ROMANIAN ACADEMY
BUCHAREST INSTITUTE OF BIOLOGY

DOCTORAL THESIS

Ex situ conservation of some species of pteridophytes from Romania which have conservative and biotechnological interest

- resume -

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Contents

INTRODUCTION

The unity, diversity and interdependence between unicellular and multicellular organisms are the underpinnings for the survival of the entire biological world. Deterioration and loss of biodiversity - as a result of the industrial revolution at the beginning of the nineteenth century (leading to the decrease of natural resources), and massive deforestation – together with the accumulation of waste of any kind from water and soil (resulting in increased soil erosion), in addition to excessive urbanization are the most serious threats to the environment on a global scale, along with climate change, endangering the existence and development of human society as a whole (Brânzan, 2013).

Notwithstanding the present scientific and technical capacity, the extinct species can no longer be "recreated", so we have a moral obligation to ensure the preservation of biological diversity. Biodiversity values compose the natural heritage, which is the legacy of the past that we enjoy today and that we ought to pass on to future generations.

Given the fact that the extinction of a considerable number of species results in severe perturbation of biodiversity, a worldwide network of international bodies and organizations has emerged in recent years, to which Romania is also a party, aimed at contributing to nature conservation as a whole.

Amongst its objectives, proclaimed principles and recommended measures, the Convention on Biological Diversity (CBD), signed in Rio de Janeiro on 5 June 1992 and entered into force on 29 December 1993, is the most important international instrument for coordinating national policies and strategies in the field, its provisions being ratified and adopted by 193 states (SNPAB, 2011).

The general objectives of the Convention are: the preservation of biological diversity, the sustainable use of the components of biological diversity, the fair and equitable sharing of the benefits arising from the use of genetic resources (SNPAB, 2011).

At the 10th Conference of the Parties to the Convention on Biological Diversity, held in October 2010 in Nagoya, Japan, the Strategic Plan 2011-2020 on Biodiversity was adopted, which includes targets and objectives for the preservation of plant diversity.

Romania ratified the **CBD** by Law no. 58/1994.

Three national strategies and action plans on biodiversity conservation (SNPAB) have been developed so far, the third one in 2011 referring to the period 2011-2020, Romania

granting significant importance to sustainable *ex situ* collections, to the recovery of *ex situ* endangered resources and expansion of *ex situ* conservation (SNPAB, 2011).

Conservation programs aim at species bearing economic value, wild species related to cultivated species that are the source of genes resistant to diseases and pests, medicinal plants, rare, endangered or vulnerable plants (Blându and Holobiuc, 2007).

Genetic heritage of great value, which appeared more than 300 million years ago, despite the fact that pteridophytes are known for their resistance, they are now being threatened.

Some ferns are used as ornamental plants, as a source of food or for phytoremediation of contaminated soils (*Ptteris vittata* and *Pityrogramma calomelanos* are fern species that accumulate large amounts of arsenic) (Niazi et al., 2012). At the same time pteridophytes have a rich content in secondary metabolites (terpenoids, alkaloids, flavonoids, phenols, etc.) their isolation and identification contributing to the understanding of the pharmacological properties of this group (Fernandez et al., 2010).

In 2012, the International Union for Conservation of Nature (IUCN) estimated that 12,000 species of pteridophytes exist globally, with 167 species being threatened out of the 311 species of pteridophytes evaluated (IUCN, 2012).

As compared to previous years, a continuous increase in the number of threatened species has been observed. Uncontrolled overexploitation, inappropriate forestry practices, urbanization, pollution are the main threats to pteridophyte diversity.

Romania's flora comprises 76 species of pteridophytes, out of which six species with uncertain presence (Ciocârlan, 2009), 10 species of pteridophytes being vulnerable, endangered or critically endangered (Dihoru and Negrean, 2009).

As a result of the expanding degradation of natural ecosystems, a network of protected natural areas has been established in order to contribute to the maintenance of biological diversity in the affected areas.

Among these there is the natural reservation “Vâlsan Valley” situated in Arges county. Declared a natural area of Community interest in 2007, unfolding on a surface of 10.000 ha and comprising 300 species of protected plants and animals, it is characterized by a high diversity of Pteridophytes, with over 40 % of the total species that can be found in Romania being identified in this area (Soare and colab., 2011).

In this framework, the research was conducted within the PN II Project no. 32 - 174/2008: "Pilot project for the identification and conservation ex situ and in situ of the

biodiversity of pteridophytes in the protected area "Vâlsanului Valley" carried out in partnership between the University of Pitești, INCDBH Ștefănești Argeș, the Institute of Biology Bucharest, Romanian Academy,the University of Bucharest, in the period 2008-2011.

The personal research was carried out within the Department of Plant and Animal Cytobiology of the Institute of Biology Bucharest, Romanian Academy, focusing on the study of four species of ferns from the Vâlsan Valley: *Asplenium trichomanes, Athyrium filix-femina, Polypodium vulgare* and *Asplenium scolopendrium* , and of other species from other areas. The researches species are valuable due to their potential as ornamental plants as well as that of biosynthesizing secondary metabolites of pharmaceutical interest.

Other experiments targeted at the analysis of total proteins, based on the use of two-dimensional electrophoresis in species of ferns typical of Romania and Poland's flora (*Asplenium trichomanes, Athyrium filix-femina, Polypodium vulgare*) were conducted in the Biodiversity Conservation Center within the Botanical Garden in Warsaw during the interacademic exchanges with the theme: "*Ex situ* conservation of rare and threatened plant species from Carpathian Flora".

The level of ploidy in gametophytes and sporophytes species (*Asplenium trichomanes, Athyrium filix-femina, Polypodium vulgare*) was determined in the Flowcytometry Laboratory of the Institute of Botany of the Slovak Academy of Sciences, during the interacademic exchanges

We would like to thank both institutions for the fruitful collaboration developed over time.

Our thanks also go to the management of the Institute of Biology of the Romanian Academy in Bucharest, which assured me the optimal context for the doctoral activity, to my colleagues dr. Cristian Banciu, dr.Elena-Monica Mitoi, dr. Florența-Elena Helepciuc, dr. Gina-Carmen Cogălniceanu dr. Anca Manole, dr. Mihaela-Irina Holobiuc, dr. Carmen-Rodica Maximilian dr. Rodica Catană as well as to Assoc. professor PhD. Liliana-Cristina Soare, from the University of Pitești, for the useful discussions and suggestions given during the training.

In a very special way, I thank Ms. CS I PhD. Aurelia Brezeanu, a huge model of researcher and very kind person, who was by my side with her invaluable guidance, both in the elaboration of the thesis and personally, in the more difficult context of my life. I will remain deeply grateful to her!

Last but not least, I would like to thank the family for ensuring an optimal climate during the period time dedicated to the thesis preparation.

The present paper comprises six chapters, three of which contain data from the specialty literature on plant conservation and general presentation of pteridophytes, important legislative aspects at global and national level aimed at preserving biodiversity and environmental protection by legislating international conventions and European policies to which Romania is a party and the general presentation of the pteridophytes, and the following two chapters highlight the practical part of the work represented by a description of the materials used and the research methodology, as well as the results of the carried out research; the conclusions are presented at the end of the work.

The chapter analysis of the thesis highlights the following aspects:

I. THEORETICAL PART – THE STAGE OF KNOWLEDGE

CHAPTER 1

GENERAL CONSIDERATIONS REGARDING THE CONSERVATION OF PLANT SPECIES

1.1. Conservation of plant genetic resources. Conservation methods

The aspects of major interest in the **first chapter** are described below:

- the importance of *in situ* and *ex situ* preservation of plants is highlighted, which has gained international recognition through its inclusion in Article 8 of the Convention on Biological Diversity and through the provisions of the Objectives VII (75% of endangered species to be preserved *in situ*) and VIII. (at least 20% of endangered plants are included in recovery, restoration programs), the Global Plant Conservation Strategy, and *ex situ* conservation by Article 9 of the CBD and Objective VIII of the Global plant Conservation Strategy (at least 75% of endangered species should be accessible in *ex situ* collections, preferably in the country of origin).
- *in situ* and *ex situ* conservation methods are briefly described

1.2. International bodies involved in nature conservation

- the main international bodies active in the field of biodiversity **IUCN**, **UNEP** and **WWF** are reviewed. Through their action on March 5, 1980, the **World Conservation Strategy** was set up, which has as main goals: conservation of natural resources necessary to maintain life, conservation of genetic diversity by preventing the extinction of species and ensuring a judicious exploitation of resources (Păunescu, 2008).

1.3. Plant conservation strategies

This sub-chapter lists the main objectives of the conservation strategies that have been adopted at global, European and national level.

The Global Strategy on plant Conservation reconsiders the importance of plants in the overall context of biodiversity as an essential element in the functioning of ecosystems and aims to halt the continuing loss of plant diversity and to ensure a safe, sustainable future where human activities support plant diversity, and plant diversity supports and improves human livelihoods.

The European Union ratified the *Convention on Biological Diversity* (CBD) on 21 December 1993 and, in implementing the provisions of the Convention, it took a leading role at international level by adopting a series of strategies and action plans to help halt biodiversity loss.

As a signatory of the CBD, Romania is required to apply the provisions of Article 6, which stipulates that the Parties shall *"draw up national strategies, plans and programs for the conservation of biological diversity and the sustainable use of its components, or adapt existing strategies, plans or programs to that end"*.

The 3rd SNPAC refers to the period 2010 - 2020 and considered the assessment of the current situation of species, habitats and ecosystems.

CHAPTER 2
PTERIDOPHYTES – AN OVERVIEW

Chapter two begins with a brief history of pteridology research at international and national level, in our country being remarked the activity of PhD Associate Professor Liliana Cristina Soare, President of the Romanian Pteridology Association, author of the work *Zygotic and somatic embryogenesis in some Pteridophytes*, coordinator of the book entitled

Conserving the Diversity of Pteridophytes in the Vâlsan Valley, as well as author of many articles on ferns. The chapter continues with a classification according to Smith et al. (2006) and life cycle in pteridophytes.

CHAPTER 3
METHODS OF PRESERVATION OF PTERIDOPHYTES

Chapter three describes methods of preservation in pteridophytes and highlights the need to encourage research, in particular to develop methodologies and techniques aimed at optimizing conservation practices, in accordance with the GSPC 2011-2020.

II. EXPERIMENTAL PART

CHAPTER 4
OBJECTIVES, MATERIAL AND METHODS

4.1. Research objectives

I order to highlight their roles in medicine, the bioactivity of ferns has been studied internationally. The studies confirmed various benefits such as antioxidants, antimicrobials, antivirals, anti-inflammatory, anti-tumor, anti-HIV, etc. a promising effect has been observed on cell proliferation inhibition and stimulation of apoptosis in the cell line of human liver cancer (Goswami et al., 2016).

The research focused mainly on the study of four species of ferns from the protected area "Vâlsan Valley": *Asplenium trichomanes, Athyrium filix-femina, Polypodium vulgare, Asplenium scolopendrium* valuable for their potential as ornamental plants as well as that of biosynthesizing secondary metabolites of pharmaceutical interest.

Two other species of ferns *Osmunda regalis* and *Asplenium adulterinum* have been introduced to *in vitro* culture, their importance being given by the status of extinct species such as *Osmunda regalis* (Boscaiu et al. 1994), (Dihoru and Dihoru, 1994) or critically endangered species as in the case of the *Asplenium adulterinum* (Dihoru and Dihoru, 1994; Dihoru and Negrean, 2009). *Asplenium adulterinum* is protected at European level by an important piece of legislation, namely: *The Habitats Directive,* Annexs IIb, IVb (Mihailescu et al.., 2015).

Another criterion underlying at the selection of the researched species was the extremely scarce volume of *in vitro* studies in Romania in these species, in general and especially of preoccupation regarding *ex situ* preservation of pteridopsis by biotechnological processes. Research on cryopreservation and techniques such as two-dimensional electrophoresis for total protein analysis, flow cytometry for determination of the degree of ploidy and RAPD analysis for genetic stability assessment in pteridophones were not reported in the country's literature.

The aim of the research carried out throughout this doctoral thesis was to establish an easy and reproducible experimental system for *in vitro* multiplication for medium and long term conservation (cryopreservation) of pteridophyte species of biotechnological and / or conservative interest.

The main objectives were the following:

1. **Evaluation of the intraspecific diversity in pteridophytes from Valea Vâlsanului.**
2. **The initiation, proliferation and regeneration of *in vitro* cultures of fern species.**
3. **Carrying out biochemical analyses on gametophytes and sporophytes of the two studied species (*Athyrium filix-femina* and *Polypodium vulgare*) which aimed at highlighting the possible changes in isoenzymatic spectra.**
4. **Establishing an optimal experimental protocol for successful long-term preservation by cryostorage.**
5. **Identification of an optimal method of sterilizing spores from the endangered species *Asplenium adulterinum* and *Osmunda regalis*.**
6. **RAPD analysis on Asplenium adulterinum for the assessment of genetic stability.**

4.2. Biological material used

This subchapter includes the presentation of the pteridophytes used in the experiments, the description of the biological material and the work methodology.

Pteridophyte taxonomies used in experiments

Polypodium vulgare L., Polypodiaceae family, Vâlsan Valley

Asplenium trichomanes L. Aspleniaceae family, Vâlsan Valley

Athyrium filix-femina (L.) Roth,, Athyriaceae family, Vâlsan Valley

Asplenium scolopendrium L. Aspleniaceae family, Vâlsan Valley

Asplenium adulterinum Milde, Aspleniaceae family, Țarcu Mountains, Căldarea Mataniei,

Osmunda regalis L. Osmundaceae family, Botanical Garden *Dimitrie Brândză* in Bucharest

4.3. Work methodology

The research methodology includes several sub-chapters:

4.3.1 Short and medium term conservation consisting of:

4.3.1.1. Listing the types of explants used to initiate in vitro culture

- inocula represented by gametophyte fragments were used in parallel with homogenate obtained by grinding the above mentioned fragments in aseptic conditions (Aldea et al., 2016)
- for the initiation of *Osmunda regalis* and *Asplenium adulterinum species* in the *in vitro* culture we used biological material represented by spores.
- leaf fragments with and without spores, rizoma, petiole, radicular tips from sporophiles in the juvenile phase.

4.3.1.2. Description of the sterilization protocol of the biological material

Ethyl alcohol 700, HgCl2 0.1% or H2O2 30% (for spores) were used depending on the type of biological material used for sterilization.

4.3.1.3. Presentation of culture media

Various culture media MS (Murashige and Skog, 1962), Knopp, modified Knudson (Berg and Bustamante, 1974), Gamborg (1968) were used, supplemented or not with

different concentrations of phytohormones depending on the species and the desired purpose.

4.3.1.4. Initiation, proliferation and in vitro regeneration of the used pteridophytes species

4.3.1.4.1. Obtaining regenerants using as a source of inoculum homogenate from gametophyte fragments in the juvenile stage

The homogenate was inoculated on MS (Murashige-Skoog, 1962) medium, without hormones, liquid and under continuous stirring conditions) (75 rot/min.), solid and KD modified (Knudson, 1946) at a temperature of 22-24±2ºC, with a photoperiod of 16 hours light/8h dark and light intensity of 3000 lux.

4.3.1.4.2. Determining the growth rate of regenerants

Explants collected from gametophyte culture were inoculated on three hormone-free nutrient media (MS1 / 2, MS1 / 4 and Knopp medium) in order to establish the growth rate and determine the optimal conditions to stimulate gametophyte growth and sporophyte differentiation.

The growth rate was calculated by applying the following formula:

Growth rate= (final mass-initial mass)/initial mass

4.3.1.4.3. The effect of some physical factors on regeneration - light

In our experiments we focused on tracking the effect of lighting regime on the homogenized gametophyte culture for a period of 30 days in a solid MS1/2 medium.

4.3.1.4.4. The effect of the culture medium on the development and rooting of regenerants

In order to develop and root the obtained sporophytes, tests were performed on different variants of basal media MS, modified Knudson, with additional phytohormones and agarized water (8 g / l agar, pH 5.8) (table 2) or without these latter. For each tested variant, 6 replications were performed.

4.3.1.4.5. The effect of hormones in the culture medium on the callus or growth of the sporophyte

Starting from the disagreement of some studies according to which hormones added to culture media may or may not (Shukla, 2014) influence the callus or growth of sporophyte, for the species *Asplenium adulterinum* and *Polypodium vulgare* we added hormones to culture media. The basal medium was V1 (Gamborg) without Fe and NH4NO3 + 30g sugar to which BAP (benzyl-amino-purine) and 2.4 D (2.4 acid, dichloro-phenoxyacetic acid) were added.

4.3.1.4.6 Obtaining regenerants using as a source of inoculum leaf fragments with and without spores, rhizome, petiole, root tips from juvenile sporophytes

The explants were sterilized and inoculated on MS ½ medium in hoods with sterile air flow. The culture was maintained at a temperature of 22-24 ± 2ºC, with a photoperiod of 16 hours light / 8 hours darkness and a light intensity of 3000 lux.

4.3.1.4.7. Obtaining gametophytes using spores as a source of inoculum

Spores collected from *Osmunda regalis* and *Asplenium adulterinum* were sterilized and inoculated on MS ½. The culture was kept in the growth room at a temperature of 22-24 ± 2ºC, with a photoperiod of 16 hours light / 8 hours darkness and a light intensity of 3000 lux..

4.3.1.5. Culture conditions

The cultures were kept in the growth room at a temperature of 22-24 ± 2ºC, with a photoperiod of 16 hours light / 8 hours darkness and a light intensity of 3000 lux.

4.3.1.6. Acclimation of the resulted regenerants

It was carried out on four variants of substrate, differentiated by pH, composition and granulation. The amount of substrate was identical in all experimental variants that took place in 6 replications. The reactivity of each species was assessed on the basis of biometric determinations (morphometric and gravimetric).

4.3.2. Procedures used in the biochemical analysis of the regenerants

In order to highlight the intraspecific natural variability existing both within a population as well as between two populations located at a significant distance from each other in the species *Asplenium trichomanes* and *Asplenium scolopendrium* and also the

differences between gametophytes and sporophytes of the species *Asplenium trichomanes*, *Athyrium filix-femina* , the isoenzymatic spectrum of alkaline peroxidases, esterases and phosphatases was analyzed at biochemical level, and a comparison of proteomic profiles was made.

4.3.2.1. Extraction of soluble cytosolic proteins was performed by grinding the gametophyte in 0.1 M phosphate buffer, pH 7 at 4°C. After centrifugation at 15.000 rpm, 10 min, the supernatant thus obtained was used for the determination of enzyme activity.

4.3.2.2. Electrophoretic analyses used polyacrylamide gels in a discontinuous system with a migration gel (10%) and a concentration gel (4%) and Tris-glycine 0,05M, pH-8,3 as a migration buffer. For the separation of total proteins, SDS was added. Sample migration was performed at 20 mA for 2h.

4.3.2.3. Highlight the electrophoretic strips - Comassie brilliant Blue G250 was used in the case of proteins, for the peroxidases - a development solution containing benzidine, Na acetate, acetic acid and oxygen water, to α ester and β-naphthyl acetate in phosphate buffer K, pH 8 and Fast Blue BB and to alkaline phosphatase- acetate buffer, 0,1 M, pH 5, using α and β-naphthyl phosphate Na 0,05 M as a substrate and Fast Blue RR 0,1 % dye to which a few drops of MgCl2 0,25 M and MnCl2 0,5 M were added.

4.3.2.4. Two-dimensional electrophoresis - 500 mg of biological material was used from each grinded sample in liquid nitrogen. The proteins were isolated using the modified phenol extraction (Hurkman & Tanaka, 1986). The protein was determined using the Bradford method (1978). The protein migration was made on acrylamide gel 12,5% (v/v), Coomasie brilliant Blue 250 was used to highlight spots.

4.3.3. RAPD analysis

11 samples (10 – *Asplenium adulterinum* and 1- *Aspllenium trichomes*) were used to perform RAPD (Random Amplified Polymorfic DNA) analysis. *Asplenium adulterinum* is a natural hybrid between the species *Asplenium trichomes* and *Aspllenium viride*.

DNA was extracted from 0,5-1g / sample using DNA extraction methodology (Doyle and Doyle, 1987). A Nanodrop 2000 was used in order to assess DNA concentration and quality.

4.3.4. Flow cytometry technique

The degree of ploidy of gametophytes and sporophytes of *Asplenium trichomanes, Athyrium filix-femina* and *Polypodium vulgare* was determined using Otto I + II buffer and DAPI fluorochrome (4 ', 6-diamidino-2-phenylindol).

4.3.5. Experimental protocol used for long-term preservation (cryopreservation)

The plant material used for cryopreservation experiments is represented by fragments of gametophyte (*Asplenium adulterinum, Polypodium vulgare*) and sporophyte (*Athyrium filix-femina*) from the *in vitro* culture.

4.3.5.1. Experimental protocol used for long-term conservation in Asplenium adulterinum and Polypodium vulgare

4.3.5.1.1. Dehydration with sucrose solution of increasing concentration.

Gametophyte fragments from the species *Asplenium adulterinum* and *Polypodium vulgare* underwent a pretreatment with increasing sucrose concentration. The sucrose treatment was carried out at a temperature of 25 ° C. The pretreatment with sucrose was followed by a treatment with PVS2 solution for 30 minutes at room temperature. The PVS 2 solution was made according to the formula described by (Sakai et al., 1991) and contains glycerin, ethylene glycol, dimethylsulfoxide and sucrose in MS growth medium (Murashige & Skoog, 1962).

4.3.5.1.2. Dehydration with sucrose solution of increasing concentration followed by gradual immersion in liquid nitrogen.

The gametophyte fragments were treated as in the previous protocol, gradually immersed in liquid nitrogen.

4.3.5.1.3. Dehydration in sterile air. The biological material (gametophyte fragments) was dehydrated in sterile air for 2 hours. 2 variants of the protocol were tested:

a - the dehydration stage was followed by a treatment with PVS2 solution for 30 minutes, carried out at room temperature.

b - without treatment with PVS2 solution.

Finally, the gametophyte fragments were immersed directly in liquid nitrogen.

4.3.5.1.4. DMSO treatment in which the explants represented by gametophyte fragments were treated with 5% dimethyl sulfoxide cryoprotective substances (DMSO) in MS + 30 g / l sucrose solution at room temperature for 30 minutes. They were then immersed directly in liquid nitrogen.

4.3.5.1.5. PVS2 treatment. The explants were treated with PVS2 in MS + 30g / l sucrose solution at room temperature for 30 minutes, followed by immersion directly in liquid nitrogen.

The explants were kept in liquid nitrogen for 4 weeks. The defrosting process was performed on a water bath at 38 ° C for 1 minute. All explants were transferred to Petri dishes containing a nutrient medium MS ½ agarized with 20 g / l sucrose to continue the growth process.

The evaluation of the viability of the gametophyte fragments was analyzed after 4 weeks using 2 parameters:

• TTC test

• resumption of gametophyte growth and development

The TTC test consists of treating explants with 0.1% 2,3,5-triphenyl-tetrazolium chloride for 48 hours at room temperature in the dark.

To resume growth, the Murashige and Skoog culture medium variant was used with micro and macro minerals reduced to ½, with 3% sucrose, without growth factors.

4.3.5.2. Experimental protocol used for long-term conservation in Athyrium filix-femina species.

In the species *Athyrium filix-femina*, the viability of the sporophyte was evaluated after a pretreatment with two cryoprotectants, at different concentrations (DMSO and glycerol), followed by gradual freezing and immersion in liquid nitrogen (-196^0C) of the explants. Prior to the beginning of treatment, the biological material was kept for one week in vitro at 10 ° C.

CHAPTER 5
RESULTS AND DISCUSSIONS

5.1. *Ex situ* conservation using *in vitro* techniques

5.1.1. Obtaining regenerants using as a source of inoculum the homogenate from gametophyte fragments in the juvenile stage

The homogenization technique of the gametophyte registered positive results, being considered a promising alternative that deserves to be optimized, given the differentiation of gametophytes and sporophytes after 3 weeks of culture (Fig.1).

The results of our experiments highlighted differentiated morphogenetic responses depending on the species. The most reactive proved to be the species *Polypodium vulgare* and *Athyrium filix-femina*.

At *Asplenium trichomes*, sporophilus developed much later, after 7 months on the solid MS medium, which proves that the use of the gametophytic homogenate as a source of inoculum is generally not an optimal choice for the propagation of this species, especially in the liquid MS medium.

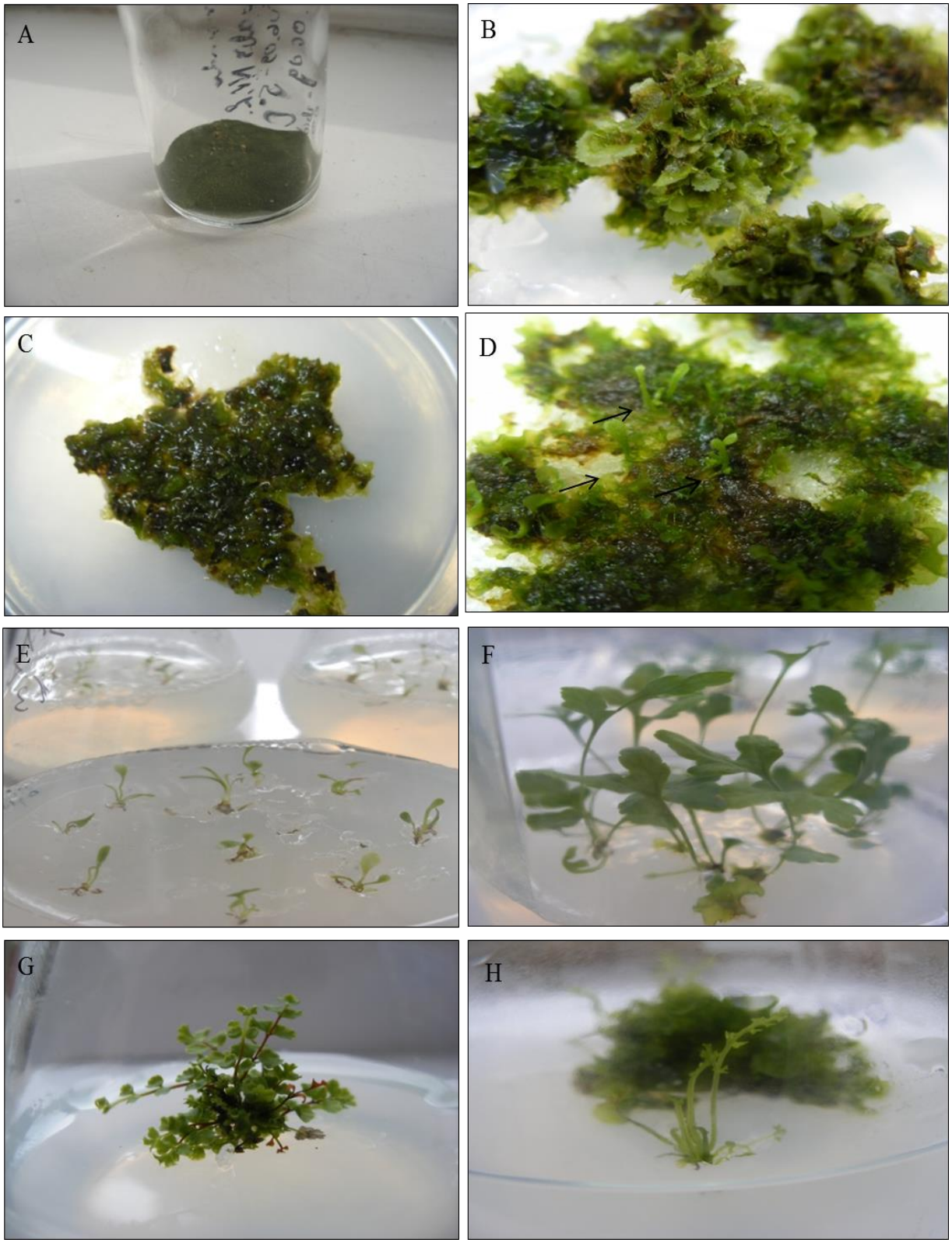

Fig.1.Sporophytes obtained by homogenizing gametophytes. A. spores; B. gametophytes; C. homogenate gametophytic; D. highlighting sporophytic formations; E. sporophytes; F. *Polypodium vulgare* (sporophytes); G. *Asplenium trichomanes* (sporophytes); H. *Athyrium filix-femina* (sporophytes).

5.1.2. Determination of the growth rate in regenerants

In the case of *Athyrium filix-femina*, the MS ½ variant stimulated the growth of the gametophyte compared to the Knop medium, while in *Asplenium trichomanes* and *Polypodium vulgare*, the registered values are close. In the case of MS ¼ medium, the effects were only positive for *Polypodium vulgare*. Among the three types of nutrients, the best results are presented by the MS ½ medium.

5.1.3. The effect of some physical factors on regeneration – light

During the experiments on ***the effects of the light regime*** on the homogenized gametophyte culture, we have found that in the species *Athyrium filix-femina* and *Asplenium trichomomes*, gametophyte protal proliferation proliferation is stimulated by light (Fig. 6 and 7), while in *Polyphium vulgare*, growth of gametophyte is reduced both in darkness and light conditions.

5.1.4. The effect of the culture medium on the development and rooting of regenerants

In regard to the **reactivity of each species** evaluated on the basis of biometric determinations (morphometric and gravimetric), optimal results were obtained in the case of *Polypodium vulgare* on environmental variants MS 1/2 with 2mg / l AIB, MS 1/2 with 0.22 mg / l kinetin + 1.8 mg / l AIA, the rhizogenesis being more accentuated on MS 1/2 media with 2mg / l AIB and agarized water (Table 1).

In the species *Athyrium filix-femina*, the optimal hormonal balance for the development of regenerants proved to be the one corresponding to the variants containing MS 1/2, MS 1/2 with 2 mg / l AIB and MS 1/2 with 0.22 mg / l kinetin + 1.8 mg / l AIA.

Table 1 Composition of culture media tested for the development and rooting of sporophytes from the species *Asplenium trichomanes, Athyrium filix-femina, Polypodium vulgare*

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

The legend:

MS1 / 2 = Murashige and Skoog basal medium, 1962, modified by halving mineral salts;

Knudson - modified Knudson culture environment (Berg and Bustamante, 1974)

AIB - indolylbutyric acid

AIA - 3 indolylacetic acid

ANA - 1-naphthylacetic acid

5.1.5. The effect of hormones in the culture medium on the callus or growth of the sporophyte

The evaluation of the survival percentage by the addition of hormones in the culture medium was performed at 1 month and 2 months. No differences in growth in area or volume were observed after 6 and 4 months, respectively (Fig. 2).

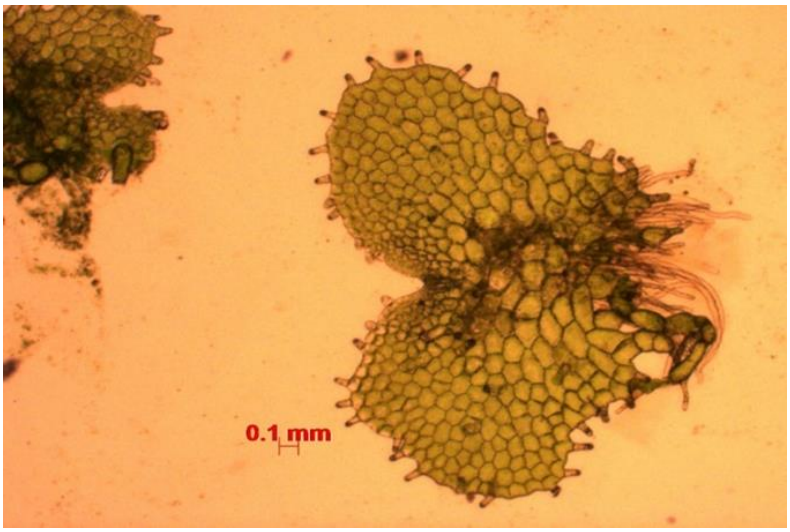

Fig.2. Chordal protals with differentiation in the basal area, trichorizoidogenic cells and secretory unicellular trichomes in the apical area (*Asplenium adulterinum*, after 2 months in vitro on the hormone medium, V3); (oc. 10x, ob.4)

5.1.6 The result of the experiment to obtain regenerants using as a source of inoculum leaf fragments with and without spores, rhizome, petiole, root tips from juvenile sporophytes

In case of use as a source of inoculum leaf fragments with and without spores, rhizome, petiole, root tips from juvenile sporophytes in *ex vitro* culture, *Polypodium vulgare*, *Athyrium filix-femina* and *Asplenium trichomanes*, after 30 days incubation the results were not positive in any of the species. However, after 60 days of culture on solid MS ½ medium, we noticed at the species *Polypodium vulgare* the germination of existing spores at the level of leaf fragments and the differentiation of filamentous formations at the level of gametophyte similar to those described by Campbell (1928) in *Osmunda claytoniana* and in more advanced stages we noticed the differentiation of typical protal cultures.

5.1.7. The result of the experiment to obtain gametophytes using spores as a source of inoculum

In the species *Asplenium adulterinum* and *Osmunda regalis* the initial result of the *in vitro* inoculation of the spores, was the acquisition of the protal about 3 weeks after the start of the culture (Fig. 3). Positive results were obtained for spores sterilized with 3 % and 2,5 % H2O2 for 7-10 minutes.

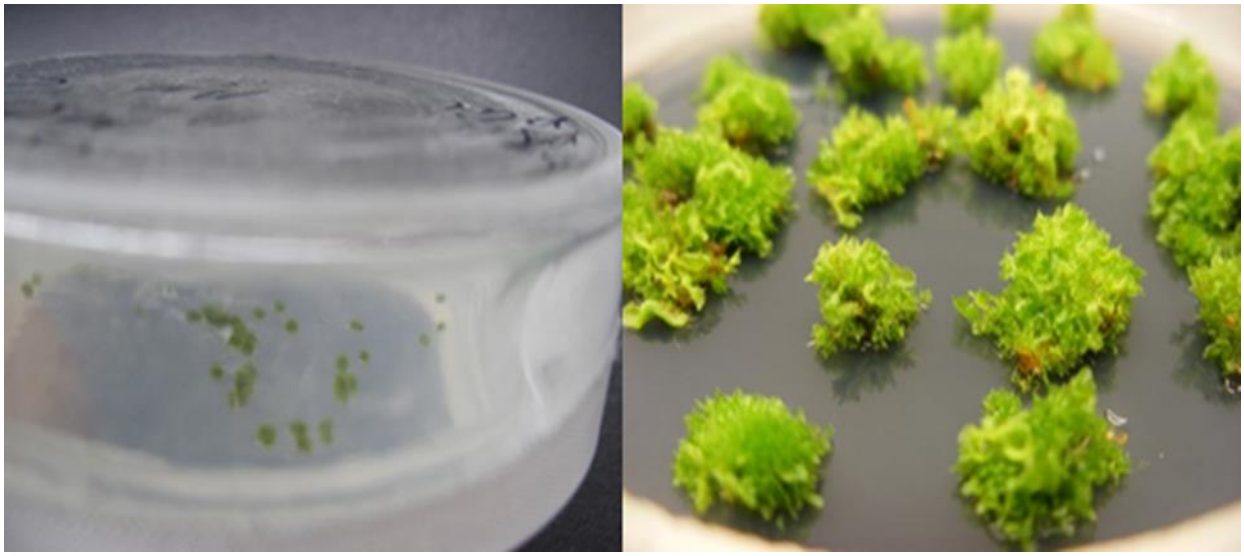

Fig. 3. Introduction of the species *Asplenium adulterinum* into the *in vitro* culture

5.2. Acclimatization of regenerators to ex vitro conditions

After acclimatization, (Fig. 4) we observed that sporophytes belonging to the species *Polypodium vulgare* adapt much more easily to *ex vitro* conditions compared to those

belonging to the species *Athyrium filix-femina*. They have different growth and development requirements, the percentage rate of plants that have survived being lower.

Fig.4. Successive stages of acclimatization of
Athyrium filix-femina and *Polypodium vulgare*

5.3. Biochemical analyses aimed at highlighting intra- and interpopulation variability and somaclonal stability of regenerants

5.3.1. Biochemical assessment of intra- and interpopulation variability in *Polypodium vulgare and Asplenium scolopendrium species*.

In the species *Asplenium trichomanes*, the isoenzymatic spectrum showed a high degree of polymorphism in esterases.

In the case of alkaline phosphatases, the samples of the second population are relatively homogeneous. The only change present is the existence of an additional isomorph in one of the individuals.

Analyzes performed on individuals of *Asplenium scolopendrium* species have similar characteristics.

In conclusion, the intra-population variability is higher in the species *Asplenium trichomanes,* highlighted by the predominant expression of isoesterases, while in the species *Asplenium scolopendrium* we highlighted an inter-population variability, individuals from the same population being relatively uniform, which denotes a predominantly vegetative reproduction in the population.

5.3.2. Evaluation of the somaclonal stability of regenerants obtained *in vitro* in the species *Polypodium vulgare, Asplenium trichomanes* and *Athyrium filix-femina*.

In both species, the enzymatic activities of sporophytes are much more intense compared to the gametophytes of the same species. This can be attributed to the lack of a high cell specialization. Both the gametophyte and the sporophyte are independent structures, capable of photosynthesis, but in terms of morphology they are very different.

The electrophoretic spectrum of the total protein revealed differences between gametophytes and sporophytes of the same species.

We noticed a somaclonal variability in gametophytes as opposed to sporophytes, which suggests a higher susceptibility to *in vitro* culture conditions.

5.4. Two-dimensional electrophoresis

For all three species studied, two-dimensional electrophoresis allowed for the separation of a large number of proteins.

Through gel analysis wes observed that most of the detected proteins had a molecular mass between 18.4-66.2 kDa and the optimal pHs were between 5 and 7, the isoelectric point ranging between 3 and 10 (Fig. 5).

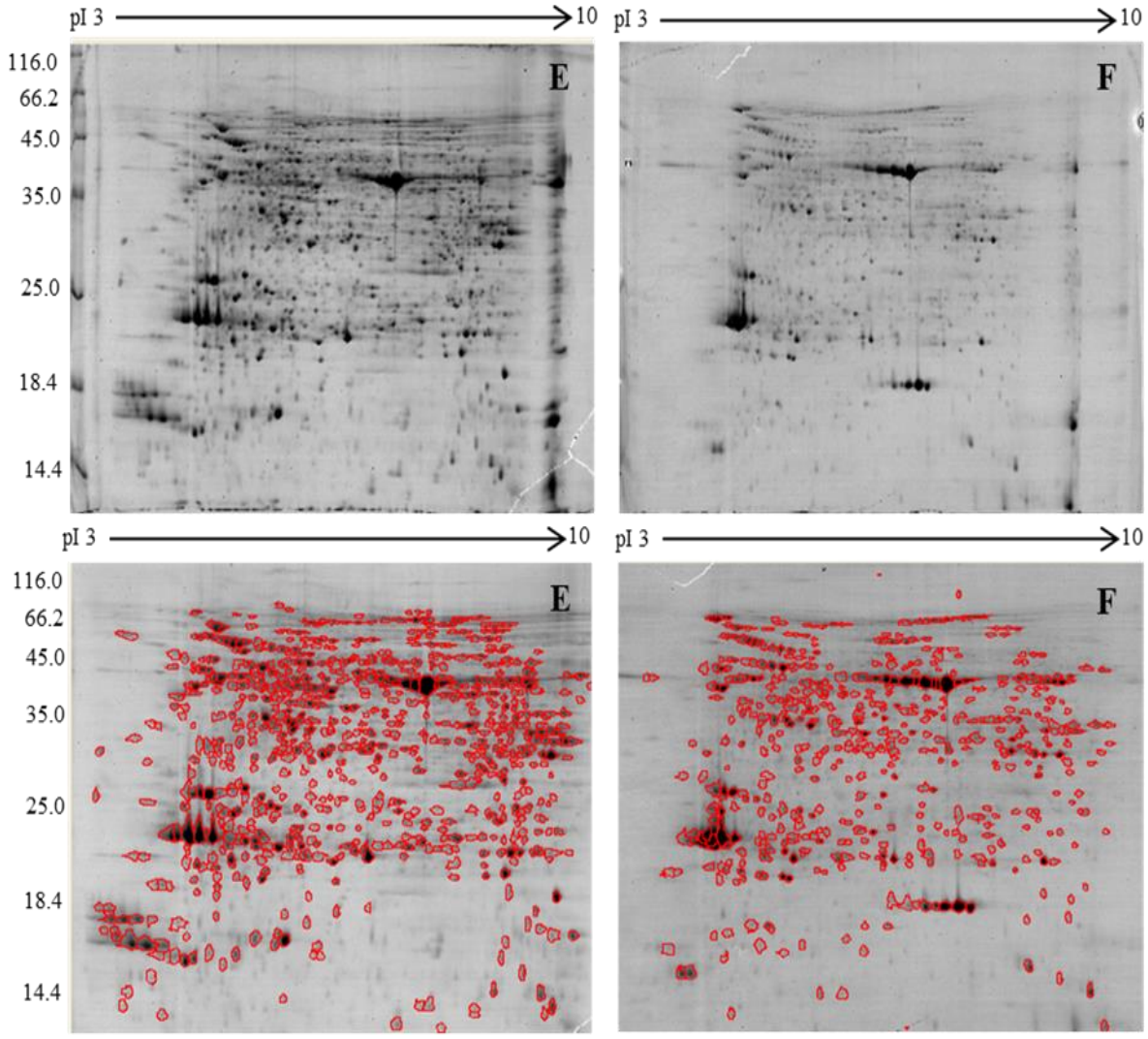

Fig.5. Highlighting of protein spots in the species *Asplenium trichomanes* by two-dimensional electrophoresis E-gametophyte; F- sporophyte

5.5. Molecular analysis of genetic stability / variability of primary regenerants obtained using in vitro culture

Since the cause of genetic variability cannot be accurately identified, it is necessary to remove individuals with variability from the *in vitro* culture or from more analyses. Our gene bank *Asplenium adulterinum* is constantly tested using molecular techniques to identify and eliminate those gametophytes that have genetic variability.

5.6. Highlighting the degree of ploidy by flow cytometry technique

By determining the **degree of ploidy**, diploid gametophytes and sporophytes were detected in the species *Atyrium filix-femina* and *Asplenium trichomanes*, and in the species

Polypodium vulgare we identified haploid gametophyte and diploid sporophyte (Fig. 6). It is possible that this diploidy found in gametophytes of *Athyrium filix-femina* and *Asplenium trichomanes* is the consequence of a false meiosis.

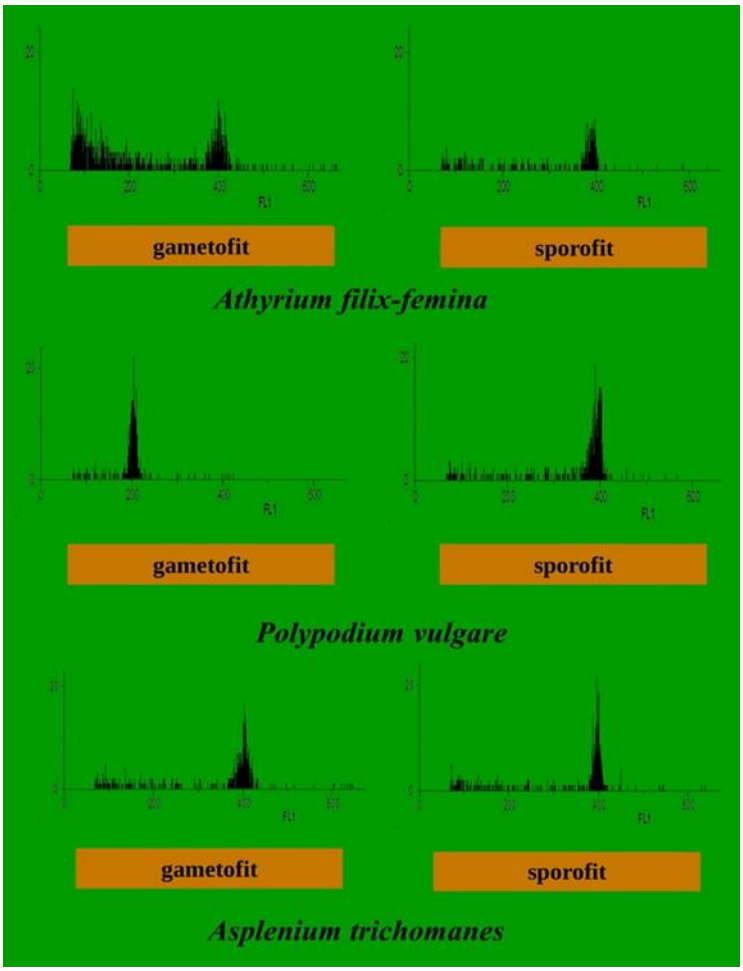

Fig.6. Highlighting the degree of ploidy

5.7. Assessment of viability of plant material under cryoconservation conditions

The first step in assessing the viability of the gametophyte fragments was TTC testing (Table 2). The results were expressed as a percentage.

Table. 2. The viability of gametophyte fragments after different pretreatments, evaluated by TTC method

Treatments	**% viability**	
	Asplenium adulterinum	***Polypodium vulgare***
Control sample	**100**	**100**
A. Dehydration sucrose solution increasing direct immersion in LN	**20**	**15**
A1. Dehydration sucrose solution increasing gradual immersion in LN	**40**	**30**
B1. Dehydration in PVS2 sterile air flow	**40**	**50**
B2. Dehydration in sterile air flow	**47**	**85**
C. Treatment with 5% DMSO in MS + 30g / l sucrose solution	**0**	**0**
D. PVS2 treatment in MS + 30g / l sucrose solution	**0**	**0**

Following the results obtained after the TTC test, for the next experiments only the dehydration treatments in sterile air were chosen, followed by the direct immersion in liquid nitrogen.

Viability testing by culturing gametophytes on regeneration medium led to the following results (Table 3). The results were recorded after 4 weeks of environmental maintenance.

Table 3. Viability percentage per medium

Treatments	% Viability			
	2 weeks		4 weeks	
	Asplenium adulterinum	***Polypodium vulgare***	***Asplenium adulterinum***	***Polypodium vulgare***
Control sample	100	100	100	100
B1. Dehydration	80	100	45	35.71
B2. Dehydration +PVS	75	64.28	10	14.28

The evaluation of the survival percentage by adding hormones in the culture medium was performed at 1 month and 2 months. No differences in growth in area or volume were observed after 6 and 4 months, respectively. sporophyte viability.

As regards the viability of *Athyrium filix-femin* sporophyte after exposure in nitrogen liquid (-196^0 C), microscopic images highlighted the survival of the plants (Fig. 7). The viability test confirmed the ability of the species to adapt to cold under experimental

conditions avoiding the formation of intracellular ice crystals, a key factor in the survival of the cells in the cryopreservation process.

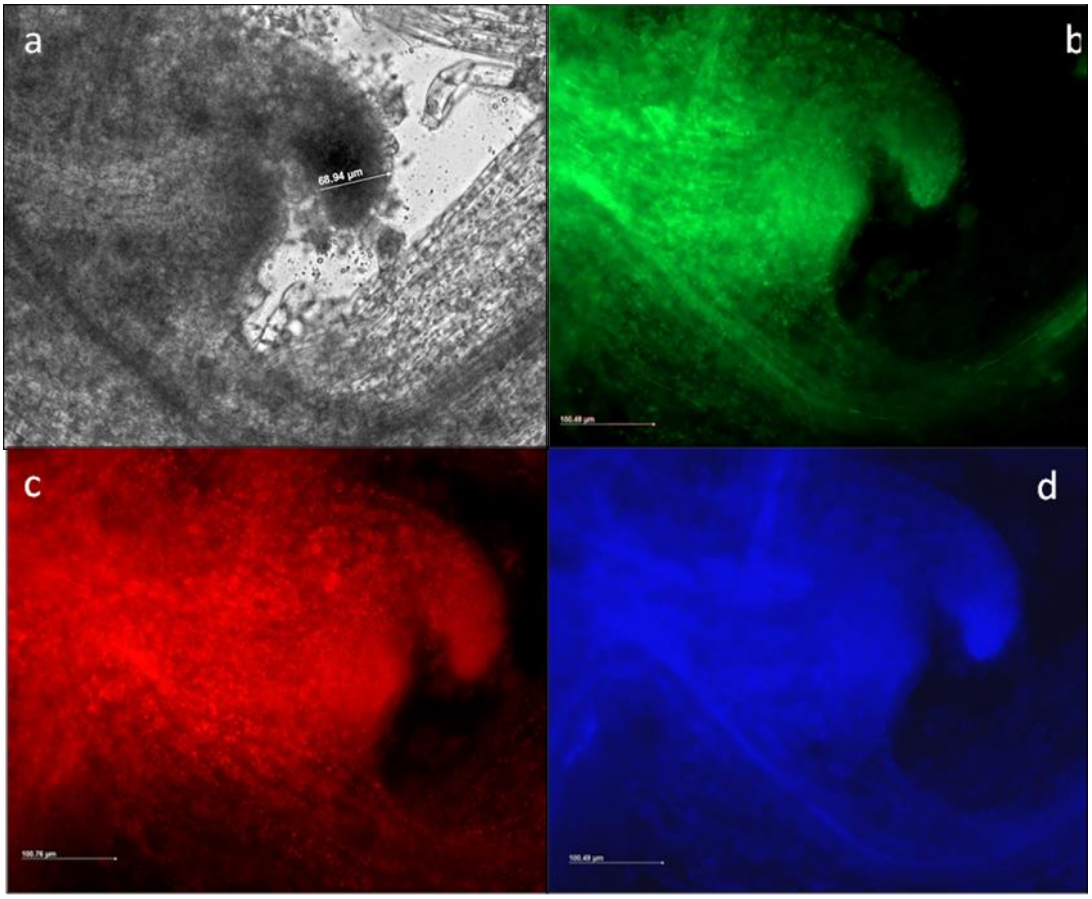

Fig.7. Meristematic apex (sporophyte of *Athyrium filix-femina*): a) in visible light; b) visualization through GFP filters; c) visualization through Rhodamine filters; d) visualization through filters for DAPI

CHAPTER 6

CONCLUSIONS

- The research carried out has revealed the possibility of successfully using *in vitro* techniques for pteridophyte species which present theoretical and biotechnological interest;
- The potential of the experimental *in vitro* system for this plant group is differentiated, species-specific, according to the reactivity of each species, as well as to the growing conditions (inoculum type, nutritive medium, physical factors)
- The homogenization technique stimulates the gametophyte's capacity to multiply and is recommendable in order to obtain a large number of sporophytes, particularly in species with a short life cycle.
- Concerning the utilized culture mediums, the results of the comparative biometric analysis conducted on the *Asplenium trichomanes*, *Athyrium filix-femina* and *Polypodium vulgare* species, using the MS $^1/_2$ liquid and Knudson mediums, have revealed the existence of a superior growth rate in favor of the MS $^1/_2$ culture medium.
- The physical state of the nutritive medium exerts a notable effect. For instance, in the case of the Polypodium vulgare species, the tests conducted on the variants of solid and, respectively, liquid consistence of the MS $^1/_2$ medium, have revealed a superior growth rate in the liquid culture medium;
- The bidimensional electrophoresis allowed for a more precise separation of the proteins in the *Asplenium trichomanes*, *Athyrium filix-femina* and *Polypodium vulgare* species in comparison with the electrophoresis polyacrylamide stacking gel;
- The analysis of the proteomic profile clearly indicates differences between the sporophytes and gametophytes of the three species;
- This study presents a preliminary analysis of proteomic profiles which may allow the spotting of possible modifications at a molecular level, which may occur during the process of *in vitro* culture, but especially during the process of cryopreservation with the purpose of long-term storage.
- Regarding the viability of the gametophytes of the *Asplenium adulterinum* and *Polypodium vulgare* species, the experiments allowed us to choose an optimal *in vitro*

stocking method. In the case of the sporophyte of *Athyrium filix-femina*, after exposure to extreme cold, microscopic images revealed the survival of the plants.

- The experiments allowed for the establishment of a facile experimental system for *in vitro* multiplication of the *Asplenium trichomanes*, *Athyrium filix-femina* and *Polypodium vulgare* species, which could be adapted to other pteridophyte species which present biotechnological and preservative interest. The experimental system allowed, as well, for the formation of an *in vitro* germplasm collection for the studied pteridophyte species.
- In the case of the RAPD analysis from the present study, we have demonstrated that there is a similarity of at least 88,88% among the *Asplenium adulterinum* gametophytes obtained from the spores of a single fern, maintained in the same physical and chemical conditions for 8 years. This variation can be linked to the somaclonal variation. For a more comprehensive analysis, a supplementary methylation of the DNA should be performed.
- Through completing the establishment-multiplication-rooting-acclimatization growth cycle, new plants have been obtained, which can be used in further studies, in three directions: biotechnological applications (extraction of secondary metabolites of pharmaceutical interest), repopulation of natural habitats, long-term conservation through crioconservation.

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