Biodiv. Res. Conserv. 3-4: 361-368, 2006

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Engagement of biotechnology in the protection of threatened plant species in Poland

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Abstract: The paper summarizes the contribution of various biotechnological techniques to the protection of the Polish flora. Polish national programmes of wild plant conservation follow the international conventions on the conservation of nature and wildlife. Among the 2750 plant species of the Polish flora, only 296 are listed in the Polish Red Book, but the list of threatened and protected plants includes about 500 species. Biotechnology could serve as a tool for plant protection in various aspects, but the most important among them are: (*i*) vegetative propagation *in vitro*; and (*ii*) protection of the morphogenetic potential of cultured cells, tissues and organs. Extended subcultures and cryopreservation in particular ensure a high level of competence in long-term cultures. For dicotyledons, monocotyledons and ferns, some examples of various biotechnological methods used are presented and discussed.

Key words: biotechnology, Polish flora, morphogenetic potential, systems of plant regeneration, cryopreservation

1. Introduction

The aim of the presented review is to give more details concerning the protection of the Polish flora with the application of biotechnology in comparison to our previous paper presented during the International Biotechnology Conference Vienna 2004 (Rybczyński & Mikuła 2004). The degradation of environmental conditions and their natural decline strongly affect the decreased number of plant species in the Polish flora. Tissue culture methods and biotechnology can serve as a tool for plant protection in various aspects, but the most important among them are: (*i*) *in vitro* vegetative propagation; and (*ii*) protection of the morphogenetic potential of cultured cells, tissues and organs. Extended subcultures and cryopreservation in particular ensure a high level of regenerative competence in long-term cultures.

Polish national programmes of wild plant conservation follow the international conventions on the conservation of nature and wildlife:

- the World Conservation Union (IUCN). Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Washington DC, USA, 3 March 1973;
- the Bern Convention of the Council of Europe (CE). Convention on the Conservation of European Wildlife and Natural Habitats. Bern, 19 September 1979, European Treaty Series 104; 1-12;
- the United Nations (UN) Conference on Environment and Development (the Rio "Earth Summit"). 3-14 June 1992. Convention on Biological Diversity – Text and Annexes; 1-34.

The Government of Poland accepted and signed all international conventions on conservation of nature and wildlife and recently two instructions have been published: by the Ministry of Environmental Protection, Natural Resources and Forestry in 1995 and by the Ministry of Environmental Protection in 2001. Simultaneously with the latter, the *Polish Red Data Book of Plants* (Kaźmierczakowa & Zarzycki 2001; Mirek *et al*. 2006) was published for the national audience.

2. Morphogenic response and its application in biotechnology

2.1. Dicotyledons

Despite the dominance of dicotyledonous plants in the Polish flora, the engagement of biotechnology in their conservation is very limited and only 3 genera: *Drosera, Dionaea* and *Gentiana* have been selected for such studies (Table 1).

In the Polish flora the genus *Gentiana* includes about 20 species and some of them are listed in the Red Book. *Gentiana cruciata*, *G. punctata* and *G. pneumonanthe* have been used for experiments concerning vegetative

Table 1. Dicotyledonous species protected with the help of biotechnology

Name of species	Type of plant	Explant used	Media	References
Drosera anglica	carnivorous	leaf segments, shoot tips	Fast (1981)+BA+NAA	Kukułczanka (1991); Kawiak & Łojkowska (2004)
*Drosera banita**	carnivorous	leaf segments, shoot tips	Vacin & Went	Kawiak & Łojkowska (2004)
*Drosera cuneifolia**	carnivorous	leaf segments, shoot tips	0.5 MS BA+NAA	Kawiak *et al.* (2003)
Drosera intermedia	carnivorous	leaf rosette	RM+Kin+NAA	Kukułczanka *et al.* (1991); Kromer *et al.* (2000)
Drosera rotundifolia	carnivorous	leaf rosette	RM+Kin+NAA	Kawiak *et al.* (2003)
*Dionaea muscipula**	carnivorous	shoot with rhizome	RM+Kin+NAA	Kukułczanka *et al.* (1991)
Gentiana cruciata	gentian	segments of seedling	MS+Kin+2,4-D MS+Dicamba+NAA+BAP+AS, MS+NAA+(Kin or BAP, Zeat)	Mikuła & Rybczyński (2001), (2003); Mikuła *et al.* (2004, 2005a, 2005b)
Gentiana pneumonanathe	gentian	leaf blade apex, axillary meristem encapsulation	MS+2,4-D or Picloram+BA	Bach & Pawłowska (2003); Pawłowska & Bach (2003); Bach *et al.* (2004)
Gentiana punctata	gentian	zygotic embryo	MS+Dicamba+NAA+BAP+AS	Mikuła *et al.* (2004)

Explanation: * – species not present in the Polish flora

plant propagation and biotechnological manipulation of their cells, tissues and organs.

Studies on *G. punctata* provided evidence that somatic embryogenesis is related to the response of primary tissues of a particular part of the analysed mature embryo. Cells of the cultured embryo divided, resulting in embryogenic callus formation. The direct induction of somatic embryo in the starch layer of callus confirmed the earlier described results on tissue culture of various explants of gentian species (Mikuła *et al.* 2004). A protocol for effective vegetative propagation of *G. cruciata* could help in its conservation.

For *in vitro* culture initiation, explants of hypocotyls, cotyledons and roots of seedlings were used. Leaf explants originated from axenic cultures of 2-month-old shoots. By using the upper part of hypocotyl, an observation of initial cell division was carried out. The epidermis and primary cortex appeared to be the tissues responding earliest by cell proliferation. The described type of cell proliferation resulted in the formation of somatic embryos (Mikuła *et al.* 2005b). The discovered morphogenetic potential of various explants, which originated from different stages of plant development, created a model plant regeneration system initiated by a single cell or its protoplast.

The application of MS medium (Murashige & Skoog 1962), supplemented with 2 sets of plant growth regulators, like: kinetin + 2,4-D (dichlorophenoxyacetic acid) or Dicamba + NAA (naphthaleneacetic acid) + BAP (benzylaminopurine) + AS (adenine sulphate), brought about a few years' maintenance of cultures characterised by the embryogenic character of agar and liquid cultures. Multidisciplinary studies of this phenomenon were undertaken to find its source and to improve the high morphogenetic potential of the species. Ultrastructural analysis revealed that intensively dividing cells of various tissues lead to a development of proembryogenic mass (PEM), easily maintained during long-term cultures as a cell suspension. Prolific embryogenic culture required 7-day-long subcultures. The age of the culture positively affected an increase in the ratio of fresh to dry mass of cell suspensions. What is more, the mitotic activity of PEM, expressed as the ratio of the cells in phase G2 to phase G1, amounted to 6%.

The transformation of embryogenic mass into embryos required a 2-step procedure with the application of liquid and agar medium. The embryo-to-plantlet conversion was initiated by elongation of cotyledons and roots. This stage of development required medium supplementation with another set of plant growth regulators: GA3 (gibberellic acid), kinetin and AS (Fig. 1).

In vitro cell and tissue culture used to be a source of various genetic alterations and finally led to a loss of embryogenic potential. Cryopreservation is an important technique for long-term preservation used at any stage of aging culture. In the case of *G. cruciata* the cell cytoplasm rearrangement, as a result of a high sucrose concentration treatment of cultures, together with cell vitrification, appeared the most successful method for preservation of embryogenic cultures of gentians. The regenerants obtained from such cultures did not show any morphological variation or changes in DNA content

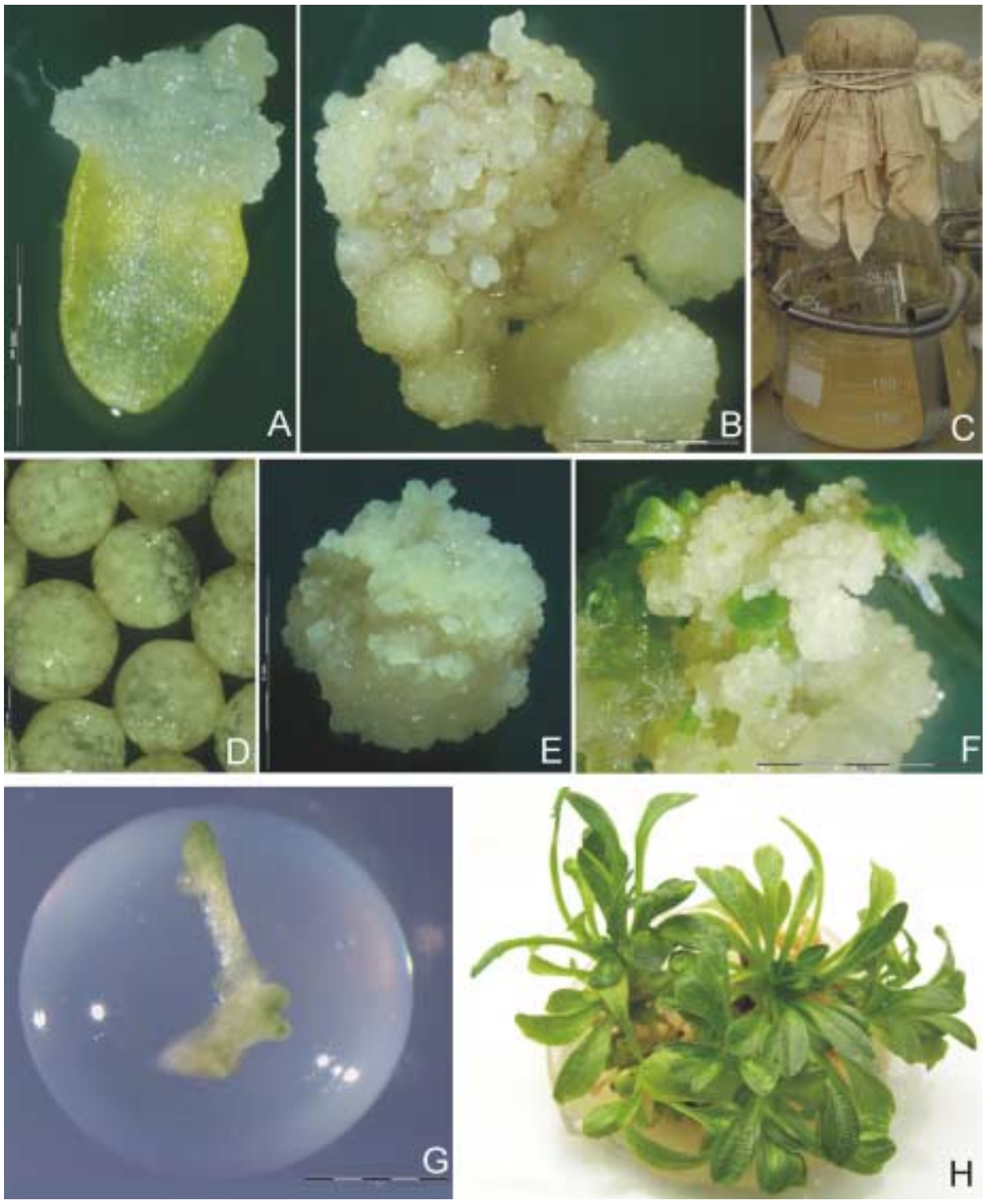

Fig. 1. *In vitro* culture of *Gentiana* sp.
Explanations: A – embryogenic callus proliferation of mature cotyledons; B – somatic embryo regeneration in callus culture; C – long–term cell suspension culture; D – cell suspension aggregates closed in alginate capsules; E – embryogenic callus regeneration after cryopreservation; F – somatic embryo regeneration in post-freezing culture, G – artificial seed; H – prolific growth of regenerant

(Mikuła & Rybczyński 2001, 2003; Mikuła *et al*. 2004, 2005a, 2005b).

In cultures of leaf and apical meristem explants of *G. pneumonanthe*, somatic embryos were directly regenerated. Embryogenic callus was initiated with the help of the media supplemented with the following plant growth regulators: BAP (0.04-8.0 µM) and Picloram or 2,4-D (4.0-8.0 µM). The greatest amount of embryogenic callus was produced on media containing 2,4-D when cultures were maintained in darkness with an extended period of regeneration. Somatic embryo development was stimulated by a decreased auxin content of the media. In their development, frequent morphological anomalies were observed. Embryo

conversion into plantlets required a hormone-free medium. Flow cytometry was used to monitor DNA content stability of embryogenic callus and plantlets converted from somatic embryos. The plantlets had the same DNA content as the *ex vitro* plants.

On the other hand, the high morphogenetic potential of primary explants, supported by their effective encapsulation, laid down the foundations for a new system of vegetative propagation. The system was developed for shoot tips and one-nodal cuttings of seedlings grown *in vitro*. Among the studied cytokinins, BAP at a concentration of 10.0 µM appeared the best for multiplication of the one-nodal cutting explants. For the shoot tips and one-nodal cuttings, the highest numbers of regenerated axillary buds per explant were 3.6 and 4.2, respectively. Root system regeneration required shoot subculture at 1.0 µM IAA (indoleacetic acid). The system can be exploited for unlimited production of individuals, which could be reintroduced into the natural habitats and used for horticultural purposes (Bach & Pawłowska 2003; Pawłowska & Bach 2003; Bach *et al*. 2004).

In experiments aimed to assess the regeneration abilities of selected *Drosera* species and *Dionaea muscipula*, a very high morphogenetic potential of the leaf and rosette explants was shown (Kukułczanka 1991). For example, *Drosera rotundifolia* leaf and leaf rosette explants annually produced 340 000 adventitious buds and 160 000 axillary rosettes, respectively. The results were achieved when RM medium (Reinert & Mohr 1967) was employed and BAP played the most important role in bud formation from rosette explants. The combination of cytokinin and auxin caused the increase in the number of buds and rosettes regenerated in leaf explant culture (Fig. 2).

Fig. 2. Vegetative propagation of *Drosera* sp. *in vitro*

With the use of the above-mentioned regeneration systems, physiological experiments were continued for germplasm storage establishment. Long-term culture required numerous manipulations of physical conditions, such as temperature. In the case of *Drosera* culture, special attention was paid to the effect of temperature decrease on plant size, biomass and chlorophyll content. In the experiments described, all those factors decreased, however the concentration of sugars, proteins and lipids increased.

An effective system of regeneration, using 2 different regeneration pathways with 2 studied explants, could be used to describe somaclonal variation on various levels of analysis. Random amplified polymorphic DNA (RAPD), employed to assess the differences between regenerants, showed that regeneration *via* adventitious bud formation from leaf explants could be a source of variation on the molecular level for morphologically uniform *Drosera anglica* regenerants. For regenerated plantlets of this species, a polymorphism frequency of 0.08% was estimated. However, regeneration *via* shoot budding showed morphological uniformity, and no variation in electrophoretic patterns of *Drosera binata* was described (Kawiak *et al*. 2003; Kawiak & Łojkowska 2004).

Experiments concerning plant hardening off before reintroduction into natural conditions showed that for the root regeneration system of *Drosera intermedia* a liquid medium with perlite ensured better conditions than an agar medium (Kromer *et al*. 2000).

2.2. Monocotyledons

In the case of monocotyledonous species, only representatives of the families Liliaceae and Orchidaceae were the objects of conservation studies (Table 2). The families Liliaceae and Amaryllidaceae proved a high morphogenetic potential in various systems of plant regeneration, since bulb scales were the source of explants. Adventitious bulblet formation appeared to be the most effective system of vegetative propagation, which required auxins and cytokinins. For example, *Leucojum vernum* formed bulblets in the presence of 1.0 mg/l kinetin and 1.0 mg/l NAA (Mikuła, unpubl. data) (Fig. 3). That type of culture was initiated from the bulb scales or their fragments and developed with the help of liquid media. An especially interesting method applied for vegetative propagation of bulb plant consisted in the culture of epidermal thin cell layers as explants isolated from bulbs of *Muscari botryoides*. The frequency of bulblet formation and their further development required medium supplementation with BAP and IBA. The Murashige & Skoog (1962) or Fast (1981) media supplemented with various plant growth regulators were employed depending on the studied species, which resulted in varied effectiveness of bulblet production. The media supplemented with cytokinins and auxins stimulated the formation of secondary bulblets. This type of plant multiplication appeared more

Table 2. Monocotyledeonous species protected by the application of biotechnology

Name of plant	Type of plant	Explant	Media	References
Crocus vernus	corm plant	corm segments	MS+Kin+NAA	Kromer (1985); Kukułczanka & Kromer (2000)
Fritillaria meleagris	bulbous plant	bulb scale	MS+Kin+NAA	Kukułczanka *et al.* (1989)
Leucoium vernum	bulbous plant	bulb scale	MS+Kin+NAA*	Kukułczanka & Kromer (2000); *Mikuła unpubl. data
Lilium martagon	bulbous plant	bulb scale	MS+NAA+BAP	Rybczyński & Gomolińska (1989); Rybczyński *et al.* (1991)
Muscari botryoides	bulbous plant	bulb scale TLExpl*	MS+IBA+BA	Kromer & Kukułczanka (1992)
Cypripedium calceolus	orchid	seeds, protocorm	Fast (1981)	Znaniecka *et al.* (2004)
Dactylorhiza majalis	orchid	seeds, protocorm	Fast+BA+ NAA or IBA	Arczewska (1998); Znaniecka *et al.* (2004)
Epipactis atrorubens	orchid	seeds, protocorm	Fast	Znaniecka *et al.* (2004)
Epipactis palustris	orchid	seeds, protocorm	Fast	Znaniecka *et al.* (2004)
Liparis loeselii	orchid	protocorm	Fast+BA+NAA or IBA	Arczewska (1998)
Orchis morio	orchid	seeds, protocorm	Fast	Znaniecka *et al.* (2004)
Gladiolus palustris	corm plant	corm	MS+2,4-D	Stefaniak (1997a)
Gladiolus imbricatus	corm plant	corm	MS+2,4-D	Stefaniak (1997b, 1998)

Explanation: * TLExpl – Thin Layer Explant

productive when it was additionally supported by liquid medium culture (Kukułczanka *et al*. 1989; Kromer & Kukułczanka 1992; Kukułczanka & Kromer 2000; Rybczyński & Gomolińska 1989; Rybczyński *et al*. 1991). In the case of the genus *Gladiolus*, 2 species were taken for experiments on somatic embryogenesis together with traditional micropropagation. Among 3 studied auxins 2,4-D seemed most effective for embryo-

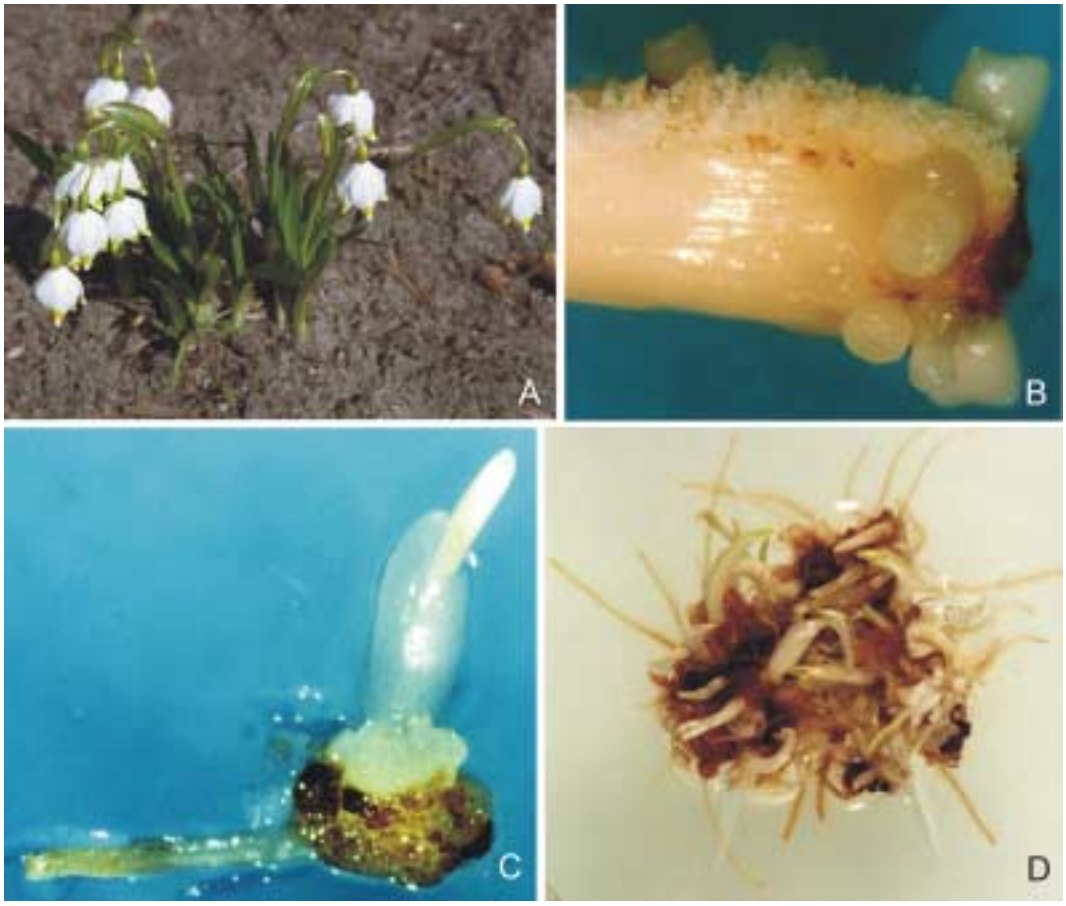

Fig. 3. Vegetative propagation of *Leucoium vernum* from bulb scales
Explanations: A – general view of blooming plant; B – bulblet formation on the primary bulb-scale explant; C and D – sprouting of the first leaf of one and numerous bulbs

genic culture induction in both *G. palustris* and *G. imbricatus*. The slices of corms regenerated embryogenic callus in the presence of 2.0 mg/l 2,4-D within 2 weeks. The somatic embryos formed on the surface of embryogenic callus, passed 2 stages of development: globular and next bipolar with distinct organization of shoot and root primordia (Stefaniak 1997a, 1998). With the application of Ca-alginate the 'synthetic seed' system of the species mentioned above was developed to create a new strategy for micropropagation and storage of genetic resources. Propagules derived from various types of culture, transferred to the MS hormone-free medium, developed into plants morphologically similar to the control (Stefaniak 1997b; Zenkteler *et al.* 2000).

The Polish flora includes 21 species of terrestrial orchids. This type of plants is more exposed to the risk of degradation than the other species that are epiphytes growing on the various natural supports. When culture is initiated by sowing seed onto an agar medium, usually numerous problems must be solved to obtain a sterile culture of seedlings. The liquid culture of green protocorms is characterized by their unlimited multiplication and high production. However, plant regeneration in that type of culture is sometimes very tricky and requires individual studies for each species. For *Dactylorhiza majalis* and *Liparis loeselii*, the effect of plant growth regulators (such as BA, IBA and NAA) was studied and the culture conditions were optimized. The response of plants to exogenous plant growth regulators was species-specific (Arczewska 1998; Znaniecka *et al.* 2004). The progress in involvement of biotechnology in this group of plants is very limited.

2.3. Ferns

Ferns are a heterogeneous group of plants with high polyploidy levels and hybrid forms. The type of reproduction consists of the sexual life cycle in which independent sporophyte and gametophyte generations alternate with each other. Vegetative multiplication with the help of *in vitro* culture usually employs haploid spores, proliferating gametophytes and the diploid apical meristem of rhizomes. All the explants could be used for fern preservation. Table 3 summarises information on the species that have already been used for vegetative propagation and conservation. Some of them, which are evergreen and grow in the crevices of the poor serpentine rocks, belong to the genus *Asplenium*. Their gene bank has been developed as a result of the collection and germination of spores on a relatively poor, sterile culture medium. After a few months on 0.5 MS medium, the spores germinated giving rise to filamentous gametophytes. For their multiplication, in the presence of a reduced salt medium, an extension of the culture in various culture vessels was required. IAA controlled the formation of generative organs with typical species response. The time required for generative organ formation was species-dependent. Sporophyte formation took up to about 17 months to reach a several-leaf stage (Marszał & Kromer 2000, 2004). The genus *Asplenium* includes *A. adulterium*, *A. cuneifolium*, *A. septentrionale*, which are endangered species in Lower Silesia (Dolny Śląsk). The studied species show a considerable variation in spore germination and the time required for gametophyte development from prothallus in 0.5 MS medium. The medium stimulated an increase in gametophyte biomass and sexual maturity. Uncontrolled fertilization resulted in spontaneous sporophyte production. Before hardening and pot culture, the sporophytes were transferred into a medium stimulating root formation (Marszał-Jagacka *et al.* 2005).

Osmunda regalis, strictly protected by law, was used for the investigation of developmental mutations that lead to a decline of its 3 lowland populations. In those studies spore germination and gametophyte cultures were carried out with the application of the relatively simple Knop mineral medium. Light and scanning electron microscopy examinations of the developmental sequence of gametophytes and young sporophytes have

Table 3. Fern species protected with the help of biotechnology

Name of plant	Explant	Medium	References
Asplenium adiantum-nigrum	spores	0.25 MS	Marszał & Kromer (2000)
Asplenium adulternium	spores	0.25 MS	Marszał & Kromer (2000)
Asplenium cuneifolium	spores	0.25 MS	Marszał & Kromer (2004)
Asplenium septentrionale	spores	0.25 MS	Marszał & Kromer (2004)
Polypodium vulgare	rhizome,	0.5 MS + PGR	Zenkteler (2000);
	spores	0.25 MS	Marszał & Kromer (2004)
Osmunda regalis	spores		
Matteuccia struthiopteris	spores		
Blechnum spicant	spores	0.125, 0.25, 0.5 and 1.0 x concentration of Hoagland;	
Cryptogramma crispa	spores	Knop	Zenkteler (1992)
Polystichum braunii	spores	Knudson	
Polystichum aculeatum	spores	White medium	
Asplenium adiandum-nigrum	spores		
Phyllitis scolopendrium	spores		
Osmunda regalis	spores	0.5 Knop's	Zenkteler (2002)

led to the conclusion that lethality is connected with homozygosity of the somatic generation (Zenkteler 1999). In order to solve this problem, heterozygotic sporophytes derived from *in vitro* fertilization, after hardening and a 2-year culture in a greenhouse, were successfully reintroduced into natural sites (Zenkteler 2002).

In natural habitats the growth and development of ferns result in mitotic activity of the meristematic zone of the rhizome. *Polypodium vulgare* was vegetatively propagated with the application of the apical meristem of rhizomes cultured in the presence of 0.5 MS medium supplemented with various plant growth regulators. The best results of culture were achieved when the medium was enriched with 0.05 µM thidiazuron + 0.5 mg/l NAA. In such culture conditions, adventitious and lateral buds were formed. Sporophyte bud cultures required changes of the medium for their rooting (Zenkteler 2000). The above-mentioned examples of experiments show that with the application of the consecutive stages of fern development, i.e. spores, prothalli, gametophytes and apical meristems of rhizomes, a gene bank of ferns could be established.

3. Conclusions

The species protected by biotechnological methods constitute only a small proportion of the total number of threatened species in the Polish flora. The majority of the studied systems of plant multiplication were based on commonly employed media with a limited use of plant growth regulators.

Some species revealed their very high morphogenetic potentials, which were exploited for their multiplication and reintroduction to natural habitats. Due to their morphological potential some of the species could play the role of model plants for the description of plant differentiation initiated by a single cell or its protoplast (Rybczyński *et al*. 2004).

Vitrification, supported by the rearrangement of cell cytoplasm, which resulted in successful cryopreservation of embryogenic cell suspension, could serve as a tool for establishment of an *in vitro* gene bank of protected species.

Acknowledgement. We thank Prof. Dr. E. Zenkteler for her critical reading of the manuscript.

References

Arczewska A. 1998. Studies on the growth and development of European orchids in cultures *in vitro*. Acta Univ. Wrat. 2038 Prace Bot. 74: 15-51.

Bach A. & Pawłowska B. 2003. Somatic embryogenesis in *Gentiana pneumonanthe* L. Acta Biologica Cracoviensia seria Botanica 45: 79-85.

Bach A., Pawłowska B. & Malik M. 2004. Plantlets from encapsulated meristems of *Gentiana pneumonanthe* L. Acta Physiol. Plant. 26: 53-57.

Fast G. 1981. Orchideen Kultur. 222 pp. Verlag Eugen Ulmer, Stuttgart.

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.

Kawiak A. & Łojkowska E. 2004. Application of RAPD in the determination of genetic fidelity in micropropagated *Drosera* plantlets. In Vitro Cell. Dev. Biol. – Plant 40: 592-595.

Kaźmierczakowa R. & Zarzycki K. (eds.). 2001. Polska czerwona księga roślin. Paprotniki i rośliny kwiatowe, wyd. 2, 664 pp. PAN, Instytut Botaniki im. W. Szafera, Instytut Ochrony Przyrody, Kraków.

Kromer K. D. 1985. Regeneration of some monocotyledons plants from subterranean organs *in vitro*. Acta Agrobot. 38: 65-87.

Kromer K. & Kukułczanka K. 1992. Control of morphogenesis in the thin cell layer explants of *Muscari botryoides*. Acta Horticulturae 325: 505-512.

Kromer K., Nowak T., Wojtuń B. & Poturała D. 2000. *In vitro* propagation of the populations of *Drosera intermedia* from Izerskie Mountains. Biuletyn Ogrodów Botanicznych, Muzeów i Zbiorów 9: 147-152.

Kukułczanka K. 1991. Micropropagation and *in vitro* germplasm storage of *Droseraceae*. Botanic Gardens Micropropagation News 1: 37-42.

Kukułczanka K. & Kromer K. 2000. Investigation on propagation of some ornamental plants in tissue culture conditions. Zeszyty Naukowe Instytutu Sadownictwa i Kwiaciarstwa 7: 49-58.

Kukułczanka K., Kromer K. & Cząstka B. 1989. Propagation of *Fritillaria meleagris* L. through tissue culture. Acta Horticulturae 251:147-153.

Marszał J. & Kromer K. 2000. The use of *in vitro* culture methods for the protection of rare and endangered serpentine fern species. Biuletyn Ogrodów Botanicznych, Muzeów i Zbiorów 9: 141-146.

Marszał J. & Kromer K. 2004. The application of the tissue cultures in protection of rare and threatened species of ferns from Lower Silesia. Biotechnologia 2(65): 237-234.

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.

Mikuła A., Fiuk A. & Rybczyński J. J. 2005. Induction, maintenance and preservation of embryogenic competence of *Gentiana cruciata* L. cultures. Acta Biologica Cracoviensia seria Botanica 47: 227-236.

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 Physiol. Plant. 23: 15-25.

Mikuła A. & Rybczyński J. J. 2003. Krioprezerwacja zawiesin komórkowych wybranych gatunków z rodzaju *Gentiana*. Biotechnologia 3(62): 39-51.

Mikuła A., Rybczyński J. J., Skierski 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, The Netherlands.

Mikuła A., Tykarska T., Kuraś M. & Rybczyński J. J. 2005b. Somatic embryogenesis of *Gentiana cruciata* (L.): Histological and ultrastructural changes in seedling hypocotyls explants. In Vitro Cell Dev. Biol-Plant 41: 686-694.

Mikuła A., Tykarska 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, seria Botanica 46: 109-120.

Mirek Z., Zarzycki K., Wojewoda W. & Szeląg Z. (eds.). 2006. Red list of plants and fungi in Poland, 99 pp. W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków.

Murashige T. & Skoog A. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum 15: 473-497.

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

Reinert R. A. & Mohr H. C. 1967. Propagation of *Cattleya* by tissue culture of lateral bud meristems. Proc. Am. Soc. Hort. 91: 664-671.

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. 2004. Plant biotechnology in the protection of Polish flora. Biotechnology Proceedings of the International Review Conference, Nov. 14-18, 2004, pp. 51-60. Polish Academy of Sciences, Scientific Center in Vienna, Austria.

Rybczyński J. J., Mikuła A. & Fiuk A. 2004. Endangered species – model plants for experimental botany and biotechnology. Bulletin of Botanical Gardens, Museums & Collections 13: 59-63.

Rybczyński J. J., Turzyński D., Podyma E. & Orszulak J. 1991. The using of *in vitro* culture for vegetative propagation of protected species of Polish flora – *Lilium martagon* L.. Prace Ogrodu Botanicznego 1: 47-54.

Stefaniak B. 1997a. Successful plant regeneration by somatic embryos of *Gladiolus palustris* Gaud. VIII Conference of plant embryologists, 16-18 Sept. 1997, Gdańsk. Scientific programme and abstracts, p. 79.

Stefaniak B. 1997b. Regeneracja roślin z zarodków somatycznych *Gladiolus imbricatus* L. Zeszyty Naukowe AR Kraków 318, Z50: 253-255.

Stefaniak B. 1998. Analiza procesu somatycznej embriogenezy u dwóch gatunków z rodzaju *Gladiolus*. In: J. Miądlikowska (ed.). Materiały sympozjum i obrad sekcji 51 Zjazdu Polskiego Towarzystwa Botanicznego Gdańsk, 15-19 września 1998, p. 456.

Znaniecka J., Maszczyszyn K. & Łojkowska E. 2004. Establishment of *in vitro* culture collection of endangered European orchids. Abstracts of Czech-Polish-Slovak Scientific Conference of Botanical Gardens 'Biodiversity conservation and ecological education – the challenges in the united Europe', p. 2.

Zenkteler E. K. 1992. Metoda in vitro w rozmnażaniu i okresowym przechowywaniu chronionych oraz rzadkich ginących gatunków paproci. Hodowla roślin i nasiennictwo 5: 20-29.

Zenkteler E. K. 1999. Sporophytic lethality in lowland populations of *Osmunda regalis* L in Poland. Acta Biologica Cracoviensia seria Botanica 41: 75-83.

Zenkteler E. K. 2000. Systemy wegetatywnego rozmnażania paproci *in vivo* oraz *in vitro*. Wyd. Nauk. UAM, seria Biologia, 62, 150 pp. Poznań.

Zenkteler E. K. 2002. *Ex situ* breeding and reintroduction of *Osmunda regalis* L. in Poland. Fern Gaz. 16: 371.

Zenkteler M., Stefaniak B. & Mól R. 2000. Experimental plant embryology at A. Mickiewicz University Poznań. Botanical Guidebook 24: 65-73.