

COMPARATIVE STUDIES BETWEEN FERNS GAMETOPHYTE AND SPOROPHYTE BY BIDIMENSIONAL ELECTROPHORESIS

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Abstract. *Athyrium filix-femina* (L.) ROTH, *Polypodium vulgare* L. and *Asplenium trichomanes* L. are three valuable ferns species due to their potential as ornamental plants and secondary metabolite production and they originate from Valsan Valley protected area. Our experiments were focused on developing an optimal *in vitro* multiplication method and medium term conservation for these species. The reactivity of each species has been evaluated on the basis of biometric (morphometric and gravimetric) and biochemical determinations. The current study showed the differences between gametophytes and sporophytes of the mentioned species by comparing the proteomic profiles. We used gametophytes and sporophytes from *A. filix-femina*, *P. vulgare* and *A. trichomanes* obtained by *in vitro* culture and multiplied on MS 1/2 medium. Proteins were isolated according to a phenol extraction procedure and the protein content was determined by Bradford assay. The protein samples were separated in two dimensional technique and stained with Coomassie. Although using PAGE technique we have not observed significant differences between gametophyte and sporophyte, the patterns obtained by two-dimensional gel electrophoresis showed the existence of distinct spots for both gametophyte and sporophyte extracts.

Keywords: *Athyrium filix-femina*, *Polypodium vulgare*, *Asplenium trichomanes*, two-dimensional gel electrophoresis.

Rezumat. Studii comparative privind gametofitul și sporofitul ferigilor prin electroforeză bidimensională. *A. filix-femina* (L.) ROTH, *P. vulgare* L. și *A. trichomanes* L. sunt trei specii de ferigi valoroase datorită potențialului lor ca plante ornamentale și sintezei de metaboliți secundari, fiind originare din zona protejată Valea Vâlsanului. Experimentele noastre s-au axat pe dezvoltarea unei metode optime de multiplicare *in vitro* și conservarea pe termen mediu pentru aceste specii. Reactivitatea fiecărei specii a fost evaluată pe baza determinărilor biometrice (morfo-metrice și gravimetrice) și biochimice. Studiul actual arată diferențele dintre gametofitii și sporofitii speciilor menționate prin compararea profilurilor proteomice. Am folosit gametofii și sporofii de la *A. filix-femina*, *P. vulgare* și *A. trichomanes* obținuți prin cultura *in vitro* și multiplicați pe mediu MS 1/2. Proteinele au fost izolate în conformitate cu procedura de extracție cu fenol iar concentrația de proteine a fost determinată prin testul Bradford. Proteinele au fost separate prin electroforeză bidimensională și colorate cu Coomassie. Deși folosind electroforeza în gel de poli-acrilamidă nu am observat diferențe semnificative între gametofit și sporofit, modelele obținute prin electroforeză bidimensională au arătat existența unor spoturi distincte pentru ambele extracte din gametofit și sporofit.

Cuvinte cheie: *A. filix-femina*, *P. vulgare*, *A. trichomanes*, electroforeză bidimensională.

INTRODUCTION

Pteridophytes represent a very important group of vascular plants. They have been known for more than 300 million years, and present an enormous diversity of forms, growing in many different habitats around the world. During the Carboniferous Period, they dominated the vegetation. While most of them became extinct, some evolved and their currently number is now around 12,000 species all over the world (FERNANDEZ & REVILLA, 2003).

In time, the ferns stimulated the interest of many research teams. After *in vitro* techniques developed, numerous interesting approaches from scientific as well as from biotechnological point of view appeared.

Using *in vitro* culture system, the fern life cycle was analysed in detail, representing a powerful tool for studying the mechanisms underlying plant development. In the same time many fern species present a great biotechnological interest because of their economic value for ornamental purposes (*A. filix-femina*) as well as for the bioactive components produced. Among the metabolites synthesized by ferns there are triterpenoids and flavonoids (ecdysone-20E, abutasterone, polypodine B, inokosterone, 24-hydroxyecdysone, catechin, saponin, osladin) isolated from *P. vulgare* and phenolic compounds (4-vinyl-phenol, 4-vinyl-phenol-1-O-[α -L-rhamnopyranosyl-(1->6) β -D-glucopyranose], arctigenin) extracted from *A. trichomanes* (HO et al., 2010).

Proteins execute and control essentially all functions in living organisms (FRAUENFELDER & MCMAHON, 1998). Proteomics allows to obtain a quantitative description of protein expression and its changes under the influences of biological perturbations, the occurrence of post-translational modifications and the distributions of specific proteins within the cell (LOPEZ, 2007). Two-dimensional gel electrophoresis is one of the most widely used techniques for resolving complex protein extracts (SHEORAN et al., 2009).

MATERIAL AND METHODS

The biological material was represented by gametophytes and sporophytes from *A. filix-femina*, *P. vulgare* and *A. trichomanes* obtained by *in vitro* culture on MS 1/2 medium (MURASHIGE & SKOOG, 1962). Plants grew under the conditions of 20 ± 2 °C and a photoperiod of 16 hours of light and 8 hours of darkness.

Proteins were isolated using a phenol-based extraction procedure (HURKMAN & TANAKA, 1986). The protein content was determined by BRADFORD (1976) assay. The protein samples were focused using 3-10 nonlinear IPG strips for the 1st dimension separated on 12.5% (v/v) acrylamide gel and stained with Colloidal Coomassie Brilliant Blue 250.

For molecular mass determination, there were used protein molecular weight markers in the size range of 14.4–116 k Da from Fermentas. Gels were scanned and calibrated with LabScan 6 software (GE Healthcare). Image analysis was performed with Image Master 2D Platinum 6.0 (GE Healthcare).

For SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) extraction of total proteins from cytosol was performed by grinding the tissue in 0.1 M phosphate buffer, pH 7 at 4°C. The supernatant obtained after centrifugation at 15,000 rpm for 10 minutes, was used for electrophoretic analysis. Analyses were based on the principle of electrophoretic migration of samples in the electrical field at 20 mA for 2 hours in a batch system. The samples were run in a 10% concentration polyacrylamide gel (SDS-PAGE), a 4% polyacrylamide stacking gel and a 0.05M Tris-Gly buffer, pH 8.3. Samples were loaded in the wells of stacking gel and subjected to electrophoretic migration process at a voltage of 10 mA through the stacking gel for 30 minutes and then at a voltage of 20 mA through the separating gel for 90 minutes, at 4°C. As a marker highlighting the front of migration, bromphenol blue was used. It was used a Biometra electrophoresis apparatus. The spectra of total proteins extracted from each sample were showed by gel staining with Coomassie Brilliant Blue solution. The gels were photographed and interpreted by comparison of the similar bands.

RESULTS AND DISCUSSIONS

Spore-derived gametophytes maintained on MS medium were homogenized in aseptic conditions to obtain a high number of sporophytes (Fig. 1).

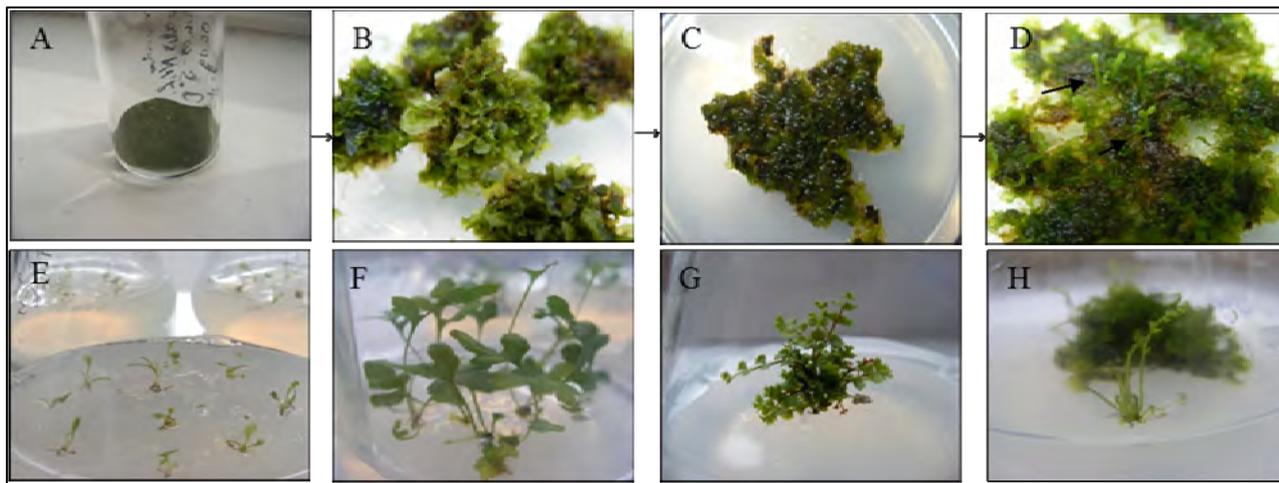


Figure 1. Sporophytes of ferns arising from homogenates of gametophytes: A, spores; B, gametophytes; C, cultures homogenates of gametophytes; D, formation of sporophytes; E, sporophytes; F, *P. vulgare* (sporophytes); G, *A. trichomanes* (sporophyte); H, *A. filix-femina* (sporophyte).

In case of *A. trichomanes* sporophyte, the development was slower and generating of enough biological material took a long time (seven months), for these reasons we used them only for two-dimensional electrophoresis. We could not observe evident differences between gametophytes and sporophytes of some species using SDS-PAGE analysis (Figs. 2; 3). However, in case of *A. filix-femina* intensity differences were observed (Fig. 2).

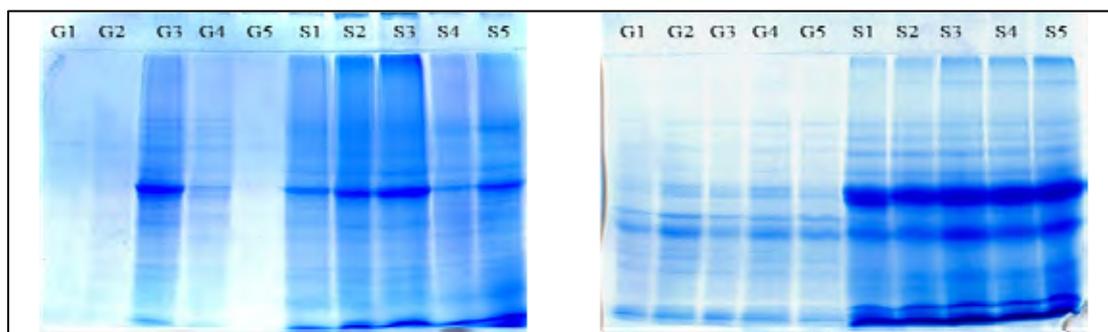


Figure 2. Protein patterns after SDS-PAGE *A. filix-femina* (left, G1-G5 gametophytes; right, S1-S5 sporophytes)

Figure 3. Protein patterns after SDS-PAGE *P. vulgare* (left, G1-G5 gametophytes; right, S1-S5 sporophytes)

SDS-PAGE is a simple method used to estimate the molecular weight of proteins, but it cannot resolve more than 80-100 different protein components.

Although using PAGE technique we have not observed significant differences between gametophyte and sporophyte, the patterns obtained by two-dimensional gel electrophoresis showed the existence of distinct spots for both gametophyte and sporophyte extracts (Figs. 4; 5; 6).

Two-dimensional gel electrophoresis separates proteins according to two independent parameters, isoelectric point (pI) in the first dimension and molecular mass in the second dimension by coupling isoelectric focusing (IEF) and SDS-PAGE (LOPEZ, 2007). Two dimensional electrophoresis is capable of resolving over 2,000 proteins in a single gel being the primary tool for proteomics research where multiple proteins must be separated for parallel analysis.

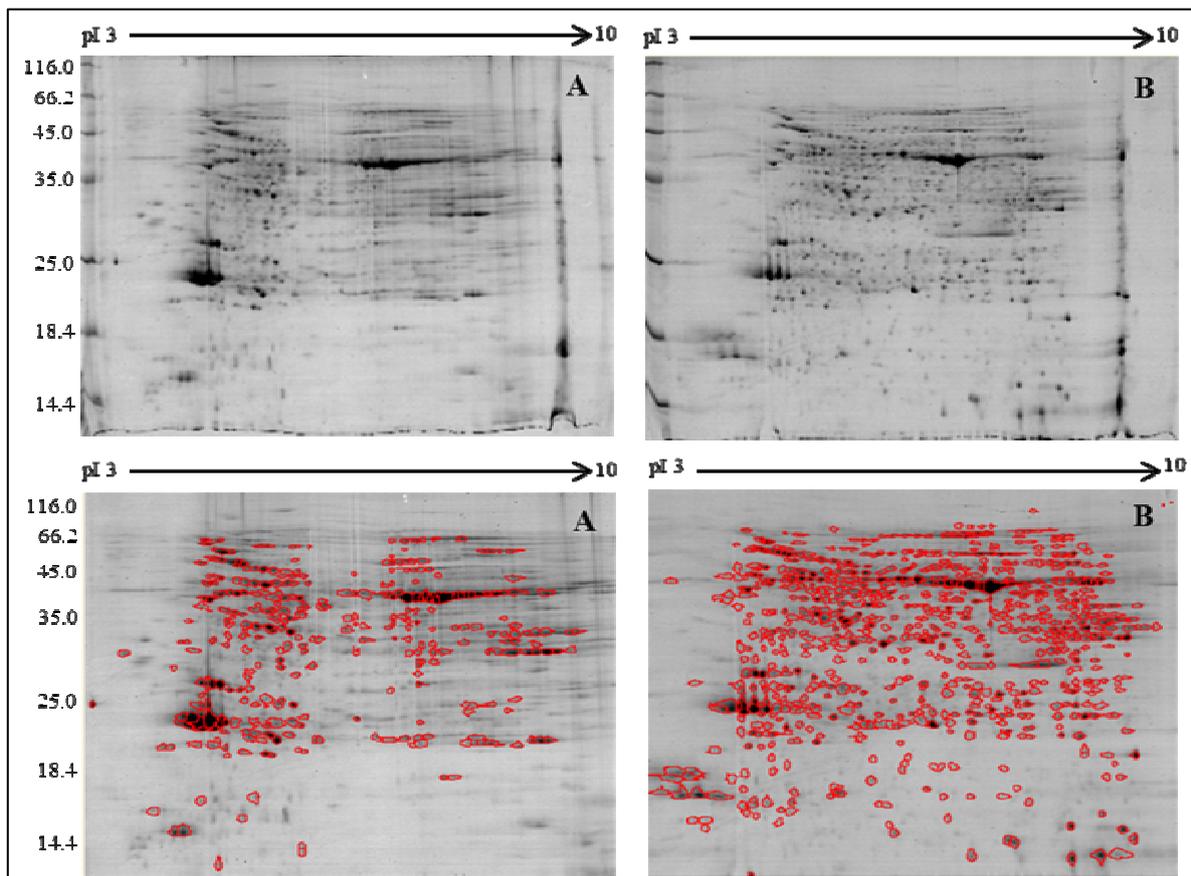


Figure 4. The comparison between Coomassie blue-stained 2-D protein maps.

Protein was extracted from *A. filix-femina* (A) gametophyte and (B) sporophyte separated on 24 cm IPG strip (pH 3-10 linear gradient) through isoelectric focusing (IEF) in the first dimension, followed by 12,5% SDS-PAGE gels in the second dimension.

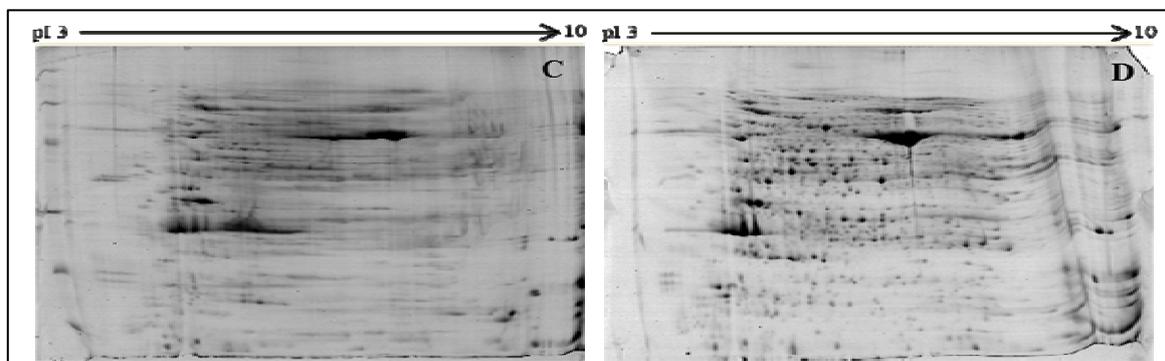


Figure 5. The comparison between Coomassie blue-stained 2-D protein maps.

Protein was extracted from *P. vulgare* (C) gametophyte and (D) sporophyte separated on 24 cm IPG strip (pH 3-10 linear gradient) through isoelectric focusing (IEF) in the first dimension, followed by 12,5% SDS-PAGE gels in the second dimension.

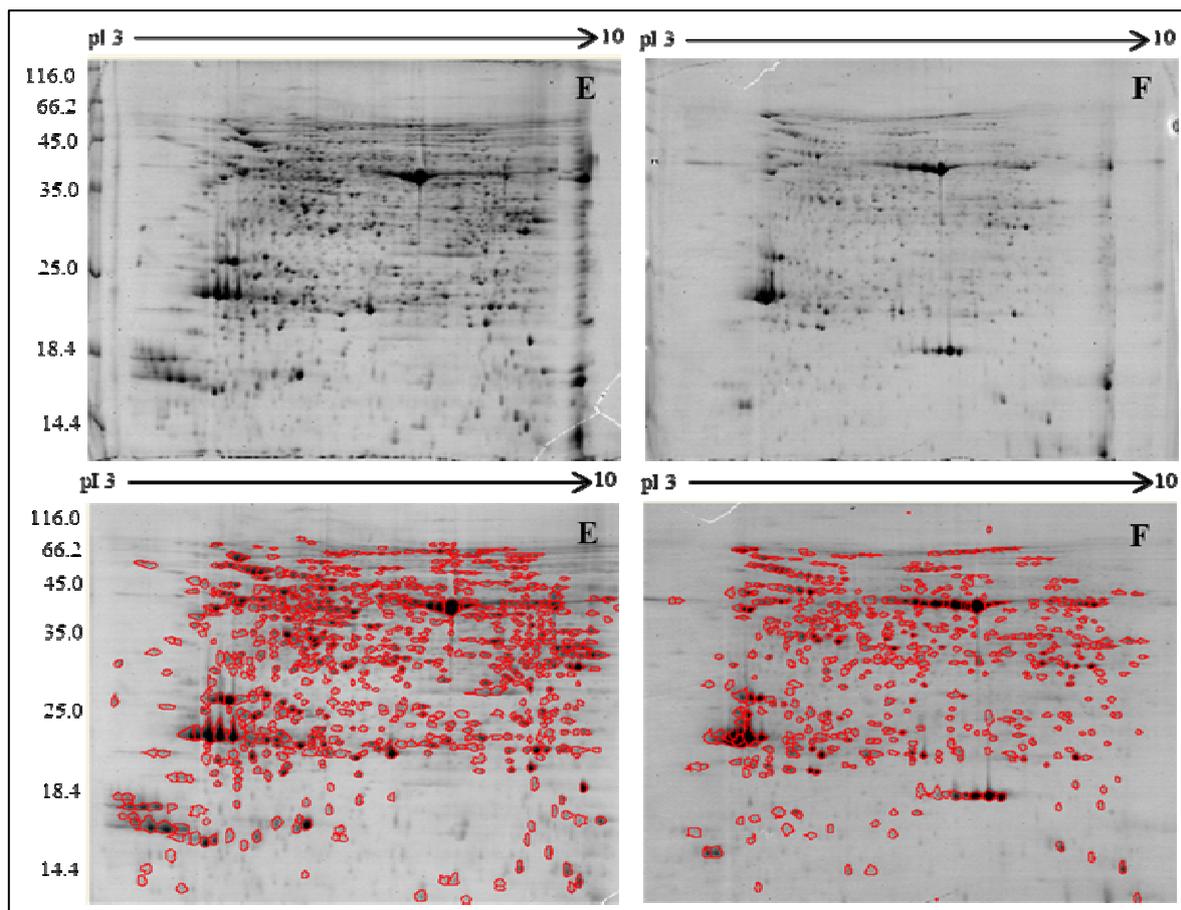


Figure 6. The comparison between Coomassie blue-stained 2-D protein maps.

Protein was extracted from *A. trichomanes* (E) gametophyte and (F) sporophyte separated on 24 cm IPG strip (pH 3-10 linear gradient) through isoelectric focusing (IEF) in the first dimension, followed by 12,5% SDS-PAGE gels in the second dimension.

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

The highest number of spots was detected in *A. trichomanes* gametophyte (approximately 950 spots), while *A. filix-femina* had the smallest number of spots (approximately 400 spots).

The molecular mass of the proteins detected was in the range of 18,4-66,2 kDa. The gametophyte of *A. filix-femina* had a very low number of spots (approximately 20 spots) in the 14,4-18,4 molecular mass interval (Fig. 4A). For all the analyzed species, the highest number of spots was found in 5-7 pH range.

In case *P. vulgare* and *A. trichomanes* we could observe that the gametophyte had generally an increased number of protein spots comparing with the sporophyte. It is known that different important compounds are synthesized in greater amounts in gametophyte comparing with the sporophyte (LAFONT et al., 2010).

In *A. filix-femina*, which was not associated until now with valuable compounds production, the gametophyte had the lowest number of spots. It is possible that the presence of a higher number of spots to be correlated with increased synthesis of valuable secondary metabolites, as in case of ecdysteroids produced by *P. vulgare* gametophyte (LAFONT et al., 2010).

CONCLUSIONS

From the analysed data we concluded that 2D gel electrophoresis technique allowed a better separation of the proteins from *A. trichomanes*, *P. vulgare* and *A. filix-femina* comparing with PAGE method.

Proteomic profiles showed much clearer differences between sporophyte and gametophyte of each species.

Also, the higher number of protein spots in *A. trichomanes* and *P. vulgare* gametophyte could be explained by an increased synthesis of secondary metabolites.

This study presents a preliminary analysis of proteomic profiles for further detection of biochemical changes that may occur during conservation (*in vitro* culture, cryopreservation).

For protein identification interesting spots must be excised from preparative gels then identified using mass spectrometry and database mining.

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