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Acclimation and photoprotection of young gametophytes of *Acrostichum danaeifolium* to UV-B stress

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Abstract

The effect of ultraviolet B radiation (UV-B) on cellular ultrastructure, chlorophyll (Chl), carotenoids, and total phenolics of *Acrostichum danaeifolium* gametophytes was analyzed. The control group of spores was germinated under standard conditions, while the test group of spores was germinated with additional UV-B for 30 min every day for 34 d. The cell characteristics were preserved in gametophytes irradiated with UV-B, but the number of starch grains increased in the chloroplasts and the more developed grana organization in contrast to the chloroplasts of the control group. Chl *a* content decreased, while Chl *b* content increased in the gametophytes cultivated with UV-B for 34 d. Contents of lutein and zeaxanthin decreased and trans-β-carotene concentration was enhanced in the gametophytes irradiated with UV-B. The content of total phenolic compounds increased in the gametophytes cultivated with UV-B. Therefore our data suggest that the gametophytes of *A. danaeifolium,* a fern endemic to the mangrove biome, were sensitive to enhancement of UV-B radiation at the beginning of their development and they exhibited alterations in their ultrastructure, pigment contents, and protective mechanisms of the photosynthetic apparatus, when exposed to this radiation.

Additional key words: carotenoids; chlorophyll; gametophyte development; phenolic compounds; ultrastructure.

Introduction

Acrostichum danaeifolium Langsd. & Fisch. (Polypodiopsida, Pteridaceae), also known as the giant leather fern, is an endemic plant from the mangrove biome and it occurs in the tropics and subtropics of the New World (Smith *et al.* 2006, Lloyd and Gregg 1975, Tryon and Tryon 1982, Moran 1995). The mangrove biome has a few endemic species, which are structurally and physiologically adapted to survive in soils that are very compacted, poorly oxygenated, and frequently flooded by tidal water (Soares 1999).

Mangroves are found almost exclusively in tropical regions. The largest continuous mangrove in the world is located along the Brazilian coast from Cape Orange (4°26'0"N, 51°31'0"W) in Amapá to Laguna (28°21'57"S, 48°46'51"W) in Santa Catarina. It has been estimated that this region contains 1.38 million ha of mangroves, which runs along the coastline for approximately 6,800 km (Schaeffer-Novelli 1995).

Ferns comprise a significant part of the vascular flora of the Earth. They are sensitive to small climatic variations and are good indicators of the degree of environmental preservation (Xavier and Barros 2003). They are distributed worldwide from tropical to temperate regions, from the sea level to high elevations, and are found in many habitats, such as sandbanks and mangroves (Page 1979, Rathinasabapathi 2006). According to Raghavan (1989), fern gametophytes are potentially useful for experimental studies, providing impetus for significant

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Abbreviations: Car – carotenoid; Chl – chlorophyll; FM – fresh mass, PAR – photosynthetically active radiation; TEM – transmission electron microscopy; UV – ultraviolet radiation; UV-B – ultraviolet B radiation.
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interdisciplinary research on its growth, differentiation, and sexuality.

The ozone layer is the only natural protection the Earth has against ultraviolet radiation (UV). Ozone is very active gas and readily reacts with several substances near the Earth surface. These reactions hurt plants and damage human lung tissues. However, ozone also absorbs harmful components of sunlight, such as UV-B (Falkowski and Raven 1997). High above the surface of the Earth, a tenuous layer of ozone absorbs UV-B (NASA 2011). In spite of natural fluctuations in recent years, the amount of ultraviolet radiation, which reached the Earth surface, has increased considerably due to the depletion of the ozone layer both in the southern and northern hemispheres (Hilal *et al.* 2004). The severity of the ozone hole in the southern polar region varies somewhat from year to year. The largest ozone hole ever observed, since 1979, was on 24 September 2006 (NASA 2011), and the ozone hole reached land and populated areas in Argentina, Chile, and the Falkland Islands on September 25–28, October 9 and 16–19, and November 17–20, 2011 (NASA 2011). The decrease of ozone in the atmosphere is a consequence of high concentrations of chlorofluorocarbons (CFCs) in the upper atmosphere, and it is possible that this triggers various morphological changes in plants due to elevated levels of UV radiation (Döhler *et al.* 1989, Kulandaivelu and Daniell 1989, Sullivan and Teramura 1989, Tevini and Teramura 1989, Nogués and Baker 1995, Whelan and Glaser 1997, Wilhelm *et al.* 1997).

The potential harm caused by UV-B (280–320 nm) is primarily associated with a damage to DNA, chloroplasts, photosynthesis, pigments, membranes, proteins, transpiration, and stomata number (Greenberg *et al.* 1989, Tevini and Teramura 1989, Rozema *et al.* 1997, Hilal *et al.* 2004, Sarghein *et al.* 2008, Ranjbarfordoei *et al.* 2009, Kumari *et al.* 2009, Qian and Qing 2009, Sarghein *et al.* 2010, Piri *et al.* 2011). UV-B radiation affects plant morphology, anatomy, biochemistry, and physiology at different levels. Thus, the reduction of the leaf area, the increased parenchyma thickness, the increased cell length and the cell number of the parenchyma and mesophyll, changes in the chloroplast structure (such as an increase in grana, dilation of the thylakoid membranes and disintegration of the envelope), changes in cellular metabolism, such as deterioration of photosynthesis, and an increase in antioxidant capacity and accumulation of phenolic compounds were observed (Rozema *et al.* 1997, Laakso *et al.* 2000, Jayakumar *et al.* 2002, Hilal *et al.* 2004). However, the research in this area was focused mainly on agricultural plants.

A. danaeifolium is an endemic plant in the mangroves. In southern Brazil, these biomes are exposed to high levels of UV-B in certain seasons of the year. This study aimed to analyze the impact of this radiation on the gametophytic development of this fern species.

Materials and methods

Plants: Fertile fronds of *A. danaeifolium* were collected in the ecological reserve of mangrove in Itacorubi (*ca.* 150 ha), which is situated on Santa Catarina Island (27°34'13"–27°35'31"S, 48º30'07'–48º31'33"W). The sporophylls were dried at room temperature on a filter paper in order to induce dehiscence. The spores were removed and separated from the sporangia by filtering them through a lens paper and then stored in glass jars at 7 ± 1ºC.

Spore germination and gametophyte cultivation: The spores were sterilized in a 10% commercial bleaching solution (with 2% active chlorine) for 20 min, filtered (*Whatman* N° 1) and washed three times with distilled water. For the germination assays, about 10 mg of spores were sown in four conical flasks containing 20 mL of autoclaved *Mohr*'s liquid medium, which was modified according to Dyer (1979) and supplemented with *Benlate* (25 mg L^{-1}). The standard germination was carried out in a growth room at 25 ± 1ºC with a 12-h photoperiod (photosynthetically active radiation, PAR, was 30 µmol m^{-2} s^{-1}) provided by two fluorescent white lamps (*Philips TLD*® 30 W/75, Germany) arranged horizontally 30 cm above the samples. The PAR irradiance was measured with a *LI-COR–250* quantometer (*LI-COR Inc.*, Lincoln, NE, USA). The gametophytes were cultivated for 34 d. For the UV-B treatment, two lamps (*Philips TL* 20W/12RS, The Netherlands) were placed horizontally 30 cm above the samples, under the above standard germination conditions (PAR 30 µmol m^{-2} s^{-1}), but they were exposed to UV-B (10 W m^{-2}) for 30 min a day (Jayakumar *et al.* 2002) for 34 d. The UV-B irradiance was measured using a UV-B radiometer (*Cole-Parmer Internacional*, IL, USA) attached to the UV-B sensor (*VLX* – 312 nm, *Cole-Parmer Internacional*, IL, USA) at the surface of the samples.

Cell ultrastructure analysis: The samples (four replicas of approximately 10 mg of 34-d-old gametophytes for each treatment) were soaked in a 0.1 M phosphate buffer with 2.5% glutaraldehyde, pH 7.0, for 9 h under refrigeration (5 ± 1°C). They were then centrifuged (5 min, 450 × *g*) and the pellets were placed in 2% agar. They were washed three times in 0.1 M phosphate buffer, at pH 7.0, and post-fixed in a 0.1 M phosphate buffer with 1% OsO_4 at pH 7.0, for 5 h at room temperature. Then they were dehydrated in an acetone gradient (30, 50, 70, 90, and 100%) two times, for 30 min each. The samples were then slowly infiltrated into a series of acetone and *Spurr*'s resins (Spurr 1969) in a seven step sequence that lasted 12 h each: 100% acetone, acetone-resin mixture (v/v of 3:1, 2:1, 1:1, 1:2, 1:3), and 100% resin (twice). Finally,

the samples were polymerized in horizontal molds in an oven at 70°C for 24 h, sectioned with an ultramicrotome (*Leica EM UC78, Leica Microsystems*, Viena, Austria) equipped with a diamond knife (Ultra 45°, *Diatome*, Bienne, Switzerland), stained with 1% uranyl-acetate followed by 1% lead-citrate according to Reynolds (1963) and evaluated with a transmission electron microscope (*JEM 1011*, *Jeol*, Germany). More than 30 images were taken for each treatment.

Chl, carotenoids (Car), and total phenolic compounds: Samples (100 mg) of the gametophytes, both the control and UV-B treated, cultivated for 34 d, were frozen in liquid N_2, and they were extracted in dimethylsufoxide. Chl *a* and Chl *b* were analyzed according to Wellburn (1994).

For the Car analysis, the samples were mixed in 4 mL of hexane: acetone (v/v of 1:1) containing 1 mg of butylated hydroxytoluene. The extracts were centrifuged (5 min, 1,000 × *g*) and the solvent was evaporated under N_2 flow. The pellet was dissolved in 3 mL of hexane and an aliquot (10 µL) of each sample was analyzed in an HPLC (*Shimadzu*, *LC-10A*, Kyoto, Japan), equipped with a precolumn (*Vydac 218GK54,* Guelph, Ontario, Canada) and column (*Vydac 201TP54*, 25 cm × 4.6 mm) C18 reversed phase and attached to a UV-Vis spectrophotometric detector. The samples were eluted in methanol:acetonitrile (90:10, v/v) as the mobile phase at a 1 mL min^{-1} flow. The identification of compounds of interest was made by comparing the retention times of the Car patterns: lutein, zeaxanthin, cis- and trans-β-carotene. The quantification of Car was made using an external standard curve of both lutein (2.5 to 50 µg mL^{-1}, r^2 = 0.99, Y = 7,044 X) and β-carotene (0.01. to 5 µg mL^{-1}, r^2 = 0.99, Y = 1,019 X). The peak areas were calculated to obtain the concentrations of Car, which were expressed as µg g^{-1} (FM) according to Wellburn (1994).

For the total phenolic compounds analysis, samples were extracted in 5 mL of acidified methanol (1% HCl) and incubated in an ice bath for 30 min. The methanol extract (1 mL) was added to 5.0 ml of 95% methanol (v/v) and shaken in a vortex for 30 s. Then, 1 mL of the solution was transferred to tubes with 1.0 mL of 95% ethanol, 5.0 mL of distilled water, and 0.5 mL of *Folin-Ciocalteau* reagent. This solution was shaken in a vortex and left to rest for 5 min. Then, 1 mL of 5% Na_2CO_3 was added to the assay. After 1 h, the absorbance (725 nm) was measured with a UV-Vis spectrophotometer (*U-1800, Hitachi*, Japan). To quantify the total phenolic compounds, a standard curve of gallic acid (50 to 800 µg mL^{-1}, r^2 = 0.99, Y = 0.0001 X) was employed. The results were expressed as µg g^{-1}(FM) according to Randhir *et al.* (2002).

Statistical analysis: The *t*-test was employed to compare data of the standard cultivation condition and UV-B treatments. When the data did not show normality or homoscedasticity, the nonparametric *Kruskal-Wallis* (H) test was performed followed by *Dunn*'s test (Zar 1996). Data were expressed as mean ± standard deviation. The analysis of variance (multifactor *ANOVA*) was followed by the mean comparison test (*Tukey*'s, 5%) for data with normal distribution and homoscedasticity (Zar 1996). Data were analysed by *Excel* and *BioEstat* softwares.

Results

Cell ultrastructure: The gametophytes cultivated under standard conditions during 34 d showed the protonemal cells with the large nucleus, one large vacuole and several small, globular vacuoles. Several elliptical chloroplasts were observed with a few starch grains (Fig. 1*A*). The cell wall showed a microfibrillar texture with microfibrils structured in concentric layers; some microfibrils were dispersed in the internal layer of the cell wall (Fig. 1*B*). Mitochondria with conspicuous cristae and part of chloroplasts were observed in the protonemal cell (Fig. 1*C*). A globular organelle, probably peroxisome without crystalline structures, was observed between the chloroplast and the mitochondria (Fig. 1*D*). The membrane system of the chloroplasts consisted of long, stacked thylakoids that formed the grana (Fig. 1*E*,*H*), and the stroma of some chloroplasts showed dark globular structures, the plastoglobuli (Fig. 1*E*,*F*). The protonemal cells of the gametophytes cultivated under UV-B treatment during 34 d presented a large central vacuole, small vacuoles, and elliptical and dilated chloroplasts with several large starch grains (Fig. 2*A*). Details of the dilated chloroplasts with large starch grains and small plastoglobuli are shown in Fig. 2*C*,*D*. A globular peroxisome was observed beside the chloroplast (Fig. 2*E*). Details of the chloroplasts showing a well organized grana system and small vesicles between grana are shown in Fig. 2*F*.

After 15 d of the cultivation under PAR, the gametophyte presented 25–30 cells and had a spatula shape. In contrast, the gametophytes subjected to PAR+UV-B were smaller, having 18–23 cells, and they were present in early spatula stage. After 34 d of the cultivation, morphological differences were observed between the gametophytes under PAR and PAR+UV-B. The UV-B treated ones showed delayed development and the presence of branching, which was not observed in the control treatment (PAR only). The control gametophytes showed an oval shape and the apical meristem was noted (Fig. 3*A*). These gametophytes presented an organized apical meristem. On the other hand, some gametophytes developed under PAR+UV-B (Fig. 3*B*) showed deformed three-dimensional shapes with multiple lateral branches. These gametophytes did not show an organized apical

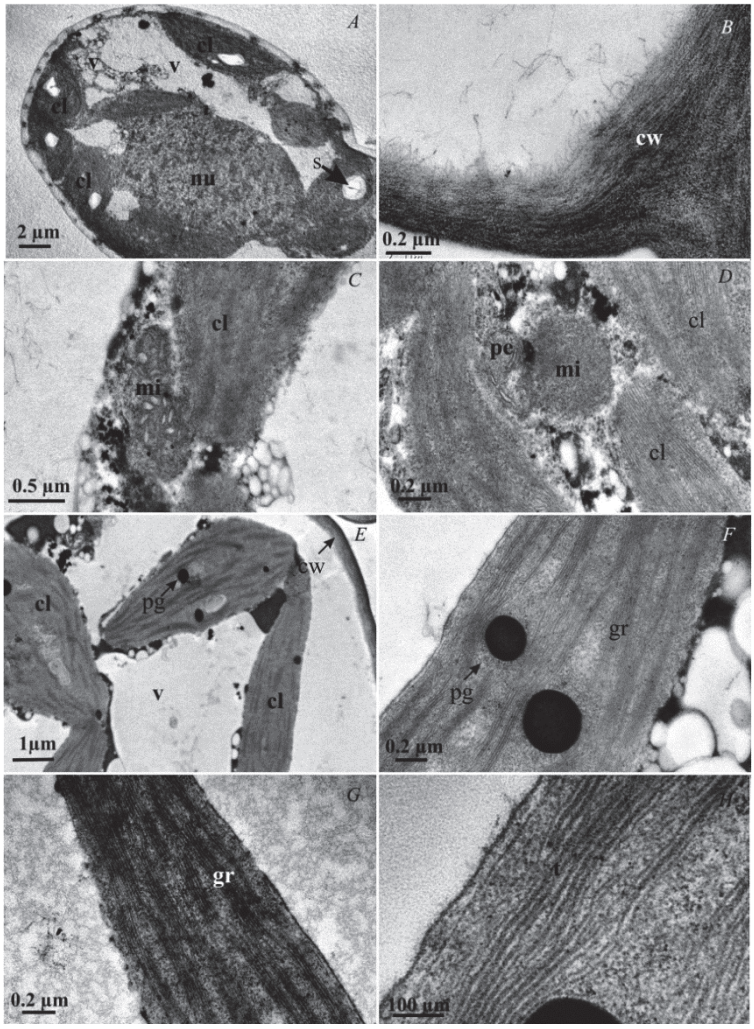

Fig. 1. Transmission electron microscopy (TEM) images of 34-d-old gametophytes of *Acrostichum danaeifolium* Langsd. & Fisch**.** cultivated under PAR. (*A*) Detail of protonemal cell with a large nucleus and several chloroplasts. (*B*) Detail of the cell wall showing microfibril texture. (*C*) Part of a chloroplast and mitochondria showing mitochondrial cristae. (*D*) Globular peroxisome without crystalline structures between mitochondria and chloroplast. (*E*) Chloroplasts with plastoglubuli. (*F*) Detail of chloroplasts with plastoglobuli. (*G*) Detail of chloroplast grana. (*H*) Detail of chloroplast thylakoids. cl – chloroplasts, nu – nucleus, mi – mitochondria, pe – peroxisome, pg – plastoglobule; s – grain starch, v – vacuole.

meristem. They also showed a severe reduction in growth when compared with the control gametophytes cultivated under PAR only.

Chl, Car, and total phenolic compounds: The Chl *a* content was higher in the control gametophytes than in the gametophytes cultivated under UV-B for 34 d. Meanwhile, there was an increase in the Chl *b* content in the gametophytes cultivated under PAR+UV-B. The Chl *a*/*b* ratio was almost two times higher in the control gametophytes than under PAR+UV-B (Table 1). The contents of lutein and zeaxanthin were reduced in the gametophytes irradiated with PAR+UV-B; however, in these gametophytes, an increase in trans-β-carotene concentration was found (Table 2). The concentrations of total phenolic compounds were more than 2-fold higher in the gametophytes cultivated under PAR+UV-B when compared with the gametophytes cultivated under standard conditions (Table 2).

Discussion

Ultrastructural changes in the cell gametophytes, mainly in the chloroplasts of *Acrostichum danaeifolium*, were observed as a result of stress caused by UV-B. Under such conditions, the chloroplasts were dilated and showed several large starch grains and small vesicles. The nuclei, mitochondria, and peroxisomes seemed to be preserved. Similar results were also observed in the chloroplasts of *Gracilaria domingensis* (Kützing) Sonder ex Dickie (Rhodophyta, Gracilariales) exposed to UV (Schmidt *et al.* 2010). However, experiments with UV radiation and four species of red algae showed broken thylakoid membranes of chloroplasts (Poppe *et al.* 2003). In vascular plants, cotyledons of *Chenopodium quinoa* Willd (Amaranthaceae) irradiated with UV-B showed a remarkable increase in the grana thylakoid/stroma ratio and dilatation of spaces between the stroma and thylakoids (Hilal *et al.* 2004). Additionally, the cells of seedlings of *Capsicum annuum* var. *longum* (Bailey)

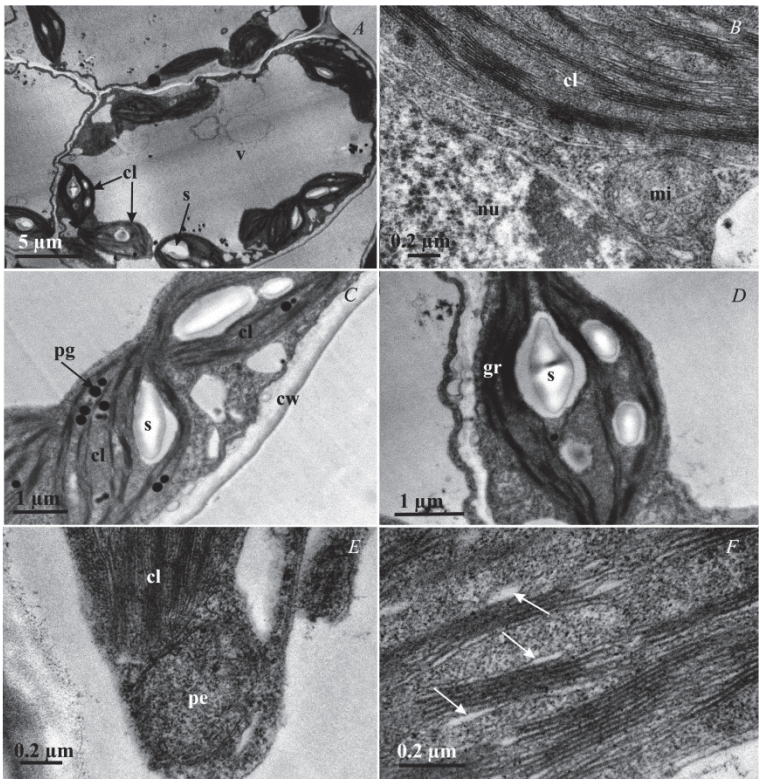

Fig. 2. Transmission electron microscopy (TEM) images of 34-d-old gametophytes of *Acrostichum danaeifolium* Langsd. & Fisch**.** cultivated under PAR+UVB. (*A*) Detail of protonemal cell with a large vacuole and several chloroplasts with starch grains. (*B*) Part of well preserved chloroplast, mitochondria with cristae and nucleus. (*C*) Aspect of preserved nucleus. (*D*) View of a chloroplast with large starch grains. (*E*) Detail of a globular peroxisome. (*F*) Details of chloroplast grana. cl – chloroplasts, nu – nucleus, mi – mitochondria, pe – peroxisome, pg – plastoglobule, s – grain starch, small vesicles (*arrow*).

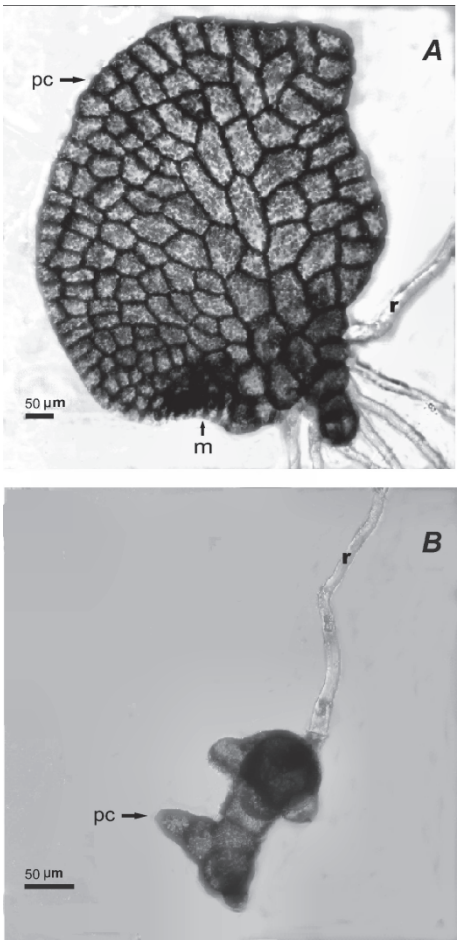

Fig. 3. Light microscopy (LM) images of 34-d-old gametophytes of *Acrostichum danaeifolium* Langsd. & Fisch**.** cultivated under PAR (*A*) and PAR+UV-B (*B*). m – apical meristem, pc – protonemal cell, r – rhizoid.

Table 1. Chl *a* and *b* contents and Chl *a*/*b* ratio from *Acrostichum danaeifolium* Langsd. & Fisch**.** gametophytes cultivated under PAR and PAR+UV-B for 34 d. *Lowercase letters* indicate the groups differentiated by *t*-test at $p = 0.05$. Mean ± SD.

Treatment	Chl *a* [µg g^{-1}(FM)]	Chl *b* [µg g^{-1}(FM)]	Chl *a*/*b* ratio
PAR	213.73 ± 1.10^a	138.98 ± 13.66^a	1.55 ± 0.12^a
PAR+UV-B	132.23 ± 0.78^b	163.7 3 ± 1.21^b	0.81 ± 0.01^b

Table 2. Contents of carotenoids and phenolic compounds from *Acrostichum danaeifolium* Langsd. & Fisch**.** gametophytes cultivated under PAR and PAR + UV-B for 34 d. *Lowercase letters* indicate the groups differentiated by *t*-test at p=0.05. Mean ± SD.

Carotenoid [µg g^{-1}(FM)]	PAR	PAR+UV-B
Lutein	220.50 ± 3.32^a	196. 90 ±1.92^b
Zeaxantin	382.50 ± 0.93^a	378.20 ± 1.27^b
α-carotene	8.30 ± 0.71^a	8.40 ±0.38^a
trans-β-carotene	15.40 ± 6.69^a	23.30 ± 0.25^b
Phenolic compounds	567.00 ± 57.74^a	1,200.00 ± 360.56^b

(Solanaceae) exposed to UV presented dilated thylakoids and showed a decrease of amyloplasts and the formation of catalase crystals in the peroxisomes, indicating stressful conditions (Sarghein *et al.* 2010). The gametophytes of *A. danaeifolium* developed under PAR + UV-B showed morphological modifications expressed as the severe growth reduction and abnormal ramifications. Kato (1964) observed the reversal of protonema polarity, modification of the axis, swelling of rhizoids and protonemal cells, and the presence of filamentous protomenata as the effects of UV in fern gametophytes of *Pteris vittata* (Polypodiopsida, Pteridaceae), *Osmunda japonica* (Polypodiopsida, Osmundaceae), and *Dryopteris* sp. (Polypodiopsida, *Dryopteridceae).*

In the present study, the decline in the content of Chl *a*, the enhancement of the accessory pigment contents in the antenna complex, mainly Chl *b* and trans-β-carotene, and the decrease in the Chl *a/b* ratio were observed in the gametophytes of *A. danaeifolium* exposed to PAR+UV-B for 34 d. We also observed the increase in the content of phenolic compounds in the gametophytes of *A. danaeifolium* exposed to UV-B, which could be related to an adaptation to the stress caused by the radiation. The presence of large starch grains in the chloroplasts could be related to the elevated content of phenolic compounds. Data obtained for crop plants showed that an increase in carbohydrates leads to an increase in the synthesis of carbon-based secondary metabolites, such as phenolic compounds and terpenes (Hamilton *et al*. 2001). Cuadra *et al.* (1997) observed rises in concentrations of total phenolics, flavonoids, and Chl *a* and *b* in plants of *Gnaphalium luteo-album* L. (Asteraceae) exposed to UV-B, but the largest increase was observed in the content of Chl *b*. Phenolic compounds are common protectors against UV-B in higher plants (Temura 1983) and depending on the species, Chl concentrations can increase or decrease in response to this radiation. In *Canavalia ensiformis*, exposure to UV-B caused a reduction in the Chl *a*/*b* ratio, which could be a result of lowered synthesis or intensified degradation of Chl *a* (Choi and Roh 2003). The same results were observed in *Jaborosa magellanica* (Griseb.) Dusen (Solanaceae) and *Prunus dulcis* (Mill.) D.A. Webb (Rosaceae), which showed enhancement in flavonoids and anthocyanins (Cuadra *et al.* 2004, Ranjbarfordoei *et al*. 2009). A study of the UV-B effect carried out with two cultivars of *Phaseolous vulgaris* L. (Fabaceae) showed that Chl contents, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and leaf photosynthesis were not significantly altered by environmental levels of UV-B (Pinto *et al.* 1999). A long UV-B exposure period using two species from boreal peat bogs, *Eriophorum russeolum* Fr. ex Hartm. (Cyperaceae) and *Warnstorfia exannulata* Loeske (Bryophyta, Amblystegiaceae), did not alter the rate of CO_2 assimilation and did not affect the structure of cell organelles (Haapala *et al.* 2010). In *Crotalaria juncea* L. (Fabaceae), additional doses of UV-B reduced significantly the plant growth and photosynthetic pigments, but contents of anthocyanins and flavonoids increased significantly (Balakrishnan *et al.* 2005). *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) seeds exposed to UV-B showed a decrease in leaf area and fresh mass and presented greater leaf thickness and greater concentrations of total Chl and phenolic compounds (Boeger and Poulson 2006). On the other hand, the contents of Chl *a* and *b* slightly declined in *Capsicum longum* (L) (Sarghein *et al.* 2008).

In the present study, we observed that the young gametophytes of *A. danaeifolium,* the fern endemic to the mangrove biome, were sensitive to the increase in UV-B during the beginning of their development. These gametophytes showed ultrastructural changes and a protective mechanism of the photosynthetic apparatus against this radiation, which has also been observed in some algae and several other vascular species.

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