Spore germination and gametophyte development of *Cyathea atrovirens* (Langsd. & Fisch.) Domin (Cyatheaceae) under different pH conditions

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(With 3 figures)

Abstract

Cyathea atrovirens (Langsd. & Fisch.) Domin, an intensely exploited tree fern, is found inside forests in several succession stages, as well as in swamps, roadsides and unused fields in the Rio dos Sinos basin, in the state of Rio Grande do Sul, southern Brazil. This study evaluated the in vitro germination and gametophyte development of *C. atrovirens* under different pH conditions, as well as spore viability after different storage times at 7 ºC. The lowest germination rate of spores was obtained at pH 7.0. At pH 5.0 to 6.5, laminar gametophyte development started at 20 to 30 days of culture. Antheridia and archegonia were first observed at 35 and 128 days, respectively. Storage at 7 ºC did not affect germination rates. The capability of germination at different pH levels may explain the occurrence of the species in a wide range of habitats. The present study contributes to the understanding of the full life-cycle of *C. atrovirens* and to the analysis of the influence of abiotic components, providing information for the cultivation, management and conservation of the species.

Keywords: tree ferns, gametophyte, pH, reproduction, conservation.

Germinação de esporos e desenvolvimento gametofítico de *Cyathea atrovirens* (Langsd. & Fisch.) Domin sob diferentes condições de pH

Resumo

Cyathea atrovirens (Langsd. & Fisch.) Domin, uma samambaia arborescente alvo de exploração extrativista, é encontrada no interior de florestas em diferentes estádios de sucessão, banhados, margens de estradas e campos abandonados da bacia do rio dos Sinos, no Estado do Rio Grande do Sul, sul do Brasil. Este estudo avaliou a germinação e o desenvolvimento gametofítico in vitro de *C. atrovirens* sob diferentes condições de pH, bem como a viabilidade de esporos após diferentes períodos de armazenamento a 7 ºC. A menor taxa de germinação de esporos foi obtida no pH 7,0. Entre os pHs 5,0 e 6,5, o início de desenvolvimento laminar dos gametófitos ocorreu entre 20 e 30 dias de cultura. Anterídios e arquegônios foram observados a partir do 35º e do 128º dia, respectivamente. A estocagem a 7 ºC não afetou as taxas de germinação. A capacidade de germinação em diferentes pHs pode explicar a ocorrência da espécie em um amplo espectro de hábitats. O presente estudo contribui para a compreensão do ciclo de vida completo de *C. atrovirens* e para a análise da influência de fatores abióticos, fornecendo subsídios para o cultivo, o manejo e a conservação da espécie.

Palavras-chave: samambaias arborescentes, gametófito, pH, reprodução, conservação.

1. Introduction

Tree ferns are important understory components of tropical rainforests (Page, 1979; Tryon, RM. and Tryon, AF., 1982). Most tree ferns species belong to Cyatheaceae and Dicksoniaceae (Fernandes, 2003), and 20 of them occur in the south and southeastern regions of Brazil. Seven species are found in the state of Rio Grande do Sul, and five, in the Rio dos Sinos basin: *Alsophila setosa* Kaulf., *Cyathea atrovirens* (Langsd. & Fisch.) Domin, *Cyathea delgadii* Sternb., *Cyathea phalerata* Mart. and *Dicksonia sellowiana* Hook. (Lorscheitter et al., 1999).

Because of its ornamental characteristics, *Cyathea atrovirens* is the object of intense exploitation. The caudices of older plants, when presenting a sheath of adventitious roots at the base, are used to manufacture fibre handicrafts (Fernandes, 2000). The leaves are used for ornamental purposes (Tryon, RM. and Tryon, AF., 1982). In the Rio dos Sinos basin, this species may be found inside forests at different succession stages, as well as in swamps, and in areas largely affected by human action, such as roadsides and abandoned fields.

Understanding tree fern germination and early development requirements could provide a basis for establishing alternative propagation methods that may contribute to their conservation. In vitro culture methods provide controlled conditions for propagating sporophytes and studying fern biology, since germination and growth requirements of the Neotropical species are poorly understood (Fernández et al., 1999; Cassanego et al., 2010).

In several cases pH conditions might be a limiting factor to fern establishment (Petersen, 1985). Studies have demonstrated that fern species present varied responses to pH in vitro. While spores of some species germinate in strong acidic medium (Whittier and Moyroud, 1993) or in a broad pH range (Nondorf et al., 2003; Viviani and Randi, 2008), some fern spores are not able to germinate in strong acidic conditions (Nester and Coolbaugh, 1986). No information could be found in the literature on the effect of pH on *Cyathea atrovirens* germination and initial growth.

This study investigated the effect of different pH and storage times on in vitro germination and gametophyte development of *Cyathea atrovirens*, in order to understand the establishment of gametophytes and young sporophtes in nature, as well as to provide ecophysiological information which could be useful for plant production for reintroduction into nature, assisting species conservation and environment management programmes.

2. Material and Methods

Fertile leaves of *Cyathea atrovirens* were collected in the Parque Municipal Henrique Luis Roessler (29° 40' 54" S and 51° 06' 56" W; altitude: 16.4 m), which is a conservation area of the Rio dos Sinos basin in the municipality of Novo Hamburgo, Rio Grande do Sul, Brazil. The leaves were wrapped in smooth paper and kept at room temperature to induce dehiscence of sporangia. Spores were filtered through lens cleaning tissue. Part of the spores were stored in tubes at 7 ± 1 °C for later viability analysis. Spores did not undergo asepsis to keep experimental conditions close to those found in nature.

To evaluate the effect of pH on germination and initial gametophyte development of fresh spores, seven different pH levels were used: 4.0; 4.5; 5.0; 5.5; 6.0; 6.5; and 7.0. In a laminar flow hood, 10-mg samples of spores were distributed in flasks with 50-mL autoclaved Meyer solution (Meyer et al., 1955). Six replicates were used for each pH level, totalising 42 flasks. The cultures were maintained in a growth chamber at 26 ± 1 °C for a 16-hour photoperiod and photon flux density of 100 $\mu mol.m^{-2}.s^{-1}$ provided by cool white fluorescent tubes.

Germination was scored at three, six, nine and 12 days of in vitro culture. Six slides (one slide per flask) from each treatment were analysed, verifying 100 spores on each slide. To follow up initial gametophyte development at different pH levels, 10 individuals on each slide (one slide per flask) were photographed and classified according to their developmental stage in the following categories at six, nine and 12 days of culture: a) rhizoid and chlorocyte; b) filamentous stage; and c) laminar stage. At 104, 128 and 150 days, the morphology of one individual from each flask was described.

To describe the in vitro development of this species, only gametophytes obtained at pH 6.0 were evaluated at up to 128 days of culture. Representative individuals at different development stages were photographed, and the images were digitalised using a Canon EOS 220 camera coupled to an Olympus CX4 microscope.

In a second experiment, the germinability rates of fresh and stored spores (90 and 180 days at 7 °C) were compared, using cultures with 10 mg of spores placed in flasks with 50-mL autoclaved Meyer solution at pH 6.0. Six replicates were used for each storage-period. Germination rates were scored at three, six, nine and 12 days of in vitro culture.

Variance analysis (ANOVA) was used for statistical analyses, and the Tukey test was used to evaluate the differences between means, using the SPSS 15.0 software. The level of significance was set at 5% (Zar, 1999).

3. Results

Cyathea atrovirens spores under light microscopy were yellow and tetrahedral-globose (trilete). Germination began on the third day after inoculation of spores onto the culture media (Figure 1a), although the process was asynchronous within each pH culture. The criterion for germination was the emergence of the chlorocyte or the rhizoid (Ranal, 1999).

The lowest germination rate was observed in the pH 7.0 culture, which was significantly different from the results found for the other pH cultures on the 6th day. On the 9th day, the result obtained in the pH 7.0 differed from pH 5.0 to 6.5, and, on the 12th day, from all pH levels, except pH 4.5 (Figure 2).

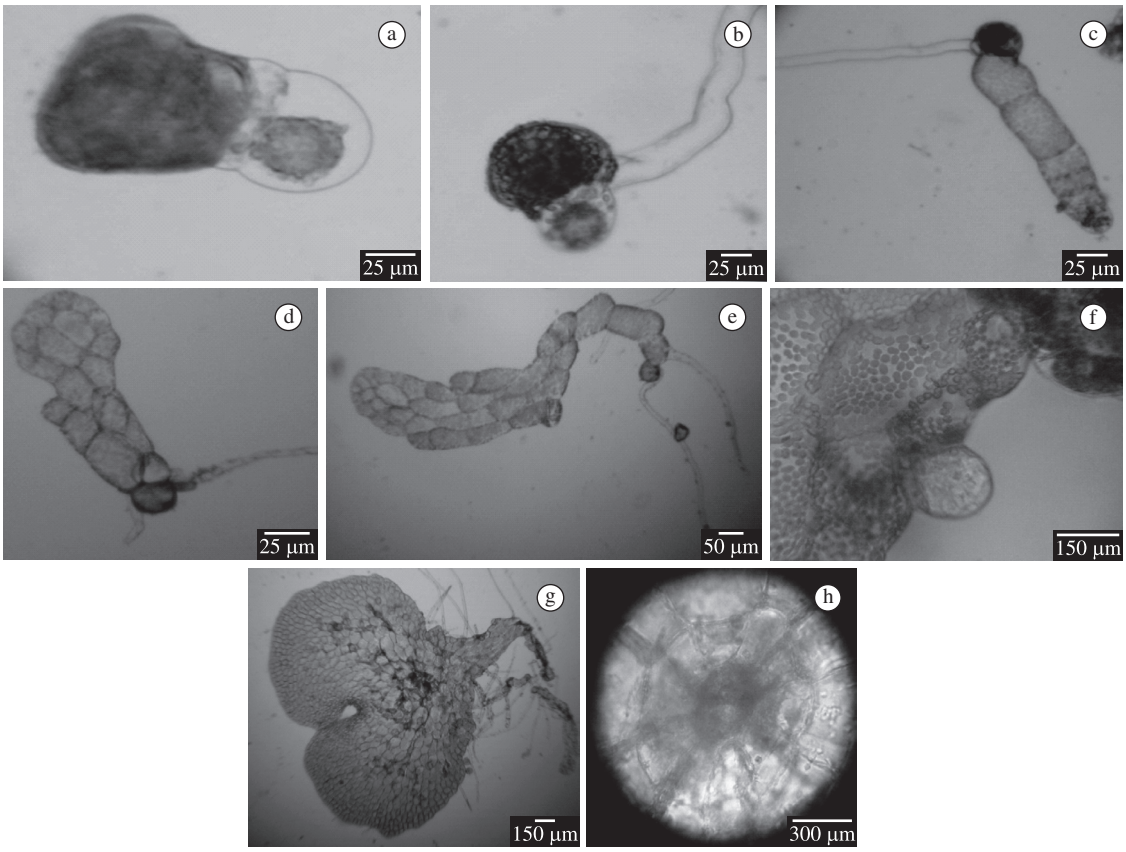

Figure 1. *Cyathea atrovirens* gametophyte development: a) gametophyte with chlorocite (3 days) (bar: 25 µm); b) gametophyte with chlorocyte and rhizoid (6 to 9 days) (bar: 25 µm); c) gametophyte in filamentous stage (bar: 25 µm); d) gametophyte in laminar stage (12 days) (bar: 25 µm); e) gametophyte in laminar stage (20 to 35 days) (bar: 50 µm); f) antheridia (bar: 150 µm); g) cordate gametophyte (50 days) (bar: 150 µm); and h) archegonia (bar: 300 µm).

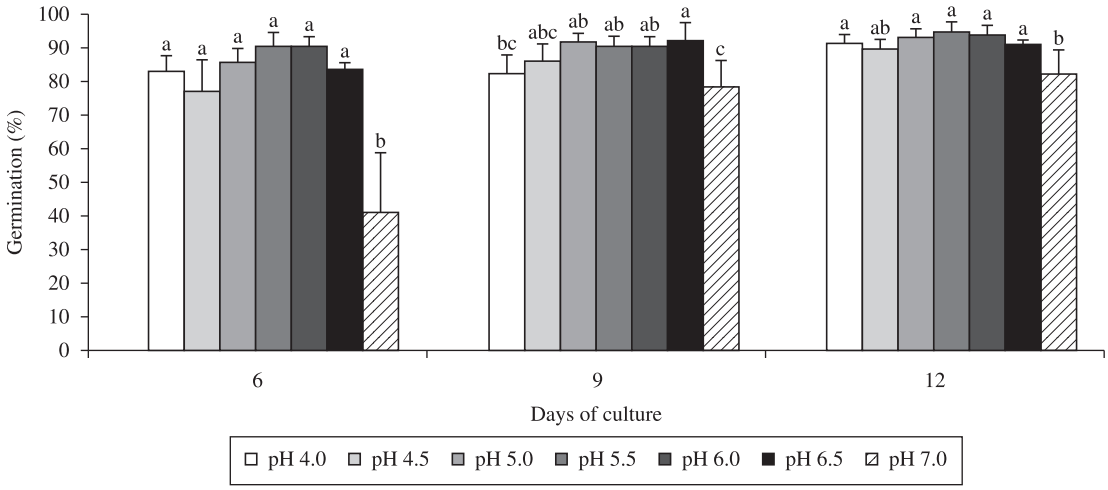

Figure 2. In vitro germination of *Cyathea atrovirens* spores in different pH levels after 6, 9 and 12 days. Same letters within each time point indicate that difference is not statistically significant according to Tukey test at a 5% level of significance (6^{th} day: $F = 23.692$; $p < 0.001$) (9^{th} day: $F = 5.948$; $p < 0.001$) (12^{th} day: $F = 6.524$; $p < 0.001$). Vertical bars indicate standard deviations.

On the 6th day, gametophytes in the pH 4.0 and 7.0 cultures were still in the rhizoid and chlorocyte stage (Figure 1b), whereas in the pH 4.5 to 6.5, gametophytes were already in the filamentous stage (Table 1), characterised by the presence of two to eight protonemal cells (Figure 1c). After nine days of culture, individuals in the rhizoid and chlorocyte stage were found in all cultures except those of pH 6.0 and 6.5. The highest number of individuals in all cultures, except at pH 7.0, was in the filamentous stage. Despite this, gametophytes in the laminar stage could be observed in the cultures of pH 4.0, 5.0, 5.5, 6.0 and 6.5 (Figure 1d). On the 12th day, individuals in the rhizoid and chlorocyte stage were found only in the pH 7.0 culture. Comparing with the cultures with lowest and highest pH, the highest mean number of individuals in the laminar stage was found in the pH 6.5 culture.

Gametophytes germinated in pH 6.0 cultures were used to follow up development for 128 days. The beginning of laminar development occurred at 20 to 30 days of in vitro culture (Figure 1e). Antheridia were observed at the surface of gametophytes from the 35th day on (Figure 1f). The evaluation at 50 days showed cordate gametophytes (Figure 1g), a large number of antheridia and an increased number of rhizoids irregularly distributed on both surfaces and on the margins of the gametophytes. The individuals were bisexual and the archegonia were first seen at 128 days (Figure 1h) next to the apical notch of the cordate prothalli.

To compare the development of the gametophyte structure under different pH conditions, evaluations were done at 104, 128 and 150 days in all pHs tested. On the 104th day, gametophytes in the pH 4.5 and 7.0 cultures were in the laminar stage. The cordate stage was observed only at 128 days, although these structures still had a smaller size than those in the other pH cultures and no archegonia were observed up to the end of the observation period. In cultures of intermediate pH levels (5.0 to 6.5), gametophytes were cordate as early as the 104th day, visibly larger and with a higher number of antheridia than in the cultures of pH 4.0, 4.5, and 7.0. Archegonia were observed at 128 days. No further morphological changes were observed in the gametophytes after this point.

In the second experiment, the germination of fresh *Cyathea atrovirens* spores and spores stored for 90 and 180 days at 7 °C were not statistically different after seven and nine days of culture. On the 12th day, spores stored for 90 days presented a higher percentage of germination than spores stored for 180 days, but the difference was not statistically significant (Figure 3). There were no statistically significant differences in the number of individuals in the stage of rhizoid and chlorocyte and the filamentous stage at six and nine days. On the 12th day, however, the spores stored for 180 days formed the lowest mean number of gametophytes in the filamentous stage. Storage did not significantly affect the number of individuals in the laminar stage after 9 and 12 days of culture (Table 2).

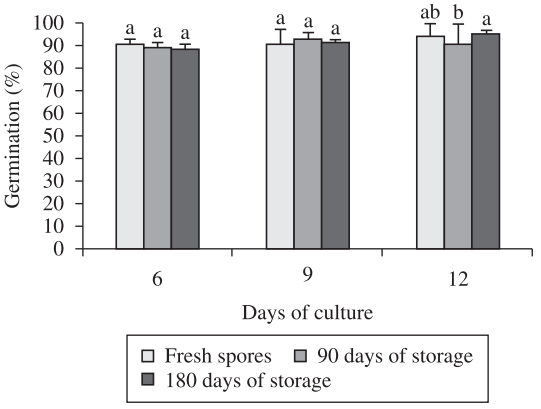

Figure 3. Germination of fresh *Cyathea atrovirens* spores and spores stored for 90 and 180 days at 7 °C. Same letters within each time point indicate that difference is not statistically significant according to Tukey test at a 5% level of significance (6th day: F = 0.127; p = 0.882), (9th day: F = 0.901; p = 0.427), (12th day: F = 3.969; p = 0.041). Vertical bars indicate standard deviations.

Table 1. Number of *Cyathea atrovirens* gametophytes (mean ± standard deviation) at different development stages after 6, 9 and 12 days in culture under different pH levels. Same letters on each line indicate that difference is not statistically significant according to Tukey test at a 5% level of significance.

Days	Stage	pH							F	P
		4.0	4.5	5.0	5.5	6.0	6.5	7.0		
6	Rhizoid/chlorocyte	8.7 ± 1.2^{a}	1.2 ± 1.2^{b}	5.2 ± 1.5^{ab}	5.7 ± 1.9^{a}	5.0 ± 3.7^{ab}	5.7 ± 1.4^{a}	5.3 ± 3.6^{ab}	5.385	0.001
	Filamentous stage	0	6.8 ± 3.1^{a}	2.8 ± 2.4^{b}	3.3 ± 2.3^{ab}	3.6 ± 2.7^{ab}	3.5 ± 1.5^{ab}	0	7.606	< 0.001
9	Rhizoid/chlorocyte	0.6 ± 1.2^{b}	0.5 ± 0.5^{b}	1.2 ± 1.2^{b}	0.5 ± 0.8^{b}	0	0	5.7 ± 2.2^{a}	19.843	< 0.001
	Filamentous stage	4.5 ± 2.7^{bc}	8.8 ± 0.7^{a}	6.7 ± 1.5^{ab}	7.8 ± 1.2^{a}	7.7 ± 2.0^{a}	6.4 ± 1.9^{ab}	1.7 ± 1.2^{c}	12.075	< 0.001
	Laminar stage	1.2 ± 1.2^{abc}	0	2.8 ± 0.7^{ab}	0.8 ± 0.7^{bc}	1.8 ± 1.9^{abc}	3.2 ± 2.3^{a}	0	5.812	< 0.001
12	Rhizoid/chlorocyte	0	0	0	0	0	0	2.3 ± 1.4^{a}	17.500	< 0.001
	Filamentous stage	6.5 ± 3.3^{a}	7.3 ± 1.5^{a}	4.2 ± 2.2^{a}	7.2 ± 2.3^{a}	5.5 ± 1.6^{a}	4.0 ± 2.5^{a}	5.0 ± 2.6^{a}	2.004	0.091
	Laminar stage	1.2 ± 1.5^{bc}	1.0 ± 1.3^{c}	5.0 ± 1.9^{ab}	2.8 ± 2.3^{ab}	4.2 ± 1.5^{ab}	5.5 ± 2.4^{a}	0.2 ± 0.4^{c}	8.959	< 0.001

Table 2. Number of *Cyathea atrovirens* gametophytes (mean ± standard deviation) at different development stages after 6, 9 and 12 days in culture, germinated from fresh spores and spores stored for 90 and 180 days. Equal letters on each line indicate that difference is not statistically significant according to Tukey test at a 5% level of significance.

Days	Stage	Fresh spores	90 days of storage	180 days of storage	F	P
6	Rhizoid/chlorocyte	5.0 ± 3.7[a]	2.0 ± 2.0[a]	2.5 ± 2.0[a]	1.334	0.293
	Filamentous stage	3.6 ± 2.7[a]	5.0 ± 2.0[a]	5.5 ± 2.7[a]	1.129	0.349
9	Rhizoid/chlorocyte	0	0.20 ± 0.4[a]	0.2 ± 0.4[a]	0.500	0.616
	Filamentous stage	7.7 ± 2.0[a]	6.0 ± 2.3[a]	5.5 ± 1.6[a]	1.969	0.174
	Laminar stage	1.0 ± 1.0[a]	3.0 ± 2.8[a]	4.3 ± 1.7[a]	1.899	0.184
12	Filamentous stage	5.0 ± 1.0[a]	3.3 ± 2.0[ab]	2.7 ± 1.7[b]	4.100	0.038
	Laminar stage	4.0 ± 1.0[a]	5.3 ± 2.8[a]	6.5 ± 1.8[a]	1.865	0.189

4. Discussion

Germination and gametophyte development of *Cyathea atrovirens* were observed in the pH range from 4.0 to 7.0 after 12 days of culture, indicating tolerance concerning the pH factor. *Cyathea atrovirens* germination occurred until the middle of the germination time (1-14 days) observed for most leptosporangiate ferns (Howland and Edwards, 1979). These plants exhibit *Cyathea*-type germination: the filament grows along the polar axis and the first rhizoid appears along the equatorial axis (Chen et al., 2008). In this study, spore size and colour were in agreement with findings by Azevedo et al. (2008). The yellow colour indicates the presence of lipids as an energy source (Dyer, 1979).

In the present study, acidic pH promoted higher germination rates than neutral pH, since the germination rate was significantly lower in the pH 7.0 culture than in all other cultures. Similarly, Nondorf et al. (2003) found that *Cheilanthes feei* Moore (Pteridaceae) presented highest germination rates in acidic pH (pH 4.5 and 5.5) and a reduction in neutral and alkaline pH (pH 6.5 and 8.5). *Ophioglossum palmatum* L. (Ophioglossaceae) spores also presented higher germination in acidic pH (4.0) with reduction of germinability in neutral conditions (Whittier and Moyroud, 1993). However, these results do not reveal any fern spore germination pattern. For *Anemia mexicana* Hook. and *Anemia phyllitidis* Hassl. (Schizaeaceae) optimum germination occurred at pH 5.0 to 6.5, with substantial reduction of germination and gametophyte development below pH 4.5 (Nester and Coolbaugh, 1986). Viviani and Randi (2008) did not find any statistically significant differences in the germination rates for *Polypodium lepidopteris* (Langsd. & Fisch.) Kunze (Polypodiaceae) in the pH range from 4.0 to 6.7.

The gametophyte laminar stage of *Cyathea atrovirens* occurred between 20 and 30 culture days, and antheridia were first detected on the 35th day. In another study, in which the authors did not indicate the pH tested, gametangia of *C. atrovirens* were observed later, on the 50th day, while for *Alsophila setosa*, the laminar stage was also observed on the 20th day, and gametangia could be visualised on the 35th day (Azevedo et al., 2008).

Differently from this study, the cordate gametophyte stage of *Cyathea corcovadensis* (Raddi) Domin and *C. delgadii* Sternb. was observed at 105 and 133 culture days, respectively (Hiendlmayer, 2004). However the culture conditions in that experiment differed from those used in the present study.

Cyathea atrovirens spores remained viable after storage. The capacity to germinate after a long storage time is not exclusive of the Cyatheaceae, and was also observed for *Dicksonia sellowiana* (Dicksoniaceae) by Filippini et al. (1999). Lipids are the main energy supply for the spores in these species. The low water content (below 5%) keeps them viable for a longer time (Dyer, 1979). However, *Blechnum brasiliense* Desv., *Cyathea corcovadensis*, *C. delgadii* and *Saccoloma inaequale* H. Christ spores, stored for 240 days at 7 °C, presented a maximum germination percentage of 65, 31, 75 and 60%, respectively (Hiendlmayer, 2004). These values are lower than the one found for *C. atrovirens* in this study. The maturation stage at the moment of the collection of the sporophytic fertile material may account for substantial differences in the spore samples.

Optimal conditions for spore germination often reflect optimal growth conditions for subsequent developmental stages (Nondorf et al., 2003), due to different physiological tolerances of the species to environmental alterations (Paciencia and Prado, 2004). Combined with other environmental factors, pH may affect the initial developmental stage and may be restrictive for the entire life cycle of the fern. The sporophyte establishment is strongly limited by the existence of microhabitats suitable to the gametophytic generation. Especially in tropical species, gametophytes can be very sensible to environmental changes (Page, 1979; Kornás, 1985; Ranal, 1995). The obtained data suggest that the substrate pH is nonrestrictive for the germination and initial development of *Cyathea atrovirens*.

The knowledge of germination characteristics and the stages of gametophyte development are important to understand the entire life cycle of *Cyathea atrovirens* and to analyse the effect of abiotic components, such as pH. Moreover, these findings provide information to support the cultivation, management and conservation of this species.

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