

Spore Germination of *Gleichenella pectinata* (Willd.) Ching (Polypodiopsida-Gleicheniaceae) at Different Temperatures, Levels of Light and pH

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ABSTRACT

*This study investigated the effects of different temperatures (25 and 30 ± 2 °C), light levels (62, 42, 22 and 5% of natural light), and pH (4.0, 4.5, 5.0, 5.5, 6.0 and 6.7) on the spore germination of *Gleichenella pectinata*. To accomplish this, the spores were surface sterilized and sown in bottles containing mineral medium. The spores of *G. pectinata* are monolets and the perispore surface can show large and irregular deposits. During germination, filamentous gametophytes with and without developing rhizoids were observed. Only gametophytes which presented developed rhizoids were able to reach the heart-shaped developmental stage. Based on the collection, the rate of and gametophyte development were generally very low. The relative germination rates were higher at 25 °C than at 30 °C. Moreover, the highest percentages of gametophytes with developed rhizoids were observed at 22 and 5% of natural light (8.8 ± 2.3% and 11.3 ± 2.2% respectively) and the highest percentage of heart-shaped gametophytes were observed at pH 4.5 and 5.0 (1.2 ± 0.8% and 2.2 ± 0.8%, respectively).*

Key words: *Gleichenella pectinata*, germination, light, pH, temperatures

INTRODUCTION

Both the degradation of forest fragments and posterior regeneration during the sucesional stages lead to increased soil erosion and leaching of mineral nutrients. However, some species of the fern family Gleicheniaceae are resurgent during the natural regeneration of degraded areas and could, therefore, be used as part of the plant covering in restoration projects, mainly of hillsides and slopes. According to Heinrich (1986), some ferns belonging to the *Pteridium*

(Dennstaedtiaceae) and *Gleichenella* (Gleicheniaceae) genera, are found growing in degraded forest fragments in some Brazilian regions. Reis et al. (1999) reported that *Gleichenella pectinata* (Willd.) Ching was one of the first species to colonize degraded areas. The Gleicheniaceae family (Polypodiopsida, Gleicheniales) (Smith et al. 2006) presents five genera and 120 to 140 species of pantropical distribution. Four genera occur in the Americas and are represented by 40 to 50 species (Ostergaard-Andersen and Ollgarard 2001).

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Gleichenella genus is geographically distributed from southern Mexico and the Antilles to Chile, Argentina, Uruguay and Brazil (Tryon and Tryon 1982, Prado 2004). According to Queiroz (1994), *Gleichenella pectinata* (Willd) Ching (Prado 2004, Prado 2005) forms associations called *Gleichenietum pectinata* or “gleichenietum” characterized by the strong predominance of this species (Queiroz 1994). Frequently, these associations survive for several years growing in abrupt declivities on roadsides and in steep slopes where *G. pectinata* is easily identified for the identical ramifications and number of pinnas. As such, *G. pectinata* is probable the most frequent species in southern Brazil (Sehnem, 1977). The germination of fern spores and the gametophyte development are strongly controlled by the light, mineral nutrition and temperature. Photosensitive spores need mild temperatures for the inductive processes (Towill 1978, Chen and Ikuma 1979, Haupt 1990, 1991, 1992, Fernández et al. 1996, 1997). According to Camloh and Gogola (1992) and Camloh (1993, 1999), liquid media are superior to solid media for early gametophyte development. Camloh (1993) and Simabukuro et al. (1998) also pointed out the necessity of spore sterilization before its inoculation in “in vitro” procedures. Therefore, the aim of this paper was to identify the optimal ecophysiological conditions for spore germination of *Gleichenella pectinata* (Willd.) Ching.

MATERIAL AND METHODS

Fertile leaves of several “gleichenietos” were collected in March, April, May and November of 2004, as well as December of 2005 in the “Unidade de Conservação Ambiental Desterro” (UCAD), situated between 27°30'48” to 27°32'34” S and 48°29'38” to 48°30'42” W in Florianópolis, Island of Santa Catarina, State of Santa Catarina State, Brazil (Ladwig 1998). The collection site was a 491-ha natural Atlantic forest fragment. Sporophylls were dried at 30°C for three days on filter paper in order to induce the dehiscence. The spores were removed and separated from the debris by filtering through lens paper, then stored in glass jars under refrigeration at 7±1°C. The spores of *Gleichenella pectinata* were surface sterilized using a 10 % (v/v) solution of

commercial bleach (2% of active chlorine) for 15 min. before filtering through sterile filter paper and washed several times with sterile distilled water. The spores were sown in conical flasks containing 20 mL of Mohr’s nutrient media solution (Dyer 1979), supplemented with Benomyl 0.01% or they were inoculated in four Petri dishes, with 20 mL of nutrient media solidified with 0.8% Agar. The flasks were plugged with two layers of autoclaved transparent commercial polypropylene film (7 x 7 cm) and fixed with a rubber band. All the procedures were carried out in a laminar hood. Because a preliminary study showed that there was no difference in spore germination between liquid media and solidified media the liquid media was used in this work.

To study the effects of light levels on the germination of *G. pectinata*, trays containing conical flasks in which surface sterilized spores were sown, were kept in 50 cm³ boxes covered with black shade net to provide 62, 42, 22 and 5% of light. A maximum and minimum thermometer was placed inside the boxes. The irradiance levels inside the boxes were measured daily using a quantameter LICOR 250, equipped with a PAR sensor (400 to 700 nm), periodically at 2 PM, except on rainy days. On a typical sunny March day, the density of photon flux reached 1400 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ in Florianópolis, SC. Using the spores collected in November 2004, which showed higher germination rates than the spores collected in the other months, the test was conducted in October 2005. The effects of different temperatures (25 and 30 ± 1 °C) and pH values (4.0, 4.5, 5.0, 5.5, 6.0 and 6.7- control) were studied in a growth chamber, at a photon flux density of 30 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and a 16-h photoperiod. The effects of different pH values were determined at 25 ± 1°C.

Dry spore samples were transferred to the stubs and then gold-coated with 20nm of gold in a Baltec-CED 030. Examination was performed with a Philips-XL 30 scanning electron microscope. Specimens were collected after 14, 34 and 48 days of spore inoculation in the mineral solution. For light microscopy (LM), gametophytes were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer at a pH of 7.2 (Bozzola and Russel 1991). All the samples were fixed in Eppendorf tubes for 3h, centrifuged for 3 min, dehydrated with an ethanol series and then stored in ethanol. The samples were mounted on

glass slides with ethanol and photographed with a Leika-MPS 30 light microscope.

Data were analyzed by Excel and BioEstat for Windows. For each treatment, eight slides (8 replications) with 100 specimens each were analyzed. Gametophytes which showed chlorosis were considered dead. The results expressed as percentages were arcsine transformed. An ANOVA analysis of variance followed by a Tukey test was applied to normal homoscedastic data. A Kruskal-Wallis test (H) followed by the Dunn test was performed whenever data did not show normality or homogeneity of variances, after angular transformations and the Student's t test was performed to compare two means (Santana and Ranal 2004, Zar 1999).

RESULTS

The spores of *G. pectinata* (Fig. 1) were monoete, ellipsoidal and reniform, presenting a prominent scar, and the perispore surface was smooth or partially irregular and showed large and irregular

deposits as described by Prado (2005). The spores considered unviable were transparent and did not present any storage substance, whereas the spores considered viable were partially or totally filled with reserve grains and showed an opaque aspect (Fig. 2). Three classes of developmental stages were observed after the spore germination: spores with chloroplasts, but without rhizoids (Fig. 3), filamentous gametophytes without developed rhizoids (Fig. 4) and filamentous gametophytes with developed rhizoids (Fig. 5). After 34 days of spore cultivation, some gametophytes remained filamentous and others presented a spatulated protallus (Fig. 6). After 48 days, some gametophytes were heart-shaped (Fig. 7). Only gametophytes which presented developed rhizoids were able to reach the heart-shape. Reproductive structures were not observed during the period of gametophyte cultivation. Table 1 shows the time of spore storage under refrigeration and the percentages of gametophytes with developed rhizoids, without rhizoids and viable spores of *G. pectinata*.

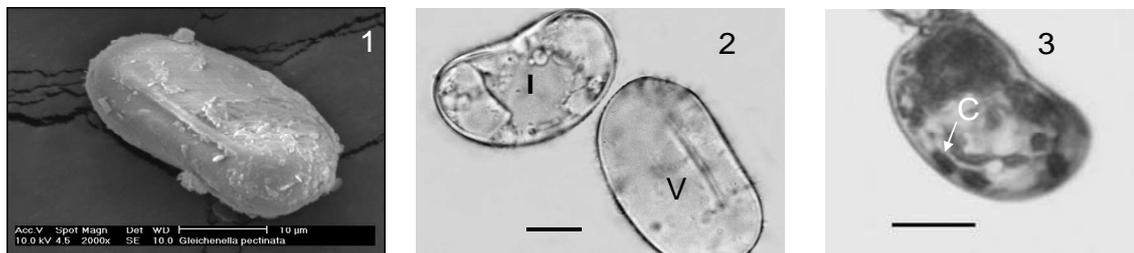


Figure 1 – Spore of *Gleichenella pectinata* under SEM (Bar = 20 µm). **Figure 2**- Spore of *Gleichenella pectinata* under LM. I -unviable; V- viable (Barras = 10µm). **Figure 3**- Spores of *Gleichenella pectinata* after 14 days of cultivation; the arrow indicate starch grains stained with lugol inside chloroplasts ; c- chloroplast (Bar = 20µm).

Table 1 - Percentage of gametophytes with developed rhizoid, without rhizoid and viable spores of *Gleichenella pectinata* (Willd.) Ching collected in 2004. Columns with different letters denote statistical differences (Dunn tests). n = 32, a = 0.05, d = 0.5.

Collection	Storage at 7 ± 1 °C (Months)	Gametophytes with developed rhizoids	Gametophytes without developed rhizoids	Viable spores
% (Mean ± standard deviation)				
March	2.8	4.4 ± 2.7 b	53.6 ± 17.3 a	89.2 ± 2.4a
April	2.4	2.8 ± 3.1 b	48.5 ± 6.4 a	83.1 ± 7.6a
May	1.6	1.5 ± 1.0 b	52.8 ± 4.3 a	55.9 ± 6.7b
November	11.0	18.2 ± 3.5a	3.5 ± 2.0 b	61.1 ± 10.2 b
H		16.73	14.97	22.84

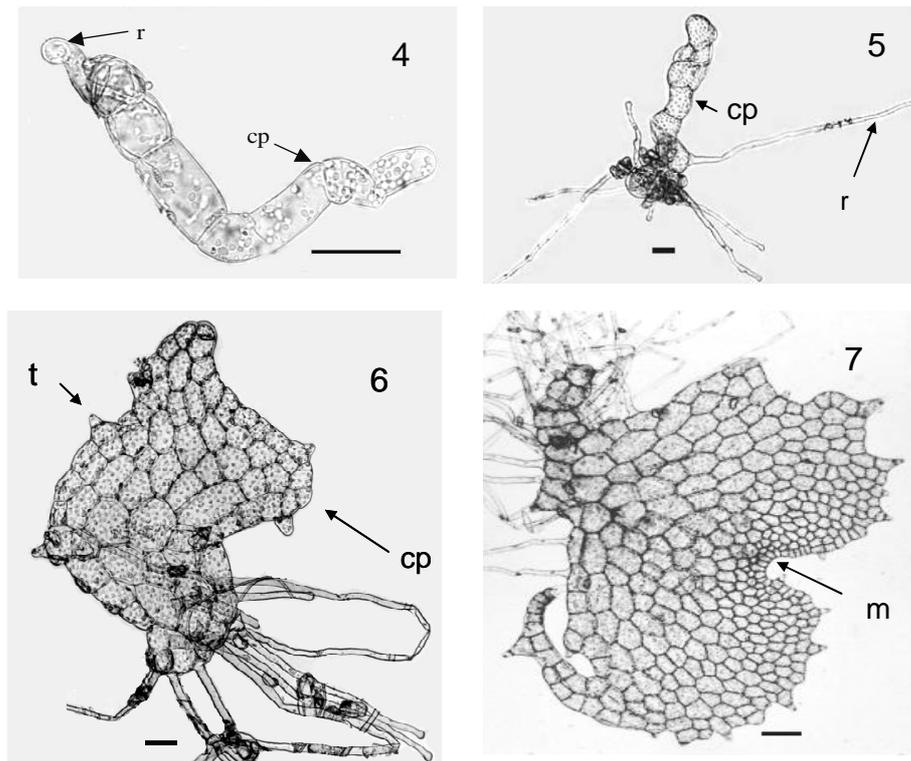


Figura 4 - Filamentous gametophyte of *Gleichenella pectinata* without developed rhizoid after 34 days of cultivation (Bar = 50 μ m, 20x). **Figure 5** - Filamentous gametophyte of *Gleichenella pectinata* with developed rhizoid after 34 days of cultivation (Bar = 50 μ m). **Figure 6** - Spatulate gametophyte (Bar = 50 μ m). **Figure 7** - Heartshaped gametophyte after 48 days of cultivation (Bar = 50 μ m). r-rhizoid, t- trichome, cp- protonemal cell, m- apical meristem.

The highest percentage of gametophytes with developed rhizoids and the lowest percentage of gametophytes without rhizoids were observed in spores collected in November 2004. There was no statistical difference between the percentages of gametophytes with developed rhizoids in the spores collected in March, April and May 2004. The highest percentages of viable spores were observed in the collection of March and April 2004, which showed a low germination percentage. Therefore, spores collected in May presented a low percentage of viable spores and an extremely low germination percentage. However, the percentage of viable spores collected in November 2004 was also lower than the percentage of viable spores collected in March and April, and similar to those collected in May 2004.

A temperature of 30 °C partially inhibited the germination of *G. pectinata* (Table 2). The lowest percentage of gametophytes with developed rhizoids was observed in 62% of sunlight; the highest percentage of gametophytes with developed rhizoids was found for the spores that germinated in 5% of sunlight. The maximum temperatures inside the boxes ranged from 30 to 40°C and minimum temperatures ranged from 12 to 19°C (Fig. 8 A). The minimum irradiances inside boxes ranged from 15 to 107 μ mol m⁻²s⁻¹, while the maximum irradiances ranged from 77 to 553 μ molm⁻²s⁻¹ (Fig. 8B). During this period, the days were generally rainy, and the highest irradiances were registered in October 19 and 22.

No statistical differences were observed between the percentages of gametophytes with and without developed rhizoids of *G. pectinata*, in all the pH values analyzed. The percentages of heart-shaped.

gametophytes were very low for all of the pH values studied, but the highest gametophyte production was found at pH 5.0, which did significantly differ from the pH 4.5 (Table 3).

Table 2 - Effect of temperature on gametophytes with developed rhizoids and without developed rhizoids of *Gleichenella pectinata* (Willd.) Ching from spores collected in November (2004), stored at $7 \pm 1^\circ\text{C}$ for 11 month. Columns with different letters denote statistical differences (Student's *t* test).

Treatment	Gametophyte with developed rhizoids	Gametophytes without developed rhizoids
Temperature ($^\circ\text{C}$)	% (Mean \pm standard deviation)	
25	$18.2 \pm 3.5\text{a}$	$16.58 \pm 5.17\text{a}$
30	$4.8 \pm 3.4\text{b}$	$14.21 \pm 9.6\text{a}$
<i>t</i>	6.6	0.49

Table 3 - Effect of light levels on gametophytes with developed rhizoids and without developed rhizoids of *Gleichenella pectinata* (Willd.) Ching from spores collected in November (2004), stored at $7 \pm 1^\circ\text{C}$ for 11 month. Columns with different letters denote statistical differences (Tukey).

Light level (% of sunlight)	Gametophytes with developed rhizoids	Gametophytes without developed rhizoids
	% (Mean \pm standard deviation)	
62	$0.5 \pm 0.7\text{c}$	$4.5 \pm 3.7\text{a}$
42	$6.2 \pm 2.3\text{b}$	$7.8 \pm 1.8\text{a}$
22	$8.8 \pm 2.3\text{ab}$	$6.9 \pm 5.5\text{b}$
5	$11.3 \pm 2.2\text{a}$	$8.2 \pm 5.3\text{a}$
<i>F</i>	31.50	0.86

Table 4 - Effect of pH on gametophytes with developed rhizoids and total gametophytes of *Gleichenella pectinata* (Willd.) Ching from spores collected in December (2005), stored at $7 \pm 1^\circ\text{C}$ for 04 months. Columns with different letters denote statistical differences (Tukey test).

pH	Gametophyte with developed rhizoids	Heartshaped gametophytes	Gametophyte without developed rhizoids
	% (mean \pm standard deviation)		
4.0	$6.8 \pm 2.3\text{a}$	$0.8 \pm 0.4\text{b}$	$21.2 \pm 5.5\text{a}$
4.5	$9.2 \pm 7.3\text{a}$	$1.2 \pm 0.8\text{ab}$	$26.7 \pm 8.9\text{a}$
5.0	$13.5 \pm 10.4\text{a}$	$2.2 \pm 0.8\text{a}$	$22.3 \pm 5.9\text{a}$
5.5	$9.7 \pm 2.9\text{a}$	$0.8 \pm 0.4\text{b}$	$20.5 \pm 2.7\text{a}$
6.0	$6.3 \pm 3.8\text{a}$	$0.5 \pm 0.5\text{b}$	$19.7 \pm 3.8\text{a}$
Control (6.7)	$4.5 \pm 4.8\text{a}$	$0.3 \pm 0.5\text{b}$	$22.5 \pm 2.7\text{a}$
<i>H</i>	6.84		
<i>F</i>		7.5574	1.2587

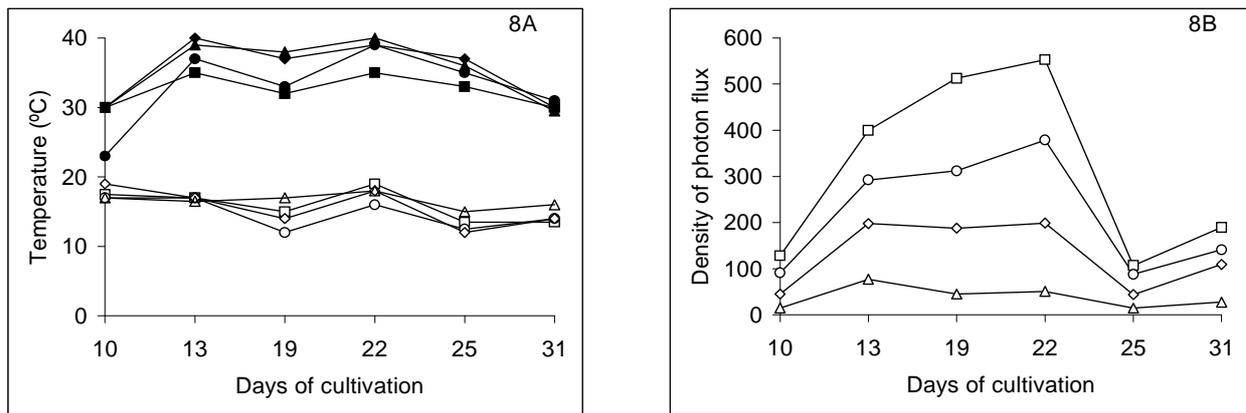


Figure 8 - Minimum and maximum temperatures (8A) and density of photon flux ($\mu\text{mol m}^{-2} \text{s}^{-1}$) (8B) measured at 2 pm during the month of October 2005 inside black shade netting boxes. Empty symbols are minimum temperatures and full symbols are maximum temperatures (8A). \square - 62% light - \circ - 42% light; \diamond , 22% light; \triangle - 5% light.

DISCUSSION

As noted above, three classes of developmental stages were observed after the germination of spore: spores with chloroplasts, but without rhizoids, filamentous gametophytes without developed rhizoids and filamentous gametophytes with developed rhizoids. The first class of development of *G. pectinata* seemed to be similar to the dark germination of *Tmesipteris tannensis* (Spreng.) Bernh., *T. elongata* Dang. and *Psilotum x intermedium* Wagner (Psilotaceae), all of which produced proplastids, but without differentiation of rhizoids (Whittier and Braggins 1993). This suggested the inhibition of spore germination and rhizoid development.

Both phenomena could be explained by the fact that not all spores considered viable were capable of germinating and that gametophytes without developed rhizoids were incapable of reaching the heart-shape and died during the cultivation. It could also be possible that the production of viable spores was influenced by the seasonal alterations, such as temperature, photoperiod and rainy or dry periods. In *Adiantum capillus - veneris* L. (Pteridaceae) the first mitosis is induced by the red light, through the phytochrome system, and the spore nucleus migrates to the spore aperture. However, if this movement is inhibited, the rhizoid does not differentiate.

Indeed, the second mitosis is induced by the blue light, but is inhibited by the red light (Furuya et al.

1997; Uchida and Furuya 1997). Moreover, during the course of this study, it was found that the spores which were collected in November 2004, and which were stored over the course of 11 months under refrigeration, showed the highest percentages of gametophyte with developed rhizoids. In this case, it was possible that the dry storage under low temperatures, promoted the germination of spores of *G. pectinata*. Data also showed variation in spore viability, total gametophytes and gametophytes with developed rhizoids among the different collections. This was supposed by Sheffield et al. (2001) who reported the species - ecotype - specific germination of fern spores in liquid mineral medium. This means that the same species could show different germinative potential depending on local collection, period of collection and time of spore storage in refrigeration.

Peres et al. (1998) and Soares et al. (2000) observed that the leaf extracts of *G. pectinata* and other Glicheniaceae collected in different seasons of the year produced fenolic compounds that presented allelopathic activity. Auto-allelopathy or auto-toxicity occurs when a plant produces toxic substances that inhibit the germination and the growth of the same species (Waller et al. 1986). These extracts delayed and inhibited the germination of *Lactuca sativa* L. (Asteraceae) and *Clidemia hirta* (L.) D. Don (Melastomataceae), and reduced the cellular length of plantlet hypocotyls, also suggesting the inhibition of

cellular divisions. Depending on the season of leaf collection and age of the leaf, they have demonstrated that extracts could induce or inhibit these seed germinations. Therefore, the allelopathic substances could also be produced by the own spores and by young gametophytes of *G. pectinata* cultivated “*in vitro*” causing auto-allelopathy or auto-toxicity and, finally, resulting in gametophytes without developed rhizoids.

G. pectinata showed the highest germination percentage from 5 to 20% of sunlight. Similar effects of light were found in the spores of *Dicksonia sellowiana* Hook (Dicksoniaceae), *Rumohra adiantiformis* (Forst.) Ching (Dryopteridaceae), *Cyathea delgadii* Sternb. (Cyatheaceae), *Blechnum brasiliense* Desv. (Blechnaceae) and *Polypodium lepidopteris* (Langsd. and Fisch.) Kunze (Pteridophyta, Polypodiaceae) (Filippini et al 1999, Renner and Randi 2004, Brum and Randi 2002, Hiendlmayer and Randi 2007, Viviani and Randi 2008). According to Haupt (1990, 1991, 1992) and Raghavan (1989) high temperatures seem to inhibit the gene expression and protein synthesis mediated by phytochrome during fern spore germination. In the present work, the germination of *G. pectinata* was partially inhibited at 30°C.

For *R. adiantiformis* and *P. lepidopteris*, the germination was also partially inhibited at 30 °C (Brum and Randi 2002, Viviani and Randi 2008). Similar results were observed by Perez-García and Riba (1982) with some tree ferns: *Lophosoria quadripinnata* (Gmel.) C. Chr (Lophosoriaceae), *Trichipteris bicrenata* (Liebm) Tryon, *T. scabriuscula* (Maxon) Tryon, *Nephelea mexicana* (Schl. and Cham.) Tryon and *Cyathea fulva* (Mart. and the Gal.) (Cyatheaceae). Ranal (1999) worked with several fern species of the mesophytic semideciduous forest of S. Paulo State and observed that the highest germinability was generally found between 21 and 29 °C.

High daily fluctuations in the minimum and maximum temperatures were observed inside the boxes covered with the black shade net during October 2005. Such oscillations were probably a consequence of the air circulation in windy days, considering that the box receiving 62% of sunlight was more ventilated than the others, due to the larger size of holes in the black shade net. The boxes receiving lower light levels reached the highest temperatures (40 °C) probably due to less ventilation. However, the highest percentages of gametophytes with developed rhizoids were found

under the lower light levels. Daily temperature alternation associated with the low levels of light seems to be beneficial for the germination of this species in this environment. Similar results were also found by Hiendlmayer and Randi (2007) for *B. brasiliense* and *C. delgadii*.

Nondorf et al. (2003) reported that the optimal conditions for spore germination were often a reflection of optimal growth conditions for the entire life cycles of ferns. However, as noted above, *G. pectinata* could grow, even in the degraded fragments of forests, where light incidence was intense and where mineral nutrition and water were limiting factors. Thus, in its natural habitat, possibly, a very low percentage of spores very low percentage of spores of *G. pectinata* would be able to germinate, but only during the rainy periods, with exposure to low light, moderate temperatures and just enough water retention in the soil pores to make the gametophyte development and sporophyte formation possible.

The present data agreed with the literature, for the spores of several ferns, which showed high germination percentages in acid or neutral pH conditions (Miller 1968). Nevertheless, the spores of some species are not able to germinate in strong acid conditions or show very low germination percentage (Hevly 1963, Mohr 1956). In other species, moderate germination levels occur, but the gametophyte development is quite limited where pH are low (Coubert 1955, Otto et al. 1984). On the other hand, the spores of *Ophioglossum palmatum* L. germinate better in strong acid conditions, and the germination is reduced or eliminated in acid or slightly neutral conditions (Whittier 1981, Whittier and Moyroud 1993). For the spores of *Cheilanthes feei* T. Moore (Polypodiaceae), higher germination percentages were observed in acidic pH (4.5 and 5.5) (Nondorf et al. 2003). This work showed that the germination percentage of *G. pectinata* spores was very low in comparison to several other fern species that could reach around 100% of spore germination as reported by Gomes et al (2006) for spores of *Dicksonia sellowiana* Hook. (Dicksoniaceae). Besides the low germination percentage, gametophytes without developed rhizoids are not able to grow and only gametophytes with developed rhizoids continue their development and reach the heart-shape. Therefore, the production of sporophytes through spore cultivation is extremely low. Consequently,

we suggest alternative methods of vegetative and *in vitro* culture in order to obtain sporophytes of *G. pectinata*.

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RESUMO

A finalidade deste estudo foi analisar o potencial germinativo dos esporos de *G. pectinata* e verificar os efeitos de níveis de luz (62, 42, 22 e 5% da luz natural), das temperaturas de 25 e 30 °C e de pHs entre 4,0 e 6,7 na germinação. Esporos de *G. pectinata* são monoletes e a superfície apresenta depósitos grandes e irregulares. Observamos três classes de desenvolvimento: esporos contendo cloroplastos, gametófitos filamentosos sem rizóides desenvolvidos e gametófitos filamentosos com rizóides desenvolvidos. Somente os gametófitos com rizóides desenvolvidos foram capazes de alcançar a fase cordiforme. As porcentagens de germinação foram mais elevadas à temperatura de 25°C do que 30 °C. As mais elevadas porcentagens de gametófitos com rizóides desenvolvidos foram observadas sob 22 e 5% da luz natural e as porcentagens mais elevadas de gametófitos cordiformes foram observadas no pH 5,0.

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