Spore viability of fern species of the genera *Amauropelta*, *Blechnum*, and *Physematium* from central Argentina after low temperature storage

Viabilidad de esporas de helechos de los géneros *Amauropelta*, *Blechnum* y *Physematium* del centro de Argentina luego del almacenamiento a baja temperatura

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Summary

Background and aims: Ferns are sensitive to environmental changes, inhabiting ecosystems susceptible to degradation. Spore banks and *in vitro* spore culture are useful tools employed in their conservation. The objective was to test the ability of the spores to germinate after being stored in a freezer and to develop gametophytes and sporophytes, in native fern species of genera *Amauropelta, Blechnum* and *Physematium*.

M&M: Spores of *A. argentina*, *B. auriculatum* and *P. montevidensis* were stored at -20 ºC in dry conditions for 6 and 12 months. They were sown *in vitro* in Dyer liquid medium and incubated in a growth chamber. The means of germination percentages were calculated and a one-way Student's t-test was employed. Gametophyte and sporophyte development was registered under light and stereoscopic microscopes.

Results: Depending on the species, statistical differences were recorded in germination percentages (viability) between both storage periods. The spores of *A. argentina* kept in freezer for 12 months completely lost viability. In *B. auriculatum*, viability decreased over time, and in *P. montevidensis* it remained constant. Gametophytes and sporophytes developed in all cultures; except in *A. argentina* spores, with 12 months in freezer.

Conclusions: The viability of the spores under dry storage at low temperature could be conditioned by the ecological requirements of the species. Protocols used for spore storage and culture allowed obtaining gametophytes and sporophytes in trials.

Key words

Amauropelta, Blechnum, ex-situ conservation, fern, freezing, *Physematium,* spore longevity.

Resumen

Introducción y objetivos: Los helechos son sensibles a cambios ambientales, habitando en ecosistemas susceptibles a degradación. Los bancos de esporas y el cultivo *in vitro* son herramientas útiles para su conservación. El objetivo fue probar la capacidad de las esporas para germinar luego del almacenamiento en freezer y de desarrollar gametofitos y esporofitos, en especies de helechos nativos de los géneros *Amauropelta, Blechnum* y *Physematium*.

M&M: Esporas de *A. argentina*, *B. auriculatum* y *P. montevidensis* se almacenaron deshidratadas a -20 ºC durante 6 y 12 meses. Se sembraron *in vitro* en medio Dyer líquido y se incubaron en estufa. Se calcularon las medias de los porcentajes de germinación y se empleó una prueba t Student unidireccional. El desarrollo de gametofitos y esporofitos fue registrado con microscopio óptico y lupa.

Resultados: Se registraron diferencias estadísticas en los porcentajes de germinación (viabilidad) entre ambos períodos de almacenamiento. Las esporas de *A. argentina* mantenidas durante 12 meses en freezer perdieron completamente la viabilidad. En *B. auriculatum*, la viabilidad disminuyó con el tiempo y en *P. montevidensis* que permaneció constante. Se desarrollaron gametofitos y esporofitos en todos los cultivos; excepto en *A. argentina* almacenadas 12 meses en freezer.

Conclusiones: La viabilidad de esporas almacenadas deshidratadas a baja temperatura podría estar condicionada por los requerimientos ecológicos de las especies. Los protocolos utilizados para el almacenamiento y cultivo de esporas permitieron obtener gametofitos y esporofitos en todos los ensayos.

Palabras clave

Amauropelta, Blechnum, congelación, conservación *ex situ*, helecho, longevidad de espora, *Physematium.*

Introduction

Ferns are one of the oldest lineages of vascular plants, highly sensitive to environmental changes. They usually inhabit ecosystems susceptible to degradation, so their protection and conservation contribute to ensuring the maintenance of these ecosystems and the biodiversity therein (Ballesteros *et al*., 2006; Arcand & Ranker, 2008; Mehltreter, 2010). Ferns have diverse ecological roles and many species are of economic interest as ornamental, food or medicinal plants (Mannan *et al*., 2008; Lee & Shin, 2010; Sharpe *et al*., 2010; Liu *et al*., 2012; Goswami *et al*., 2016; Huang *et al*., 2019). Furthermore, various fern taxa are considered as indicators of environment disturbance, climate changes and pollution (Della & Falkenberg, 2019).

Procedures for biodiversity plant conservation can be *in situ* (protection of natural habitats), or *ex situ* (botanic gardens, germplasm banks, *in vitro* culture), both not mutually exclusive but complementary (Dyer, 1994; Ibars & Estrelles, 2012; Breman *et al*., 2021). Fern spore banks (analogous to seed banks for angiosperms) are an *ex-situ* conservation tool that allows storing large quantities of germplasm with high genetic variation in a small space (Dyer, 1994; Pence, 2008; Ibars & Estrelles, 2012; Ballesteros & Pence, 2018). Ballesteros *et al*. (2006) claimed that the spores of ferns that inhabit threatened ecosystems should be included in germplasm banks for experimental purposes and for long-term biodiversity conservation.

The sporophytes of most ferns produce large quantities of spores that are readily available every year, and their viability plays an important role for the successful establishment of individuals and populations in new habitats (Dyer, 1994; Sheffield, 1996). Due to their desiccation tolerance, spores can be stored for long periods of time at low temperatures maintaining their viability (Dyer, 1979; Ballesteros, 2010). The reduction in spore viability can be measured by the decrease in germination percentage over time (Gabriel y Galán & Prada, 2010).

Various works address the analysis of the optimal conditions to preserve the viability of the spores over time, using variables as wet or dry storage (i.e. spores stored in capsules with mineral agar vs. kept in Eppendorf tubes), or different temperatures (most common 20 °C, 5 °C, -20 °C or -196 °C in liquid nitrogen) (Pence, 2002, 2008; Quintanilla *et al*., 2002; Aragón & Pangua, 2004; Ballesteros *et al*., 2006, 2012; Gabriel y Galán & Prada, 2011; Mikula *et al*., 2009, 2015; Vargas & Droste, 2014; Tomiczak *et al*., 2023). Usually, the viability of fern spores is retained when they are stored in dry and cold conditions (Pence, 2008; Gabriel y Galán & Prada, 2010).

As part of a project that aims to investigate the optimal methods for *ex situ* conservation of ferns that inhabit Argentina, we began our studies in the central region of this country, one of the four centres of fern diversity in Southern Cone (Arana *et al*., 2013). In this area, fern populations are concentrated in the mountain systems (called "Sierras Pampeanas centrales"), which act as islands immersed in pampean plains (Arana & Ponce, 2004; Oggero & Arana, 2012; Arana *et al*., 2021). Despite habitat heterogeneity, the biota inhabiting isolated mountain systems is less likely to survive catastrophic events (Taylor & Kumar, 2016). In central Argentina, the mountain systems are frequently disturbed by fires which cause strong impacts on species composition, vegetation structure, and biogeochemical and hydrological cycles (Whelan, 1995; Morgan *et al*., 2001; Argarañaz *et al*., 2020).

The objective of the present work was to test the ability of the spores to germinate after being stored in a freezer and to develop gametophytes and sporophytes, in native fern species of genera *Amauropelta*, *Blechnum*, and *Physematium* from central Argentina with different ecological requirements.

Materials and Methods

Three taxa were studied: *Amauropelta argentina* (Hieron.) Salino & T.E. Almeida (Thelypteridaceae), *Blechnum auriculatum* Cav. (Blechnaceae), and *Physematium montevidensis* (Spreng.) Shmakov (Woodsiaceae). Materials were collected in four localities of the Chaco serrano district, Chaco biogeographic province, Neotropical region: Las Jarillas (31° 32′ 03.16′′ S, 64° 32′ 14.98′′ W), Atos Pampa (31° 57′ 49.34′′ S, 64°40′13.19′′ W), Las Albahacas (32° 53′ 59.02′′ S, 64° 50′′ 17.97′′ W), and Achiras (33° 09′ 07.38′′ S, 64° 59′ 32.78′′W).

The characteristic vegetation of the district is the xerophytic forest alternating with shrublands. The analysed taxa occur in different microhabitats: *A. argentina* is a hygrophilous species that lives associated with water courses, usually along stream banks, whereas *B. auriculatum* and *P. montevidensis* grow in xeric microhabitats of deep cracks and rock crevices (Fig. 1A-D). Voucher specimens were deposited in the herbarium RCVC of the National University of Río Cuarto, Argentina (Thiers, 2024).

Spore storage conditions

Portions of fronds with mature closed sporangia were taken from ten individuals per species and transported to the laboratory in paper envelopes. Materials were air-dried at room temperature (~24 °C) and ambient RH until spore release. Spores were filtered using a mesh with pores 88 µm in diameter, in order to eliminate remnants of indusia, scales, sporangia or leaf material. The spores were kept for 2 months in a refrigerator (4 ºC) before sowing. To test the viability of the spores before their storage at low temperature, samples were sown *in vitro* following the methodology described below. The spores were considered viable when they germinated (rupture of the spore wall and emergence of the first rhizoid).

Once the viability of the fresh spores was verified, subsamples were placed in Eppendorf tubes, labelled and stored in a freezer at -20 ºC. The viability of the spores was evaluated at two storage times: 6 and 12 months. Six replicates for each storage period per species were employed. The final spore viability was calculated according to Quintanilla *et al*., (2002) and Aragon & Pangua (2004), by analysing the germination percentage after 30 days from spore sown (no further germination was observed after). Germination percentage was calculated from a random count of 100 spores from each dish (following Aragón & Pangua, 2004).

In vitro spore culture and gametophyte development

The spores were cultured in previously autoclaved (120 °C for 20 minutes) Petri dishes 9 cm in diameter, containing Dyer liquid medium (Dyer, 1979). Spores were not disinfected before sowing. The capsules where sealed with Parafilm and placed in a growth chamber with a photoperiod of 12 hours, under white fluorescent illumination 28 µmol m-2 s-1, at 20 ± 2 °C. The cultures were examined every 2-3 days under a stereoscopic microscope Nikon SMZ 1000.

The different stages of gametophyte development were photographed employing a Nikon E200 light microscope (LM). For this, samples were placed on microscopic slides and mounted in water. When the gametophytes reached the cordiform phase they were transferred to Petri dishes containing previously autoclaved soil extracted from the natural habitats. Cultures were kept in the growth chamber until sporophyte emergence.

Statistical analysis

First, the means of germination percentages were calculated in the three fern species at the two cold storage conditions and then, a one-way Student's t-test was employed to determine if the differences in percentages were statistically significant at the distinct storage times. A Student's test calculator (InfoStat) was used to calculate the T-value, which can be employed to compare the variability of data and to determine whether means were significantly different or not. Last, p-values were calculated using the difference in mean at the 5% significance level, for our data from 6 and 12 months of storage in each species.

Results

Fresh spores germinated quickly, between 5 days (*Blechnum auriculatum*) and 7-9 days after sowing (*Amauropelta argentina* and *Physematium montevidensis*) (Table 1). In the frozen materials, a variation in germination time was recorded. The spores of *B. auriculatum* stored for 12 months in freezer took longer to germinate than those kept for 6 months, whereas in *P. montevidensis* germination occurred in both trials 15 days after sown. The spores of *A. argentina* completely lost viability after 12 months of storage at -20 ºC.

There were significant differences in germination percentages between the two storage periods in freezer depending on the species analysed (Table 2). *B. auriculatum* showed a greater spore germination percentage after a 6-months of storage in comparison with *A. argentina* and *P. montevidensis*. The storage at low temperature during 12 months damaged or reduced the viability

Fig. 1. Sampling area and individuals in their habitats. **A**: Typical landscape of the Chaco serrano district. **B**: individual of *Amauropelta argentina* growing on a stream bank. **C**: population of *Blechnum auriculatum* growing among rocks. **D**: individuals *of Physematium montevidensis* with rupicolous habit. Scales= B-D: 20 cm.

of the spores (expressed as germination percentage) in *A. argentina* and *B. auriculatum*, meanwhile storage period did not affect the germination percentage in *P. montevidensis*.

Gametophyte and sporophyte development was registered in all cultures, except for those corresponding to the spores of *A. argentina* kept for 12 months in the freezer. The morphological characteristics of the gametophytes, their sexual expression and the emergence of the sporophytes are described in Table 1 and Figs. 2-4. The developmental characteristics of gametophytes and sporophytes for each species were similar in all trials (i.e. fresh spores and under different storage periods). Briefly, in *A. argentina* the filamentous gametophyte phase consisted of short filaments 2-3 cells long (Fig. 2A-B). During the laminar phase, trichomes developed first on the margins and then on the gametophyte surface (Fig. 2C-F). Both smaller elongated and larger symmetrical gametophytes were recorded in the cordiform phase (Fig. 2G-J). Smaller ones were unisexual male (Fig. 2H-I), whereas larger gametophytes were bisexual (Fig. 2J-K). In some instances, branched male gametophytes were observed (Fig. 2L). Sporophytes developed in trials where fresh spores and those stored in a freezer for 6 months were used (Fig. 2M-N). In *B. auriculatum*, the filaments were 3-5 cells long and some of them developed a terminal trichome (Fig. 3A-B). The bi-dimensional growth phase showed elongated gametophytes that bore trichomes on the margins and on the prothallus

Table 1. Spore germination, gametophyte and sporophyte development in *Amauropelta argentina*, *Blechnum auriculatum*, and *Physematium montevidensis* laboratory trials. References= GCP: gametophyte cordiform phase; GFP: gametophyte filamentous phase; GLP: gametophyte laminar phase; SG: spore germination.

Species	Fresh spores	Spores 6 months storage	Spores 12 months storage
Amauropelta argentina	SG: 8-9 days from sown. TG: 60 %	SG: 10 days from sown.	Spores did not germinate
	GFP: 2-5 days from SG. Filaments 2-3 cells long.	GFP: 4-6 days from SG. Filaments 2-3 cells long.	
	GLP: 5 days from SG. Trichomes on the margins.	GLP: 6 days from SG. Trichomes on the margins.	
	GCP: 24 days from SG. Trichomes on the margins and the prothallus surface.	GCP: 20 days from SG. Trichomes on the margins and the prothallus surface.	
	Antheridia: 50 days from SG.	Antheridia: 60 days from SG.	
	Archegonia: 60 days from SG.	Archegonia: 67 days from SG.	
	Gametophyte sexuality: bisexual and male unisexual.	Gametophyte sexuality: bisexual and male unisexual.	
	Sporophyte: 90-100 days from SG.	Sporophyte: 120 days from SG.	
Blechnum auriculatum	SG: 5 days from sown. TG: 70%	SG: 6 days from sown.	SG: 14 days from sown.
	GFP: 2-6 days from SG. Filaments 3-5 cells long, some with terminal trichome.	GFP: 2-7 days from SG. Filaments 3-5 cells long, some with terminal trichome.	GFP: 2-7 days from SG. Filaments 3-5 cells long, some with terminal trichome.
	GLP: 7 days from SG. Trichomes on the margins.	GLP: 7 days from SG. Trichomes on the margins.	GLP: 7 days from SG. Trichomes on the margins.
	GCP: 18 days from SG. Trichomes on the margins and the prothallus surface.	GCP: 17 days from SG. Trichomes on the margins and the prothallus surface.	GCP: 18 days from SG. Trichomes on the margins and the prothallus surface.
	Antheridia: 40 days from SG.	Antheridia: 75 days from SG.	Antheridia: 120 days from SG.
	Archegonia: 48 days from SG.	Archegonia: 78 days from SG.	Archegonia: 140 days from SG.
	Gametophyte sexuality: bisexual and male unisexual.	Gametophyte sexuality: bisexual and male unisexual.	Gametophyte sexuality: bisexual and male unisexual.
	Sporophyte: 60 days from SG.	Sporophyte: 90 days from SG.	Sporophyte: 210 days from SG.
Physematium montevidensis	SG: 6-8 days from sown. TG: 90%	SG: 15-26 days from sown.	SG: 15-30 days from sown.
	GFP: 2-4 days from SG. Filaments 3-5 cells long.	GFP: 2-5 days from SG. Filaments 3-5 cells long.	GFP: 2-7 days from SG. Filaments 3-5 cells long.
	GLP: 10 days from SG. Trichomes on the margins.	GLP: 10 days from SG. Trichomes on the margins.	GLP: 8 days from SG. Trichomes on the margins.
	GCP: 25 days from SG. Trichomes on the margins and the prothallus surface.	GCP: 30 days from SG. Trichomes on the margins and the prothallus surface.	GCP: 30 days from SG. Trichomes on the margins and the prothallus surface.
	Antheridia: 25 days from SG.	Antheridia: 43 days from SG.	Antheridia: 35 days from SG.
	Archegonia: 35 days from SG.	Archegonia: 49 days from SG.	Archegonia: 38 days from SG.
	Gametophyte sexuality: bisexual and male unisexual.	Gametophyte sexuality: bisexual and male unisexual.	Gametophyte sexuality: bisexual and male unisexual.
	Sporophyte: 52 days from SG.	Sporophyte: 60 days from SG.	Sporophyte: 65 days from SG.

Table 2. Significance of storage time on spore germination percentage using Student's t-test. References= n: number of capsules. In each one, 100 spores were counted. Porcentage germination (mean±standar error). T: t-valor. **: p>0,05.

Species	Source variation	n	Storage time of 6 months	Storage time of 12 months	T	p-valor
Amauropelta argenina	% of germination	6	30±7	0	10,39	0,000
Blechnum auriculatum	% of germination	6	72±3	30±3	26,37	0,000
Physematium montevidensis	% of germination	6	40±7	40±7	0,32	0,7537**

surface (Fig. 3C-E). The cordiform gametophytes were mostly elongated throughout their development (Fig. 3F-I). Both unisexual male (Fig. 3G-H) and bisexual gametophytes were recorded, the latter with a wide notch behind which the gametangia differentiated (Fig. 3I-K). Sporophytes developed in all cultures using fresh spores as well as those stored in freezer (Fig. 3L-N). The filamentous phase of *P. montevidensis* gametophytes consisted of filaments 4-5 cells long, with or without a terminal trichome (Fig. 4A). During the laminar phase, more trichomes developed on the margins and on the gametophyte surface (Fig. 4B-C). As occurred in the other analysed species, a sexual dimorphism was observed being the male gametophytes smaller in size and cordiform elongated, whereas bisexual ones were larger and symmetrical cordiform (Fig. 4D-F). Sporophytes developed in all cultures (Fig. 4G).

Discussion and Conclusions

The spores of *Blechnum auriculatum* and *Physematium montevidensis* maintained their viability for up to 12 months when they were stored in the freezer. Both fern species completed the life cycle in laboratory conditions and sporophytes developed in all essays (after 6 and 12 months of storage). Quintanilla *et al*. (2002) analysed spore germination in five threatened ferns after different storage conditions, and stated that dry storage at -20 °C or 5 °C is an effective technique to maintain spore viability, requiring low preparation time and storage space. Aragón & Pangua (2004) also found dry storage at -20 °C fairly effective to maintain spore viability in rupicolous taxa of *Asplenium*. These authors reported a decrease in the percentage of viable spores after 12 months of storage, as occurred in our cultures of *B. auriculatum*. In the case of *P. montevidensis*, this percentage remained constant over storage time.

By other hand, the spores of the hygrophilous species *A. argentina* completely lost viability when they remained 12 months stored in the freezer. Our results agree in general with those of Quintanilla *et al*. (2002), who found in other hygrophilous ferns that dry storage killed the spores after 6 months at -20 °C. According to these authors, hygrophilous species require high levels of soil moisture and relative humidity, thus wet storage seems more appropriate for them. In our study, the spores of *A. argentina* remained viable for up to 6 months of dry storage in the freezer, and gametophytes and sporophytes developed in these cultures. This indicates that the spores of *A. argentina* resist desiccation to a certain period of time. Ballesteros *et al*. (2012) also observed in some fern taxa, that shortest lived spores belonged to species from riparian or shaded forests of the Iberian Peninsula. As Aragón & Pangua (2004) suggested for other ferns, the ecological requirements of this species seem to have influenced to certain extent spore viability.

Various studies have shown the beneficial effects of preserving spores or tissues in liquid nitrogen, in particular to preserve endangered fern species (Mikula *et al*., 2009; Ballesteros, 2012; Pence, 2015; Filipin *et al*., 2016; Pence, 2018; Tomiczak *et al*., 2023). However, for other purposes, storage in a freezer is much less expensive and requires less preparation time. Our findings demonstrate that the spores of *B. auriculatum* and *P. montevidensis* can be stored in freezer (at -20 °C) for at least one year, whereas in the case of *A. argentina*, new essays

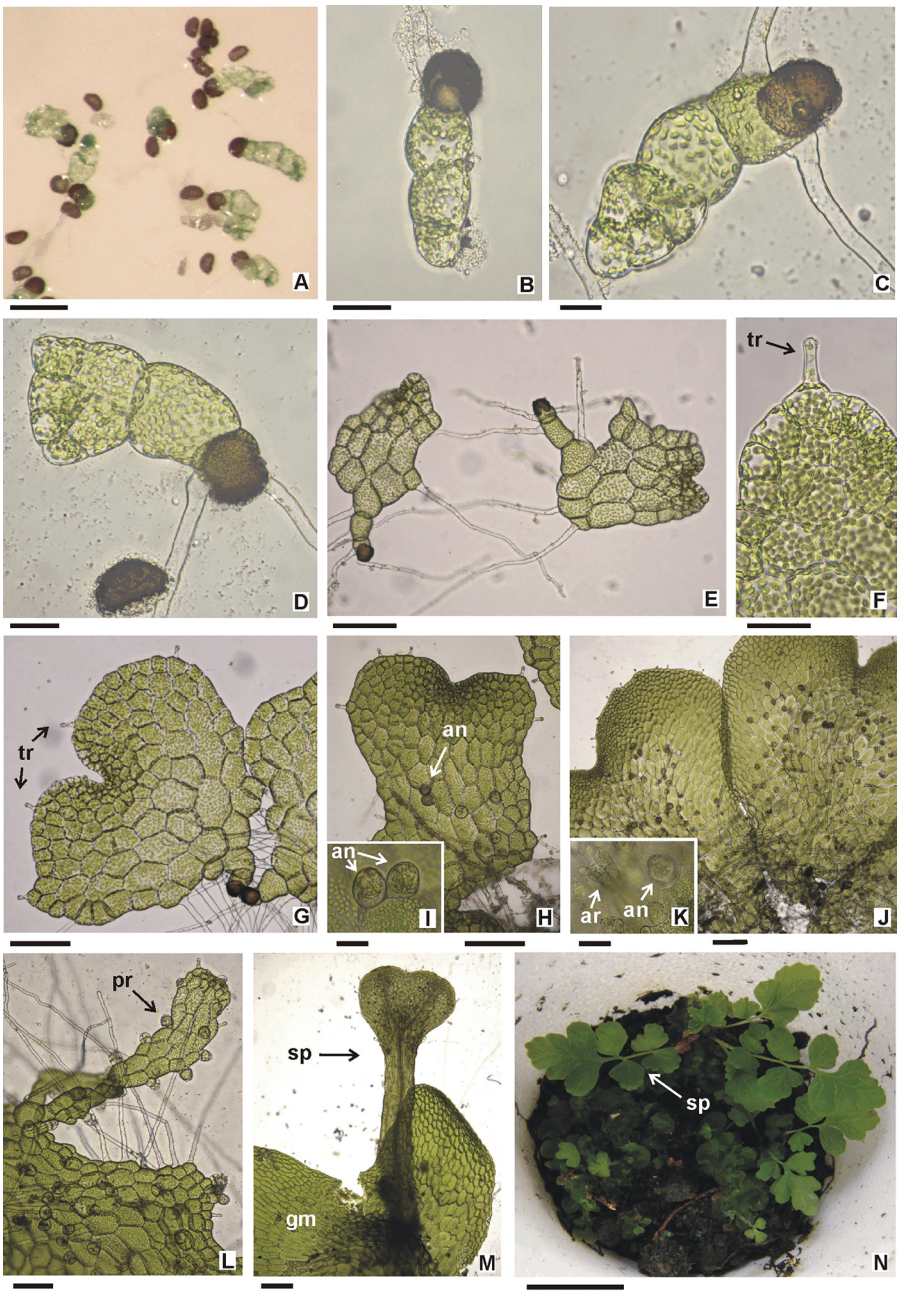

Fig. 2. *Amauropelta argentina* gametophyte and sporophyte development in cultures of spores stored 6 months in freezer. **A**: Earlier stages of gametophyte development. Some spores have not germinated. **B**: Three-celled filamentous gametophyte. **C**: Gametophyte at the beginning of the laminar phase. **D**: Gametophyte slightly wider after successive mitotic divisions. **E**: Laminar phase (left) and early cordiform phase (right). **F**: Detail of gametophyte margin with a unicellular glandular trichome. **G**: Cordiform gametophyte phase with scarce marginal trichomes. **H**: Elongated cordiform male gametophyte. **I**: Antheridia in detail. **J**: Symmetrical cordiform bisexual gametophytes. **K**: Gametangia (archegonia and antheridia) in detail. **L**: Proliferation of male gametophyte bearing antheridia. **M**: Sporophyte first leaf. **N**: More developed sporophyte with several leaves. Abbreviations= an: antheridium; ar: archegonium; gm: gametophyte; pr: proliferation; sp: sporophyte; tr: trichome. Scales= A, H, J, M: 200 µm; B, F: 50 µm; C-D, I, K: 25 µm; E, G, L: 100 µm; N: 2 cm.

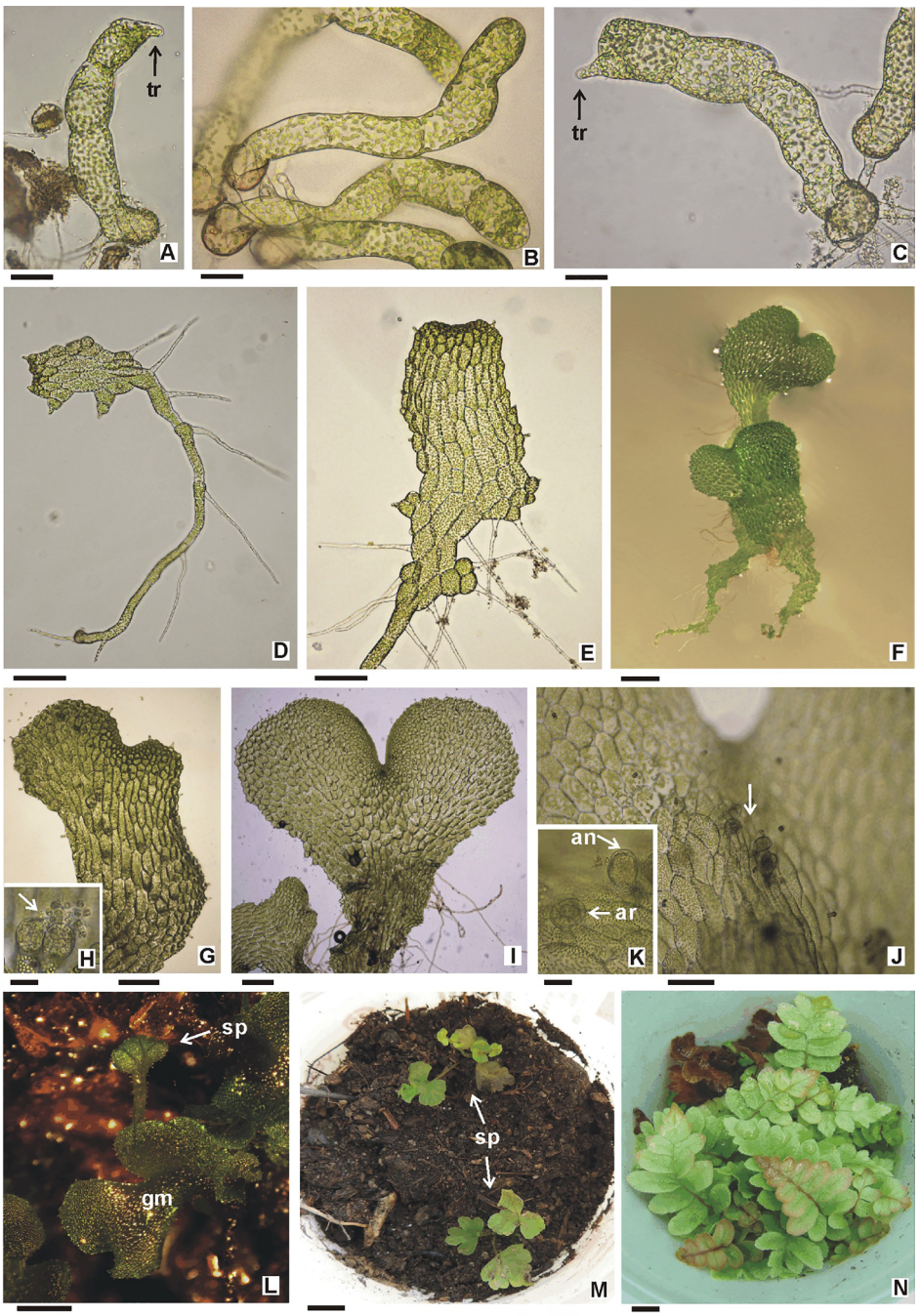

Fig. 3. *Blechnum auriculatum* gametophyte and sporophyte development in cultures of spores stored 12 months in freezer. **A**: Filamentous gametophyte phase with terminal trichome. **B**: Filaments without terminal trichome. **C**: Early laminar gametophyte phase. **D**: Laminar phase after successive mitosis. **E**: Early cordiform gametophyte phase. **F**: Mature cordiform gametophytes. **G**: Male gametophyte. **H**: Antheridia in detail (arrow). **I**: Symmetrical cordiform bisexual gametophyte. **J**: Gametangia near the notch (arrow). **K**: Magnified imagen of gametangia. **L**: Young sporophyte with first leaf. **M**: Sporophytes with 2-3 leaves. **N**: Sporophytes with several leaves with divided blade. Scales= A-C: 50 µm; D-E, G, I: 200 µm; F: 400 µm; H, K: 25 µm; J: 100 µm; L: 3 mm; M: 5 mm; N: 1 cm.

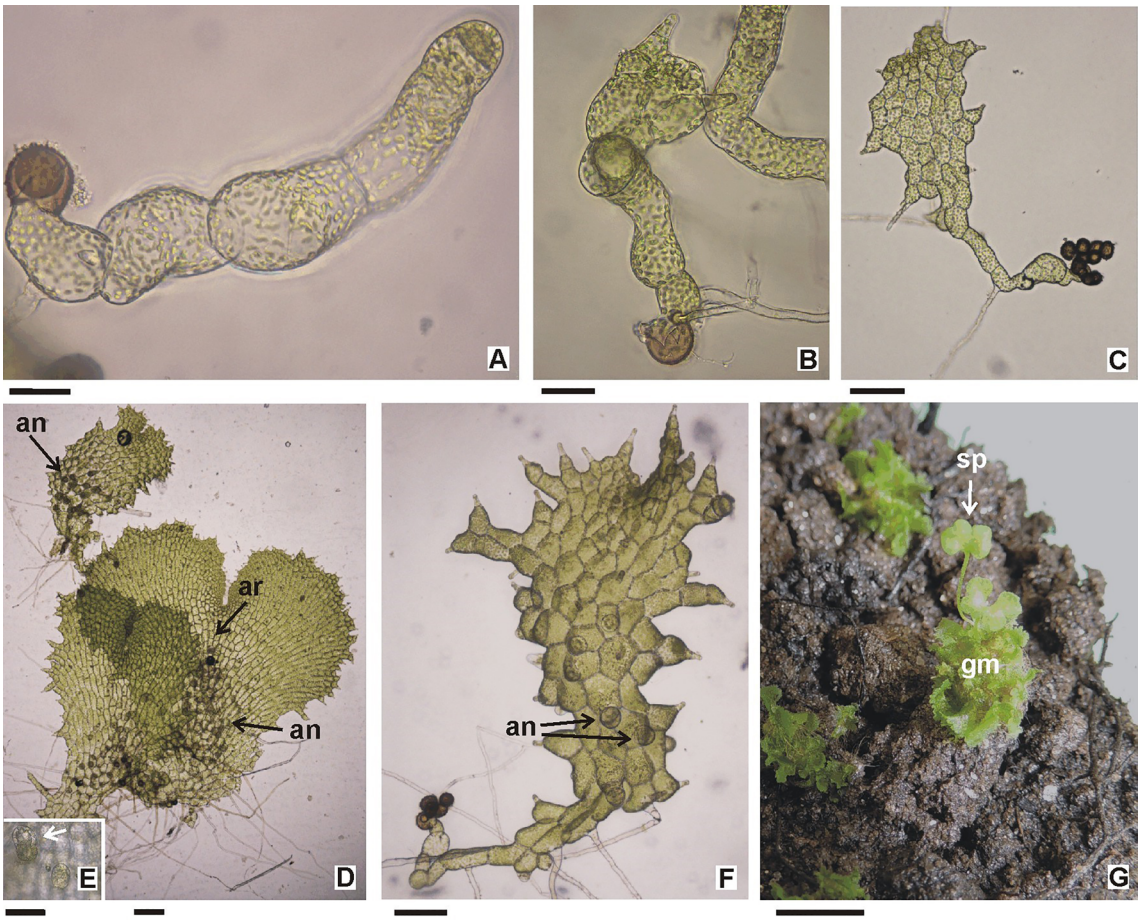

Fig. 4. *Physematium montevidensis* gametophyte and sporophyte development in cultures of spores stored 12 months in freezer. **A**: Filamentous gametophyte phase. **B**: Early laminar phase. **C**: Early cordiform phase. **D**: Unisexual male and bisexual gametophytes. **E**: An archegonium in detail. **F**: Magnified image of a male gametophyte. **G**: Young sporophyte with first leaves. Scales= A-B, E: 50 µm; C-D, F: 200 µm; G: 5 mm.

employing other storage and culture conditions will be tested to achieve a longer spore viability.

Concerning the developmental and morphological features of *B. auriculatum* and *P. montevidensis* gametophytes, they were previously described by Durán (1997) and Martinenco *et al*., (2023), and our observations are in coincidence with them. For *A. argentina*, this information is provided here for the first time. The spore germination pattern is *Vittaria*-type and the gametophyte development follows the *Aspidium*-type (according to Nayar & Kaur, 1971). The gametophytic development of each species was similar in the different trials (i.e. using fresh spores and those stored at low temperatures).

This work constitutes the first report on the viability of spores of fern taxa native to Argentina conserved at low temperature. We intend to provide baseline information for future propagation and conservation studies in native ferns, particularly those which inhabit areas with severe periodic disturbances, as occurs in the central Mountains systems of Argentina.

Author Contributions

MLM and MLL made the general conceptualization of the manuscript. MDA collected

the materials used in the essays. MLM and MLL realized the experimental tests and collected the data. All authors participated in the interpretation of data and in the preparation and writing of the manuscript.

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