IN VITRO MICROPROPAGATION OF FERN SPECIES (PTERIDOPHYTA) OF BIOTECHNOLOGICAL INTEREST, FOR *EX SITU* CONSERVATION

ALDEA Florentina, BANCIU Cristian, BREZEANU Aurelia, HELEPCIUC Florenţa Elena, SOARE Liliana-Cristina

Abstract. *Asplenium adulterinum* (Milde), *A. lepidum* C. Presl, *Osmunda regalis* L., *A. filix-femina* (L.) Roth, *Polypodium vulgare* L., *A. trichomanes* L. are fern species, of conservative, biotechnological or economic interest, growing in various habitats. The presented studies were based on *in vitro* culture optimization method, involving sterilisation, inoculation, multiplication for medium term conservation of these species. The reactivity of each species was evaluated by biometrical methods (morphometrics and gravimetrics) and biochemicals (peroxidase, esterase, alkaline phosphatase and total protein spectra). *In vitro* experiments revealed a species-specific reactivity, *P. vulgare* presenting the highest reactivity followed by *A. filix-femina*. Research allowed establishing a viable experimental system for *in vitro* multiplication of the following species - *A. trichomanes*, *P. vulgare* and *A. filix-femina* that may be adapted to other fern species of biotechnological and conservative interest. The developed experimental system also allowed creating an *in vitro* germplasm collection for the studied fern species.

Keywords: *in vitro* culture, isoenzymatic spectra, bidimensional electrophoresis, cryoconservation.

Rezumat. Multiplicarea *in vitro* a unor specii de ferigi (Pteridophyta) de interes biotehnologic, pentru conservarea *ex situ*. *Asplenium adulterinum* (Milde), *A. lepidum* C. Presl, *Osmunda regalis* L., *A. filix-femina* (L.) Roth, *Polypodium vulgare* L., *A. trichomanes* L., sunt specii de ferigi, unele cu valoare conservativă ridicată, biotehnologică şi economică, ce provin din diferite habitate. Studiile s-au axat pe elaborarea unei metode optime de introducere în cultura *in vitro* ce implică sterilizare, inoculare, multiplicare în vederea conservării pe termen mediu a acestor specii. Reactivitatea fiecărei specii a fost evaluată pe baza determinărilor biometrice (morfometrice şi gravimetrice) şi biochimice (peroxidaze, esteraze, fosfataze-alkaline şi proteine totale). Experimentele *in vitro* au relevat o reactivitate specie-specifică a acestora: *P. vulgare* manifestând o reactivitate mai mare urmată de *A. filix-femina*. Cercetările noastre au permis stabilirea unui sistem experimental facil pentru multiplicare *in vitro* a speciilor *A. trichomanes*, *P. vulgare* şi *A. filix-femina* ce ar putea fi adaptat şi la alte specii de pteridofite de interes biotehnologic şi/sau conservativ. Sistemul experimental elaborat de noi a permis de asemenea realizarea unei colecţii de germoplasmă *in vitro* pentru speciile de pteridofite studiate.

Cuvinte cheie: cultură *in vitro*, spectre izoenzimatice, electroforeză bidimensională, crioconservare.

INTRODUCTION

The holistic study of important changes to biodiversity by climate changes at global level and by anthropogenic impact imposed more intense concerns regarding the conservation of a significant number of endangered plant species. Ferns are one of most important groups.

In time, ferns attracted worldwide numerous research teams (FERNANDEZ et al., 2010), but also in Romania in the last years (Soare L.C., Banciu C.), due to their age, biotechnological potential and due to a low number of national studies that concern *ex situ* conservation by *in vitro* culture.

Appeared over 300 million years ago ferns are a valuable gene source. Although many species of that period disappeared, some have evolved in species found today, representing a material for studying the genetic mechanisms underlying plant development in general.

In 2012, the International Union for Conservation of Nature (IUCN) estimated that, globally, there were 12,000 species of pteridophytes; 167 species of the 311 evaluated species are threatened (IUCN, 2012).

Compared to previous years, it has been a constant increase in the number of threatened species. Uncontrolled overexploitation, inappropriate forestry practices, urbanization, pollution are the main threats to the diversity of the pteridophytes.

Romanian flora includes 76 species of pteridophytes, of which six species with uncertain presence (CIOCÂRLAN, 2009), 10 species being vulnerable, endangered or critically endangered (DIHORU & NEGREAN, 2009).

In this context, our research focused on the study of three fern species from the Vâlsan Valley: *A. trichomanes*, *A. filix-femina*, *P. vulgare* valuable because of their potential as ornamentals and biosynthesis of secondary metabolites of pharmacological interest (ecdysone - 20E, abutasterone, polypodine B inokosterone 24 - hydroxyecdysone, catechins, osladin isolated from *P. vulgare* species, 4 - vinyl phenol, 4 - vinyl phenol -1 - O - [α - L- rhamnopyranosyl - (1 - > 6) β -D - glucopyranose], arctigenin extracted from *A. trichomanes* species (HO et al., 2010) and the introduction of the species *A. lepidum*, *O. regalis* and *A. adulterinum*, *in vitro* culture.

O. regalis and *A. adulterinum* are rare species, the latter being included in Annex II of the Habitats Directive (Directive 92/43/EEC), the main legal instrument of the European Union created for nature conservation purposes in the European Community.

The aim of this study was to develop an experimental system useful for the *in vitro* multiplication of *A. trichomanes, P. vulgare, A. filix-femina, A. lepidum, O. regalis* and *A. adulterinum* species that could be adapted to other fern species of biotechnological and / or conservative interest. The new developed experimental system has also allowed the realization of an *in vitro* collection of germplasm from the studied species.

MATERIAL AND METHODS

The research took place in the period 2009-2015 and took into account different species of ferns characteristic to various habitats:

- *P. vulgare*, Polypodiaceae family, the Vâlsan Valley;
- *A. trichomanes*, Aspleniaceae family, the Vâlsan Valley;
- *A. filix-femina*, Athyriaceae family, the Vâlsan Valley;
- *A. lepidum*, Aspleniaceae family, Herculane, Vânturătoarea Falls;
- *A. adulterinum*, Aspleniaceae family, Ţarcu Mountains, Căldarea Mătaniei;
- *O. regalis*, Osmundaceae family, Dimitrie Brândză Botanical Garden in Bucharest.

The biological material used for introduction of *in vitro* culture of *A. lepidum, O. regalis* and *A. adulterinum* species was represented by spores that were sterilized in filter paper packets using H_2O_2 30 % as a sterilizing solution or 70% ethanol and 0.1% NaClO, with different sterilizing times.

For *A. trichomanes, A. filix-femina, P. vulgare* species, the biological material was represented by gametophyte obtained by *in vitro* cultivation on ½ MS medium (MURASHIGE & SKOOG, 1962), at a temperature of 22 to 24 ± 2 °C, with a photoperiod 16 hours light / 8 hours dark and a light intensity of 3,000 lux.

For initiating a primary culture, there were used in parallel inoculum represented by gametophytes fragments originating from natural habitats and / or collection of ferns from the University of Pitesti and homogenate obtained by grinding them under aseptic conditions.

When using biological material from nature, superficial sterilization was a prerequisite. This was achieved with 70 % ethanol and a solution of calcium hypochlorite ($CaCl_2O_2$) 6% (SOARE et al., 2011).

The homogenate was inoculated to the media, MS without hormones, liquid, under continuous stirring (75 rot / min.), solid and modified KD (KNUDSON, 1946), at a temperature of 22-24 ± 2 °C, with a photoperiod 16 h light / 8 hours dark and a light intensity of 3,000 lux.

In order to grow and root the obtained sporophytes, there were tested on different media variants of MS basal, KD modified with or without phytohormones and water with agar (8 g / l agar, pH 5.8) (Table 1). Tests for each variant were done in 6 replications.

Table 1. Composition of media tested for the development and rooting of sporophytes of *A. trichomanes, A. filix-femina, P. vulgare* species.

Medium/ hormones	M1	M2	M3	M4	M5	M6	M7
Basal media	MS ½	MS ½	MS ½	MS ½	KD	KD	H_2O
AIB (mg/L)	-	2	-	-	-	-	-
Kinetin (mg/L)	-	-	0,22	1	-	-	-
AIA (mg/L)	-	-	1,8	-	-	-	-
ANA (mg/L)	-	-	-	0,1	-	1	-

Legend:
MS1 / 2 = Murashige and Skoog basal 1962 medium, as amended by reducing halfway mineral salts;
KD - modified Knudson culture medium;
AIB - indolelbutyric acid;
AIA - 3 indoleacetic acid;
ANA - 1- naphthylacetic acid;

Reactivity of each species was evaluated based on biometric determinations (morphometric and gravimetric).

To highlight the differences between gametophytes and sporophytes and to evaluate the maintenance of the fidelity of genetic characters of the regenerants, biochemical markers were used, as well as analysis of isoenzyme spectrum of peroxidases, esterases and alkaline phosphatase and a comparison of the protein profiles.

The extraction of the soluble cytosolic proteins was carried out by grinding gametophyte in 0.1 M phosphate buffer, pH 7 at 4°C. After centrifugation at 15,000 rpm for 10 min, the supernatant was used for the determination of enzyme activity.

Electrophoretic analysis of polyacrylamide gels used a batch system, with a migration gel (10%) and a stacking gel (4%) and the Tris-glycine running buffer 0.05 M, pH 8.3. To separate the total protein SDS was added. The migration of the samples was carried out at 20 mA for 2 hours.

To highlight the electrophoretic bands Coomassie Brilliant Blue G250 was used in the case of proteins, a developing solution containing benzidine, Na-acetate, acetic acid and hydrogen peroxide in the case of peroxidase, and α and β - naphthyl acetate in K-phosphate buffer, pH 8 and Fast Blue BB for the esterase. Alkaline phosphatase was

viewed using acetate buffer, 0.1 M, pH 5, with Na α and β - naphthyl phosphate 0.05M as substrate and Fast Blue RR 0.1% as dye adding few drops of $MgCl_2$ 0.25 M and 0.5 M $MnCl_2$. For two-dimensional electrophoresis, it was used 500 mg of each sample of biological material milled in liquid nitrogen.

The proteins were isolated using the modified phenol-based extraction (HURKMAN & TANAKA, 1986). The dosage of the proteins was carried out using the method of BRADFORD (1978). The migration of the proteins was done on 12.5 % acrylamide gel (v / v) where Coomassie Brilliant Blue 250 was used to highlight the spots.

Molecular weight markers between 14.4 and 116 kDa were used from Fermentas. Gels were scanned and calibrated using the software Labscan 6 (GE Healthcare). The image was analysed with ImageMaster 2D Platinum 6.0 (GE Healthcare).

Acclimation was carried out on four different substrates, differentiated by the pH, the composition and grain size:

- A1 - peat mix for cacti Florimo, pH 6-7;
- A2 - Florimo acidic peat mixture, pH 5-6;
- A3 - Vâlsan Valley soil;
- A4 - soil for houseplants Blumeland, pH 6.5-7.5.

The amount of substrate was identical in all experimental variants that were conducted in six repetitions.

Given that our future studies will focus on cryopreservation, the sporophyte species *A. filix-femina* viability was assessed after a pretreatment with two cryoprotectants at different concentrations (DMSO and glycerol 5 and 10% each) followed by gradual freezing and immersion in liquid nitrogen of the explants (BANCIU et al., 2013).

RESULTS AND DISCUSSIONS

During the 6 years of the study they were addressed several issues. Thus, starting from the premise that proper research starts from a broad knowledge of the analysed biological material, in the first phase a comparative estimation of inter-populational genetic variability in situ of the studied species was considered useful. Also, using biochemical markers sensitive to variation of the conditions in the natural habitat (peroxidase, alkaline phosphatase, esterase) emergence of changes in enzymatic spectra of main types of isoenzymes and on this basis the estimated comparatively existence of inter-populational genetic variability of plants in their natural habitat can be expressed.

Analysis of individuals coming mainly from *A. scolopendrium* and *A. trichomanes* species did not reveal notable differences but changes were observed between gametophytes and sporophytes of each species (BANCIU et al., 2011).

Next, it was initiated a primary *in vitro* culture using various sources of inoculum from plants from natural habitats and / or *in vitro* collection of ferns from the University of Pitești. Since regeneration *in vitro* from spores, very commonly used, is longer lasting, in our research we focused on using as a source of inoculum homogenate obtained by trituration gametophyte.

The reactivity of each species was assessed by biometric measurements (morphometric, gravimetric) and biochemical as well as comparative analysis of isoenzyme spectrum of peroxidases, esterase, alkaline phosphatase, total protein spectrum and electrophoretic analysis of protein profiles (ALDEA et al., 2013).

In vitro experiments have revealed first of all, the species - specific existence of differences, depending on the reactivity species and the growing conditions (type of inoculum, nutrient medium, physical factors). The reactive species proved to be *P. vulgare* and *A. filix-femina*.

An initial finding that emerged from our studies was that the species of pteridophytes analysed *in vitro* showed a modest initial reactivity, sporophyte and gametophyte development being done in a long time. Thus, in case of the species *A. lepidum, O. regalis* and *A. adulterinum*, spore germination was achieved after approximately six weeks (Fig. 1).

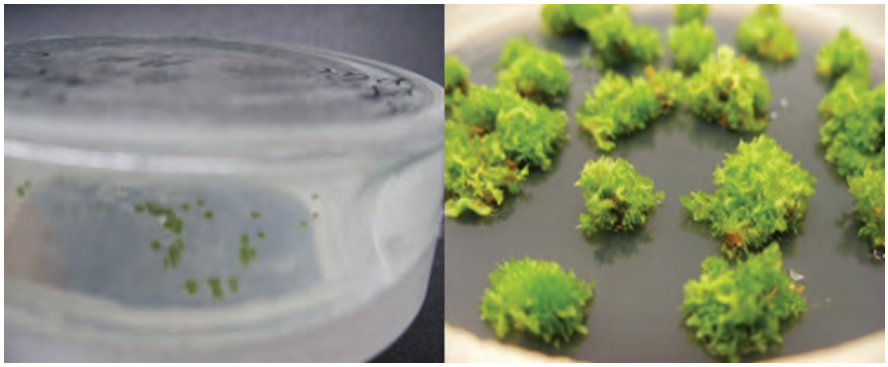

Figure 1. Introduction in *in vitro* culture of *A. adulterinum* species (original).

By using gametophytic homogenate in *A. trichomanes, A. filix-femina, P. vulgare* species, positive results were obtained, this technique constituting a promising alternative that deserves to be considered and optimized for differentiation of sporophyte from gametophyte after 3 weeks of culture (Fig. 2).

Figure 2. Sporophytes obtained by homogenizing the gametophyte a) gametophyte; b), c), d) sporophyte evidentiation at *P. vulgare*, *A. trichomanes*, *A. filix-femina*; e), f) sporophytes from *P. vulgare*; g) *A. trichomanes* (sporophytes); h) *A. filix-femina* (sporophytes) (original).

Secondly, it was noticed that, although the gametophyte can manifest a satisfactory proliferation, that is not associated with the same degree of sporophyte differentiation.

Thus, in case of *P. vulgare* species inoculated on KD solid and MS liquid medium and *A. filix-femina* inoculated on MS liquid under continuous stirring sporophytes were differentiated after 3 weeks of culture.

In the case of *A. trichomanes* sporophyte developed much later, after seven months on MS solid medium, which proves that the use of gametophytic homogenate as a source of inoculum is not generally an optimal way of propagating this species, especially in the case of MS liquid. *A. trichomanes* species material was used for comparison of protein profiles by two-dimensional electrophoresis.

Regarding the reactivity of each species, it was assessed based on biometric measurements (morphometric and gravimetric) (Fig. 3).

Figure 3. Aspects of *in vitro* culture of *P. vulgare*, *A. trichomanes*, *A. filix-femina* species b) sporophyte of *A. filix-femina* species (original).

Optimal results were obtained for *P. vulgare* species on media variants MS1 / 2 with 2 mg / l IBA, MS1 / 2 with 0.22 mg / l kinetin + 1.8 mg / l IAA, rooting is faster on media MS1 / 2 with 2mg / l AIB and water with agar. The optimal hormonal balance for the multiplication of *A. filix-femina* species proved to be in the variants that contained MS1 / 2, MS1 / 2 with 2 mg / l AIB and MS1 / 2 with 0.22 mg / l kinetin + 1.8 mg / l AIA (Figs. 4; 5).

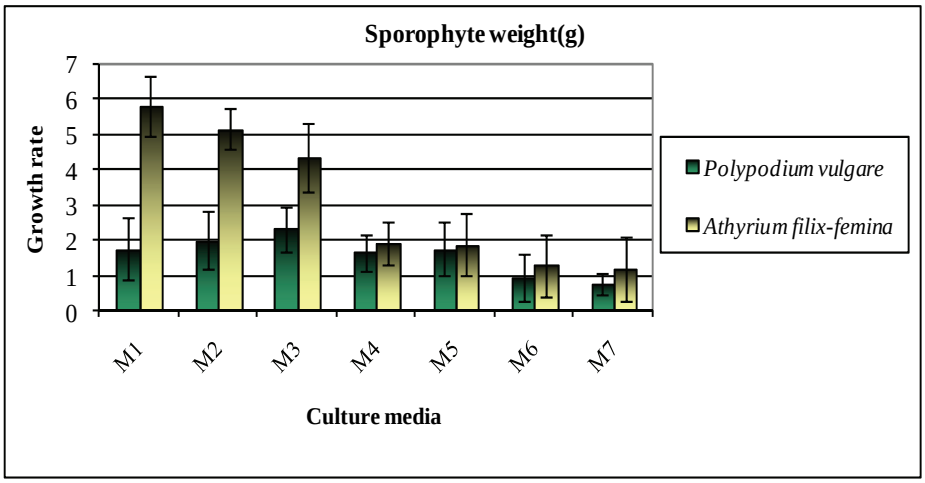

Figure 4. Graphical representation of the weight of the sporophyte.

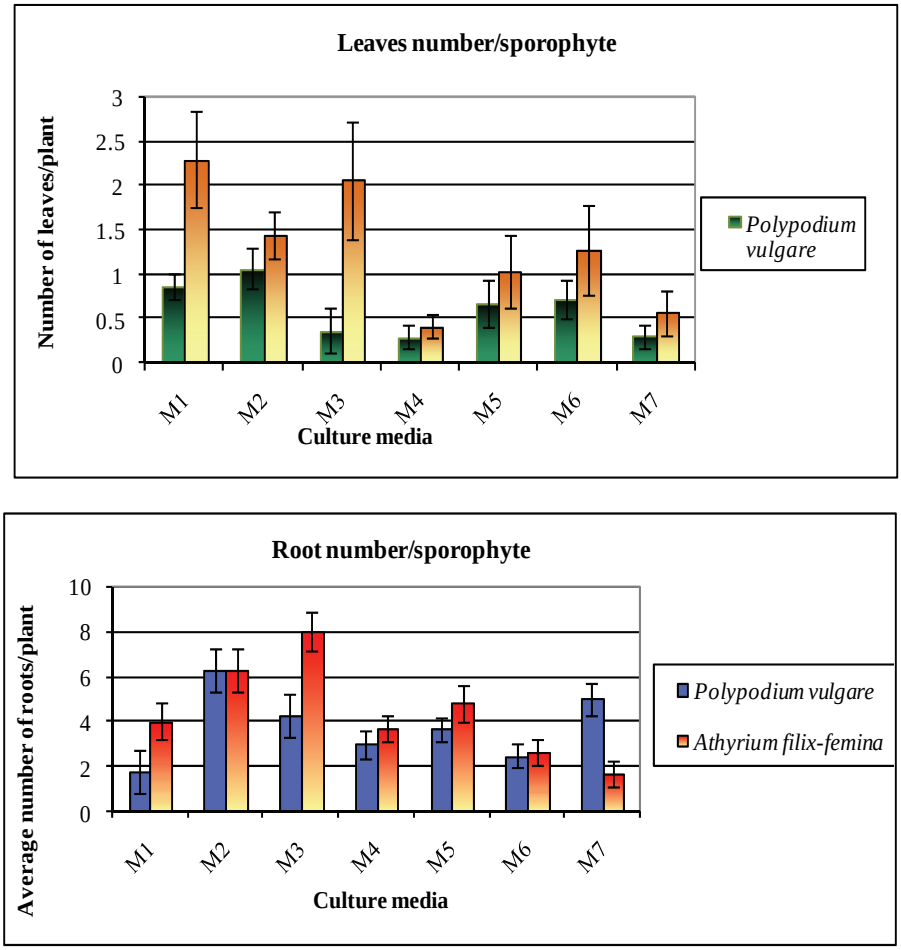

Figure 5. Graphical representation of biometric measurements (morphometric and gravimetric): - top: the number of leaves/ sporophyte, - bottom: the number of roots/sporophyte.

Biochemical investigations have shown that in both species, the enzyme activities of the parent sporophyte are more intense than the same species gametophytes. This can be attributed to a lack of highly specialized cells. Both the gametophyte and the sporophyte are independent structures capable of photosynthesis, but as regards morphology, they are very different. Biochemical analyses revealed minor differences between gametophytes of the same species and only if peroxidase and esterase (Figs. 6; 7; 8; 9; 10; 11).

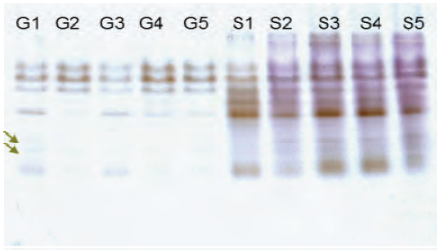

Figure 6. Isoenzymatic spectra of peroxidase on *P. vulgare*, G1-G5 gametophytes, S1-S5 sporophytes) (original).

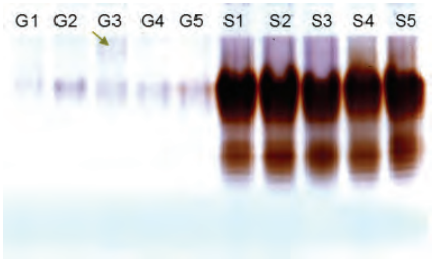

Figure 7. Isoenzymatic spectra of peroxidase on *A. filix-femina*, G1-G5 gametophytes, S1-S5 sporophytes) (original).

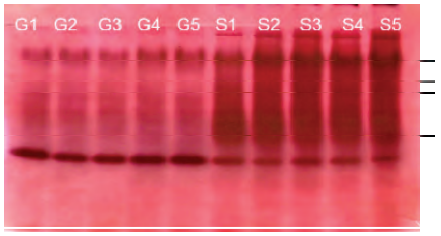

Figure 8. Isoenzymatic spectra of esterase on *P. vulgare*, G1-G5 gametophytes, S1-S5 sporophytes) (original).

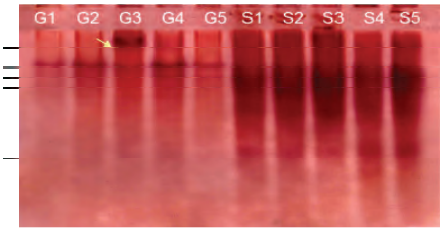

Figure 9. Isoenzymatic spectra of esterase on *A. filix-femina*, G1-G5 gametophytes, S1-S5 sporophytes) (original).

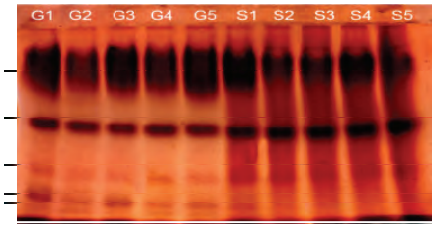

Figure 10. Isoenzymatic spectra of alkaline phosphatase on *P. vulgare*, G1-G5 gametophytes, S1-S5 sporophytes) (original).

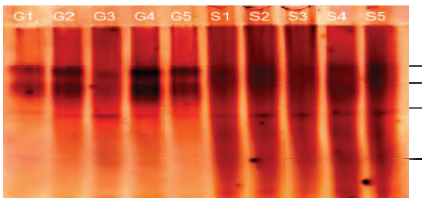

Figure 11. Isoenzymatic spectra of alkaline phosphatase on *A. filix-femina*, G1-G5 gametophytes, S1-S5 sporophytes) (original).

Total protein electrophoretic spectra highlight the differences between gametophytes and sporophytes of the same species. Following electrophoretic separation of the total protein extracts of *A. filix-femina* species it was observed that one of the gametophytes (G3) has a higher intensity of electrophoretic bands (Figs. 12; 13). That gametophyte presented a different isoenzyme spectrum from the others in case of peroxidase and esterase (Figs. 7; 9).

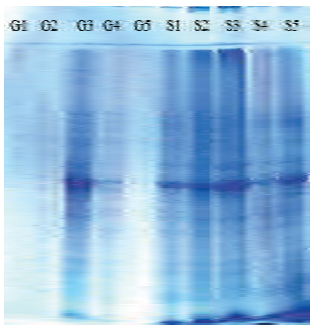

Figure 12. Cytosolic proteins spectrum of the *A. filix-femina* species (G1- G5 gametophytes, S1- S5 sporophyte) (original).

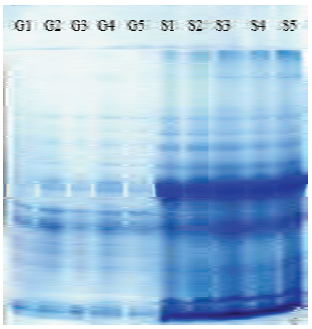

Figure 13. Cytosolic proteins spectrum of *P. vulgare* species (G1- G5 gametophytes, S1- S5 sporophyte) (original).

Preliminary analyses performed by two-dimensional electrophoresis on *A. trichomanes, A. filix-femina, P. vulgare* species allowed the separation in all cases of a significant number of proteins and revealed at the same time, the existence of clear differences between sporophyte and gametophyte (ALDEA et al., 2013). The highest number of spots was detected in *A. trichomanes* species gametophyte (about 950 spots), while *A. filix-femina* had the lowest number of spots (about 400 spots). By analyzing the gel, it was observed that most of the detected proteins had a molecular weight between 18.4 to 66.2 kDa and the optimal pH between 5 and 7 the isoelectric point ranging from 3 to 10. For *A. trichomanes* and *P. vulgare* we could notice that the gametophyte generally had a large number of protein spots compared to sporophyte. It is possible that the presence of a larger number of spots to be correlated with increased synthesis of secondary metabolites, such as in the case of *P. vulgare* gametophyte.

Future studies are are required to explore these issues. We may detect possible changes at the molecular level, which may occur during *in vitro* culture especially during cryopreservation for long term storage of biological material.

Acclimatization

An important element in successful experiments and conservation and micro multiplication is setting up the acclimatization process to *ex vitro* conditions. Our research has shown that for each studied biological material, acclimatization required differentiated conditions specific to each species. Thus, comparative observations have revealed, for instance, that sporophytes of *P. vulgare* species accommodate much easier to *ex vitro* conditions than those belonging to *A. filix-femina* species. They often exhibit differentiated requirements for growth and development, which is why the percentage of plants that survived was lower.

Following acclimation, it was observed that the sporophytes of *P. vulgare* species accommodate much easier to *ex vitro* conditions than those belonging to *A. filix-femina* species, due to different requirements for growth and development, the percentage of plants that survived being lower (Table 2) (Fig. 14).

Table 2. The percentage of acclimatization on 4 types of substrate for sporophytes of *A. filix-femina* and *P. vulgare* species.

Substrate/species	A1	A2	A3	A4
P. vulgare	100%	100%	100%	83%
A. filix-femina	83%	66%	66%	83%

Figure 14. Successive stages of acclimatization: a), b), c) *P. vulgare*, d) *A. filix-femina* (original).

Long-term preservation of biological material by cryopreservation

In order to establish an optimal experimental protocol for successful long term preservation by cryopreservation, we considered necessary preliminary tests aiming to maintain cell viability inoculum subject to treatments with extreme negative suboptimal temperatures (-196^{o}C) over different periods of time.

Regarding the viability of *A. filix-femina* sporophyte, after exposure to low temperatures, microscopy images have revealed the survival of plants. This is a first step, very important for the success of the process as a whole.

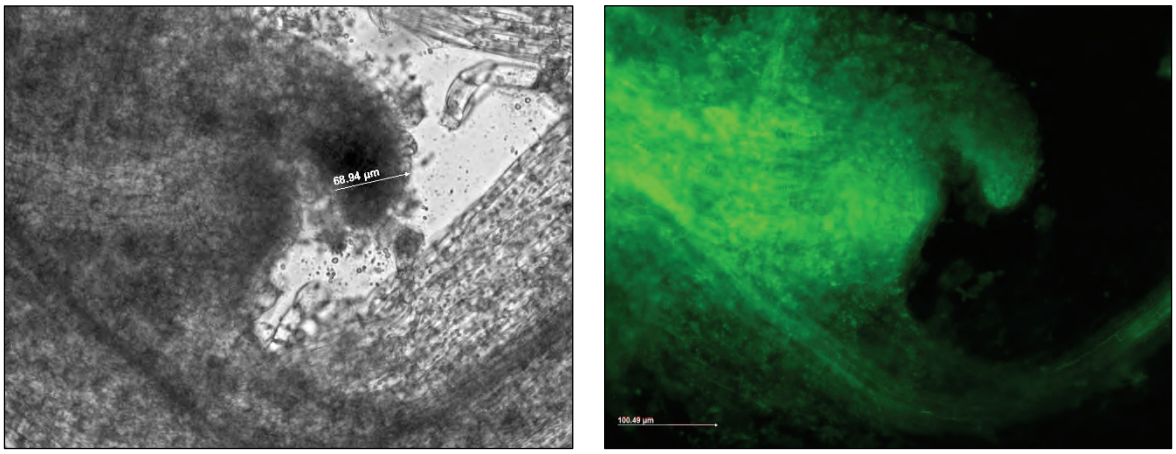

Figure. 15 Left: Meristematic apex of fern sporophytes in visible light (original), Right: Meristematic apex in UV light visualized by GFP filter (original).

Viability tests based on the use FDA (REINERT & BAJAJ, 1977) carried out on fragments belonging to *A. filix-femina* sporophyte have confirmed their ability to adapt to the experimental conditions (BANCIU et al., 2013). The process used by us, based on the application of pretreatment with two cryoprotectants in varying concentrations (with possible cytotoxic effects on cytoplasmic content), followed by freezing and gradual immersion in liquid nitrogen, have given positive results. The images obtained in fluorescence microscopy, after applying the vital dye mentioned, showed a satisfactory viability rate and revealed the successful possibility of applying this method to other fern species of biotechnological and / or conservative interest (Fig. 15).

CONCLUSIONS

Our research over six years of study successfully showed the possibility of using *in vitro* techniques on ferns of biotechnological and/or conservative interest.

The potential of the *in vitro* system developed for this group of plants, based on biometric and biochemical analyses is differentiated, depending on the reactivity of each species and the growing conditions (type of inoculums, nutrient medium, physical factors).

The use as a source of inoculums for initiation of the *in vitro* culture of homogenate obtained by the trituration of the gametophyte has boosted the capacity to disseminate them and it is advisable to obtain a large number of sporophytes, in all cases, especially for the species with a short life cycle.

The optimal nutrient media for both initiation and proliferation of the gametophyte and sporophyte were KD and variants of basal MS (1962) diluted in half.

The physical state of the nutrient medium has played an important role. In the case of *P. vulgare* species, MS1 / 2 liquid revealed a high growth rate.

Two-dimensional electrophoresis allowed a more accurate separation of the proteins of the species *A. trichomonas, P. vulgare* and *A. filix-femina* compared to polyacrylamide gel electrophoresis.

Proteomic profile analysis clearly shows the differences between the three sporophytes and gametophytes species.

This study presents a preliminary analysis of proteic profiles that can allow the detection of possible changes that may occur at the molecular level during the *in vitro* culture, especially in the cryopreservation for long term storage.

Our research allowed an easy experimental system for *in vitro* multiplication of *A. trichomanes, P. vulgare* and *A. filix-femina* species that can be adapted to other fern species of biotechnological and conservative interest.

The experimental system developed by us has also allowed the realization of an *in vitro* germplasm collection of studied species of pteridophytes.

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REFERENCES

ALDEA FLORENTINA, HELEPCIUC FLORENTA ELENA, BANCIU C., BREZEANU AURELIA, SOARE LILIANA CRISTINA. 2013. Comparative studies between ferns gametophyte and sporophyte by bidimensional electrophoresis. *Oltenia. Studii şi comunicări. Ştiinţele Naturii*. Muzeul Olteniei Craiova. **29**(1): 120-124.

BANCIU C., ALDEA FLORENTINA, HELEPCIUC FLORENTA ELENA, SOARE LILIANA CRISTINA. 2011. Characterization by biochemical markers of the interpopulational variability of ferns species with biotechnological properties. *Proceedings of the third international Conference "Research people and actual tasks on multidisciplinary sciences"*. Lozenec, Bulgaria. **3**: 266-270

BANCIU C., ALDEA FLORENTINA, SOARE LILIANA CRISTINA. 2013. Assessing the viability of *Athyrium filix-femina* (L.) sporophyte after extreme cold exposure. *Oltenia. Studii şi comunicări. Ştiinţele Naturii*. Muzeul Olteniei Craiova. **29**(2): 80-83.

BRADFORD M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72**: 248-254. Available on line at: http://hoffman.cm.utexas.edu/courses/bradford_assay.pdf (Accesed: August 7, 2012).

CIOCÂRLAN V**.** 2009. *Flora Ilustrată a României. Pteridophyta et Spermatophyta*. Edit. Ceres. București: 78-96.

DIHORU GH. & NEGREAN G. 2009. *Cartea Roșie a Plantelor Vasculare din România*. Edit. Academiei Române, București. 630 pp.

FERNANDEZ HELENA, KUMAR A., REVILLA MARIA ANGELES. 2010. *Working with Ferns: Issues and Applications*, Springer. New York. 386 pp.

HO R., TEAI T., BIANCHINI J., LAFONT R., PHILA RAHARIVELOMANANA. 2010. Ferns: From traditional Uses to Pharmaceutical development, Chemical Identification of Active Principles. In: Fernandez et al. (Eds.) *Working with Ferns: Issues and Applications*. Springer. New York: 321-347.

HURKMAN W. J. & TANAKA C. K. 1986. Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiology*. American Society of Plant Biologists. **81**(3): 802-806, http://www.plantphysiol.org/content/81/3/802.full.pdf+html. (Accesed: December 3, 2012).

KNUDSON L. 1946. A new nutrient solution for germination of orchid seeds. *American Orchid Society Bulletin.* **15**: 214–217.

MURASHIGE T. & SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum*. Wiley-Blackwell. New Jersey. **15**: 473-497.

REINERT J. & BAJAJ Y. P. S. 1977. *Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture*. Springer-Verlag Berlin-Heidelberg. 803 pp.

SOARE LILIANA CRISTINA & MOTOUNU MONICA. 2011. *Conservarea diversității pteridofitelor din Valea Vâlsanului*. Edit. Universității din Pitești. 214 pp.

***. EC .1992. Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora http://eur-lex.europa.eu/ (Accessed: March 22, 2015).

***. IUCN. 2012. The IUCN Red List of Threatened Species. Version 2012.2. http://www.iucnredlist.org/ (Accesed: May 8, 2012).

Aldea Florentina, Banciu Cristian, Brezeanu Aurelia, Helepciuc Florenţa-Elena
Institute of Biology Bucharest, Romanian Academy,
296 Splaiul Independenței, 060031 Bucharest, Romania.
E-mail: florentina.aldea@ibiol.ro

Soare Liliana-Cristina
University of Pitesti, Faculty of Sciences,
1 Târgul din Vale St., 110040, Pitești,Argeș.Romania.
E-mail: soleil_cri@yahoo.com

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