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Germination and gametophyte development of *Cyathea corcovadensis* (Raddi) Domin (Cyatheaceae) from spores stored at low temperatures

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ABSTRACT. The tree fern *Cyathea corcovadensis* (Raddi) Domin is an endangered species in the state of Rio Grande do Sul, Brazil. It currently occurs only in the northern segment of the coastal region. Spore storage would help in conservation programs since it maintains genetic variability and provides material for *in vitro* cultures. Current study evaluates the effect of low temperatures combined to different spore storage times on the germination and initial gametophyte development of *C. corcovadensis*. Spores were divided into two groups: spores of the first group were sowed immediately in Meyer culture medium with nystatin, at pH 4.0, while spores of the second group were stored at 7, -20 and -196°C during 60, 120, 180, and 365 days and then sowed in the same medium. Spore storage at 7 and -196°C for 365 days not only provided higher germination percentages than those reported for recently-collected spores but also stimulated gametophytic development. The latter was demonstrated by the higher percentages of laminar gametophytes in these treatments. The possibility of storing spores provides material for *in vitro* experiments, which is of special interest for *C. corcovadensis* due to its ornamental potential and conservation status.

Keywords: conservation, *in vitro* culture, tree fern, cryopreservation, abiotic factor.

Germinação e desenvolvimento gametofítico de *Cyathea corcovadensis* (Raddi) Domin (Cyatheaceae) com esporos armazenados em baixas temperaturas

RESUMO. A samambaia arborescente *Cyathea corcovadensis* (Raddi) Domin está em perigo de extinção no Estado do Rio Grande do Sul, Brasil, ocorrendo somente no norte da região costeira. O armazenamento de esporos pode auxiliar programas de conservação, uma vez que oferece a possibilidade de manutenção da variabilidade genética e fornece material para culturas *in vitro*. O objetivo deste estudo foi avaliar o efeito de temperaturas baixas combinadas com diferentes tempos de armazenamento de esporos sobre a germinação e o desenvolvimento inicial de gametófitos de *C. corcovadensis*. Os esporos foram divididos em dois grupos: os do primeiro grupo foram semeados imediatamente em meio de cultura Meyer com nistatina, com pH 4,0 e os do segundo grupo foram armazenados a 7, -20 e -196°C durante 60, 120, 180 e 365 dias e então semeados no meio supra mencionado. O armazenamento de esporos a 7 e -196°C durante 365 dias propiciou porcentagens de germinação superiores àquelas observadas para esporos recém coletados e também estimulou o desenvolvimento gametofítico, o que foi demonstrado pelos elevados valores de porcentagem de gametófitos laminares observados nestes tratamentos. A possibilidade de armazenar esporos oferece material para experimentos *in vitro*, o que é de especial interesse para *C. corcovadensis*, considerando seu potencial ornamental e estado de conservação.

Palavras-chave: conservação, cultura *in vitro*, samambaia arborescente, criopreservação, fator abiótico.

Introduction

Cyatheaceae and Dicksoniaceae represent most tree ferns (FERNANDES, 2003), which are an important component of tropical forests (TRYON; TRYON, 1982). Approximately 480 species belong to Cyatheaceae, with pantropical distribution (TRYON; TRYON, 1982), or rather, a group with predominantly arborescent plants growing up to 25 m high (SPORNE, 1970). This family's most marked characteristic is its high value as an ornamental plant used in landscapes. However, the species were so extensively extracted that some were placed on the endangered list (WINDISCH, 2002). There are six species of Cyatheaceae in the Brazilian State of Rio Grande do Sul: *Alsophila capensis* J. Sm., *A. setosa* Kaulf., *Cyathea atrovirens* (Langsd. and Fisch.) Domin, *C. corcovadensis* (Raddi) Domin, *C. delgadii* Sternb. and *C. phalerata* Mart. (WINDISCH; SANTIAGO, 2014).

Cyathea corcovadensis occurs in the northeastern, southeastern and southern regions of Brazil

(WINDISCH; SANTIAGO, 2014) and may be found at 2,050 m above sea level, in environments with varying characteristics, such as dry and clearing sites of hygrophilous forests, gallery forests and riparian vegetation, and in humid areas in seasonal forests (FERNANDES, 2003). Since the species may only be found in the northern coastal region of Rio Grande do Sul State (LORSCHEITTER et al., 1999), it is listed as a threatened species and classified as endangered in the State of Rio Grande do Sul (RIO GRANDE DO SUL, 2003). *Cyathea corcovadensis* forms caudices as tall as 5 m and has persistent petiole bases. The leaves are up to 3 m long, leaf blades are bipinnate and the petioles are tuberculate or muricate. Secondary veins are bifurcate or forked, and sori are distributed medially on the pinnulae (FERNANDES, 2003).

Fern species may be protected *in situ*, in the areas of their natural occurrence, or *ex situ*, in especially prepared environments. They may be cultivated from spores by vegetative propagation or by *in vitro* tissue culture (MEHLTRETER, 2010). *In vitro* cultures offer the chance to study the plant's development stages, from gametophyte to sporophyte, and also the opportunity to better understand its abiotic demands, thereby providing data to develop efficient propagation strategies (RANKER; HAUFLER, 2008) and contribute to conservation and sustainable use of the species (HARDING et al., 1997). However, if *in vitro* culture is to be effective, the ecophysiological demands that must be met so that the plants may develop should be thoroughly understood.

Gametophytes may be cultured *in vitro* since spores maintain their germination capacity and physiological integrity. Spore storage at low temperatures has proved to be an important tool for *ex situ* fern conservation (LI et al., 2010; SIMABUKURO et al., 1998b). However, longevity has been related to abiotic and biotic factors, such as spore type, traditionally named as green (chlorophyllous) and non-green (non-chlorophyllous) (LLOYD; KLEKOWSKI JR., 1970), and considered recalcitrant or orthodox, respectively, based on the typical classification used for seeds (ROBERTS, 1973). Although orthodox spores tolerate desiccation and low temperatures and although recalcitrant spores have high water content and do not tolerate droughts, fern species present different responses when stored at low temperatures (ARAGON; PANGUA, 2004; LI et al., 2010; QUINTANILLA et al., 2002; SIMABUKURO et al., 1998b). In fact, storage efficacy has still to be better understood (BALLESTEROS; WALTERS, 2007). Since green and non-green spores appear to be tolerant to desiccation, dry storage at room or low temperatures has received attention, although there are important behavior differences in relation to spore aging among species (ARAGON; PANGUA, 2004; BALLESTEROS et al., 2012; LI et al., 2010; LLOYD; KLEKOWSKI JR., 1970; PENCE, 2000; QUINTANILLA et al., 2002). In Brazil, the influence of storage on spore germination was investigated for *Acrostichum danaeifolium* Langsd. and Fisch. (RANDI, 1996), *Dicksonia sellowiana* Hook. (FILIPPINI et al., 1999; ROGGE et al., 2000; BEGNINI; RANDI, 2009), *Rumohra adiantiformis* (G. Forst.) Ching (BEGNINI; RANDI, 2009; BRUM; RANDI, 2006) and *Cyathea atrovirens* (RECHENMACHER et al., 2010; VARGAS; DROSTE, 2014).

Current study evaluates the effect of low temperatures combined with different spore storage times on the germination and initial gametophyte development of *Cyathea corcovadensis*. Due to the potential of the species for ornamental use and its conservation status, this study will contribute towards the establishment of an *in vitro* propagation protocol that may be employed for its conservation and management.

Materials and methods

Cyathea corcovadensis population from which the spores were collected grows in a 5 ha forest fragment in the municipality of Três Cachoeiras (29° 25' 04.54" S and 49° 54' 47.37" W; 15 m height) in the State of Rio Grande do Sul, Brazil. The forest fragment is located on the coastal physiographic region (FORTES, 1959) with vegetation classified as ombrophylous dense forest (TEIXEIRA et al., 1986) in the phytogeographic domain of the Atlantic Rainforest (WINDISCH; SANTIAGO, 2014).

Fertile leaves, collected from ten specimens of a natural population in August 2012, were placed on trays and stored at 26°C for a minimum of 72 hours until sporangia dehiscence, following Brum and Randi (2006), with adaptations. Spores were separated from the sporangia by filtration with interleaved paper (Melpaper™) and divided into two groups. The spores in the first group were sowed immediately and considered as the reference group. Spores in the second group were stored in Eppendorf tubes (1.5 mL) at temperatures 7, -20, and -196°C, during 60, 120, 180, and 365 days. The spores frozen to -196°C were not treated with any type of cryoprotective substance. A slow defrosting

method was used, consisting of removing the Eppendorf tube from the liquid nitrogen and placed at room temperature (25°C) during 24 hours before sowing (AGRAWAL et al., 1993; BRUM; RANDI, 2006).

Spore viability

Three microscopy slides were prepared with approximately 5 mg of spores on 100 μL of distilled water for each of the above temperatures at 365 days of storage and for the reference group. The first 100 spores observed were scored. Transparent spores with no reserve substances were scored as non-viable, whereas spores with these substances were scored as viable, following Gomes et al. (2006).

Spore germination and gametophyte development

Spores of the reference group and spores that had been stored at different temperatures for different periods were disinfected in 1 mL of sodium hypochlorite at 2% (v v^{-1}) for 15 min. and rinsed four times in distilled autoclaved water (VARGAS; DROSTE, 2014).

Immediately after asepsis, the spores were sowed in liquid Meyer culture medium (MEYER et al., 1955) that had been pH-adjusted to 4.0 prior to sterilizing in an autoclave. Germination conditions have been shown to be suitable for *C. corcovadensis* in previous experiments (unpublished data). Disinfectant agent nystatin (50,000 units) was placed in each flask (200 mL capacity) containing 30 mL Meyer medium and 10 mg of spores were sowed. Three repetitions were prepared for each temperature and storage time, as well as for the reference group, with one repetition defined as one flask. Cultures were maintained at 26 ± 1°C, with a photoperiod of 12 hours and a light intensity of 70 μmol m^{-2} s^{-1}.

Spore cultures of all treatments and spores of the reference group were evaluated for germination 28 days after sowing, when laminar gametophytes were observed in a previous study (unpublished data). One microscopy slide was prepared per repetition, and the first 100 specimens, observed on each slide by a binocular optical microscope (Nikon, Eclipse E200), at 400 x magnification, were scored. The criterion adopted for defining a germinated spore was the emergence of the chlorocyte or the rhizoid (RANAL, 1999). The cultures from spores of all treatments and from spores of the reference group were assessed for gametophyte development 28 days after sowing. Slides were prepared similarly as for the verification of spore germination. Gametophytes were classified according to the development stages described by Rechenmacher et al. (2010), as follows: gametophyte with chlorocyte and rhizoid; filamentous gametophyte and laminar gametophyte.

Statistical analysis

Data of spore germination and gametophyte development were given in percentages and analyzed with SPSS 20. Normality of data was verified by Shapiro-Wilk test. Germination of spores and gametophyte development were tested by analysis of variance (ANOVA) and differences between means were tested by Tukey's test at 5% probability.

Results and discussion

In the reference group, 88% of the spores were considered viable due to their yellow-brown color typical of non-green spores, which evidenced the presence of reserve substances. After 365 days, spores stored at 7, -20, and -196°C showed viability means of 93, 89.7, and 94.3%, respectively. The fact that spores of *Cyathea corcovadensis* stored at low temperatures did not lose their viability is of relevance in the case of *in vitro* conservation efforts (QUINTANILLA et al., 2002) and may be explained, at least partially, by their chemical composition. The species´s spores are classified as non-green, lipids make up 44 to 50% of their weight, while proteins account for 1% (ESTEVES; FELIPPE, 1985). Owing to this composition and their low humidity level, lipidic spores have a mean viability ranging between several months and years, in contrast with green spores which lose their viability within two to six weeks because of the chlorophyll´s fast catabolization (SHARPE et al., 2010).

Germination of spores and development of gametophytes after storage at different temperatures at each time tested were compared. The significantly higher germination of spores stored at 7 and -196°C was observed for all storage times tested when compared with germination of spores stored at -20°C (Table 1). The percentage of gametophytes in the third developmental stage or laminar stage was numerically higher in treatments at -196°C, which differed significantly from the percentages of this type of gametophytes observed after storage at 7°C (60 and 365 days) and -20°C (60, 180, and 365 days).

Table 1. Percentages (mean ± standard deviation) of *Cyathea corcovadensis* (Raddi) Domin gametophytes at different stages after 28 days, developed from spores stored at different temperatures, for 60, 120, 180 and 365 days. Letters in the same row indicate significant difference according to Tukey´s test at 5% probability.

		Temperature				
Storage time	Stage	7°C	-20°C	-196°C	F	p
60	Chlorocyte and rhizoid	7.0 ± 2.0^{ab}	10.0 ± 3.6^{a}	3.0 ± 1.0^{b}	6.167	0.035
	Filamentous	24.0 ± 1.7^{a}	16.3 ± 3.5^{ab}	11.0 ± 3.6^{b}	13.565	0.006
	Laminar	68.0 ± 5.0^{b}	9.7 ± 3.5^{c}	87.0 ± 2.6^{a}	329.692	<0.001
	Total	99.0 ± 1.7^{a}	35.3 ± 2.1^{b}	99.3 ± 1.1^{a}	1410.500	<0.001
120	Chlorocyte and rhizoid	12.7 ± 14.1^{a}	9.7 ± 1.2^{a}	14.7 ± 6.0^{a}	0.239	0.794
	Filamentous	22.3 ± 6.6^{a}	18.0 ± 5.3^{a}	9.0 ± 6.0^{a}	3.843	0.084
	Laminar	63.0 ± 19.7^{a}	57.7 ± 2.1^{a}	73.7 ± 9.3^{a}	1.251	0.352
	Total	98.0 ± 2.6^{a}	85.3 ± 3.5^{b}	97.3 ± 2.3^{a}	18.541	0.003
180	Chlorocyte and rhizoid	13.3 ± 2.8^{a}	16.7 ± 3.5^{a}	11.3 ± 3.2^{a}	2.108	0.203
	Filamentous	19.3 ± 10.0^{a}	16.0 ± 2.0^{a}	19.7 ± 3.5^{a}	0.317	0.740
	Laminar	62.7 ± 12.2^{ab}	45.3 ± 1.5^{b}	63.7 ± 1.5^{a}	6.210	0.035
	Total	95.3 ± 2.3^{a}	78.0 ± 2.6^{b}	94.7 ± 0,6^{a}	68.526	<0.001
365	Chlorocyte and rhizoid	14.3 ± 3.8^{ab}	18.7 ± 4.9^{a}	7.7 ± 3.2^{b}	5.639	0.042
	Filamentous	23.7 ± 5.7^{a}	24.3 ± 9.2^{a}	16.3 ± 5.0^{a}	1.231	0.356
	Laminar	57.7 ± 4.9^{b}	40.0 ± 3.0^{c}	71.7 ± 6.1^{a}	32.071	0.001
	Total	95.7 ± 2.1^{a}	83.0 ± 7.2^{b}	95.7 ± 2.1^{a}	7.934	0.021

For each temperature tested, the influence of storage time on germination and development of laminar gametophytes was evaluated and compared with the reference group. Results obtained at 7 and -196°C showed that germination percentages were significantly higher than those in the reference group and no difference in germination between storage times 60 and 365 days was registered. Germination was higher than 95% after storage at 7°C, and ranged between 94.7 and 99.3% after storage at -196°C, whereas in the reference group germination was 82% (Figure 1). At -20°C, germination after 120, 180, and 365 days of storage ranged between 78 and 85%, significantly higher than that reported after 60 days of storage (35%), although it did not differ from the germination percentage of the reference group (Figure 1). Other species of *Cyathea* have been assessed for their spores' capacity to germinate after storage at low temperatures above zero and data corroborate with those of the species investigated in current study. Spores of *C. atrovirens* (RECHENMACHER et al., 2010) stored during 180 days at 7°C exhibited 95% germination. However, germination percentages of spores of *C. delgadii* stored at long term (for up to 3 years) at 4°C gradually decreased from 86% for recently-collected spores, through 25% for spores stored for two years, to close to zero after 32 months (SIMABUKURO et al., 1998b). Filippini et al. (1999) tested the effects of different storage periods (15 to 731 days) at approximately 10°C on the germination of another tree fern, *Dicksonia sellowiana*, and found that the spores remained viable after 731 days, developing a slight decrease in germination percentage after the period. Further, storage of *D. sellowiana* spores at -196°C for 90 days resulted in 90% germination, while recently-collected spores exhibited 80% germination, suggesting that cryopreservation caused dormant spores to germinate (ROGGE et al., 2000). Dormancy plays an important role in nature, since it makes asynchronous germination possible (FILIPPINI et al., 1999; ROGGE et al., 2000; SCHNELLER, 1998), reduces competition during the initial phases of development, and increases the chances of reproductive success (GOMES et al., 2006) and formation of spore banks (ESTEVES, 2013), which is highly important in seasonal climates.

The behavior of spores observed in current study corroborate previous reports on sensitivity and faster aging of spores from many species to temperatures between -12 and -25°C (ARAGON; PANGUA, 2004; BALLESTEROS et al., 2012; LI et al., 2010; QUINTANILLA et al., 2002; SIMABUKURO et al., 1998b). Sensitiveness to this temperature range may be related to the spores´ chemical composition. The amount of triacylglycerols (TAG) with saturated and monounsaturated fatty acids, and their interaction with water may damage the cell in non-green spores when crystallization occurs (BALLESTEROS et al., 2012; VOLK et al., 2006). Differential Scanning Calorimetry (DSC) (BALLESTEROS; WALTERS, 2007) has been applied to detect TAG in non-green spores, and estimates were consistent with a previous study that TAG compromised up to 50% of the dry mass of *Cyathea delgadii* spores (RANDI; FELIPPE, 1988; SIMABUKURO et al., 1998a). The detrimental effect of freezer storage may be avoided by cryogenic storage conditions below -80°C, associated with rapid cooling, since crystallization of TAG and water is prevented (BALLESTEROS et al., 2012). In fact, cryogenics has been pointed out as an effective

alternative for seed and spore storage (AGRAWAL et al., 1993; BRUM; RANDI, 2006; ROGGE et al., 2000). Unfortunately, most studies are focused on spore survival, rather than quantification of germination and gametophyte development. Germination alone was considered an insufficient parameter to evaluate the effect of storage on spore longevity (BALLESTEROS et al., 2011). Monitoring the early stages of development may actually provide important information on the influence of storage with regard to gametophytes´ ontogeny and morphology.

Laminar gametophytes in all treatments in current study were observed after 28 days of culture and indicated that germination was successful and the third stage of gametophyte development was achieved. After storage at 7°C, there was no significant difference between the percentages of laminar gametophytes recorded for the different storage times, ranging between 57 and 68% (Figure 1). These percentages did not differ from the percentage in the reference group too (52.67%). After 60 days of storage at -20°C, only 9.67% of laminar gametophytes were reported, or rather, a percentage significantly lower than that in other treatments and in the reference group (Figure 1).

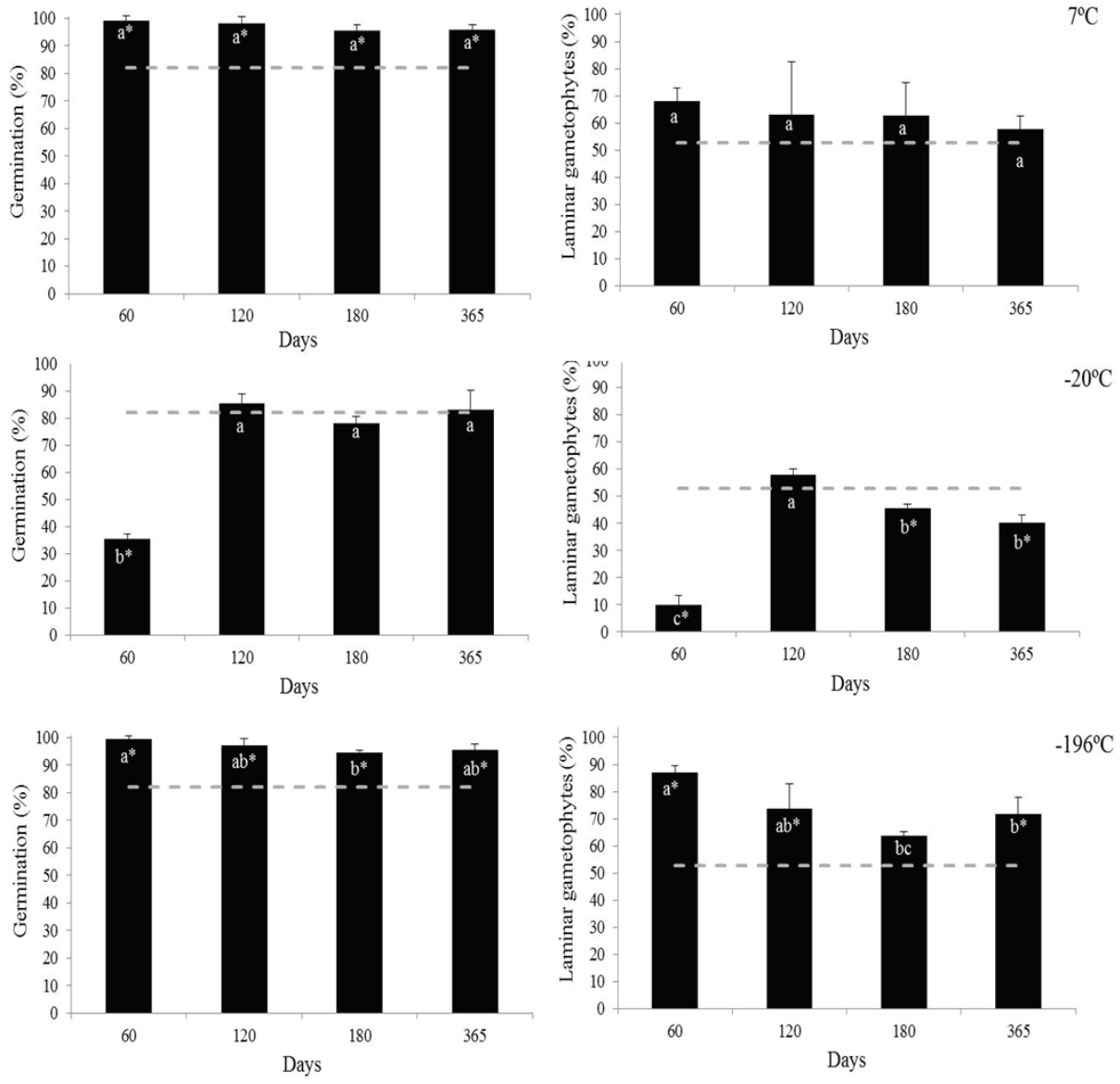

Figure 1. Percentages of germination and laminar gametophytes of *Cyathea corcovadensis* (Raddi) Domin from spores stored during different storage times at 7, -20 and -196°C. Dotted lines indicate percentages of the reference group. Letters and * indicate, respectively, significant difference of germination and laminar gametophytes between treatments and between each treatment and the reference group, according to Tukey´s test at 5% probability. Columns show means ± standard deviations.

After 120 days of storage, 57.67% of laminar gametophytes were recorded. This percentage did not differ from the percentage in the reference group (52.67%), but was significantly higher than that of the other treatments (Figure 1). Quintanilla et al. (2002) reported that wet storage at -20°C killed the spores of *Culcita macrocarpa* C. Presl (Dicksoniaceae), *Dryopteris aemula* (Aiton) Kuntze, *D. corleyi* Fraser-Jenk., *D. guanchica* Gibby and Jermy (Dryopteridaceae) and *Woodwardia radicans* (L.) Sm. (Blechnaceae) after one month of storage, whereas spores maintained their viability in dry storage at the same temperature. The data obtained in current study for -20°C storage suggested that the metabolism of *C. corcovadensis* spores was impaired by this storage method, although results have to be confirmed by further experiments.

In cultures from spores stored at -196°C for 180 and 365 days, the percentages of laminar gametophytes were significantly lower (63.67 and 71.67%) than those after 60 days of storage (87%) (Figure 1). These results suggest that longer storage times at -196°C may lead to minor gametophyte development. Nevertheless, the percentages of laminar gametophytes after 60, 120 and 365 days of storage were significantly higher than those of the reference group (Figure 1), which were previously reported in the literature for other fern species (BRUM; RANDI, 2006; ROGGE et al., 2000). Cryogenic treatment is known to improve absorption by spores in culture medium, since it increases water uptake due to the breaking up of the spore coat causing faster germination (BRUM; RANDI, 2006). Fern spores may be subjected to cryopreservative treatments without the need to add cryoprotective substances (AGRAWAL et al., 1993; PENCE, 2000; ROGGE et al., 2000) since the rapid cooling to -196°C inhibits the formation of ice crystals. The above occurs because of the spores' chemical composition, particularly in spores with lipid content, by diminishing the probability of damaging events and irreversible injury caused by crystallization (KAVIANI, 2011).

Since developmental abnormalities in older spores may be considered as limiting the success of *in vitro* cultures (QUINTANILLA et al., 2002), no morphological abnormal gametophytes were reported at any stage in current study. In fact, this is in line with observations for *C. spinulosa* Wall. ex Hook. (AGRAWAL et al., 1993) and for *D. sellowiana* (ROGGE et al., 2000), both of which exhibited normal development after at least six years' storage at -196°C.

Considering the conservation status of *C. corcovadensis*, spore storage could be of help in conservation programs since it offers the chance to maintain genetic variability and provides material for more in-depth studies about the species's behavior. Greater discrimination among storage conditions may be achieved by testing additional storage conditions and longer storage times in future studies.

Conclusion

Storage of *Cyathea corcovadensis* spores at -196 and 7°C for 365 days caused higher germination percentages than those observed for recently-collected spores and for spores stored at -20°C. Gametophyte development was faster after storage at -196°C during 365 days. Cryopreservation provides a feasible alternative for spore germoplasm conservation of *C. corcovadensis*, considering the storage time evaluated in current study.

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