ASSESSING THE VIABILITY OF *Athyrium filix-femina* (L.) ROTH. SPOROPHYTE AFTER EXTREME COLD EXPOSURE

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Abstract. The present study concerns the viability assessment after revitalization of sporophyte of *Athyrium filix-femina* species from the Vâlsan Valley, central Romania. After a pre-treatment with two cryoprotectants in different concentrations, the fern explants have been slowly cooled and immersed in liquid nitrogen. Fluorescence microscopy images done on viability coloured sections revealed plant survival.

Keywords: ferns, cryopreservation, fluorescence, viability.

Rezumat. Evidenţierea viabilităţii sporofitului de *Athyrium filix-femina* (L.) ROTH. după expunerea la frig extrem. Studiul de faţă prezintă evaluarea viabilităţii sporofitului speciei *A. filix-femina* din aria protejată Valea Vâlsanului, situată în centrul României. După un pretratament cu doi crioprotectori, la concentratii diferite, explantele de pretridofite au fost congelate gradat şi imersate în azot lichid. Imaginile de microscopie prin fluorescenţă realizate la preparate cu coloranţi de viabilitate au reliefat supravieţuirea plantelor.

Cuvinte cheie: ferigi, crioconservare, fluorescenţă, viabilitate.

INTRODUCTION

Ferns are a plant category that live all over the world, consisting in approximately 15,000 species. The Red List of IUCN (2004) considers 67% of the evaluated species as endangered (140 from 210 species) (BAILIE et al., 2004).

In Romania, there were identified 76 fern species (6 of them uncertain) (CIOCÂRLAN, 2009); 11 of them are rare according to the national red list (OLTEAN et al., 1994), 10 are endangered according to the Red Book of Vascular Plants of Romania (DIHORU & NEGREAN, 2009) and 5 species are of community interest according to the European Red List, Habitat Directive or Bern Convention.

The Vâlsan Valley is one of the protected areas habituated by more than 30 fern species, situated in the centre of Romania in Argeş county. Previous researches on ferns from the Vâlsan Valley have been performed for *ex situ* conservation trough *in vitro* cultures as a tool for short and medium term preservation methods. The present study is important for long term conservation of this fern as an economical tool for preserving the germplasm. The species *A. filix-femina* was identified in 29 sites in the Vâlsan Valley as protals, plants and juvenile individuals (SOARE et. al, 2011).

Cryopreservation is a time saving method to maintain the original variability of the germplasm for long and very long time (from days to years and even hundreds of years) in independent conditions.

The results are a model for preserving other fern species with ornamental, pharmacological or conservative importance.

MATERIALS AND METHODS

The analysed material is represented by sporophytes of *A. filix-femina* from *in vitro* culture (Fig. 2) maintained for one week at cold (10^{o}C). The cryoprotective treatment consisted in a solution of liquid half mineral concentration MS medium (MURASHIGE & SKOOG, 1962) supplemented with 60 g/L sucrose, and two versions of cryoprotectants: 5% Dimethyl sulfoxide (DMSO) + 5% glycerol respectively 10% Dimethyl sulfoxide (DMSO) + 10% glycerol, applied each for 30 minutes to 10 samples of ferns (WITHERS & WILLIAMS, 1985). In the following step the cryotubes containing 0.5 mL of solution and a 5 mm fragment of plant material were loaded to the controlled rate unit (from CryoLogic) using a computerized program (CryoGenesys) as in the following graphic (Fig.1).

The steps of the program consisted in cooling with 2^{o}C/min. to 0^{o}C, then with 1^{o}C/min. to -6^{o}C, waiting for nucleation process 7 minutes and cooling with 0.3^{o}C/min. to -32^{o}C and then with 0.5^{o}C/min. to -42^{o}C. After the treatment, the explants have been immersed in (LN) liquid nitrogen (at -196^{o}C) for 24 hours.

The thawing process took place in a water bath at 37^{o}C followed by recultivation on MS medium supplemented as described above, without cryoprotectants. The viability test was performed by treating the explants for 5 minutes with fluorescein diacetate (FDA) in concentration of 0.1% (w/v) at room temperature (REINERT & BAJAJ, 1977) and observing the sample on optical microscope Imager M1 from Leica. During the reaction, the FDA is hydrolysed to fluorescein (coloured in UV light) and malic acid (PĂUNESCU, 2008).

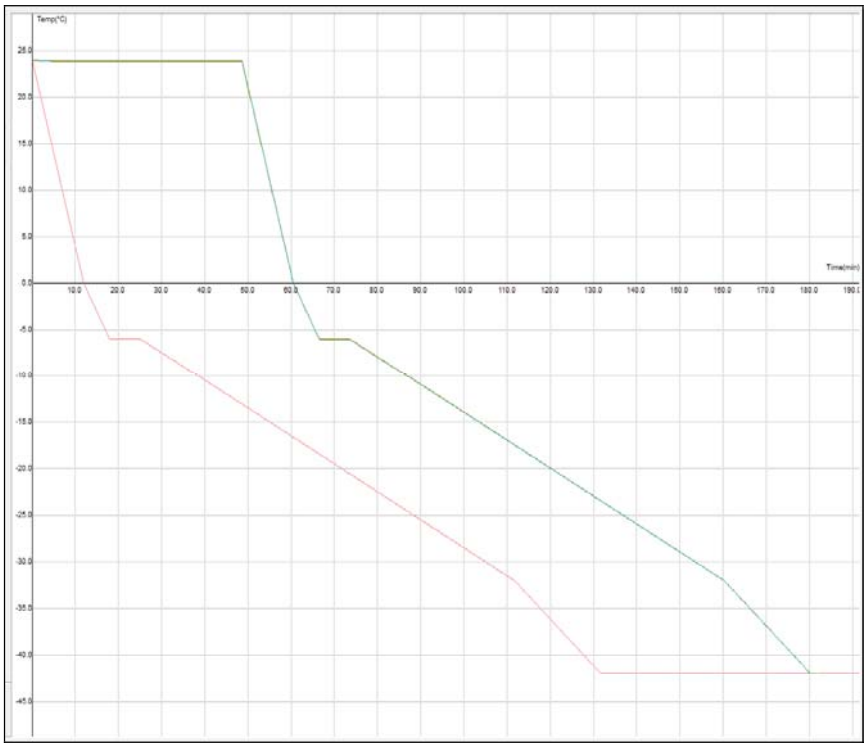

Figure 1. Slow freezing temperature graphic (CryoGenesys 5). Y-temperature (^{o}C), X-time (minutes); red line-protocol parameters, green line-achieved parameters.

Figure 2. Sporophytes of *A. filix-femina* from *in vitro* culture (original).

RESULTS AND DISCUSSIONS

The protocol used for fern cryopreservation consisted in two treatments: a chemical one with two cryoprotective solutions (DMSO and glycerol) and a thermal treatment by gradually cooling the specimens. The chosen program allows the most important phenomena (the ice nucleation) to take part in the intercellular space avoiding ice crystals to grow in the cells and break the cell membrane. In this way, the cooling program permits the extracellular water to ice, reducing the concentration of liquid phase and attracting the intracellular water outside the cell by osmosis which is replaced by cryoprotectants as DMSO and glycerol.

The fern explants appeared green after thawing and were visualized on fluorescence microscopy using 3 fluorochromes: DAPI (4', 6-diamidino-2-phenylindole), GFP (green fluorescence protein) and ROD (Rhodamine). The fluorescence reflected by the tissues confirms the viability of the cells.

The structure of the meristematic apex as seen in figure 3 presents a meristematic dome with vascular tissue undeveloped yet and foliar primordia.

In UV light, using **GFP** filter, vascular bundles are visible emphasizing the tracheidal structure; thus, these tissues with intense transport activity have survived and the esterase from intracellular space has hydrolysed FDA (Fig. 4).

In figure 5 the parenchymal tissue displayed in UV light using **ROD** as filter is intense red coloured, revealing that internal tissues have survived with the help of cryoprotective agents.

The picture coloured in blue visualized by UV light using **DAPI** filter give a general image on the viable tissue with the intensity on central vascular bundles and meristematic tissues (Fig. 6).

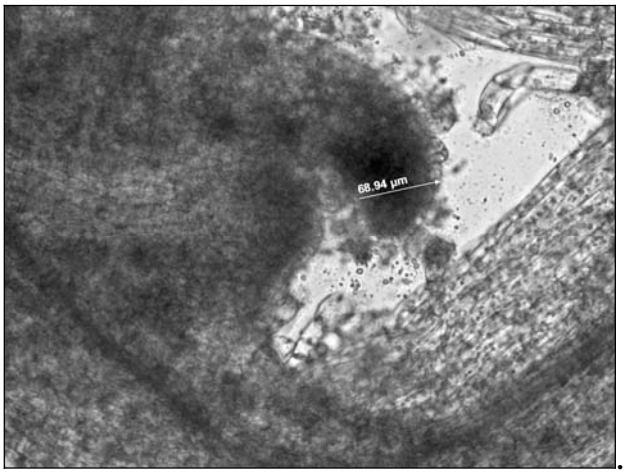

Figure 3. Meristematic apex of fern sporophytes in visible light (original).

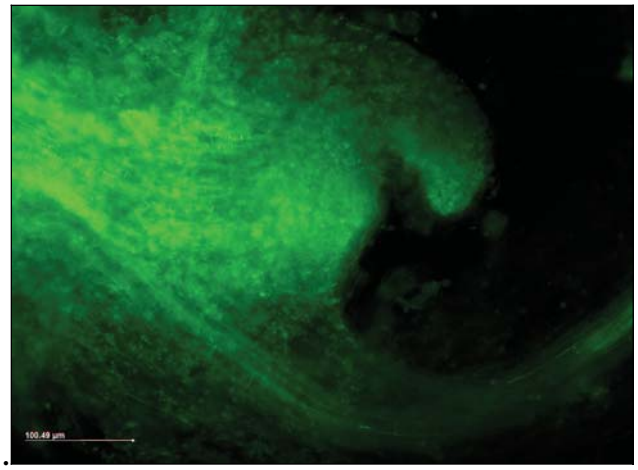

Figure 4. Meristematic apex in UV light visualized by GFP filter (original).

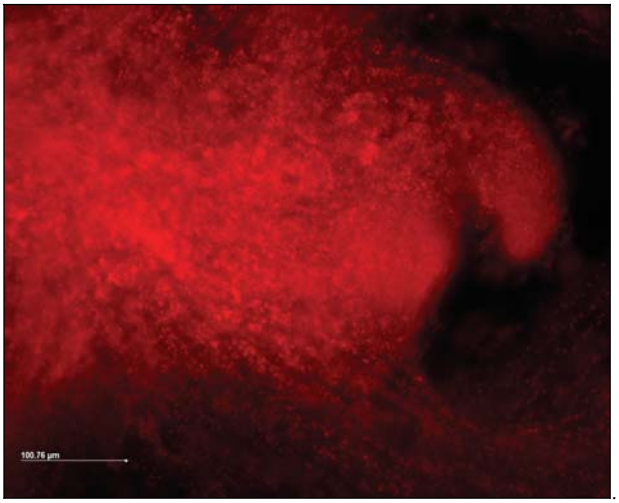

Figure 5. Meristematic apex in UV light visualized by Rhodamine filter (original).

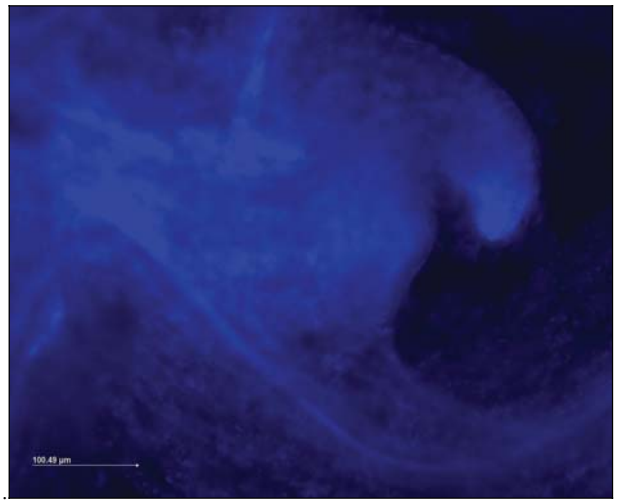

Figure 6. Meristematic apex in UV light visualized by DAPI filter (original).

The viability test of fern specimens exposed to extreme cold conditions in LN at -196^{o}C confirmed the capacity of ferns to adapt to cold in experimental conditions. Avoiding the intracellular ice crystal formation is the key factor in cells survival in cryopreservation processes.

The thawing procedure is also important due to the risk of repeating the same phenomena during ice melting and water re-entering in the cells. If the thawing process is too slow the water has enough time to form ice crystals in the vacuom and the cell membranes are broken.

Other authors used encapsulation method for cryopreservation of ferns (MIKUŁA et al., 2009) starting with spores. In those conditions, exposure to LN had even a stimulating role for the immature spores. We have used the slow freezing procedure due to the low concentration of cryoprotectants (avoiding in this way cyto-toxicity of the cytoplasm content) and less shocking treatment of the cells that avoids osmotic stress.

These results are a good start for long term conservation for a wide category of plant species threatened by natural and anthropogenic factors, but also for special germplasm collections with economic importance.

CONCLUSIONS

The protocol established for extreme cold treatment for *A. filix-femina* species was successful, as after thawing the explants maintained the viability. It allows further experiments on other fern species or on gametophyte and spores as a source of explants.

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