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Ferns and Lycophytes as new challenges

In vitro spore germination and gametophyte development of two *Cyathea* species of South America in response to nutrient media

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Abstract

Cyathea corcovadensis and *Cyathea phalerata* are tree ferns native to Brazil, endangered in the state of Rio Grande do Sul. Spore germination and gametophyte development in media with different nutrient formulations and activated charcoal were evaluated, aiming to develop a process for obtaining plants of the two species. Spores were sown in four semi-solid culture media: Meyer, Dyer, MS with 50% and MS with 25% of the original macronutrient concentration. For each medium, 10 replicates were carried out (flasks with 5 mg of spores/30 mL of medium), with and without 1% activated charcoal, respectively. Spore germination and gametophytic development (laminar and cordate stages) were quantified at 30, 60 and 90 days of culture. *Cyathea corcovadensis* and *C. phalerata* germinated and developed gametophytes in all media. For both species, the highest percentages of germination and cordate gametophytes (more advanced development stage) were recorded in Meyer medium without activated charcoal, which has higher concentrations of macronutrients and no micronutrients compared to the other evaluated media. We recommend cultivating the plants in Meyer medium for greater gametophytic development and subsequent sporophyte obtention, as a biotechnological tool for *C. corcovadensis* and *C. phalerata* conservation and for environmental restoration and enrichment using these tree ferns.

Key words: conservation, Cyatheaceae, endemic species, *in vitro* culture, reproduction.

Resumo

Cyathea corcovadensis e *Cyathea phalerata* são samambaias arborescentes nativas do Brasil, ameaçadas de extinção no estado do Rio Grande do Sul. A germinação de esporos e o desenvolvimento de gametófitos em meios com diferentes formulações de nutrientes e carvão ativado foram avaliados, visando ao desenvolvimento de um processo para obtenção de plantas das duas espécies. Esporos foram semeados em quatro meios de cultura semi-sólidos: Meyer, Dyer, MS com 50% e MS com 25% da concentração original dos macronutrientes. Para cada meio, foram realizadas 10 repetições (frascos com 5 mg de esporos/30 mL de meio), respectivamente com e sem 1% de carvão ativado. A germinação dos esporos e o desenvolvimento gametofítico (estádios laminar e cordiforme) foram quantificados aos 30, 60 e 90 dias de cultivo. *Cyathea corcovadensis* e *C. phalerata* germinaram e desenvolveram gametófitos em todos os meios. Para ambas as espécies, as maiores porcentagens de germinação e de gametófitos cordiformes (estádio de desenvolvimento mais avançado) foram registradas no meio Meyer sem carvão ativado, que se caracteriza por maiores concentrações de macronutrientes e ausência de micronutrientes em comparação com os demais meios avaliados. Recomendamos cultivar as plantas em meio Meyer para maior desenvolvimento gametofítico e subsequente obtenção de esporófitos, como uma ferramenta biotecnológica para a conservação de *C. corcovadensis* e *C. phalerata* e restauração e enriquecimento ambiental usando estas samambaias arborescentes.

Palavras-chave: conservação, Cyatheaceae, espécie endêmica, cultura *in vitro*, reprodução.

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Introduction

Tree ferns play an important role in forest dynamics and ecosystem function (Brock *et al*. 2016), contributing to the maintenance of moisture in the forest interior and to biomass stocks (Smith 1972; Medeiros & Aidar 2011). This group of plants also participates in the ecological succession process, influences the regeneration of woody species, nutrient cycling (Arens & Baracaldo 1998; Brock *et al*. 2016) and provides microhabitats for the epiphytic flora, including species that occur exclusively on their caudices (Moran *et al.* 2003; Schneider & Schmitt 2011).

Even if the sporophytes produce and disperse their spores, the main fern habitats degradation and the consequent changes in the biotic and abiotic conditions prevent the sporophytes from finding the ideal environmental conditions for their establishment (Page 1979). Since their reproduction occurs without pollinator and dispersers action, and the establishment in nature is directly related to environmental abiotic factors (Ferrer-Castán & Vetaas 2005; Silva *et al.* 2011), ferns are good ecological indicators of habitat fragmentation and loss (Grime 1985; Silva *et al.* 2018). The Atlantic Forest, the Amazon and the Cerrado are the three Brazilian phytogeographic domains with the greatest diversity of fern species (Prado *et al.* 2015). The Atlantic Forest and the Cerrado are considered global hotspots, due to the high degree of biodiversity and endemism, as well as being among the most threatened environments by human activities (Mittermeier *et al.* 2005; IBGE 2015; MMA 2018; Fundação SOS Mata Atlântica 2023).

Tree ferns, owing to their morphological characteristics, have high commercial value in landscaping, handicrafts, ornamentation and as substrates for the cultivation of other plants (Fernandes 2000; Eleutério & Perez-Salicrup 2006; Shukla & Khare 2014; Oliveira *et al.* 2015). Irregular exploitation, associated with the slow growth of individuals and the loss of habitats, leads to the erosion of natural populations (Santiago *et al.* 2013) and the loss of epiphytic species that occur on them (Schwartz & Gasper 2020).

Cyatheaceae is an outstanding tree fern family and presents a high degree of endemism. This family comprises 643 species with pantropical distribution, of which 53 are found in Brazil, six in the state of Rio Grande do Sul (Tryon & Tryon 1982; Large & Braggins 2004; Smith *et al.* 2006; Pietrobom *et al.* 2023). The species of Cyatheaceae occupy different habitats, such as hillsides, watercourse edges, forest edges, sandy coastal plain vegetation, and roadsides, although most occur in the forest interior (Tryon & Tryon 1982; Fernandes 2003; Weigand & Lehnert 2016).

Cyathea corcovadensis (Raddi) Domin is endemic to Brazil and occurs in the Northeast, Southeast and South regions of the country, at altitudes of up to 2,050 m, in the different forest formations of the Atlantic Forest and Caatinga, with a preference for understory environments (Fernandes 2003; Lehnert & Weigand 2013; Pietrobom *et al.* 2023). In Rio Grande do Sul, there are records of its occurrence in the north of the coastal region and in the Central Depression (speciesLink 2023). *Cyathea phalerata* Mart. occurs in all regions of Brazil, predominantly in the phytogeographic domains of the Atlantic Forest and Cerrado (Pietrobom *et al*. 2023), and in Bolivia (Lehnert 2006). This fern grows preferentially in the shady interior of forests, next to watercourses and in the interfluves of humid forests, or close to streams in drier forests (Fernandes 2003). In Rio Grande do Sul, *C. phalerata* settles mostly in the coastal region, in the northeast hillside and in Campos de Cima da Serra (fields up the mountain) (speciesLink 2023). *Cyathea phalerata* is used in popular medicine to treat various diseases associated with inflammatory processes, due to the presence of cyathenosin A, a spiropyranosyl derivative of protocatechuic acid, which proved to have antioxidant and hepatoprotector activity on rats (Pizzolatti *et al.* 2007; Hort *et al.* 2008). The presence of kaempferol 3-O-neohesperidoside, a natural substance which can mimic the action of insulin, is also reported for this species (Yamasaki *et al.* 2011). According to State Decree 52.109/2014, *C. corcovadensis* and *C. phalerata* are listed as endangered in Rio Grande do Sul, respectively as vulnerable and critically endangered (Rio Grande do Sul 2014).

The life cycle of ferns has two distinct phases: the gametophytic, haploid stage, and the sporophytic stage, which is longer, with significantly larger individuals and thus much better known. Both stages are chronologically and spatially separated (Menéndez *et al.* 2011). In the gametophytic stage, successive mitotic divisions after spore germination (which involves the emergence of the chlorocyte and rhizoid) give rise to gametophytes, which undergo different stages until sporophyte formation (Fernández & Revilla 2003). *In vitro* spore germination is widely

used for fern propagation and for studies about the biology of tree ferns from the early stages of their life cycle (Menéndez *et al.* 2011), which are difficult to recognize in nature due to the small size of their structures. The main advantages of starting aseptic cultures from mature spores collected in the wild and sown *in vitro* are (i) avoiding contamination of the culture with microorganisms, which is common in cultures started from sporophytic explants, (ii) the rescue and maintenance of the genetic pool of natural populations that donate spores, through the production of individuals for conservation or sustainable use. Specifically, in dealing with endangered species or populations and degraded environments, *in vitro* culture can provide plants for projects of translocation, environmental restoration and enrichment, in addition to awareness-raising and education (Fay 1994; Pence 2008; Soare 2008; Barnicoat *et al*. 2011; Baker *et al*. 2014).

Nevertheless, *in vitro* spore germination and gametophyte development of ferns are strongly affected by abiotic factors, such as light, pH, temperature, concentration of mineral salts and sugar, whose effects are not yet well understood (Chang *et al.* 2007; Hua *et al.* 2010; Rechenmacher *et al*. 2010; Barnicoat *et al.* 2011; Marcon *et al*. 2015, 2017; Medeiros *et al*. 2017). Specially, medium composition and mineral content is considered one of the main factors that influence spores, gametophytes and sporophytes, as nutritional supply affect directly growth and development in each stage of the life cycle (Fernández *et al.* 1997; Fernández & Revila 2003; Suo *et al*. 2015). So, the source and concentration of nutrients applied depend on the plant species and each culture step (Besson *et al.* 2010; Rybczyński & Mikuła 2011; Suo *et al*. 2015). Culture media commonly used include MS (Murashige & Skoog 1962; Borelli *et al*. 1990), Dyer (Dyer 1979; Gomes *et al*. 2006), Jones (Jones 1987; Borelli *et al*. 1990), Knop (Knop 1865; Chen *et al*. 2008), Knudson (Knudson 1946; Agrawal *et al*. 1993), White (White 1951; Alves *et al*. 2019) and Meyer (Meyer *et al*. 1955; Marcon *et al*. 2015). The latter has been used to investigate the influence of temperature, photoperiod, and pH on *C. corcovadensis* and *C. phalerata* (Marcon *et al*. 2014, 2017; Medeiros *et al*. 2017). Plants can benefit from the addition of activated charcoal to the culture medium. Its action is mainly linked to the adsorption of toxic compounds in the culture medium, drastically reducing the bioavailability of the exudates. Activated charcoal also provides a dark culture medium, simulating the substrate in nature and contributing to the establishment and growth of the aerial apical axis (Thomas 2008; Fagundes *et al.* 2017). Although often used in *in vitro* culture to improve cell growth and development of spermatophyte plants, there is little evidence in the literature of the use of activated charcoal in culture media for ferns (Teng 1997; Avila-Pérez *et al*. 2011; Nofal *et al*. 2022).

Aiming to develop a process for obtaining plants of *Cyathea corcovadensis* and *C. phalerata* for the purpose of conserving populations *in situ* and for environmental restoration and enrichment, this study investigated the germination of spores and the development of gametophytes in different culture media and in the presence or absence of activated charcoal. Our assumption was that higher rates of germination and developed gametophytes are obtained in a medium with lower nutrient content (Zhang *et al.* 2007; Silveira *et al.* 2015) and in the presence of activated charcoal (Avila-Pérez *et al*. 2011; Nofal *et al*. 2022).

Material and Methods

Collection and processing of spores

The spore donor population of *Cyathea corcovadensis* lives in the understory of a 6 hectares forest fragment (29º25'04.54''S and 49º54'47.37''W, alt. 21 m) located in Três Cachoeiras, in the northeast of Rio Grande do Sul (RS), in Southern region of Brazil (Fig. 1). The municipality is located in the Tramandaí River Basin and belongs to the Coastal physiography (Comitê Tramandaí 2023). Its vegetation is classified as Dense Ombrophylous Forest of Lowlands, phytophysiognomy of the Atlantic Forest domain (IBGE 2012; Atlas Socioeconômico do Rio Grande do Sul 2021). The average annual temperature in the region ranges from 18.9 °C to 20.4 °C and the annual precipitation ranges from 1,342 mm to 1,998 mm (Neumann *et al.* 2014).

The spore donor population of *Cyathea phalerata* lives in a forest fragment of the Área de Preservação Ambiental de Caraá (Caraá Environmental Preservation Area), on the margins of Miguel stream (29º42'25.0''S and 50º17'27.8''W, alt. 420 m), located 16.8 km from the center of Caraá municipality (Fig. 1). Caraá is located in the upper stretch of Rio dos Sinos Hydrographic Basin, between the Coastal Region and the Mountain range of Rio Grande do Sul state, with vegetation formed by Dense and Mixed Ombrophylous Forest characteristic elements

(IBGE 2012; Atlas Socioeconômico do Rio Grande do Sul 2021). According to data from the Davis Vantage PRO 2 VP USB NS Mobile Weather Station (29°44'15.88''S and 50°21'34.52''W, alt. 375 m), installed at 7.5 km (in a straight line) away from the area where *C. phalerata* occur, the monthly temperature varies between 15 ºC and 25 ºC and the annual accumulated precipitation is 3,273 mm (Cunha *et al.* 2023). Based on the Köppen classification, the climate in both areas of spore donor populations occurrence is Cfa type, temperate subtropical climate, with temperatures above 22 °C during the summer (Alvares *et al.* 2013).

Fertile mature leaves of *Cyathea corcovadensis* and *C. phalerata* presenting sori before opening were collected from five individuals of each population in 2017. The criterion adopted to classify the leaf as mature was the color of the sori, being dark brown for *C. corcovadensis*, and light brown for *C. phalerata*. In the laboratory, the leaves were placed in plastic trays and kept at room temperature for 72 hours, for sporangia dehiscence. Material from each species was mixed and filtered through interleaf paper (Melpaper®), in order to separate spores and sporangia. The spores of each species were stored for 10 months in 1.5 mL Eppendorf tubes, at a temperature of 7 ºC, in the dark (Marcon *et al.* 2014).

In vitro culture

In a horizontal laminar flow chamber, 60 mg of each species spores were sterilized in an Eppendorf tube (1.5 mL) with 1 mL of 2.5% sodium hypochlorite solution for 15 minutes (Marcon *et al.* 2017). After removing the disinfesting agent, 1 mL of autoclaved distilled water was added to wash the spores, and then centrifugation was carried out for 3 minutes at 3,000 rpm. The washing and centrifugation step was performed in four repetitions. Spores were sown in different culture media (Tab. 1), which were prepared according to the original formulations published by Meyer *et al.* (1955), Murashige & Skoog (1962) and Dyer (1979) (Tab. 2).

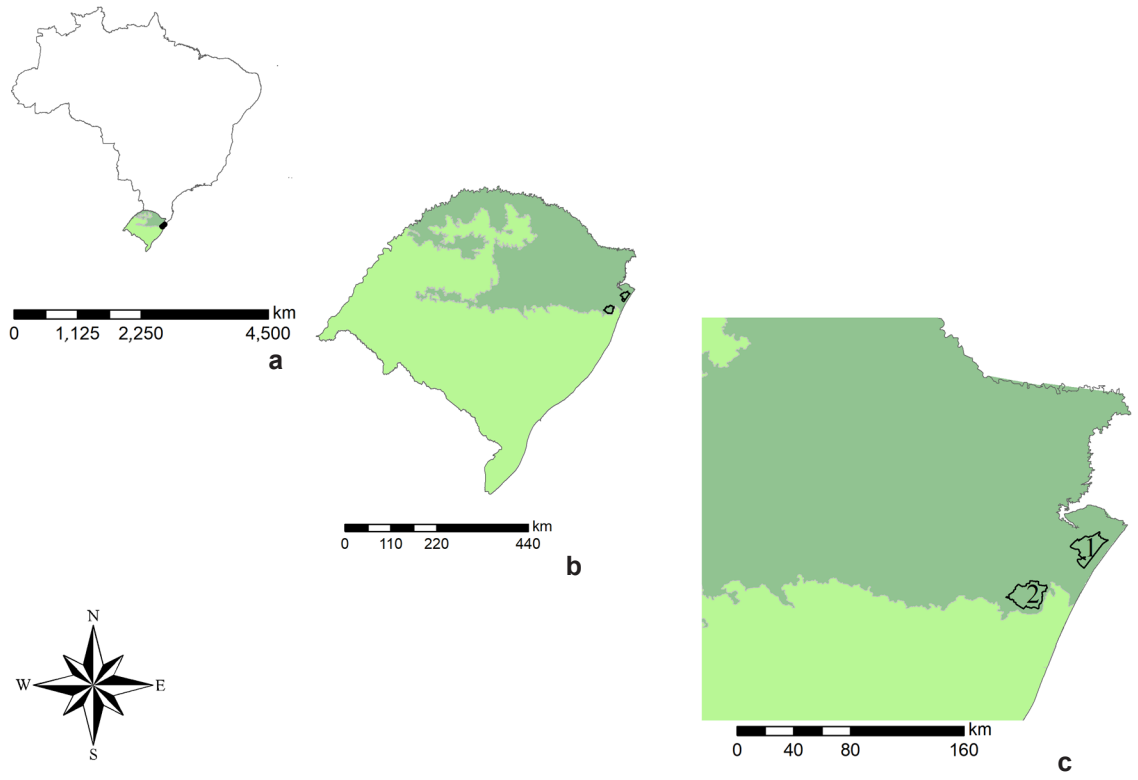

Figure 1 – a-c. Location of *Cyathea corcovadensis* and *Cyathea phalerata* spore donor populations – a. Brazil, highlighting the state of Rio Grande do Sul in shades of green; b. state of Rio Grande do Sul, highlighting the Atlantic Forest biome in green, the Pampa biome in light green and the location of the municipalities of Três Cachoeiras and Caraá in black; c. in highlight, the municipalities of Três Cachoeiras (1) and Caraá (2) in the Atlantic Forest.

Table 1 – Meyer, Dyer and MS culture media with and without charcoal used for spore germination and gametophyte development of *Cyathea corcovadensis* and *Cyathea phalerata*.

Culture medium	Activated charcoal	Abbreviation
Meyer	Absence	M
Meyer	Presence	MC
Dyer	Absence	D
Dyer	Presence	DC
MS [1] with 50% of the original macronutrient concentration	Absence	50MS
MS with 50% of the original macronutrient concentration	Presence	50MSC
MS with 25% of the original macronutrient concentration	Absence	25MS
MS with 25% of the original macronutrient concentration	Presence	25MSC

[1] MS: Murashige & Skoog (1962)

Table 2 – Formulations of Meyer, Dyer and MS culture media used for spore germination and gametophyte development of *Cyathea corcovadensis* and *Cyathea phalerata*.

Component	Culture medium			
	Meyer	Dyer	50MS [1]	25MS [2]
Macronutrients (mg L^{-1})				
KH_2PO_4	1000	250	85	42.5
NH_4NO_3	1000	-	825	412.5
$MgSO_4 \cdot 7H_2O$	300	510	185	92.5
$CaCl_2 \cdot 2H_2O$	80	-	220	110
NaCl	100	-	-	-
$FeCl_3 \cdot 6H_2O$	10	-	-	-
KNO_3	-	120	950	475
$Ca(NO_3)_2 \cdot 4H_2O$	-	1,440	-	-
Na_2 EDTA [3]	-	37.3	37.3	37.3
$FeSO_4 \cdot 7H_2O$	-	27.8	27.8	27.8
Micronutrients (mg L^{-1})				
H_3BO_3	-	-	6.200	6.200
$MnSO_4 \cdot H_2O$	-	-	16.900	16.900
$ZnSO_4 \cdot 7H_2O$	-	-	8.600	8.600
KI	-	-	0.830	0.830
$Na_2MoO_4 \cdot 2H_2O$	-	-	0.250	0.250
$CoCl_2 \cdot 6H_2O$	-	-	0.025	0.025
$CuSO_4 \cdot 5H_2O$	-	-	0.025	0.025

[1] = with 50% of the macronutrient concentration and without vitamins and amino acids present in the original formulation.

[2] = with 25% of the macronutrient concentration and without vitamins and amino acids present in the original formulation.

[3] = EDTA: ethylenediamine tetra-acetate.

For each species and culture medium, 10 glass flasks (200 mL capacity) were used, each containing 5 mg of spores and 30 mL of semi-solid culture medium (Phytagel™ 0.4%) supplemented with nystatin (Sigma-Aldrich) 50,000 U mL^{-1} after autoclaving (Marcon *et al.* 2017; Medeiros *et al.* 2017). The pH was adjusted to 4.0 for *C. corcovadensis*, according to Medeiros *et al*. (2017), and to 5.0 for *C. phalerata*, according to Marcon *et al*. (2017). The cultures were placed at temperature of 25±1 °C, photoperiod of 12 hours of light and light intensity of 70 µmol m^{-2} s^{-1} (Marcon *et al.* 2017; Medeiros *et al.* 2017).

Spore germination and gametophyte development were evaluated after 30, 60 and 90 days of *in vitro* cultivation. A microscopic slide was prepared from each flask containing 150 µL of nutrient medium with culture material (10 slides per species and culture medium). The first 100 individuals observed on each slide were classified as: non-germinated spore (Fig. 2a), gametophyte with chlorocyte and rhizoid (GCR) (Fig. 2b), filamentous gametophyte (FG) (Fig. 2c), laminar gametophyte (LG) (Fig. 2d-e) and cordate gametophyte (CG) (Fig. 2f). The quantification of germinated spores (G) was calculated by the formula G=GCR+FG+LG+CG. Gametophytic development was quantified by the percentages of LG and CG, corresponding to the most advanced stages (Marcon *et al.* 2017).

Statistical analysis of the data was performed using the SPSS version 28 program, with significance set at 5%. G, LG and CG data were transformed into percentages. As they met the assumptions of normality, verified using the Shapiro-Wilk test, the data were submitted to a two-way analysis of variance (two-way ANOVA; four culture media and the activated charcoal absence or presence), followed by the Bonferroni test.

Results

Cyathea corcovadensis and *C. phalerata* spores germinated, and gametophytes developed in all culture media tested. On day 30 of the *C. corcovadensis in vitro* cultivation, a difference was observed between the tested treatments (Tab. 3). In the assay without added activated charcoal, 71.9% of spores germinated in the M media, a value significantly higher than the percentages in the 50MS (58%) and 25MS media (59%). Over time, this difference continued to be observed, and on day 90 there were 94% of spores germinated in the M medium (Tab. 3).

In culture media with activated charcoal, the highest percentage of *C. corcovadensis* germination at 30 days was also observed in MC medium (45.4%), which differed significantly from the values observed in the other treatments (Tab. 3). At 90 days, in the 50MSC and 25MSC media, only 45.1% and 48.1% of spores germinated, respectively, whereas, in the MC medium, there were 75.8% of germinated spores. In cultures with D and DC media, intermediate values of spore germination were observed over time compared to values in the other media. Regardless of the culture medium and the period evaluated, higher percentages of germination were recorded in media without added activated charcoal than in the media with this component (Tab. 3).

The *C. corcovadensis* development of gametophytes was also influenced by the culture medium, with gametophytes in more advanced stages in M and D media (Tab. 3). On day 30 of cultivation, there were already approximately 60% of laminar gametophytes in the M medium, which differs significantly from the other media without activated charcoal. Even in the presence of charcoal, the MC medium provided a higher percentage of laminar gametophytes (36.6%) than the other treatments. In the 50MSC medium, less than 10% of the individuals were in this stage. Comparing the absence and presence of activated charcoal, all treatments with charcoal showed significantly lower percentages of laminar gametophytes than cultures without this component (Tab. 3). At the end of the experiment, on day 90, there was interaction between the tested treatments. In the D medium, there was an average of 70.8% of laminar gametophytes, statistically differing only from 25MS. However, in treatments with activated charcoal, the 50MSC medium provided the lowest average of laminar gametophytes (22%), whereas, in the MC and DC media, the averages were significantly higher. The same significant difference between the absence and presence of activated charcoal observed for germinated spores was recorded for laminar gametophytes development (Tab. 3).

Cordate gametophytes (more developed gametophytic stage) were observed after 60 days of *C. corcovadensis* cultivation. Considering the media without activated charcoal, about 7% of cordate gametophytes were recorded in M medium, significantly higher percentage than those recorded in 50MS and 25MS media (averages close to 1%) (Tab. 3). In the presence of activated charcoal,

there was no significant difference among media, with means between 4.6% and 8.4% of cordate gametophytes. Cordate gametophytes developed in higher percentages in DC and 25MSC media compared to D and 25MS media, respectively. At 90 days, 23.7% of cordate gametophytes were observed in M medium, a significant higher percentage compared to the percentages recorded in the other media without charcoal (between 6.3 and 10.0%). A comparable result was observed in the media with activated charcoal, with 24.5% of cordate gametophytes in MC, a significantly higher percentage than those in all other media. The averages of cordate gametophytes in DC and 50MSC media were higher, respectively, than the averages in D and 50MS (Tab. 3).

For *C. phalerata*, a low percentage of spore germination and low gametophytic development were verified at 30 days in all tested media. Though, there was an interaction between the

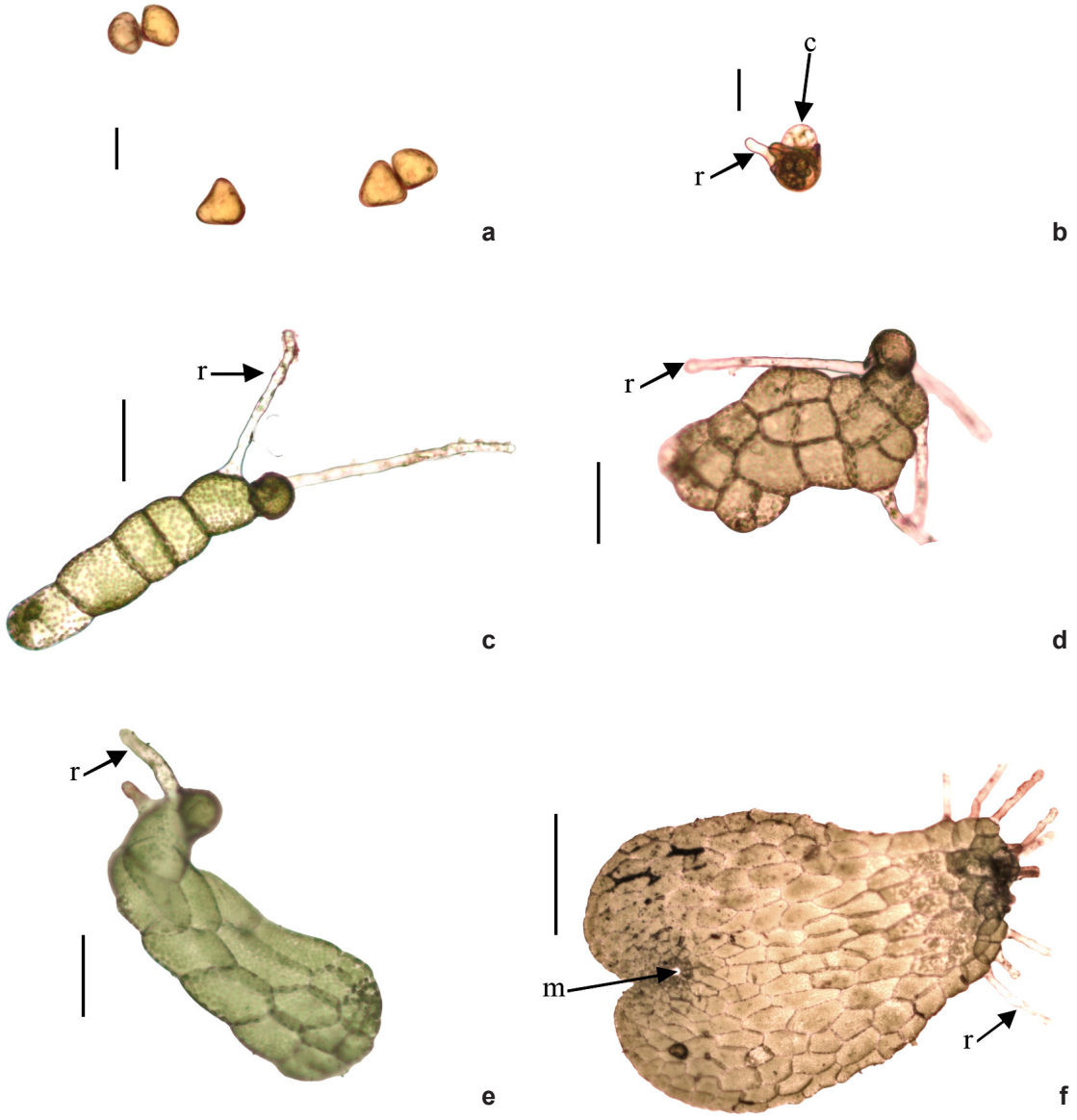

Figure 2 – a-f. Spores and gametophyte development stages of *Cyathea corcovadensis* – a. non-germinated trilete spore; b. gametophyte with chlorocyte and rhizoid; c. filamentous gametophyte; d-e. laminar gametophyte; f. cordate gametophyte. (c = chlorocyte; r = rhizoid; m = meristematic region). Bars: a-b = 50 µm; c-e = 100 µm; f = 150 µm.

Table 3 – Percentage (mean ± standard deviation) of germinated spores (G), laminar gametophytes (LG) and cordate gametophytes (CG) of *Cyathea corcovadensis* cultivated for up to 90 days in Meyer, Dyer and MS culture media in the absence or presence of activated charcoal. Different letters in the rows and asterisks in the columns indicate that the data differ significantly from each other, according to the Bonferroni test, at 5% significance.

	Day	Activated charcoal [1]	Meyer	Dyer	50MS [2]	25MS [3]	F	p
G	30	A	71.90±5.04^{a*}	67.20±2.57^{ab*}	58.10±2.5^{b*}	59.10±2.71^{b*}	27.154	<0.001
		P	45.40±9.01^{a}	36.60±10.93^{b}	16.00±13.82^{c}	12.20±13.88^{c}		
			F = 357.663				Interaction [4]	
			p < 0.001				3.835	0.013
	60	A	89.70±3.83^{a*}	87.50±3.63^{a*}	60.10±5.13^{b*}	66.90±12.38^{b*}	42.368	<0.001
		P	62.50±9.08^{a}	50.80±10.96^{a}	26.90±17.63^{b}	28.50±15.49^{b}		
			F = 191.078				Interaction	
			p < 0.001				1.019	0.389
	90	A	94.00±1.56^{a*}	86.80±4.13^{ab*}	74.40±6.45^{bc*}	70.00±12.23^{c*}	16.941	<0.001
		P	75.80±6.35^{a}	60.60±9.38^{ab}	45.10±27.58^{b}	48.10±18.04^{b}		
			F = 63.304				Interaction	
			p < 0.001				0.688	0.562
LG	30	A	59.70±6.78^{a*}	43.70±3.68^{b*}	35.90±4.17^{b*}	37.80±6.14^{b*}	51.799	<0.001
		P	36.60±8.18^{a}	25.10±7.65^{b}	9.80±8.21^{c}	12.80±9.86^{c}		
			F = 212.626				Interaction	
			p < 0.001				1.080	0.363
	60	A	76.80±6.30^{a*}	73.10±5.40^{a*}	50.60±5.50^{b*}	58.00±12.60^{b*}	43.678	<0.001
		P	45.70±9.68^{a}	34.50±11.98^{b}	14.80±9.61^{c}	17.20±8.49^{c}		
			F = 324.369				Interaction	
			p < 0.001				1.061	0.371
	90	A	64.60±3.02^{ab*}	70.80±5.59^{a*}	60.40±3.56^{ab*}	56.00±11.40^{b*}	9.612	<0.001
		P	43.00±7.36^{a}	35.90±11.78^{ab}	22.00±13.30^{c}	29.60±14.07^{bc}		
			F = 195.901				Interaction	
			p < 0.001				3.154	0.030
CG	60	A	7.10±3.1^{a}	3.70±2.49^{ab}	0.90±1.28^{b}	1.70±2.54^{b}	10.150	<0.001
		P	8.40±3.95^{a}	8.30±3.19^{a*}	5.10±4.65^{a}	4.60±3.5^{a*}		
			F = 20.124				Interaction	
			p < 0.001				1.056	0.373
	90	A	23.70±2.31^{a}	10.40±3.20^{b}	6.30±5.10^{b}	7.60±3.62^{b}	33.340	<0.001
		P	24.50±7.15^{a}	17.60±5.50^{b*}	13.70±9.15^{bc*}	9.20±4.34^{c}		
			F = 12.100				Interaction	
			p = 0.001				2.096	0.108

[1] = A: Absence; P: Presence.

[2] = With 50% of the macronutrient concentration and without vitamins and amino acids present in the original formulation.

[3] = With 25% of the macronutrient concentration and without vitamins and amino acids present in the original formulation.

[4] = Indicates interaction between the different culture media and the absence/presence of activated charcoal.

culture medium and the presence or absence of charcoal in most stages and periods analyzed (Tab. 4). In media without activated charcoal, spore germination ranged from 6.5% to 12.2%, a value significantly higher in medium D, as observed in media with activated charcoal. However, when absence and presence of activated charcoal were compared in each medium, only 50MS (8.4%) and 25MS (9.8%) differed significantly from 50MSC (2.1%) and 25MSC (5.80), respectively. At 90 days, between 41.9% and 51.3% of spores had germinated in media without charcoal, and between 28.3% and 44.3% in media with charcoal. Both in the presence and in the absence of charcoal, the M medium provided significantly greater germination than the 50MS, 50MSC, 25MS and 25 MSC media. In the cultivation with medium D, the percentage of germination did not differ from those of the other treatments, whereas, in the medium DC, germination was significantly higher than in 50MSC and 25MSC (Tab. 4).

Laminar gametophyte development of *C. phalerata* became more significant only at 60 days (between 19.4 and 24.3%), with no significant difference between media without charcoal (Tab. 4). The presence of charcoal negatively affected the laminar gametophyte formation in the 50MSC and 25MSC media. At 90 days, the percentages of laminar gametophytes in the medium without charcoal remained between 17.9 and 25.6%. In the presence of activated charcoal, the highest percentage of laminar gametophytes was observed in medium D (19.4%), although the medium without charcoal allowed higher values than the medium with charcoal (Tab. 4).

As in *C. corcovadensis*, the occurrence of cordate gametophytes in *C. phalerata* was observed only after 60 days of cultivation. In the M medium, an average of approximately 21% of gametophytes was recorded at this stage, differing significantly from the other media, which presented cordate gametophyte averages of less than 11% (Tab. 4). The same behavior was recorded for cultures with the presence of charcoal. At the end of the experiment, on day 90, the M medium, regardless of activated charcoal, continued to be the greatest promoter of the formation of cordate gametophytes, with more than 20% of individuals in this stage, significantly differing from the other media (Tab. 4). Comparing each medium in the presence and absence of charcoal, there was no significant difference between cordate gametophytes at 60 and 90 days (Tab. 4).

Discussion

Cyathea corcovadensis and *C. phalerata* initial development was directly affected by the culture medium composition, which reinforces the importance of this factor for spore germination and gametophyte development (Suo *et al.* 2015). The non-chlorophyllous spores of certain fern species, similar to orthodox seeds, contain the necessary nutrients for their initial growth (Pence 2008; Li *et al.* 2010) and are composed mainly of reserve content. Therefore, spore germination and the initial development of gametophytes are successful in culture media with low concentrations of nutrients, such as Knop (1865), Knudson (1946) and MS (Murashige & Skoog 1962), the less with minor salt concentrations than the original formulation (Cox *et al.* 2003; Menéndez *et al.* 2011). In addition, the lower osmotic pressure in media with low nutrient concentrations benefits water absorption by spores, which is a fundamental requirement to start the germination process (Whittier 1975).

Cyathea corcovadensis and *C. phalerata*, despite having non-chlorophyllous spores (Hirai & Prado 2014), showed the highest germination in Meyer medium, which is composed of major concentrations of macronutrients compared to the evaluated MS formulations. The germination rates obtained are considered high and corroborate those described in the literature for both species. Medeiros *et al*. (2017) observed more than 90% of germinated spores of *C. corcovadensis* and Marcon *et al*. (2017) obtained more than 70% of germinated spores of *C. phalerata* after 30 days of cultivation in Meyer medium. However, this preference for high concentration of nutrients is surprising for Cyatheaceae species. The germination of *C. atrovirens* (Langsd. & Fisch.) Domin and *Alsophila podophylla* Hook. spores increased as the concentration of macronutrient salts in the MS medium decreased (Zhang *et al.* 2007; Silveira *et al.* 2015). For *Cyathea spinulosa* (Wall. *ex* Hook.), spores germinated more when they were cultivated in modified Knudson medium, a medium also with lower nutrient concentrations (Agrawal *et al*. 1993). *Cyathea schanschin* Mart. showed higher germination when cultivated in Knop medium modified by Dyer with low salt concentrations (Borelli *et al.* 1990).

The higher spore germination recorded in cultures using the Meyer medium compared to the modified MS media may be related to the significantly higher macronutrient concentrations and micronutrient absence in the former. The

Table 4 – Percentage (mean ± standard deviation) of germinated spores (G), laminar gametophytes (GL) and cordate gametophytes (CG) of *Cyathea phalerata* cultivated for up to 90 days in Meyer, Dyer and MS culture media in the absence or presence of activated charcoal. Different letters in the rows and asterisks in the columns indicate that the data differ significantly from each other, according to the Bonferroni test at 5% significance.

	Day	Activated charcoal [1]	Meyer	Dyer	50MS [2]	25MS [3]	F	p
G	30	A	6.50±3.89^{b}	12.20±4.34^{a}	8.40±3.40^{ab*}	9.80±2.70^{ab*}	13.793	<0.001
		P	6.30±3.68^{b}	11.50±3.10^{a}	2.10±1.19^{b}	5.80±4.31^{b}		
				F = 13.049			Interaction [4]	
				p = 0.001			3.448	0.021
	60	A	46.60±7.06^{a}	33.50±11.99^{b}	38.60±4.06^{ab*}	35.80±5.24^{b*}	17.932	<0.001
		P	42.60±4.81^{a}	38.80±8.47^{a}	19.20±9.25^{b}	23.80±7.07^{b}		
				F = 19.322			Interaction	
				p < 0.001			9.610	<0.001
	90	A	51.30±6.24^{a*}	46.10±4.56^{ab}	44.70±3.95^{b*}	41.90±3.14^{b*}	25.865	<0.001
		P	42.90±2.13^{a}	44.30±4.64^{a}	28.30±6.41^{b}	32.30±5.65^{b}		
				F = 71.082			Interaction	
				p < 0.001			7.761	<0.001
LG	30	A	3.10±1.59^{b}	5.80±2.78^{ab}	5.00±3.39^{ab*}	7.40±2.67^{a*}	9.418	<0.001
		P	3.50±2.37^{b}	7.30±2.36^{a}	0.90±1.37^{b}	3.40±2.83^{b}		
				F = 7.674			Interaction	
				p = 0.007			6.817	<0.001
	60	A	19.80±6.89^{a}	21.50±4.72^{a}	24.30±5.33^{a*}	19.40±6.38^{a*}	4.523	0.006
		P	15.80±4.86^{ab}	17.30±6.65^{a}	4.10±3.14^{c}	9.60±3.68^{bc}		
				F = 63.828			Interaction	
				p < 0.001			10.084	<0.001
	90	A	17.90±5.74^{b*}	23.40±3.56^{a*}	25.60±3.63^{a*}	22.70±4.37^{a*}	10.124	<0.001
		P	13.40±2.30^{b}	19.40±3.53^{a}	6.80±2.15^{c}	12.20±2.44^{b}		
				F = 134.261			Interaction	
				p < 0.001			17.892	<0.001
CG	60	A	20.90±4.43^{a}	6.30±3.02^{b}	8.60±3.06^{b}	10.60±3.69^{b}	30.510	<0.001
		P	19.30±3.33^{a}	12.90±3.81^{b}	9.60±7.18^{bc}	6.60±5.52^{c}		
				F = 0.251			Interaction	
				p = 0.618			5.193	0.003
	90	A	28.10±1.72^{a}	14.50±5.12^{b}	14.40±3.09^{b}	14.90±4.12^{b}	28.957	<0.001
		P	23.90±4.04^{a}	17.10±5.70^{b}	13.40±5.10^{b}	13.90±7.91^{b}		
				F = 0.670			Interaction	
				p = 0.416			1.597	0.198

[1] = A: Absence; P: Presence.

[2] = With 50% of the macronutrient concentration and without vitamins and amino acids present in the original formulation.

[3] = With 25% of the macronutrient concentration and without vitamins and amino acids present in the original formulation.

[4] = Indicates interaction between the different culture media and the absence/presence of activated charcoal.

wide use of MS medium for *in vitro* cultivation of vascular plants and different fern species (Borelli *et al.* 1990; Avila-Pérez *et al.* 2011; Bharati *et al.* 2013; Suo *et al.* 2015; Alves *et al.* 2019; Pu *et al.* 2023) is related to the broad diversity of nutrients in its composition, which includes vitamins, amino acids and essential macronutrient and micronutrient salts for plant development (Rybczyński & Mikuła 2011). However, depending on the physiology of the cultivated species, metabolic processes can be impaired by this nutrient composition (Murashige & Skoog 1962; Sakuta *et al.* 1987; Grattapaglia & Machado 1998), as seen in the present study for *C. corcovadensis* and *C. phalerata*.

Cyathea corcovadensis and *C. phalerata* responded similarly to cultivation in Dyer medium, presenting spore germination considered intermediate in relation to the other treatments. However, in a study carried out with *Dicksonia sellowiana* Hook. (Dicksoniaceae), another tree fern, there was more than 70% of spore germination in Dyer and MS medium with original nutrient formulation (Renner & Randi 2004). High germination rates in media with low nutrient concentration, such as 1/8 Knop, 1/8 MS, 1/4 MS e 1/2 MS, were also observed for Dryopteridaceae [*Dryopteris varia* (L.) Kuntze], Osmundaceae (*Osmunda japonica* Thunb.), Polypodiaceae [*Pyrrosia lingua* (Thunb.) Farw. and *Drynaria fortunei* (Kunze) J. Sm], Pteridaceae (*Adiantum reniforme* var. *sinense*, *Pteris tripartita* Sw., *Pteris wallichiana* J. Agardh and *Pteris cretica* L.) and Schizaeaceae [*Schizaea dichotoma* (L.) J. Sm.] species (Yuan *et al.* 2002; Cox *et al.* 2003; Xu *et al.* 2005; Chang *et al.* 2007; Ouyang *et al.* 2008; Zhang *et al.* 2008; Du *et al.* 2009; Hua *et al.* 2010; Baskaran & Jeyachandran 2012). The cultivation efficiency in these media is attributed to the probability that these species spores store the necessary nutrients for initial development (Suo *et al.* 2015).

In the present study, the germination of *C. corcovadensis* and *C. phalerata* spores were negatively affected by the presence of activated charcoal. This compound is considered an *in vitro* growth inhibitor for some species (George & Sherrington 1984). Even so, in studies with other fern species, activated charcoal was beneficial for germination, such as for *S. dichotoma*, in which a higher germination percentage was observed in MS medium with 25% of macronutrients supplemented with activated charcoal (Cox *et al.* 2003). For *Rumohra adiantiformis* (G. Forst.) Ching, the activated charcoal addition to the Knop medium accelerated the germination process, leading to 100% spore germination after 18 days of cultivation, whereas, without this compound, this percentage was reached only on the twenty-third day (Avila-Pérez *et al*. 2011).

Studies aimed at evaluating the influence of nutrient media focus mainly on spore germination and/or sporophyte formation (Avila-Pérez *et al*. 2011; Suo *et al*. 2015; Alves *et al*. 2019; Pu *et al*. 2023), and the development of gametophytes is lacking in discussion. However, the newly germinated spores, with chlorocyte and rhizoid, show fast growth and rapidly develop into gametophytes (Rybczyński & Mikuła 2011), and the gametophytic stage is determinant for the success of fertilization and sporophyte formation. The development of *C. corcovadensis* and *C. phalerata* gametophytes was also influenced by the culture medium, as well as by the presence of activated charcoal. For *C. corcovadensis*, M medium proved to be the greatest promoter of laminar gametophytes when compared to nutrient-reduced media (25MS and 50MS) at 30 and 60 days. At 90 days, this was not maintained, as many gametophytes were already in the cordiform stage, proving the benefit of this medium. Similar findings were recorded for *C. atrovirens*, a drastic reduction of laminar gametophytes was observed as the concentration of macronutrients in the MS medium increased (Silveira *et al.* 2015). In MS medium with 100% of macronutrient salts, less than 6% of laminar gametophytes were obtained, and in cultures with 25%, 50% and 75% of macronutrients, this number ranged from 35 to 42% (Silveira *et al.* 2015). For *Osmunda regalis*, the growth of gametophytes was higher in Knop medium, whereas in original MS medium and with 50% of macronutrients, growth was inhibited, which was attributed to higher osmotic levels (Fernández *et al.* 1997).

The presence of activated charcoal did not appear to be beneficial, since at 90 days, considerably more laminar gametophytes of both species were formed in the media without this component. If the lower percentages of laminar gametophytes were accompanied by higher percentages of cordiform gametophytes, it could be inferred that activated charcoal was an enhancer of gametophyte development, but, in general, the data did not indicate that. An exception was the cordiform gametophytes of *C. corcovadensis* in the DC and 50MSC media,

which, to be validated, should be investigated repeatedly. Activated charcoal promotes a dark substrate, which reduces the passage of light, just like in nature, guiding the plant tissues. It also acts in the adsorption of phenolic substances released by plants in the medium, in stimulating the rhizoid and root production, in reducing or even preventing the seedling browning, as well as in improving the vegetative aspect of individuals (Fridborg *et al.* 1978; Van Waes 1987; George & Ravishankar 1997; Pan & Van Staden 1998; Paul *et al.* 2012; Kim *et al.* 2019). Thus, it is likely that its beneficial effect occurs mainly on sporophytes, in the final stage of *in vitro* culture and in the *ex vitro* acclimatization of plants.

When analyzing the composition of the culture media used in this study in relation to the source of nitrogen (N), an important limiting nutrient for plants (Walch-Liu *et al.* 2000), the M medium provides N in the form of ammonium nitrate (NH_4NO_3), while the D medium provides it in the form of potassium nitrate (KNO_3). MS medium presents both forms equivalently in its formulation. NH_4NO_3 is an important source of N and is considered to stimulate germination and development of non-photosynthetic gametophytes growing *in vitro* (Whittier 1989), such as in *C. corcovadensis* and *C. phalerata*. Nevertheless, if used as the sole source of N, NH_4NO_3 may have a negative effect on growth and morphogenesis (Walch-Liu *et al.* 2000), a fact that has not been proven for the species studied here, since in M medium, the initial ontogenetic development was greater.

All treatments tested in this study led to spore germination and gametophytic development of *C. corcovadensis* and *C. phalerata*. However, by determining the nutrient medium that allows the production of the greatest number of developed gametophytes, we can establish a successful plant propagation process. Our initial assumption was not proven since the medium with the highest concentrations of macronutrients (Meyer medium) without the addition of activated charcoal provided the most suitable conditions for both species. The relevance of the results lies in their contribution to the establishment of a biotechnological tool for the conservation of *C. corcovadensis* and *C. phalerata* and their use in restoration and environmental enrichment programs, as well as revealing previously unknown abiotic preferences that are distinct from those of other fern species.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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