and serve as tissue banks.

Currently, biotechnological methods based on *in vitro* techniques are being used as plant protection tools for various purposes. The most important are vegetative reproduction *in vitro* and protection of the morphogenetic potential of cells, tissues, and organs (Rybczyński & Mikuła, 2006).

For almost 30 years, Polish scientists from universities, research institutes, and botanical gardens have been working on developing micropropagation protocols for endangered species in Poland. However, only a few species of Polish flora are protected using *in vitro* techniques. The number of endangered plant species is growing, hence, propagation studies must be intensified in the near future and all possible techniques must be combined to cover various aspects of biodiversity protection.

The aim of this study is to review the most significant achievements of Polish scientists in the application of *in vitro* culture and biotechnology for the protection of native taxa. There are ten notable research institutions in Poland involved in this field, including eight academic centers and two botanical gardens (Table 1). These research centers investigate the possibilities of employing *in vitro* and biotechnological techniques to protect the endangered species native to Poland.

The achievements presented in this study include fern and monocotyledon species (Table 2), herbaceous dicotyledons (Table 3), and several woody species (Table 4).

This study does not include cryopreservation. Detailed information on cryopreservation was presented by Mikuła et al. (2022).

2. Endangered national ferns and monocotyledons propagated by tissue culture

In vitro cultures of the native ferns *Asplenium adulterinum*, *Asplenium cuneifolium* (Marszał-Jagacka et al., 2005) and *Osmunda regalis* (Zenkteler, 1999; Makowski et al., 2016) were initiated by spore germination, which is the most common method for this group of plants. Attempts have also been made to grow *Matteucia struthiopteris* cultures from rhizomes and dormant buds on rhizomes and to induce callus formation and proliferation (Zenkteler, 2006). The mineral salt content of the medium plays a significant role in fern *in vitro* culture. For the growth of *A. adulterinum* and *A. cuneifolium* gametophytes, half strength nutrient Murashige and Skoog (MS) medium was used (Murashige & Skoog, 1962; Marszał-Jagacka et al., 2005). Similarly, in research carried out in the Botanical Garden in Powsin, reducing the content of mineral salts in the MS medium to half or even a quarter had a positive effect on the proliferation of gametophytes *Osmunda regalis*. The maximum sporophyte production in this fern requires 1/8 MS mineral salts (Makowski et al., 2016). *M. struthiopteris*

Table 1 The most notable research institutions involved in this field of *in vitro* conservation of protected species in Poland.

Institution	Plant species
University of Agriculture in Krakow	*Leucojum vernum, Lilium maratagon*, *Cypripedium calceolus, Dactylorhiza maculate*, *Epipactis helleborine, Goodyera repens*, *Gymnadenia conopsea, Biscutella laevigata*, *Gentiana pneumonanthe, Hepatica nobilis*, *Primula farinose, Staphylea pinnata*, *Daphne cneorum*
University of Gdańsk	*Drosera anglica, Cypripedium calceolus*, *Dactylorhiza majalis, Epipactis atrorubens*, *Epipactis palustris, Orchis morio*
Nicolaus Copernicus University in Toruń	*Carlina onopordifolia, Cirsium pannonicum*, *Inula germanica, Leontopodium alpinum*, *Taraxacum pieninicum*
Poznań University of Medical Sciences	*Eryngium maritimum, Eryngium alpinum, Eryngium planum, Linnaea borealis, Rubus chamaemorus*
Botanical Garden of the University of Wrocław	*Asplenium adulterinum, Asplenium cuneifolium, Drosera intermedia, Drosera rotundifolia*
Polish Academy of Sciences Botanical Garden – CBDC*	*Osmunda regalis, Lilium maratagon*, *Gentiana cruciate, Gentiana punctata*
Jagiellonian University	*Pulsatilla vulgaris, Viola ulginosa*, *Viola stagnina*
Adam Mickiewicz University in Poznań	*Matteucia struthiopteris, Osmunda regalis*
University of Life Sciences in Lublin	*Aldrovanda vesiculosa, Salix lapponum*
Warsaw University of Life Sciences	*Daphne mezereum*

* Center for Biological Diversity Conservation in Powsin.

in vitro culture could be initiated in the Modified Fern Multiplication Medium (Miller & Murashige, 1976).

The reviewed publications indicate that *in vitro* techniques have been used to protect the greatest number of Orchidaceae species to date, even though only two papers published in 2004 investigated the species mentioned here. The development of protocols for protocorm culture and further plant regeneration for native orchid species is particularly challenging. Pindel & Pindel (2004) initiated cultures of five orchid species from green capsule sections. Znaniecka & Łojkowska (2004) established the cultures of five other native orchid species by focusing mainly on the observations of seedling germination and development. Both teams worked on *Cypripedium calceolus*; but the most advanced development of protocorms with shoots and roots was achieved in *Dactylorhiza maculata* (Pindel & Pindel, 2004). MS medium supplemented with naphthaleneacetic acid (NAA), 6-benzyl-aminopurine (BAP), and activated charcoal was the best medium for the development of protocorms (Pindel & Pindel, 2004). In contrast, media containing peptone, yeast extract, and casein hydrolysate were the most effective for germination and development of orchid seedlings (Znaniecka & Łojkowska, 2004).

However, Polish scientists have rarely investigated bulbous plants plants. The first research results on the induction of adventitious bulbs in *Lilium martagon* were published over 30 years ago by Rybczyński & Gomolińska (1989). They cultured bulb scales as explants in MS medium containing NAA and BAP. Significant progress in the propagation of bulbous plants was made at the University of Agriculture in Krakow, where the protocols for adventitious organogenesis and indirect somatic embryogenesis were developed for *Leucojum vernum* and *L. martagon*, thus considerably increasing their propagation rate (Kędra & Bach, 2005; Ptak, 2010). In *L. martagon*, the formation of adventitious bulbs was observed in seedling bulb explants on MS medium in absence of plant growth regulators or in the presence of BAP. The best explants for embryogenic callus initiation were adventitious bulb scales in *L. martagon*

Table 2 Selected species of native fern and monocotyledonous species protected in Poland with the use of *in vitro* techniques.

Plant species	Initial explants	Morphogenetic pathway	Plant acclimatization*	Cytometric/ Molecular analysis**	References
FERNS					
Asplenium adulterinum *Asplenium cuneifolium*	spores	in vitro spore germination, gametophytes and sporophytes obtained	+	–	Marszał-Jagacka et al., 2005
Matteucia struthiopteris	rhizomes, dormant buds from rhizomes	indirect organogenesis	-	-	Zenkteler, 2006
Osmunda regalis	spores	gametophyte cultures, young sporophytes, saprophyte production	-	-	Zenkteler, 1999; Makowski et al., 2016
MONOCOTYLEDONOUS					
Amaryllidaceae family					
Leucojum vernum	scales, leaves from bulbs, ovaries and fruits	indirect somatic embryogenesis	-	CA	Ptak, 2010
Liliaceae family					
Lilium maratagon	seedling explants, adventitious bulblet scales	organogenesis, indirect somatic embryogenesis	-	CA	Rybczyński & Gomolińska, 1989; Kędra & Bach, 2005; Pawłowska et al., 2007
Orchidaceae family					
Cypripedium calceolus	seeds, capsules, young ovaries	seedling growth protocorm-like bodies obtained	-	-	Znaniecka & Łojkowska, 2004; Pindel & Pindel, 2004
Dactylorhiza maculata	capsules, young ovaries	protocorms, protocorms with shoots and roots development	-	-	Pindel & Pindel, 2004
Dactylorhiza majalis	seeds	seedling growth	-	-	Znaniecka & Łojkowska, 2004
Epipactis atrorubens *Epipactis palustris*	seeds			-	
Epipactis helleborine *Goodyera repens* *Gymnadenia conopsea*	capsules, young ovaries	indirect organogenesis	-	-	Pindel & Pindel, 2004
Orchis morio	seeds	seedling growth	-	-	Znaniecka & Łojkowska, 2004

* + acclimatization were conducted, - plants were not acclimatized.
** CA: cytometric analysis.

Table 3 Achievements of Polish scientists in the protection of native, herbaceous, dicotyledonous species by means of *in vitro* techniques.

Species	Explants	Morphogenetic pathway	Plant acclimatization*	Cytometric/Molecular analysis**	References
Herbaceous plants					
Asteraceae family					
Carlina onopordifolia	shoot tips from seedlings	organogenesis: axillary shoot multiplication and rooting	-	-	Trejgell et al., 2012a
Cirsium pannonicum	shoot tips from seedlings	organogenesis: axillary shoot multiplication and rooting	+	-	Trejgell et al., 2012b
Inula germanica	shoot tips from seedlings, fragments of cotyledons, hypocotyls and roots	organogenesis: adventitious shoot induction, multiplication and rooting	+	-	Trejgell et al., 2018
Leontopodium alpinum	shoot tips from seedlings, fragments of cotyledons, hypocotyls and roots	organogenesis: axillary and adventitious shoot proliferation and rooting	-	-	Trejgell et al., 2010
Taraxacum pieninicum	shoot tips from seedlings	organogenesis: axillary bud proliferation, synthetic seed production and storage in slow growth conditions	-	-	Kamińska et al., 2021
Apiaceae family					
Eryngium maritimum *Eryngium alpinum* *Eryngium planum*	apical and axillary buds	organogenesis: multiplication and rooting of shoots	+	CA	Kikowska et al., 2014; Kikowska et al., 2020; Thiem et al., 2013
Brassicaceae family					
Biscutella laevigata	*in vitro* shoots	organogenesis: shoot multiplication	+	-	Muszyńska et al., 2017

Continued on next page

Table 3 *Continued.*

Species	Explants	Morphogenetic pathway	Plant acclimatization*	Cytometric/Molecular analysis**	References
Droseraceae family					
Drosera anglica	leaf segments	direct organogenesis	-	-	Kawiak et al., 2003
Drosera intermedia *Drosera rotundifolia*	seeds	seed germination and seedling growth, storage in slow growth conditions	-	-	Kukułczanka & Cząstka, 1987; Kukułczanka et al., 1991
Aldrovanda vesiculosa	shoot fragments	organogenesis: shoot multiplication	-	-	Parzymies, 2021
Gentianaceae family					
Gentiana pneumonanthe	seeds	direct organogenesis: axillary shoot multiplication, somatic embryogenesis, synthetic seeds	-	CA	Pawłowska & Bach, 2003; Bach & Pawłowska, 2003; Bach et al., 2004
Gentiana cruciata	seedlings	somatic embryogenesis	-	-	Mikuła & Rybczyński, 2001; Mikuła et al., 2005a,b
Gentiana punctata	zygotic embryos	somatic embryogenesis	-	-	Mikuła et al., 2004
Primulaceae family					
Primula farinosa	seedlings	organogenesis: axillary shoot cultures and rooting	+	CA	Sitek et al., 2020
Ranunculaceae family					
Hepatica nobilis	seedlings	somatic embryogenesis	+	-	Szewczyk-Taranek & Pawłowska, 2015
Pulsatilla vulgaris	seedlings	somatic embryogenesis (direct and indirect), organogenesis - adventitious shoot, rooting	+	CA, ISSR	Żabicka et al., 2021
Violaceae family					
Viola uliginosa	fragments of leaf and petiole	organogenesis (direct and indirect), shoot rooting	+	CA, AFLP	Ślązak et al., 2015
Viola stagnina	fragments of leaf and petiole	organogenesis (direct and indirect)	+	CA, ISSR	Żabicki et al., 2019, 2021

* + acclimatization were conducted, - plants were not acclimatized.
** CA: cytometric analysis, ISSR - inter simple sequence repeat, AFLP - amplified fragment length polymorphism.

Table 4 Achievements of Polish scientists in the protection of native, woody species (including dwarf shrubs) by means of *in vitro* techniques.

Species	Explants	Morphogenetic pathway	Plant acclimatization*	Cytometric/Molecular analysis**	References
Caprifoliaceae family					
Linnaea borealis	nodal segments and shoot tips with apical meristems	organogenesis: shoot multiplication on solid and liquid media, rooting	-	CA	Thiem et al., 2021
Rosaceae family					
Rubus chamaemorus	shoot tips	organogenesis: shoot multiplication, rooting	+	CA	Thiem, 2021; Thiem & Śliwińska, 2003
Salicaceae family					
Salix lapponum	shoots	organogenesis: shoot multiplication, rooting	+	CA, ISSR	Parzymies et al., 2020
Staphyleaceae family					
Staphylea pinnata	dormant buds	organogenesis: axillary shoot propagation	-	-	Szewczyk-Taranek & Pawłowska, 2016
Thymelaeceae family					
Daphne mezereum	axillary shoots	organogenesis: shoot multiplication, rooting	-	ISSR, RAPD	Pacholczak & Nowakowska, 2019; Nowakowska & Pacholczak, 2020
Daphne cneorum	*in vitro* roots	root cultures, tolerance to *Thielaviopsis basicola*	-	-	Hanus-Fajerska et al., 2014

* + acclimatization were conducted, - plants were not acclimatized.
** CA: cytometric analysis, ISSR - inter simple sequence repeat, RAPD - randomly amplified polymorphic DNA.

and fruit tissues in *L. vernum*. The medium for culture initiation, callus propagation, and somatic embryo induction of *L. martagon* and *L. vernum* was enriched with 4-amino-3,5,6-trichloropicolinic acid (picloram) and BAP. The abscisic acid and polyethylene glycol added to the medium stimulated somatic embryo maturation in *L. vernum* (Table 2).

3. Endangered national dicotyledon herbaceous species and woody plants propagated by tissue culture

In the case of dicotyledonous species, the research mostly involved herbaceous species, 21 of which belonged to eight botanical families. Woody plants were represented by only six species belonging to five families (Tables 3 and 4).

Drosera species are among the first protected herbaceous plants propagated *in vitro*. The study was initiated by Professor Kukułczanka (Kukułczanka & Cząstka, 1987) and continued by researchers from the University of Gdańsk (Kawiak et al., 2003).

The *in vitro* cultures of *Drosera* are usually initiated from seeds, but also, more rarely, from leaf fragments (Kawiak et al., 2003). The seeds of the protected taxa are easily available, their harvesting does not destroy the mother plants, and furthermore, the seed-initiated culture contributes to maintaining the species variability and biological diversity. *In vitro* cultures of many other herbaceous taxa have been established from seeds and seedling fragments and used for further propagation. Such procedures have been implemented for species belonging to the Asteraceae family, for example, *Carlina onopordifolia, Cirsium pannonicum, Inula germanica, Leontopodium alpinum, Taraxacum pieninicum*, from the Gentianaceae family (e.g., *Gentiana pneumonanthe*), Primulaceae family (e.g., *Primula farinosa* or *Hepatica nobilis*), and from the Ranunculaceae family (*Pulsatilla vulgaris*) (Table 3). In some taxa of herbaceous plants, such as *Eryngium maritimum* (Kikowska et al., 2014), *Eryngium alpinum* (Kikowska et al., 2020), *Viola uliginosa* (Ślązak et al., 2015), *Viola stagnina* (Żabicki et al., 2019, 2021), and all investigated tree taxa (except for *Rubus chamaemorus*, which was initiated from seeds), the cultures were initiated from vegetative explants (dormant buds) (Table 4). The most recent advances within this family involved the successful application of nanosilver to maintain sterility in *Aldrovanda vesiculosa* cultures (Parzymies, 2021).

The micropropagation of protected and endangered plants is mainly based on organogenesis, and research has focused primarily on determining the influence of growth regulators on shoot multiplication and *in vitro* rooting. The research also focused on the selection of micro- and macro-elements in the nutrient medium; for example, the medium according to Reinert and Mohr (1967) was the best for *Drosera rotundifolia* seedling development (Kukułczanka & Cząstka, 1987) and the liquid Fast medium (Fast, 1981) was the best for the proliferation of *Drosera anglica* shoots (Kawiak et al., 2003).

In contrast, in studies on *C. onopordifolia*, the MS medium was modified by replacing ferric ethylenediamine-tetraacetic acid (FeEDTA) with ethylenediamine di-2-hydroxy-phenyl acetate ferric acid (Fe-EDDHA). These modifications had no effect on the rate of axillary shoot proliferation, but they enhanced leaf chlorophyll content (Trejgell et al., 2012a).

In addition to MS medium, *in vitro* propagation of woody plants has also been achieved on QL medium (Quoirin & Lepoivre, 1977) and Woody Plant Medium (WPM) (Lloyd & McCown, 1980) for *Staphylea pinnata* (Szewczyk-Taranek & Pawłowska, 2016) and WPM medium for *Daphne mezereum* (Nowakowska & Pacholczak, 2020).

During axillary shoot multiplication of *S. pinnata*, the identification and elimination of endophytic bacterial contamination was studied. VITEK®2, a rapid bacterial identification system, and the 16S rRNA gene sequencing method allowed for the identification of *Acinetobacter johnsonii* strain ATCC 17909 and *Methylobacterium rhodesianum* strain DSM 5687. The addition of gentamicin to the medium was the most effective in eliminating bacteria (Szewczyk-Taranek et al., 2020).

The cultures were maintained under fluorescent white light. Szewczyk-Taranek et al. (2017) showed that LED light may be an alternative light source for the *in vitro* shoot proliferation stage of *S. pinnata* (Szewczyk-Taranek et al., 2017).

To protect endangered plants, Polish researchers have also used the process of somatic embryogenesis for plant propagation. This process was used by Mikuła and Rybczyński (2001) in *Gentiana cruciata*, and by Bach and Pawłowska (2003) in *G. pneumonanthe*. Plantlet regeneration from somatic embryos was achieved in both studies. Supplementation of MS medium by 2,4-D (dichlorophenoxyacetic acid) was a key factor for somatic embryogenesis induction. In *G. cruciata*, embryogenic callus formation was observed in seedlings (best performance on hypocotyl and cotyledonous seedlings, 39–46 somatic embryos were formed per explant). In *G. pneumonanthe*, the explants were leaves or apical meristems derived from shoots multiplied *in vitro* (25–37% of the explants formed embryogenic callus). Additionally, embryogenic callus in marsh gentians were observed in media containing picloram. Embryo development was possible after lowering the auxin level in the culture medium, and maturation occurred in the media without growth regulators.

Moreover, Mikuła et al. (2005a) demonstrated the huge potential of embryogenic suspension cultures of *G. cruciata* for the long-term mass production of somatic embryos. Somatic embryogenesis was also performed in two species of the Ranunculaceae family. Żabicka et al. (2021) used shoot tips of *P. vulgaris* seedlings to obtain embryogenic callus and somatic embryos. A method for secondary somatic embryogenesis was developed for *H. nobilis* (a species protected since 2004) embryos derived from embryogenic callus formed on seedlings. The most efficient repetitive cyclic process of secondary somatic embryogenesis was observed in MS medium without growth regulators (on 78–87% primary somatic embryos), and the best rate of somatic embryo germination was achieved in MS medium with BAP (52%) (Szewczyk-Taranek & Pawłowska, 2015).

The final stage of micropropagation of protected plants is acclimatization. *In vitro* regenerated plants of *C. pannonicum* (Trejgell et al., 2012b), *E. maritimum* (Kikowska et al., 2014), *H. nobilis* (Szewczyk-Taranek & Pawłowska, 2015), *I. germanica* (Trejgell et al., 2018), *P. farinosa* (Sitek et al., 2020), *P. vulgaris* (Żabicka et al., 2021), *R. chamaemorus* (Thiem, 2001), *V. stagnina* (Żabicki et al., 2019), *V. ulginosa* (Ślązak et al., 2015) and *Salix lapponum* (Parzymies et al., 2020) were successfully acclimatized to greenhouse conditions. Studies of plant adaptation to *ex vitro* conditions in a greenhouse also included observations of plant performance in the field (e.g., for *Biscutella laevigata*) (Muszyńska et al., 2017).

4. Selected achievements of biotechnological research

Experiments on *G. pneumonanthe* (Bach et al., 2004), *T. pieninicum* (Kamińska et al., 2021) and *E. alpinum* (Kikowska et al., 2020) have investigated the possibility of storing plant material in the form of synthetic seeds. Sodium alginate coatings were used for lateral shoot tips derived from gentian, cloudberry, and eryngo propagated *in vitro* and for shoot tips taken from dandelion seedlings germinated *in vitro*.

In vitro cultures have also been used to investigate the protected plants with respect to their resistance to fungal pathogens, such as *Thielaviopsis basicola* attacking *Daphne cneorum* (Hanus-Fajerska et al., 2014), or tolerance to heavy metals such as Pb and Cd (*B. laevigata)* (Muszyńska et al., 2017).

To assess the usefulness of the micropropagation protocol for species protection, it is important to test the genetic stability of the regenerated plants. This is typically performed using molecular markers and flow cytometry. Recently, the genetic stability of the *in vitro* regenerated plants was analyzed using the following markers: inter simple sequence repeat (ISSR) in *S. lapponum*, *D. mezereum*, *V. stagnina* and *P. vulgaris*; randomly amplified polymorphic DNA (RAPD) in *D. mezereum*; and amplified fragment length polymorphism (AFLP) in *V. uliginosa* (Ślązak et al., 2015; Nowakowska & Pacholczak, 2020; Parzymies et al., 2020; Żabicki et al., 2019, 2021). Cytometric analysis of the regenerated plants was performed for *E. maritimum* (Kikowska et al., 2014), *E. alpinum* (Kikowska et al., 2020), *G. pneumonanthe* (Bach & Pawłowska, 2003), *L. vernum* (Ptak, 2010), *L. martagon* (Kędra & Bach, 2005; Pawłowska et al.,

2007), *Linnaea borealis* (Thiem et al., 2021), *P. farinosa* (Sitek et al., 2020), *P. vulgaris* (Żabicka et al., 2021), *R. chamaemorus* (Thiem & Śliwinska, 2003), *S. lapponum* (Parzymies et al., 2020), *V. uliginosa* (Ślązak et al., 2015) and *V. stagnina* (Żabicki et al., 2019). Majority of the studies found no differences between *in vitro* regenerated plants and mother plants, indicating that the developed micropropagation protocols are suitable for use. In some cases, as suggested by Ślązak et al. (2015), more direct methods are preferred for micropropagation, even when the process is less effective. The authors showed that the genetic uniformity of the regenerated *V. uliginosa* plants with the mother plants, as detected by AFLP analyses, clearly depended on the type of organogenesis.

Notably, biotechnological research also focuses on the use of *in vitro* cultures of protected species as a source of therapeutically important substances. For this purpose, various types of *in vitro* cultures have been performed (e.g., shoot, callus, root, and suspension cultures), and various treatments have been used to stimulate the biosynthesis of secondary metabolites (Kikowska et al., 2022). This issue has been discussed in a separate publication (Pietrosiuk et al., 2022).

5. Conclusion

Although studies on the implementation of *in vitro* multiplication and conservation procedures for protected plant species are actively carried out in Poland, they are concerned only with a small percentage of endangered species of the Polish flora. As the number of endangered plant species is constantly increasing, these studies should be intensified in the future. However, this claim has considerable limitations. First, the protected species come from various botanical families, and the development of micropropagation procedures is highly individualized and, therefore, a slow process. Second, due to a lack of legal regulations in Poland on plants propagated *in vitro*, the reintroduction of such species is not common.

Despite the wide range of possibilities provided by well-equipped laboratories and experienced researchers in Poland, the potential of *in vitro* cultures for biodiversity conservation has not been fully utilized. Currently, the use of *in vitro* cultures is usually restricted to storing plant material in *in vitro* gene banks and long-term storage under *in vitro* conditions. Future studies should focus on using *in vitro* cultures as a source of explants stored in liquid nitrogen (cryopreservation).

References

Ashmore, S. E., Hamilton, K. N., & Offord, C. A. (2011). Conservation technologies for safeguarding and restoring threatened flora: case studies from Eastern Australia. *In Vitro Cellular Developmental Biology – Plant*, *47*, 99–109. https://doi.org//10.1007/s11627-010-9320-9

Australian Government. (2021). Threatened Species Strategy 2021–2031. https://www.awe.gov.au/environment/biodiversity/threatened/publications/threatened-species-strategy-2021-2031

Bach, A., & Pawłowska, B. (2003). Somatic embryogenesis in *Gentiana pneumonanthe* L. *Acta Biologica Cracoviensia Series Botanica*, *45*, 79–85.

Bach, A., Pawłowska, B., & Malik, M. (2004). Plantlets from encapsulated meristems of *Gentiana pneumonanthe* L. *Acta Physiologiae Plantarum*, *26*, 53–57. https://doi.org/10.1007/s11738-004-0044-5

Fast, G. (1981). *Orchideen Kultur* (p. 220). Eugen Ulmer-Verlag.

Hanus-Fajerska, E., Wiszniewska, A., & Riseman, A. (2014). Elaboration of *in vitro* root culture protocol to efficiently limit Daphne Sudden Death Syndrome evoked by *Thielaviopsis basicola*. *Acta Scientiarum Polonorum, Hortorum Cultus*, *13*(1), 117–127.

Journal of Laws. (2014). Rozporządzenie Ministra Środowiska z dnia 9 października 2014 r. w sprawie ochrony gatunkowej roślin. *Dz. U.* 2014, poz. 1409.

Kamińska, M., Kęsy, J., & Trejgell, A. (2021). Abscisic acid in preservation of *Taraxacum pieninicum* in the form of synthetic seeds in slow growth conditions. *Plant Cell Tissue and Organ Culture*, *144*, 295–312. https://doi.org/10.1007/s11240-020-01924-0

Kawiak, A., Królicka, A., & Łojkowska, E. (2003). Direct regeneration of *Drosera* from leaf explants and shoot tips. *Plant Cell Tissue and Organ Culture*, *75*, 175–178. https://doi.org/10.1023/A:1025023800304

Kędra, M., & Bach, A. (2005). Morphogenesis of *Lilium martagon* L. explants in callus culture. *Acta Biologica Cracoviensia Series Botanica*, *47*, 65–73.

Kikowska, M., Thiem, B., Śliwińska, E., Rewers, M., Kowalczyk, M., Stochmal, A., & Oleszek, W. (2014). The effect of nutritional factors and plant growth regulators on micropropagation and production of phenolic acids and saponins from plantlets and adventitious root cultures of *Eryngium maritimum* L. *Journal of Plant Growth Regulation*, *33*, 809–819. https://doi.org/10.1007/s00344-014-9428-y

Kikowska, M., Śliwinska, E., & Thiem, B. (2020). Micropropagation and production of somatic seeds for short-term storage of the endangered species *Eryngium alpinum* L. *Plants (Basel)*, *9*(4), Article 498. https://doi.org/10.3390/plants9040498

Kikowska, M., Chanaj-Kaczmarek, J., Derda, M., Budzianowska, A., Thiem, B., Ekiert, H., & Szopa, A. (2022). The evaluation of phenolic acids and flavonoids content and antiprotozoal activity of *Eryngium* species biomass produced by biotechnological methods. *Molecules*, *27*, Article 363. https://doi.org/10.3390/molecules27020363

Kukułczanka, K. (1987). Kultury *in vitro* w zachowaniu gatunków roślin rzadkich i ginących. *Biuletyn Ogrodów Botanicznych Muzeów i Zbiorów*, *1*, 65–67.

Kukułczanka, K., & Cząstka, B. (1987). Rozmnażanie rosiczek (*Drosera* L.) w warunkach *in vitro*. *Wiadomości Botaniczne*, *31*(2), 61–64.

Kukułczanka, K., Kromer, K., Klonowska, B., & Cząstka, B. (1991). Wpływ temperatury na przechowywanie kultur roślin *Droseraceae in vitro*. *Prace Ogrodu Botanicznego PAN*, *1*, 63–68.

Lloyd, G., & McCown, B. (1980). Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Proc. Int. Plant Propagators*, *30*, 421–437.

Makowski, D., Tomiczak, K., Rybczyński, J. J., & Mikuła, A. (2016). Integration of tissue culture and cryopreservation methods for propagation and conservation of the fern *Osmunda regalis* L. *Acta Physiologiae Plantarum*, *38*, Article 19. https://doi.org/10.1007/s11738-015-2037-y

Marszał-Jagacka, J., Kromer, K., & Świerkosz, K. (2005). *Ex situ* protection of endangered *Asplenium* ferens species using *in vitro* culture. *Biuletyn Ogrodów Botanicznych, Muzeów i Zbiorów*, *14*, 35–42.

Miller, L. R., & Murashige, T. (1976). Tissue culture propagation of tropical foliage plants. *In Vitro*, *12*, 797–813.

Mikuła, A., Chmielarz, P., Hazubska-Przybył, T., Kulus, D., Maślanka, M., Pawłowska, B., & Zimnoch-Guzowska, E. (2022). Cryopreservation of plant tissues in poland: Research contributions, current status, and applications. *Acta Societatis Botanicorum Poloniae*, *91*, 1–26. Article 9132. https://doi.org/10.5586/asbp.9132

Mikuła, A., & Rybczyński, J. J. (2001). Somatic embryogenesis of *Gentiana* taxa. I. The effect of species, preculture and primary explant origin on somatic embryogenesis of *Gentiana tibetica, G. cruciata, G. pannonica*. *Acta Physiologiae Plantarum*, *23*, 15–25. https://doi.org/10.1007/s11738-001-0017-x

Mikuła, A., Tyrkarska, T., Zielińska, M., Kuraś, M., & Rybczyński, J. J. (2004). Ultrastructural changes in zygotic embryos of *Gentiana punctata* L. during callus formation and somatic embryogenesis. *Acta Biologica Cracoviensia series Botanica*, *46*, 109–120.

Mikuła, A., Rybczyński, J. J., Siekierski, J., Latkowska, M., & Fiuk, A. (2005a). Somatic embryogenesis of *Gentiana* genus IV: Characterization of *Gentiana cruciata* and *G. tibetica* embryogenic cell suspensions. In A. K. Hvoslef-eide & W. Preil (Eds.), *Liquid culture systems for in vitro plant propagation* (pp. 345–358). Springer.

Mikuła, A., Tyrkarska, T., Kuraś, M., & Rybczyński, J. J. (2005b). Somatic embryogenesis of *Gentiana cruciata* (L.): Histological and ultrastructural changes in seedling hypocotyls explants. *In Vitro Cellular and Developmental Biology - Plant*, *41*, 686–694. https://doi.org/10.1079/IVP2005678

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, *15*, 473–497.

Muszyńska, E., Hanus-Fajerska, E., Piwowarczyk, B., Augustynowicz, J., Ciarkowska, K., & Czech, T. (2017). From laboratory to field studies — assessment of *Biscutella laevigata* suitability to biological reclamation of areas contaminated with lead and cadmium. *Ecotoxicology and Environmental Safety*, *142*, 266–273. https://doi.org/10.1016/j.ecoenv.2017.04.019

Nowakowska, K., & Pacholczak, A. (2020). Comparison of the effect of meta-topolin and benzyladenine during *Daphne mezereum* L. micropropagation. *Agronomy*, *10*, Article 1994. https://doi.org/10.3390/agronomy10121994

Pacholczak, A., & Nowakowska, K. (2019). Micropropagtion of february daphne (*Daphne mezereum* L.). *Propagation of Ornamental Plants*, *19*(4), 106–112.

Parzymies, M., Pogorzelec, M., Głębocka, K., & Śliwińska, E. (2020). Genetic stability of endangered species *Salix lapponum* L. regenerated *in vitro* during reintroduction process. *Biology*, *9*, Article 378. https://doi.org/10.3390/biology9110378

Parzymies, M. (2021). Nano-silver particles reduce contaminations in tissue culture but decrease regeneration rate and slows down growth and development of *Aldrovanda vesiculosa* explants. *Applied Sciences*, *11*, Article 3653. https://doi.org/10.3390/app11083653

Pawłowska, B., & Bach, A. (2003). *In vitro* propagation of protected species *Gentiana pneumonanthe* L. for ornamental horticultural use. *Folia Horticulturae*, *15*, 113–122.

Pawłowska, B., Nowak, K., & Bach, A. (2007). Ploidy of Martagon lily (*Lilium martagon* L.) regenerants propagated *in vitro*. In P. Nowaczyk (Ed.), *Spontaneous and induced variation for the genetic improvement of horticultural crops* (pp. 275–281). University Press. University of Technology and Life Science in Bydgoszcz.

Pietrosiuk, A., Budzianowska, A., Budzianowski, J., Ekiert, H., Jeziorek, M., Kawiak, A., Kikowska, M., Krauze-Baranowska, M., Królicka, A., Kuźma, Ł., Łuczkiewicz, M., Malarz, J., Matkowski, A., Stojakowska, A., Sykłowska-Baranek, K., Szopa, A., Szypuła, W., Thiem, B., & Zielińska, S. (2022). Polish achievements in bioactive compound production from in vitro plant cultures. *Acta Societatis Botanicorum Poloniae*, *91*, 1–38. Article 9110. https://doi:10.5586/asbp.9110

Pindel, A., & Pindel, Z. (2004). Initiation of *in vitro* cultures of chosen endangered European species of orchids. *Folia Horticulturae*, *16/2*, 111–117.

Ptak, A. (2010). Somatic embryogenesis in *in vitro* culture of *Leucojum vernum* L. In S. M. Jain & S. J. Ochatt (Eds.), *Protocols for* in vitro *propagation of ornamental plants* (pp. 223–233). Humana Press. https://doi.org/10.1007/978-1-60327-114-1_21

Reinert, R. A., & Mohr, H. C. (1967). Propagation of *Cattleya* by tissue culture of lateral bud meristems. *Proceedings of the American Society for Horticultural Science*, *91*, 664–671.

Quoirin, M., & Lepoivre, P. (1977). Improved media for *in vitro* culture of *Prunus* sp. *Acta Horticulturae*, *78*, 437–442.

Rybczyński, J. J., & Gomolińska, H. (1989). 6-benzyladenine control of the initial bulblets formation of wild lily *Lilium martagon* L. *Acta Horticulturae*, *251*, 183–195.

Rybczyński, J. J., & Mikuła, A. (2006). Engagement of biotechnology in the protection of threatened plants species in Poland. *Biodiversity Resouces Conservation*, *3-4*, 361–368.

Sitek, E., Nowak, B., Fecowicz, M., Gajewski, Z., Dańda, P., Kapała, K., & Kozik-Dąbek, B. (2020). Application of horticultural and tissue culture methods for *ex situ* conservation of endangered *Primula farinosa* L. *Acta Societatis Botanicorum Poloniae*, *89*(1), Article 8913. https://doi.org/10.5586/asbp.8913

Szewczyk-Taranek, B., & Pawłowska, B. (2015). Recurrent somatic embryogenesis and plant regeneration from seedlings of *Hepatica nobilis* Schreb. *Plant Cell Tissue and Organ Culture*, *120*, 1203–1207. https://doi.org/10.1007/s11240-014-0661-7

Szewczyk-Taranek, B., & Pawłowska, B. (2016). Investigation on micropropagation of *Staphylea pinnata* L., a rare protected ornamental plant. *Acta Horticulturae*, *1113*, 201–206. https://doi.org/10.17660/ActaHortic.2016.1113.30

Szewczyk-Taranek, B., Pawłowska, B., Prokopiuk, B., & Żupnik, M. (2017). Effectiveness of LED and fluorescent light on in vitro shoot proliferation of *Staphylea pinnata*. *Acta Horticulturae*, *1155*, 375–380.

Szewczyk-Taranek, B., Jaglarz, A., Pałka, P., Supel, P., Kaszycki, P., Mazur, J., & Pawłowska, B. (2020). Identification and control of endophytic bacteria during *in vitro* cultures of *Staphylea pinnata* L. *Folia Horticulturae*, *32*(1), 1–9.

Ślązak, B., Śliwinska, E., Saługa, M., Ronikier, M., Bujak, J., Słomka, A., Göransson, U., & Kuta, E. (2015). Micropropagation of *Viola uliginosa* (Violaceae) for endangered species conservation and for somaclonal variation-enhanced cyclotide biosynthesis. *Plant Cell Tissue and Organ Culture*, *120*, 179–190. https://doi.org/10.1007/s11240-014-0592-3

Thiem, B. (2001). Micropropagation of cloudberry (*Rubus chamaemorus* L.) by initiation of axillary shoots. *Acta Societatis Botanicorum Poloniae*, *70*(1), 11–16. https://doi.org/10.5586/asbp.2001.002

Thiem, B., Kikowska, M., Krawczyk, A., Więckowska, B., & Sliwinska, E. (2013). Phenolic acid and DNA contents of micropropagated *Eryngium planum* L. *Plant Cell Tissue and Organ Culture*, *114*(2), 197–206. https://doi.org/10.1007/s11240-013-0315-1

Thiem, B., Kruszka, D., Turowska, N., Śliwinska, E., Berge, V., & Kikowska, M. (2021). *Linnaea borealis* L. var. *borealis—In vitro* cultures and phytochemical screening as a dual strategy for its ex situ conservation and a source of bioactive compounds of the rare species. *Molecules*, *26*, Article 6823. https://doi.org/10.3390/molecules26226823

Thiem, B., & Śliwińska, E. (2003). Flow cytometric of nuclear DNA content in cloudberry (*Rubus chamaemorus* L.) *in vitro* cultures. *Plant Science*, *164*, 129–134. doi:10.1016/S0168-9452(02)00344-8

Trejgell, A., Domżalska, L., Szczepanek, D., & Tretyn, A. (2010). *In vitro* propagation of *Leontopodium alpinum* L. from different seedling explants. *Propagation of Ornamental Plants*, *10*(2), 81–87.

Trejgell, A., Libront, I., & Tretyn, A. (2012a). The effect of Fe-EDDHA on shoot multiplication and *in vitro* rooting of *Carlina onopordifolia* Besser. *Acta Physiologiae Plantarum*, *34*, 2051–2055. https://doi.org/10.1007/s11738-012-1000-4

Trejgell, A., Dąbrowska, A., & Tretyn, A. (2012b). Micropropagation and influence of *in vitro* culture on development of *Cirsium pannonicum* (L. f.) Link regenerants. *Acta Scientiarum Polonorum Hortorum Cultus*, *11*(5), 81–90.

Trejgell, A., Kamińska, M., Lisowska, K., & Tretyn, A. (2018). Micropropagation of *Inula germanica* L. from the seedlings explants. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, *46*(1), 52–57. https://doi.org/10.15835/nbha46110810

Zenkteler, E. (1999). Sporophytic lethality in lowland populations of *Osmunda regalis* L in Poland. *Acta Biologica Cracoviensia series Botanica*, *41*, 75–83.

Zenkteler, E. (2006). Micropropagation of *Matteucia struthiopteris* (L.) Tod. through meristem proliferation from rhizomes. *Biodiversity: Research and Conservation*, *1–2*, 167–173.

Znaniecka, J., & Łojkowska, E. (2004). Establishment of *in vitro* culture collection of endangered European orchids. *Biulletin of Botanical Gardens, Museums and Collections*, *13*, 69–73.

Żabicka, J., Żabicki, P., Słomka, A., Jędrzejczyk-Korycińska, M., Nowak, T., Śliwińska, E., Kapler, A., Migdałek, G., & Kuta, E. (2021). Genotype-dependent mass somatic embryogenesis: A chance to recover extinct populations of *Pulsatilla vulgaris* Mill. *Plant Cell Tissue and Organ Culture*, *146*, 345–355. https://doi.org/10.1007/s11240-021-02074-7

Żabicki, P., Mikuła, A., Śliwinska, E., Migdałek, G., Nobis, A., Żabicka, J., & Kuta, E. (2021). Cryopreservation and post-thaw genetic integrity of *Viola stagnina* Kit., an endangered species of wet habitats – A useful tool in *ex situ* conservation. *Scientia Horticulturae*, *284*, Article 110056. https://doi.org/10.1016/j.scienta.2021.110056

Żabicki, P., Śliwinska, E., Mitka, J., Sutkowska, A., Tuleja, M., Migdałek, G., Żabicka, J., Słomka, A., Kwiatkowska, M., & Kuta, E. (2019). Does somaclonal variation play advantageous role in conservation practice of endangered species?: Comprehensive genetic studies of *in vitro* propagated plantlets of *Viola stagnina* Kit. (Violaceae). *Plant Cell Tissue and Organ Culture*, *136*, 339–352. https://doi.org/10.1007/s11240-018-1519-1