EFFECTS OF PH, TEMPERATURE AND LIGHT INTENSITY ON SPORE GERMINATION AND GROWTH ANALYSIS OF YOUNG SPOROPHYTES OF *POLYPODIUM LEPIDOPTERIS* (PTERIDOPHYTA, POLYPODIACEAE)[1]

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ABSTRACT

(Effects of pH, temperature and light intensity on spore germination and growth analysis of young sporophytes of *Polypodium lepidopteris* Kunze (Pteridophyta, Polypodiaceae)) *Polypodium lepidopteris* is a terrestrial fern from coastal vegetation, and is used as medicinal. This work analyzed the effects of pH, temperature and light levels on the spore germination and the relative growth rate (RGR) of young sporophytes. Fertile fronds were collected in Florianópolis, SC, Brazil. The effect of pH (4.0 to 6.7) on spore germinability was observed in a growing room at 25 ± 2ºC (22 mmol $m^{-2}s^{-1}$) under a 16h photoperiod. No statistical differences between treatments were found. The effect of different temperatures on the germinability was analyzed (20, 25 and 30ºC). The test was carried out in a germination chamber (17 mmol $m^{-2}s^{-1}$) under a 16h photoperiod. The germination was inhibited at 30°C. The effect of natural light levels (54, 38, 22 and 8%) was analyzed. The highest germination percentages were verified at 22 and 8% of natural light. The growth analyses show statistical differences in the number of fronds and height of the longest frond between time 1 (283 days of spore inoculation) and time 2 (343 days of spore inoculation). Sporophytes of *P. lepidopteris* produced 1.33 ± 0.09 fronds per month. The RGR (relative growth rate) was 0.15 ± 0,009 cm $cm^{-1}month^{-1}$.

Key words: germination, growth, medicinal plant, *Polypodium lepidopteris.*

RESUMO

(Efeito de pH, temperatura e intensidade luminosa na germinação de esporos e análise de crescimento de esporófitos jovens de *Polypodium lepidopteris* Kunze (Pteridophyta, Polypodiaceae)) *Polypodium lepidopteris* é uma pteridófita terrestre que ocorre nas restingas e que apresenta propriedades medicinais. Este trabalho analisou o efeito de pH, temperatura e níveis de luz na germinação de esporos e a taxa de crescimento relativo (TCR) de esporófitos jovens de *P. lepidopteris*. Frondes férteis foram coletadas em Florianópolis, SC, Brasil. O efeito do pH (4,0 a 6,7) na germinabilidade de esporos foi analisado em sala de cultivo a 25 ± 2ºC (22 mmol $m^{-2}s^{-1}$) sob fotoperíodo de 16 horas. Não houve diferença estatisticamente significativa entre os tratamentos. Diferentes temperaturas foram testadas (20, 25 e 30ºC) em câmara de germinação (17 mmol $m^{-2}s^{-1}$) sob fotoperíodo de 16 horas. A germinação foi inibida a 30°C. O efeito de diferentes níveis de luz natural (54, 38, 22 e 8%) foi analisado. As maiores porcentagens de germinação ocorreram a 22 e 8% de luz natural. A análise de crescimento dos esporófitos jovens mostra diferença significativa entre número de frondes e altura da maior fronde avaliados no tempo 1 (283 dias de inoculação de esporos) e no tempo 2 (343 dias de inoculação de esporos). Esporófitos de *P. lepidopteris* produziram 1,33 ± 0,09 frondes por mês. A TCR (taxa de crescimento relativo) foi de 0,15 ± 0,009 cm cm^{-1}mês.

Palavras-chave: germinação, crescimento, planta medicinal, *Polypodium lepidopteris.*

INTRODUCTION

Approximately 65% of the world fern species occur in the tropics (Tryon & Tryon 1982). In Brazil, ferns occur preferentially in the Atlantic and Amazonian forests (Senna & Kazmirczak 1997; Labiak & Prado 1998) but some species are also found in the Brazilian caatinga, mangroves, coastal vegetation and pantanal (Tryon & Tryon 1982; Barros *et al.* 1989; Ambrósio & Barros 1997).

In the last few decades, several ornamental or medicinal ferns have been indiscriminately exploited. *Polypodium lepidopteris* (Langsd. & Fisch.) Kunze (Polypodiaceae), a herbaceous and terrestrial species found in the Brazilian coastal vegetation called 'restinga' (CONAMA

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1999), is considered medicinal due to the presence of therapeutic substances. In order to establish immobile and half-fixed sand-dunes, sporophytes of *P. lepidoteris* are small and have numerous trichomes and scales to reduce water loss and protect the epidermis from intense sunlight. The lamina of *P. lepidopteris* is more or less densely scaly and the stem scales are long and narrow (Tryon & Tryon 1982).

Polypodium lepdopteris is used since 1924 in association with 'cainca' (*Chiococca alba* (L.) Hitchc, Rubiaceae) in a phytoterapic medicine form known as 'Rheumoflora®', which had been initially manufactured by the 'Flora Medicinal' and is currently under the responsibility of 'Natura do Brasil'. This medicine is indicated as analgesic and anti-inflammatory (Gazda *et al.* 2006). The whole plant presents therapeutically active principles, for this reason, whole plants or parts of the plants have been directly extracted from their habitat over many years.

There is a lack of information on fern germination and initial growth in literature. The knowledge of fern ecophysiology is of essential importance for the development of methods aimed at assisting their conservation and management.

The same exogenous factors needed for germination plus mineral nutrition are involved in growth and development of fern prothallus and in the formation of its sporophyte (Millër 1968; Pérez-García & Riba 1982; Whittier & Moyroud 1993; Fernández *et al.* 1996; Fernández *et al.* 1997; Ranal 1999; Nondorf *et al.* 2003).

This paper investigates the effect of pH, light levels and temperature on the germination of *Polypodium lepidopteris* spores and analyzes the relative growth rate of sporophytes produced from the spore germination. The main objective was to contribute with ecophysiological information on germinability and young sporophyte growth, which could be useful to assist in management and conservation programmes.

Material and Methods

Sporophylls of *Polypodium lepidopteris* (Langsd. & Fisch.) Kunze were collected in the 'Parque Municipal das Dunas da Lagoa da Conceição', east coast of the Santa Catarina Island, in the dunes of the Joaquina beach, Florianópolis,Brazil.

Sporophylls were dried at room temperature on filter paper in order to induce dehiscence. The spores were removed and separated from sporangia by filtering through lens paper and later stored in glass jars under refrigeration at 7 ± 1ºC. Spores were surface sterilized in a 10% (v/v) commercial bleach solution (2% of active chlorine) for 20 min before filtering through sterile filter paper and washing several times with sterile distilled water. For the germination tests, about 10 mg of the spores were sown in four conical flasks containing 20 ml of autoclaved liquid medium. The flasks were plugged with two layers of autoclaved transparent commercial polypropylene film (7 × 7 cm) and fixed with rubber bands. Spores were inoculated in Mohr medium modified by Dyer (1979) supplemented with Benlate® (25 $mg.L^{-1}$). The spore germination was carried out in growth room at 25 ± 2ºC (22 $mmol\ m^{-2}s^{-1}$) under a 16-hour photoperiod. The pH of the mineral solutions was adjusted with H_2SO_4 to 4.0, 4.5, 5.0, 5.5, 6.0 and 6.7 (control). The percentage of germination was scored once a week and four slides from each treatment containing 100 spores each slide, were analyzed. The effect of 20, 25 and 30ºC on the germinability was analyzed and the test was carried out in a germination chamber (17 $mmol\ m^{-2}s^{-1}$) under a 16-h photoperiod.

To study the light level effects on spore germination, four Erlenmeyer flasks containing 20 ml of autoclaved liquid medium were used per light treatment. Each flask was inoculated with surface sterilized spores (10 mg) and kept in 50 cm^3 boxes covered with black shade netting (sombrite) that provided 54, 38, 22 and 8% of natural light. A maximum and minimum thermometer was placed inside the boxes. Minimum and maximum temperatures were

daily recorded at noon. Irradiance inside the boxes was also daily recorded, once a day, at noon. The irradiance levels were measured through a quantameter LICOR 250, using a PAR sensor (400 to 700 nm). The boxes were kept outdoors, in a shade free open area.

After one month of cultivation in mineral solution, the spores developed into young filamentous gametophytes that were transferred to trays containing the following substrate: a) washed sand b) a mixture of sand, humus compost Aduplan® and typic hapludult soil in the proportion of 1:1:1. The substrates were previously autoclaved for 60 min at 120ºC in order to avoid contamination with spores from other fern species. The trays were kept in a growth room, at 25 ± 2ºC (22mmol $m^{-2}s^{-1}$), under a 16-hour photoperiod for sporophyte production. For growth analysis, 30 sporophytes (*ca* 4 cm tall) were transplanted individually for small vases (125 ml), which were kept in transparent polyethylene boxes (San Remo®, 28.2 L). The number of sporophyte fronds and the height of the longest frond were measured in a 60-day interval. The first measures were recorded after 283 days of cultivation (Time 1, T_1) and the second measures after 343 days of cultivation (Time 2, T_2). The relative growth rate (RGR) was estimated as (Log L_2–Log L_1) / T_2–T_1 where Log is the natural logarithm, L_2 is the length of the longest leaf at time 2 and L_1 is the length of the longest leaf when the sporophytes were transplanted into the pots, according to Bernabe *et al*. (1999).

Averages and standard deviation were calculated. The Kolmogorov-Smirnov test (*Dmax*) for normality was applied before analysis of variance, both for growth analysis and germinability data. The Kolmogorov-Smirnov test and the F test of Snedecor for the homogeneity of variance (0.05) were both applied for germinability data before the analysis of variance. The Kolmogorov-Smirnov test showed absence of normality for the germinability in different temperatures and the Bartlett's test showed that the variances were not homogeneous for the germinability in different light levels, which were submitted to arcsine transformation. The same tests were applied again to the transformed data. After this procedure, the pH data showed normality and homogeneity of variance and the pairwise comparison was analyzed by the parametric Tukey Test (5%). For the temperate and light levels data, that did not show normality or homogeneity of variance after angular transformation, the pairwise comparison was analyzed by the nonparametric Kruskal-Wallis Test (*H*) followed by the Dunn test. Averages of fronds number and the length of the longest frond between T1 and T2, that did not show normality or homogeneity of variance, were also analyzed through the Kruskal-Wallis Test followed by the Dunn Test (Santana & Ranal 2004; Zar1996). Statistical tests were performed through the Excel for Windows (Microsoft), Minitab for Windows and Biostat softwares (Microsoft).

Results and Discussion

When fern spores are cultivated in laboratory, they need to be superficially sterilized before being sowed in mineral solutions. Camloh (1993, 1999) reported the best germination of *Platycerium bifurcatum* (Cav.) C. Chr. (Polypodiaceae) with unsterilized spores, but contamination always occurred after 10 days of culture, which was the reason for the lower cell number as compared to sterilized spores. Simabukuro *et al.* (1998) comment that before the germination of dry-stored spores, in order to avoid the incidence of fungal growth, there is the need to sterilize them. In this work, spores of *P. lepidopteris* were surface sterilized in order to guarantee gametophyte development without contamination.

The pH range from 4.0 to 6.7 did not affect the germination of *P. lepidopteris* spores after 28 days of inoculation (Fig. 1a). So, *P. lepidopteris* spores show plasticity concerning the pH factor. These data are in accordance with Millër (1968), who observed the highest percentages of germination for several fern species in acidic or neutral pH. Nondorf *et al*. (2003) working with *Cheilanthes feei* Moore

(Pteridaceae) spores, a xerophyte fern, observed that spores germinated at the highest rate in acidic pH (4.5 and 5.5), but the germination occurred in a broad pH range (4.5, 5.5, 6.0 and 8.5). However, some fern spores are not able to germinate in strong acidic conditions or the germinability is very low in such situations (Mohr 1956; Hevly 1963). In other species, a moderate germination percentage is observed in acidic pH, but the gametophyte development is quite limited (Courbet 1955; Otto *et al*. 1984). Spores from terrestrial species of Ophioglossaceae also show the highest germinability in slight acidic pH (Whittier 1981). *Ophioglossum palmatum* L. spores reached the highest germinability in strong acidic medium, but the germinability was reduced in less acidic or neutral conditions. (Whittier & Moyroud 1993).

The germination of *P. lepidopteris* spores after 28 days of spore inoculation did not differ between 20 and 25ºC, but it was drastically inhibited at 30ºC (Fig.1b). Data from literature also show similar responses for several fern species. Pérez-García & Riba (1982) studied the effect of temperature on several species of Cyatheaceae and Dicksoniaceae: *Cyathea fulva* (Mart.& Gal.) Fée, *Lophosoria quadripinnata* (Gmel.) C. Chr, *Nephelea mexicana* (Schl. & Cham.) Tryon, *Trichipteris bicrenata* (Liebm) Tryon, *Trichipteris scabriuscula* (Maxon) Tryon and also observed partial inhibition of germination above 25ºC. Ranal (1999) studied the effect of temperature on several fern species from the Atlantic Forest of the state of São Paulo, Brazil, and observed similar germinability at all temperatures tested for *Polypodium hirsutissimum* Raddi, *Polypodium latipes* (L.) Watt. and *Pteris denticulata* Sw. (Polypodiaceae) but high germinability was observed between 18 and 25ºC for *Microgramma lindbergii* (Kuhn) Sota, *Microgramma squamulosa* (Kaulf.) Sota and *Polypodium polypodioides* (L.) Watt. (Polypodiaceae). For *Adiantopsis radiata* (L) Fée (Pteridaceae) and *Polypodium pleopeltifolium* Raddi (Polypodiaceae) high germinability was verified at 21 to 29ºC. For spores of *Rumohra adiantiformis* (Forst.) Ching (Dryopteridaceae) Brum & Randi (2002) observed high percentages of germination at 15, 20 and 25 ± 1ºC but germinability was partially inhibited at 30 ± 1ºC. *Cheilanthes feei* spores also germinated optimally at 25ºC (Nondorf *et al*. 2003). Therefore, data from

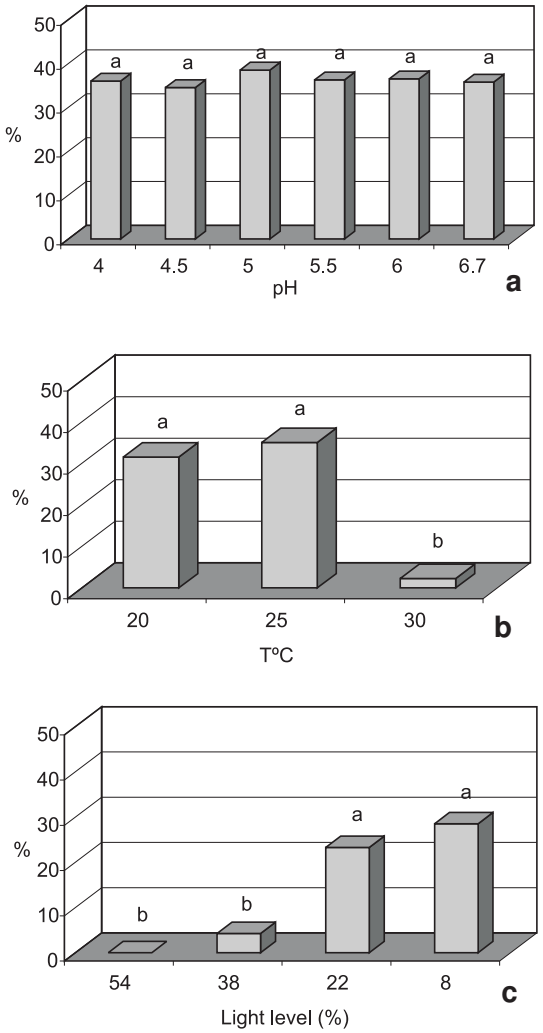

Figure 1 – Effects of pH, temperature and light levels on the germination rate (%) of *Polypodium lepidopteris* spores after 28 days of inoculation. Letters denote statistical differences. * Data did not show normality.
a. pH; D_{max} =0.084; c^2 = 3.975; F= 0.6261
b. TºC; D_{max} = 0.204*; c^2 = 4.092; H = 8.5781 (s)
c. Light levels; D_{max} = 0.243*c^2 =12.837*; H = 13.810 (s)

literature are similar to those observed for *P. lepidopteris*, with respect to the temperature required for germination. Raghavan (1989) explains that high temperatures uncouple the phytochrome during fern germination. Haupt (1991, 1992) observes that the phytochrome-mediated spore germination in *Dryopteris filix-mas* L. and *D. paleacea* (Dryopteridaceae) is inhibited by raising the temperature from 22 to 27 or 32°C. The elevated temperature is effective during the 'coupling phase' when the far-red phytochrome (Pfr) starts the processes leading to germination (Haupt 1990). It is generally accepted that Pfr starts a whole cascade of events, eventually culminating in gene-dependent protein synthesis as the immediate cause of the terminal response (Haupt 1992).

High percentages of germination of *P. lepidopteris* spores were observed at 8 and 22% of natural light and the germination was photoinhibited at 38 and 54% of light (Fig. 1c). A degradation of storage substances was also observed in spores, which were kept under 58% of light. On the other hand, under 8% of light and after 28 days of spore inoculation, laminar gametophytes were observed. The behavior of *P. lepidopteris* spores to light levels seems to be very usual for several fern species, which also presented optimal

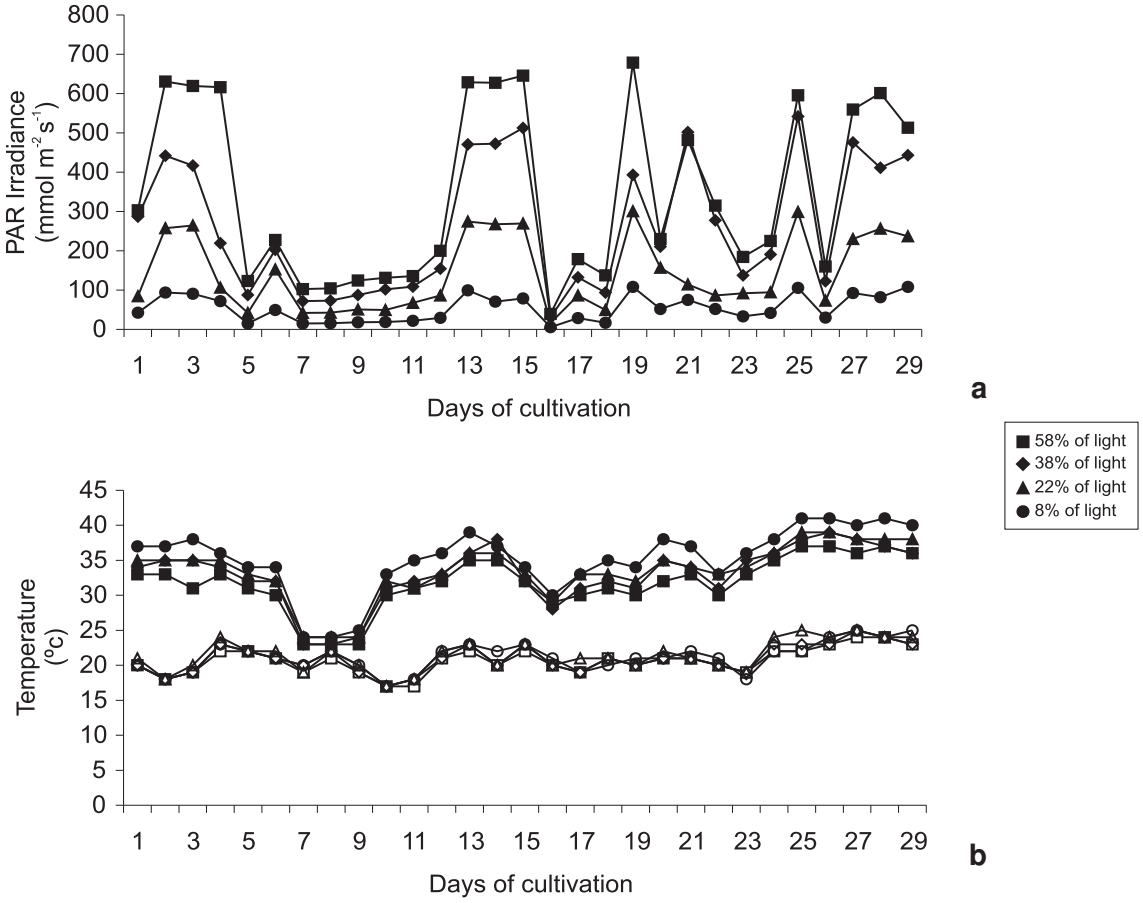

Figure 2 – Irradiances (mmol m^{-2} s^{-1}) and minimal and maximum temperatures from November 21st to December 19th measured at noon inside the boxes of black shade where spores received different light levels. Open symbols are minimum temperatures and dark symbols are maximum temperatures.

germinability at lower light levels. Filippini *et al.* (1999) and Renner & Randi (2004) observed the highest germination percentages at 5 and 20% of light for spores of *Dicksonia sellowiana* Hook. (Dicksoniaceae). Brum & Randi (2002) demonstrated that for *R. adiantiformis* spores, the highest germination percentages were achieved at 9 and 17% of light in April and August. Hiendlmayer & Randi (2007) also found the best germinability for *Cyathea delgadii* Sternb. (Cyatheaceae) and *Blechnum brasiliense* Desv. (Blechnaceae) spores at 5 and 22% of light in April and July. These authors also observed gametophyte death of both species at 62 and 42% of light, after a few days of cultivation. This effect was probably a consequence of chlorophyll oxidation at higher light levels, which reduces the photosynthetic efficiency, limiting the plant growth and eventually causing plant death (Demming-Adans & Adams 1992; Kitao *et al.* 2000). On the other hand, for *Cheilanthes feei* Moore, high germination rates were achieved under a regime of continuous light of 100 mmol m^{-2} s^{-1}; however, this species can germinate under a wide variety of conditions including constant darkness (Nondorf *et al.* 2003).The irradiance from November 21st to December 19th 2006 ranged from 39.5 to 678.8 mmol m^{-2} s^{-1} at 54% of light. At 38% of light, it ranged from 30.4 to 541.6 mmol $m^{-2}s^{-1}$. At 22% of light it ranged from 16.2 to 300.0 mmol m^{-2} s^{-1} and at 8% of light it ranged from 5.6 to 107.9 mmol m^{-2} s^{-1} (Fig. 2a). Maximum temperatures ranged from 32ºC (54% of light) to 35ºC (8% of light), but the minimum temperature average was constant (21ºC) inside the four 'sombrite' boxes (Fig. 2b). On windy days, higher temperatures were observed inside the 8% light box and lower temperatures inside the 54% light box which was more ventilated due to the larger holes in the black shade netting. The highest temperatures in the hotter days in the 8 and 22% light boxes did not inhibit the germination of *P. lepidopteris* spores, that reached the highest percentages at the same light levels. On the other hand, the germination of *P. lepidopteris* was drastically inhibited at constant 30ºC. Probably, the daily day - night temperature oscillation and the low light intensity seem to be beneficial for the germination of *P. lepidopteris* spores.

Nondorf *et al*. (2003) reported that optimal conditions for spore germination are often a reflection of optimal growth conditions for the entire fern life cycles. Considering that *P. lepidopteris* grows in coastal dunes, where light incidence is intense and mineral nutrition and water are limiting factors, the spore germination photoinhition by high light intensity seems to be a paradox. It is possible that in its habitat, spores will only be able to germinate during rainy periods, in shady areas, with moderate temperatures and with some water retention in the soil pores in order to make gametophyte development and sporophyte formation possible. On the other hand, *P. lepidopteris* spores can germinate in broad pH ranges, showing plasticity to this requirement.

Table 1 – Frond number (FN) and height of the longest frond (FH) of *Polypodium. lepidopteris* sporophytes that grew in growth room at 25 ± 2ºC (22mmol $m^{-2}s^{-1}$) under a 16-hour photoperiod. T1 = 283 days after spore inoculation; T2 = 343 days after spore inoculation. Letters denote statistical differences. * Data did not show homogeneity of variances.

	T1	**T2**
	(Mean ± *ic*)	
FN	5.00 ± 0.13a	7.67 ± 0.22b
D_{max}	0.046	0.047
F		2.842*
H		28.93
FH (cm)	4.04 ± 0.09a	8.65 ± 0.34b
D_{max}	0.051	0.076
F		3.935*
H		34.05
RGR (cm $cm^{-1}month^{-1}$.)		0.15 ± 0.009
FN $month^{-1}$		1.33 ± 0.09

The gametophytic phase shows plasticity due to large fluctuations in light intensity, light quality and mineral nutrition. Sato & Sakai (1981) observed that gametophytes of *Vittaria lineata* (L.) Smith and *Vittaria graminifolia* Kaulf (Vittariaceae) could survive in sites where sporophytes do not develop. Similar response was observed for *Trichomanes speciosum* Willd. (Hymenophyllaceae) (Makgomol & Sheffield 2001). This different gametophyte and sporophyte distribution pattern reflects the wide ecologic tolerance between both generations (Johnson *et al.* 2000). It is possible that *P. lepidopteris* gametophytes and sporophytes also present different requirements for growth and establishment.

During this experiment, gametophytes were not able to grow in washed sand. In the substrate of mixed sand, humus and typic hapludult soil (1:1:1), the number of sporophyte fronds and the height of the longest frond after 283 days of spore inoculation (Time 1) and after 343 days of spore inoculation (Time 2) were statistically different. The RGR based on the height of the longest frond was 0.15 cm cm^{-1} $month^{-1}$ (Table 1). Similar results were found by Hiendlmayer & Randi (2007) working with four fern species from the Atlantic forest: *Blechnum brasiliense* Desv. (Blechnaceae), *Cyathea corcovadensis* (Raddi) Domin and *Cyathea delgadii* Sternb. (Cyatheaceae), and *Saccoloma inaequale* (Kze.) Mett. (Dennstaedtiaceae). Young sporophytes of *P. lepidopteris* produced 1.33 ± 0.09 fronds per month (Table 1). These data are similar to the results found for *Acrostichum danaeifolium* (Pteridaceae) in the environment, which were 1.0 ± 0.03 to 1.3 ± 0.04 fronds per month (Mehltreter & Palacios-Rios 2003; Mehltreter *et al.* 2003).

According to Zamith & Scarano (2006) restinga is both a geomorphological and a botanical term. Plant communities experience a wide array of adverse environmental conditions, such as high and low temperatures, flooding, drought, constant wind, high salinity and lack of nutrients. Thus, diversity, productivity and structural complexity are lower in these communities. The restinga ecosystem is therefore unique because it comprises a pool of species with high ecological plasticity, since they colonize, survive and grow in the dry, resource-poor restingas. Paradoxically, it has been shown that few restinga plants are capable of establishing via seeds on bare sand and, therefore, the structure and function of open restinga vegetation relies on a few pioneer nurse-plants that facilitate the arrival and establishment of a number of other species (Scarano 2002).

P. lepidopteris is found in seaside vegetation (restinga) therefore it could be supposed that its gametophytes are able to develop in salty sand which is poor in mineral nutrition and organic matter. However, in the present study, sporophytes grew very well in a substrate that is a mixture of typic hapludult soil, sand and humus, which certainly is more enriched in nutrients than sand dunes. Actually, in the present paper, gametophytes were not able to develop in washed sand. So, how can gametophytes develop in this very restrictive habitat? This is a very instigating question. Possibly, these gametophytes will be able to grow only in sites that offer adequate nutrition, for example, parts of the sand soil previously colonized by other species and enriched with organic matter and recycled mineral nutrients, which is in accordance to comments on Scarano (2002). This is a matter of future investigations.

This study has shown that it is feasible to cultivate plants of *P. lepidopteris* from spores. The limiting factors for spore germination in mineral solution observed in this study were the high light levels and temperature of 30 ºC. For the establishment of sporophytes, which was performed in laboratory, it was observed that mineral nutrition and high humidity are important factors, because gametophytes did not develop in pure sand and therefore, sporophytes were not produced. Such information may be useful for management and conservation of *P. lepidopteris.*

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