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SCREENING FOR A SUITABLE CRYOPRESERVATION PROTOCOL FOR *Polypodium vulgare*

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Abstract

Cryopreservation (storage at -196°C) is one of the techniques used for ex situ conservation to guarantee the long-term storage of different biological materials. Polypodium vulgare (Common polypody) is an evergreen fern used as food and medicine. To our knowledge, no cryopreservation protocol for gametophytes from this species is available. In this context, our paper aimed to screen a suitable cryopreservation protocol for P. vulgare - an important source of secondary metabolites. Different protocols were tested to identify a suitable cryopreservation protocol. Techniques like light microscopy and biochemical assays were used to certify an appropriate protocol for this fern. Gametophyte desiccation for 3 hours in the sterilized airflow before rapid immersion in liquid nitrogen provides sufficient dehydration to assure a good rate of survival.

Key words*: conservation, common polypody, enzymes, liquid nitrogen.*

INTRODUCTION

Cryopreservation is a technique of *ex situ* conservation strategies based on *in vitro* methods. This technique guarantees the long-term storage of different biological materials. Plant material conserved through medium-term conservation techniques showed different degrees of genetic modifications due to different factors like light intensity, culture media, growth factors, temperature, etc.

The ultra-low temperature (-196°C) used assures the conservation of material without genetic changes (Gonzalez-Arnao et al., 2008). Being relatively inexpensive, independent of electricity, and chemically inert (Pence et al., 2020), cryopreservation is the only safe and cost-effective option for long-term conservation.

The establishment of a suitable regeneration and recovery of plant material techniques is mandatory for a successful conservation method (Engelmann, 2011).

Polypodium vulgare (Common polypody) **(**Polypodiaceae family) is an evergreen fern, harvested from the wild for local use as food and medicine (Jizba et al., 1971). The species belongs to poikilohydric plants, and may support a loss of 45% of its water content, from this point of view, the studied species is considered a model plant for cryopreservation (Bagniewska-Zadworna & Zenkteler, 2005).

Gametophytes of pteridophytes, the haploid stage of the fern, cannot be stored using conventional seed banking approaches, requiring alternative *ex situ* conservation approaches (Pence et al., 2020).

Being very rich in biologically active compounds (Farràs et al., 2021) may be of conservative interest, even if the EU conservation status considered the species like least concern https://eunis.eea.europa.eu/species/150071).

Starting in 2008, the European Medicines Agency recognized the medical qualities of *P. vulgare* rhizome (Farràs et al., 2021). In this context, the interest in collecting from natural habitats was increased.

Until now, the *ex situ* conservation of *P. vulgare* was realized by spore maintained at 4°C (Smith & Robinson, 1975), and medium-term conservation - on culture medium, at 24 ± 2°C with 16-h illumination (Aldea et al., 2016).

To our knowledge, no cryopreservation protocol for *P. vulgare* gametophyte is available. In this context, our paper aimed to screen a suitable cryopreservation protocol for *Polypodium vulgare* - an important source of secondary metabolites.

MATERIALS AND METHODS

Plant material was represented by *P. vulgare* gametophytes from medium-term culture (Aldea et al., 2016). Fragments of gametophytes with different dimensions from 0.1 - 1cm were used like explants. A control represented by gametophyte without any pretreatment was used.

Cryopreservation protocols

Four different pre-treatments were screened to identify a suitable cryopreservation protocol for this species (Table 1). Generally, the first step of dehydration consisted of pre-treatments with different high-density solutions (0.5 and 1 M sucrose). For the second step, Plant Vitrifying Solution 2 (PVS2) was used. The PVS2 was prepared according to Sakai et al., 1991 and contained 30% w/v glycerol, 15% w/v ethylene glycol, 15% w/v dimethyl sulfoxide (DMSO), and 0.4 M sucrose in MS (Murashige & Skoog, 1962) plant growth medium.

Table 1. Cryoconservation protocols for *P. vulgare*

	Cryoconservation protocol steps		
	Step 1	**Step 2**	**Step 3**
Protocol 1a	0.5 M and 1 M sucrose for 24 hours each at 25°C	PVS2 solution for 30 minutes at room temperature	direct immersion in LN
Protocol 1b	0.5 M and 1 M sucrose for 24 hours each at 25°C	PVS2 solution for 30 minutes at room temperature	gradual immersion in LN
Protocol 2	Air dehydration in sterile airflow for 3 hours.	-	direct immersion in LN
Protocol 3	DMSO 5% added to MS with 30 g/l sucrose 30 minutes	-	direct immersion in LN
Protocol 4	-	PVS2 solution for 30 minutes at room temperature	direct immersion in LN

For Protocol 2, dehydration in sterile airflow of laminar flux hood (Telstar AH-100) for 3 hours was tested.

The dehydration degree of inoculum was calculated after the formula:

$$[(FW - DW)/FW] \times 100$$

were: FW - fresh weight, DW - dry weight after dehydration.

A Freeze control system CryoLogic (CryoLogic Pty. Ltd. Australia) with CryoGenesys 5.0 software was used for gradual immersion in LN (Protocol 1b). The freezing program consisted of the following steps:

- cooling at 2°C/min. to 0°C,
- cooing at 1°C/min. to -6°C,
- stopping at this temperature for the ice nucleation process for 7 minutes,
- cooling at 0.3°C/min. to -32°C
- cooling at 0.5°C/min. to -42°C.

After immersion in liquid nitrogen, all explants were maintained for 1 month.

Recovery after cryostorage

The samples were thawed by plunging the cryovials into a +38°C water bath for 3 minutes. The explant recovery was evaluated using two ways represented by the TTC viability test (Towill & Mazur, 1975) and regrowth capacity. For the TTC test, the explants were maintained for 20 h in dark at room temperature in 0.8% TTC prepared in phosphate buffer at pH 7.5. The red-colored gametophyte fragments were scored and reported viability as the percentage of the total used fragments. The TTC test was made immediately after thawing.

Regrowth capacity represents the percentage of gametophytes able to regrowth in established conditions from the total of gametophytes used for the experiments. For gametophytes regrowth, an MS 1/2 medium with reduced micro and macronutrients, supplemented with 3% sucrose, without growth factors was used. The cryopreserved gametophyte cultures were maintained in a growth chamber at 24 ± 2°C with 16-h illumination, in the same conditions with medium-term conservation. The regrowth capacity was monitored at 4 weeks after inoculation.

Three replicates were performed.

Light microscopy

The squash method was used by placing the gametophyte fragments from all treatments on a drop of water on a glass slide, covered with a

cover slide, and visualized immediately. A Scope A1 Microscope, Axio Zeiss, Germany was used to visualize the cell structure of gametophytes after cryostorage treatments.

Biochemical analysis

Preparation of enzyme extract. The gametophyte fragments after cryostorage were grounded with quartz sand to obtain the homogenate. The enzymes were extracted in 50 mM K phosphate buffer, pH 7 containing 2 mM Na_2EDTA and 4% PVP, at 4°C for 2 h. After centrifugation at 15000 rpm, for 20 minutes the supernatant was used for enzymatic analysis.

Assay of enzyme activity. The assay of the enzyme activity of the catalases (CAT) was performed spectrophotometrically at 240 nm by increasing the disappearance of H_2O_2 from the reaction medium (Garcia-Limones et al., 2002). An enzyme unit decomposes one µmol of H_2O_2 in one minute at 25°C and pH 7. The enzyme activity of superoxide dismutases (SOD) was determined by the standard method of Beauchamp and Fridovich, 1971, which is based on the ability of the enzyme to inhibit the reduction of tetrazolium salt (Nitro Blue Tetrazolium) by superoxide radicals. One unit of SOD activity represents the amount of enzyme that inhibits substrate reduction by 50% at 560 nm.

RESULTS AND DISCUSSIONS

Five cryopreservation protocols were tested with the aim of cell protection and survival after cryostorage. Is well known that, before cryopreservation, the explants should be subjected to different cryoprotective treatments. Four pre-storage treatments are used (Table 1) to prepare the cells by reducing the cell water content, in order to protect them from the formation of ice crystals which can damage cell membranes.

The majority of these treatments produced dehydration which protects against lethal intracellular freezing (Chen et al., 2011). Different osmolytes such as sucrose, glycerol, ethylene glycol, or cell membrane permeabilizers such as DMSO were used alone or in certain mixtures. Also, the cooling rate helps to avoid intracellular ice formation. All of these factors are important for successful cryopreservation procedures.

In this context, the results concerning the evaluation of gametophytes viability on the first day after rewarming, using the TTC test, varied between 0% for protocols 3 and 4 up to 85% for protocol 2 (Table 2). The TTC test, based on the cellular absorption of dyes, represents a simple and rapid method to locate the surviving cells in gametophytes (Mikula et al., 2009). Almost gametophyte fragments were colored brown-red in the case of treatments applied in protocol 2, while after protocol 1, cell viability is maintained only in certain areas that appear colored brown after the TTC test (Figure 1).

The gametophyte fragments pre-treated with sucrose in the first step and PVS 2 solution in the second step managed to survive at a percentage of 15%. But if the cooling was gradual, the survival rate was doubled to 30%, while if the first step of dehydration with sucrose was skipped, the survival was canceled (Table 2). Similarly, DMSO treatment did not provide the necessary protection for cells to survive after immersion in liquid nitrogen. It seems that the best dehydration protocol in the case of *Polypodium vulgare* gametophytes was desiccation in sterile air flow for 3 hours. We tried different times of dehydration in sterile airflow and a 3h dehydration in our conditions assures 52.47% of gametophyte desiccation. After 4 weeks, only 6.35-45.28% of gametophytes resumed their ability to regrowth, the best results being observed also in the case of Protocol 2. Although on the first day after cryostorage the gametophyte's viability was higher, it seems that not all these inocula succeeded in their regrowth capacity.

Table 2. Viability explants through TTC test and regrowth capacity

	Viability (%)	**Regrowth capacity (%)**
Control	100	100
Protocol 1a	15	6.35
Protocol 1b	30	25.35
Protocol 2	85	45.28
Protocol 3	0	0
Protocol 4	0	0

Physical dehydration (sterile airflow or silica gel) represents a common approach to direct

immersion in LN (Lambardi et al., 2008). In our case, sterile air dehydration for 3 hours assures 45.28% of gametophytes survival (Table 2). Our results are comparable with those obtained by Pence, 2000, where a low survival percentage (10-30%) of gametophytes of six species exposed to drying in sterile air compared with encapsulation dehydration was obtained.

Generally, two kinds of cryoprotectants are used. DMSO and glycerol are compounds that penetrate the plasma membrane, while polyethylene glycol and sucrose are non-penetrating compounds (Lambardi et al., 2008). DMSO (5-10%) represents the most used cryoprotectant agent (alone or in combnation) for all kinds of tissue (plants, animals, human) (Kim et al., 2004).

In our case, *P. vulgare* gametophytes, treated with 5% DMSO, did not survive after cryostorage.

Observations in light microscopy were done to check the integrity of the cell after cryopreservation protocols (Figure 1).

All the explants kept their green colour after cryopreservation even in the case of protocols 3 and 4, where no survival rate was identified. Different degrees of cell plasmolyzed were observed in the case of all protocols but correlated with gametophytes' survival. All gametophytes exposed only to PVS 2 solution showed a predominance of plasmolyzed cells, which will cause irreversible necrosis (Figure 1). The severity of the stress induced by treatments may be determined by the number of surviving cells. The fern gametophytes are highly regenerative following storage under *in vitro* conditions and can be recovered from even a single living cell of cryopreserved explants (Mikula et al., 2010).

Matsumoto, 2017 showed that even if PVS2 represents the most common cryoprotective treatment (used for different types of explants), a successfully cryopreserved using only PVS2 treatment is not possible due to its harmful effects from osmotic stress or chemical toxicity. In our case, pretreatment with PVS2 for 30 minutes resulted in cell plasmolysis which was incompatible with gametophyte survival.

An important aspect of understanding the stress associated with cryopreservation may be achieved through biochemical studies of oxidative stress (Prudente & Paiva, 2017). Even if there are many studies concerning the antioxidant activities during cryopreservation (Rahmah et al., 2015; Liu et al., 2021; Burkhan et al., 2022), none of them referred to sterile airflow dehydration in gametophyte ferns. For this purpose, the activity of catalase and superoxide dismutase of cryostorage gametophytes exposed airflow dehydration and were determined.

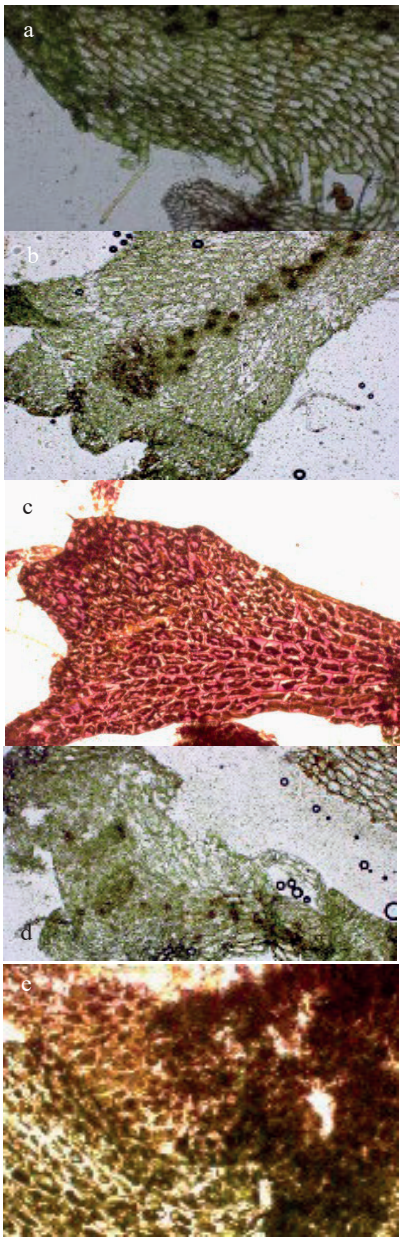

Figure 1. Microscopical aspects of *P. vulgare* gametophytes after cryopreservation: a - control, b - protocol 1a, c - protocol 1b, d - protocol 2 (air dehydration), e - protocol 4 (PVS2 treatment)

Catalase and superoxide dismutase represent the antioxidant enzymes, which are effective and efficient against various oxidative stresses,

acting to suppress/prevent the formation of free radicals or reactive species in cells (Ighodaro & Akinloye, 2017).

The gametophyte explants frozen in liquid nitrogen without dehydration, after cryostorage, had lower CAT activity and a slight increase in SOD activity than the typical culture of gametophytes. Apparently, these explants had been preserved by instantly freezing, but the regrowth of inoculum was not restored. The instant freezing at -196°C preserved the enzymatic activity, but the explant cells are dead due to the formation of ice crystals that perforated the cell membranes. The explants that were subjected to sterile airflow dehydration pretreatment before instant frozen in liquid nitrogen showed an increase in antioxidant enzyme activity approximately four times for CAT and more than ten times for SOD activity (Table 3).

Table 3. Enzymes activity of the gametophytes immersed directly in liquid nitrogen with and without dehydration pre-treatment

	Units of enzyme activity/g dry weight	
	CAT	**SOD**
Control	22.57± 0.97	22.39 ± 5.50
Gametophytes without dehydration	6.31	29.97
Gametophytes dehydrated	86.29 ± 16.37	303.32 ± 52.21

Kaczmarczyk et al. (2012) underlined that the increased antioxidant activity in the plant provides better tolerance to oxidative stress. In our case, increasing the antioxidant activities were correlated with restoring the growth capacity of cryopreserved gametophytes. The same results were obtained in the case of *Dendrobium* inocula subjected to the vitrification method where an increase in CAT activity was observed (Poobathy et al., 2013).

CONCLUSIONS

In the case of the valuable fern *P. vulgare*, a simple drying of gametophytes in sterile air before rapid immersion in liquid nitrogen provides sufficient dehydration to assure a good rate of survival.

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