CRYOCONSERVATION OF *Pseudevernia furfuracea* L. SPECIES AND ASSESSING THE VIABILITY AFTER THAWING

BANCIU Cristian, CRISTIAN Diana

Abstract. The present study concerns the viability assessment after revitalization of fragments of *Pseudevernia furfuracea* L. species from 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 lichen survival.

Keywords: lichens, cryopreservation, fluorescence, viability.

Rezumat. Crioconservarea speciei *Pseudevernia furfuracea* şi evidenţierea viabilităţii după dezgheţ. Studiul de faţă prezintă evaluarea viabilităţii speciei *P. furfuracea* din România. După un pretratament cu doi crioprotectori, la concentraţii diferite, explantele de lichen au fost congelate gradat şi imersate în azot lichid. Imaginile de microscopie prin fluorescenţă realizate la preparate cu coloranţi de viabilitate au reliefat supravieţuirea indivizilor.

Cuvinte cheie: licheni, crioconservare, fluorescenţă, viabilitate.

INTRODUCTION

Lichens are a composite category of organisms that live all over the world, consisting in approximately 20 000 species. In Romania, there live 1700 lichen species, 4 of them being of European interest according to Habitat Directive Annex IIb.

P. furfuracea (L.) Zopf belongs to the family of Parmeliaceae. The thallus of this species is characterized by a dark gray, velvety upper face and blackish lower face, a special image that distinguishes it from the Evernia and Ramalia species. It has been used to preserve the odour of species employed in embalming mummies in ancient Egypt. Large amounts of *P. furfuracea* (1 900 t/years) are processed in the perfume industry (GUVENC et al., 2012).

In some experiments of the antifungal activity of the extracts of the *P. furfuracea* chemical races, chloroatranorin and olivetoric acid showed remarkable antifungal activities (HAYRETTIN et al., 2006). It is also proved to accumulate heavy metals being a good material for studying the bioaccumulation and for biomonitoring the polluted areas (CANSARAN-DUMAN et al., 2009). The species is sensible to some air pollutants, researches indicating DNA damage in the samples analyzed (ARAS et al., 2010). It was also used to study the effects of ultrastructural trace elements (Cd, Pb, Cu and Zn) in field and *in vitro* treatments, proving to develop important changes making the species tolerant to those elements (SORBO, 2011).

In the latest years the attention of the researchers for the antioxidant activity of the plants has increased (HELEPCIUC et al., 2014) and even the lichens can be used as sources of antioxidant agents on species as *P. furfuracea* and *Platismatia glauca* (MITROVIC et al., 2014). Other researches have revealed that *P. furfuracea* is a serious source of natural products such phisodic acid, antranozin, oxiphisodic and virensic acids as bioactive compounds and proteolitic enzymes with pharmacological and biotechnological research importance (KIRMIZIGUL et al., 2003; PROSKA et al., 1994).

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 (PĂUNESCU, 2009). Conservation strategies may be adapted by species specificity and considering other important factors such as reproduction strategies (MANOLE, 2015). The results are a model for preserving other lichen species with ornamental, pharmacological or conservative importance.

MATERIAL AND METHODS

The material analysed is represented by fresh fragments of talus from *P. furfuracea* (Fig.2) from the natural habitat (as seen as a fragment in Fig. 3) taken from coniferous woods of *Picea abies* from Bucegi mountains, Romania (from two populations one from Sinaia and the second one from Baba Mare peak, Buşteni). It was maintained for one week at cold (5^oC). For cryoprotection a treatment was applied using a solution of liquid half mineral concentration MS medium (MURASHIGE & SKOOG, 1962) supplemented with 6% (w/v) 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 next stage the cryotubes containing 0.5 mL of solution and a 5 mm fragment of lichen material were inserted in the controlled cooling rate machine (from CryoLogic) using a computerized program (CryoGenesys) as in the graphic from below (Fig. 1). The program consisted in several steps: cooling with 2^oC/min. to 0^oC, then with 1^oC/min. to -6^oC, stopping at this temperature for ice nucleation process during 7 minutes, cooling with 0.3^oC/min. to -32^oC and then with 0.5^oC/min. to -42^oC. After the cooling processes, the specimens have been

immersed in (LN) liquid nitrogen (at -196^{o}C) for 24 hours. The thawing process took place rapidly in a water bath at 37^{o}C followed by storage in liquid MS medium supplemented as described above, without cryoprotectants (BANCIU et. al., 2013). The viability test was performed by keeping the lichens for 5 minutes in 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, only in the living cells, the FDA is hydrolysed to fluorescein (coloured in UV light) and malic acid (PĂUNESCU, 2009).

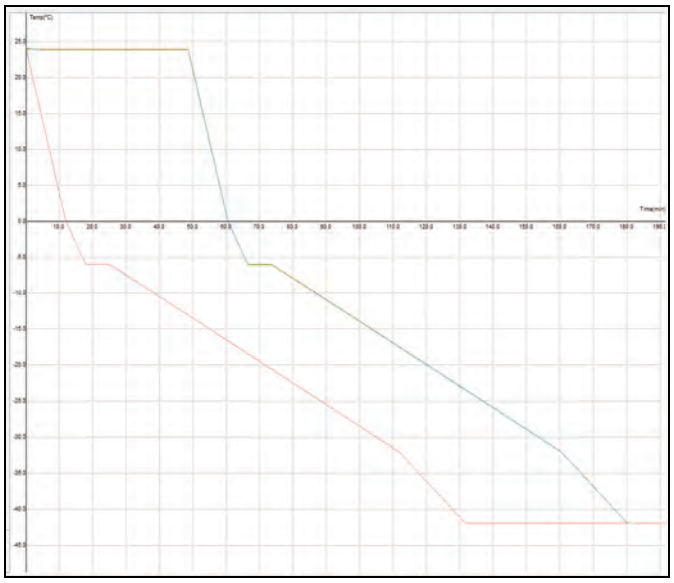

Figure 1. Slow freezing temperature graphic (CryoGenesys 5). Y-temperature (^{o}C), X-time (minutes); red line-protocol parameters, green line-achieved parameters.

Figure 2. Fragments of talus from *Pseudevernia furfuracea* (original).

RESULTS AND DISCUSSIONS

The protocol used for lichen 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 program chosen 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 allows 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 (BANCIU et al., 2013).

The lichen explants appeared grey-green coloured 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 cells confirms their viability.

The algal cells from the symbiotic tissue appear viable being coloured with all fluorochromes.

In UV light, using **GFP** filter the algal cells are green coloured being visible active; this proves that these cells have survived and the esterase from intracellular space hydrolysed FDA (Fig. 4).

In the figure 5, again the algal cells displayed in UV light using **ROD** as filter are intense red coloured, revealing that internal organelles have survived with the help of cryoprotective agents.

The picture coloured in blue visualized by UV light using **DAPI** filter gives a general image on the viable symbiotic tissue with the intensity on both algal cells and fungus filaments that are visible (Fig. 6).

Viability test of lichen specimens exposed to extreme cold conditions in LN at -196^{o}C confirmed the capacity of lichens to adapt to cold in experimental conditions, which is known from nature where lichens resist and grow in arctic and alpine regions. 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 vacuome and the cell membranes are broken (BANCIU et al., 2013).

Other authors used encapsulation method for cryopreservation of ferns (MIKUŁA et al., 2009) starting with spores. In that conditions exposure to LN had even a stimulating role for the immature spores. We have used the slow freezing procedure on lichens, due to low concentration of cryoprotectants (avoiding in this way cyto-toxicity of the cytoplasm content) and less damaging 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 *Pseudevernia furfuracea* species was successful, after thawing the explants maintaining the viability. It allows further experiments on other lichen species in the direction long term conservation and pharmacological properties.

ACKNOWLEDGEMENTS

This project was conducted through the program Partnerships in Priority Domains-PNII, developed with the support of MEN-UEFISCDI, project no. 273/2014 and project financed by the Romanian Academy no. RO1567-IBB06/2014.

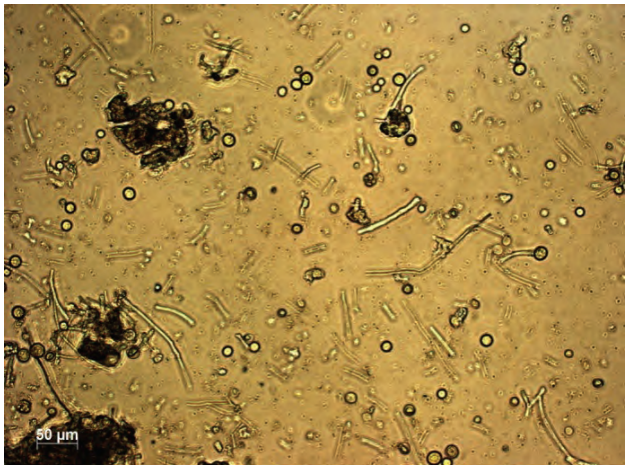

Figure 3. Revitalised talus of lichens in visible light (original).

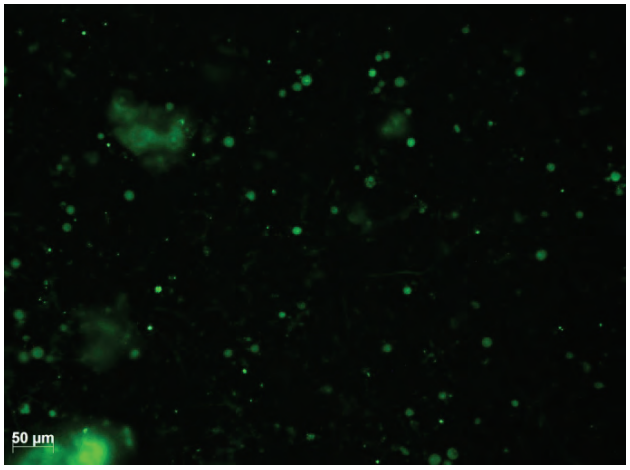

Figure 4. Revitalised talus in UV light visualized by GFP filter (original).

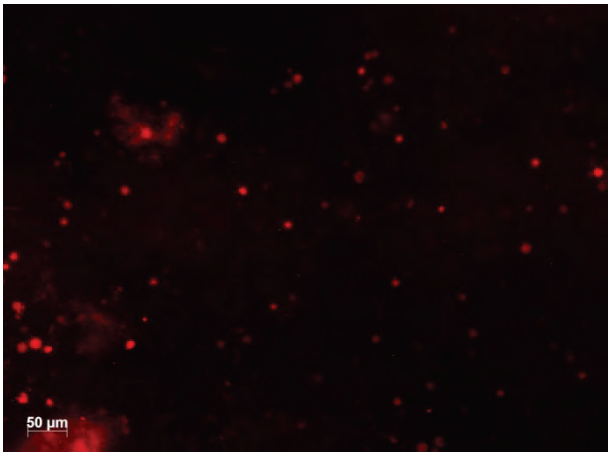

Figure 5. Revitalised talus in UV light visualized by Rhodamine filter (original).

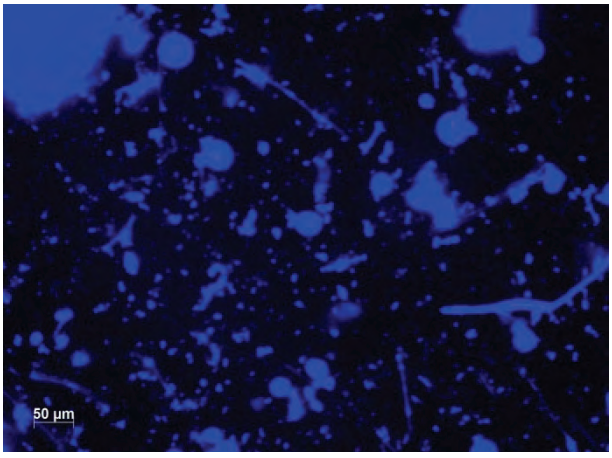

Figure 6. Revitalised talus in UV light visualized by DAPI filter (original).

REFERENCES

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

CANSARAN-DUMAN D., ATAKOL O., ATASOY I., KAHYA D., ARAS S., BEYAZTAŞ T. 2009. Heavy metal accumulation in *Pseudevernia furfuracea* (L.) Zopf from the Karabük iron-steel factory in Karabük, Turkey. *Zeitschrift fur Naturforschung C*. Edit. Max Planck Society. Berlin. **64**(9-10): 717-23.

GUVENC A., AKKOL ESRA KUPELI, SUNTAR I., KELES H., YILDIZ S., CALIS I. 2012. Biological activities of *Pseudevernia furfuracea* (L.) Zopf. extracts and isolation of the active compounds. *Journal of Ethnopharmacology*. Elsevier, Dublin. **144**: 726–734.

HAYRETTIN T., MERAL Y., TURGAY T., AYSEN Ö. T., MERIH KIVANCH. 2006. Antimicrobial Activity of Extracts of Chemical Races of the Lichen *Pseudevernia furfuracea* and their Physodic Acid, Chloroatranorin, Atranorin, and Olivetoric Acid Constituents. *Zeitschrift fur Naturforschung C*. Edit. Max Planck Society. Berlin. **61**: 499-507.

HELEPCIUC FLORENTA ELENA, MITOI MONICA ELENA, MANOLE - PĂUNESCU ANCA, ALDEA FLORENTINA, BREZEANU AURELIA, CORNEA CALINA PETRUTA. 2014. Induction of plant antioxidant system by interaction with beneficial and/or pathogenic microorganisms. *Romanian Biotechnological Letters*. Edit. Ars Docendi. Bucureşti. **19**(3): 9366-9375.

KIRMIZIGUL S., KOZ M., ANIL H., ICLI S., ZEYBEK U. 2003. Isolation and structure elucidation of novel natural products from Turkish lichens. *Turkish Journal of Chemistry*, Edited by Tubitak, Ankara, Turkey, **27** (4): 493–500.

MANOLE ANCA. 2015. First mature fruit description of Pietrosia laevitomentosa (Asteraceae) and its implications to the taxonomic position of the genus Pietrosia. *Phytotaxa*, Edited by Magnolia Press, Auckland, New Zealand, **197**(4): 282-290.

MIKUŁA ANA, JATA KARINA, RYBCZYŃSKI J. J. 2009. Cryopreservation strategies for Cyathea australis (R. Br.) Domin. *CryoLetters*. Edited by CryoLetters LLP. Lewen. UK. **30**(6): 429-439.

MITROVIĆ TATJANA, STAMENKOVIĆ SLAVISA, CVETKOVIĆ V., RADULOVIĆ N., MLADENOVIĆ M., STANKOVIĆ M., TOPUZOVIĆ MARINA, RADOJEVIĆ IVANA, STEFANOVIĆ OLGICA, VASIĆ SAVA, ČOMIĆ LJIJANA. 2014. Platismatia glauca and Pseudevernia furfuracea lichens as sources of antioxidant, antimicrobial and antibiofilm agents. *EXCLI Journal*. Edit. Leibniz Research Centre. Dortmund. **13**: 938-953.

MURASHIGE T. & SKOOG F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum*. Edited by Scandinavian Plant Physiology Society. Copenhagen, Denmark.**15**: 473-497.

PĂUNESCU ANCA. 2009. Biotechnology for endangered plant conservation: a critical overview. *Romanian Biotechnological Letters*. Edit. Ars Docendi. Bucureşti. **14**: 4095-4103.

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

WITHERS L. A. & WILLIAMS J. T. 1985. *Research on long term storage and exchange of in vitro plant germplasm*. In: Biotechnology in International Agricultural Research. Manilla: 11-24.

Banciu Cristian
Institute of Biology, Romanian Academy, Bucharest.
E-mail: cristi.banciu@ibiol.ro

Cristian Diana
Institute of Biology, Romanian Academy, Bucharest.
E-mail: diana.cristian@ibiol.ro

Received: March 12, 2015
Accepted: July 16, 2015