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Original Article

Effects of UV-B radiation on germlings of the red macroalga *Nemalion helminthoides* (Rhodophyta)

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ABSTRACT

Studies have clearly demonstrated the damaging effects of UV-B exposure on macroalgae, but few have reported the impact of UV-B on spore germination and development at juvenile stages. Therefore, this work aimed to analyze the effects of UV-B radiation on germlings of *Nemalion helminthoides* at the tetrasporophytic phase. To accomplish this, germlings of *N. helminthoides* were cultivated in the laboratory and separated into two groups. The control group was exposed onlyto photosynthetic radiation, while the treatment group was exposed to photosynthetic radiation + UV-B for 2 hours during a period of 12 days. Control germlings showed increasing cellular proliferation and accumulation of reserve substances, as well as intense ramification in the last observed stages between 9 days and 12 days of development. Moreover, the chloroplasts presented a typical globular pyrenoid, profusely traversed by thylakoid membranes. Treated germlings, by contrast, showed intracellular damage, such as cell wall thickness, loss of chloroplast organization, changes in mitochondrial cristae, and increasing atrophy of the Golgi bodies. Additionally, changes in developmental patterns were observed, including loss of polarity in the first divisions of carpospores and abnormal stem ramification. The quantification of autofluorescence data coincided with the ultrastructural changes observed in the chloroplasts of cells exposed to UV-B. It can be concluded that exposure to radiation changed the developmental pattern and morphology of the germlings of *N. helminthoides*.

1. Introduction

Increased UV-B radiation on the surface of the Earth has been of great concern worldwide. Several studies have sought to evaluate the effects of UV radiation on living organisms.

Numerous reports studying the results of UV-B exposure on algae have demonstrated negative effects on spore release and germination [1,2], a reduction in growth rate [2–6], photoinhibition of the photosynthetic apparatus [7,8], and other changes. Furthermore, at the juvenile stage, the exposure of spores to UV-B radiation causes more damage than that seen in adult plants, similar to the early developmental stage of several brown algae [7,9–12] and red algae [1,2,5,6,13–16]. Carpospores of *Gracilaria vermiculophylla* exposed to UV radiation [17] show the synthesis and accumulation of photoprotective compounds, such as mycosporine-like amino acids and

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carotenoids, which directly or indirectly absorb UV radiation energy.

The target species of the present study is *Nemalion helminthoides* (Velley in With.) Batters. This species is typically found in the exposed rocks of intertidal zones along temperate coasts worldwide [18], but it is also present in subtropical seas [19]. The species is exposed to UV-B radiation for extended periods during low tide. The thallus is a soft, gelatinous cylinder, unbranched or rarely branched, with blunt apices [20,21].

N. helminthoides is a dioecious species and presents a three-phase life cycle. The species has three morphologically distinct generations, the gametophyte, the carposporophyte growing on the female gametophyte, and the tetrasporophyte. Gametophytes are haploid ($n = 10$) and form gametes. Fertilization occurs in the carpogonial branch, subsequently forming carpospores ($2n = 20$). The carpospore germinates and gives rise to the tetrasporophyte, which is diploid ($n = 20$). Meiosis occurs in the tetrasporangium, resulting in the development of haploid tetraspores ($n = 10$), and these give rise to germinating gametophytes [21,22].

Various patterns of spore germination have been described for Rhodophyta [23–25]. While the order Nemaliales exhibits both the *Nemalion* and *Naccaria* germination patterns, most Nemaliales exhibit the *Nemalion* type of spore germination [26]. After attachment of the spore, this germination pattern is characterized by the production of a protuberance extending to the region where the whole cytoplasmatic content migrates, and this region functions as an apical cell. This apical cell produces a filament that can function as a rhizoid, or the rhizoid can later be produced from the wall of the spore [27]. In red seaweed, the spores are the main units of dispersion. As such, their ability to adhere and settle to the substratum is basic in the distribution and abundance of benthic seaweed populations [28]. Adhesion can act as an indicator of germination, as well as the accompanying morphological and physiological changes of the cells [29]. Other studies have focused on the cytochemical profile and characterization of polysaccharides involved in the adhesion of spore germination [30]. DNA barcoding studies have demonstrated diversity in the order [31].

In this paper, we describe the effects of UV-B radiation on the carpospores and germlings of *N. helminthoides*. In the life cycle of macroalgae, spores are a link between developmental stages. Therefore, it is important to study the effects of UV-B radiation at the spore stage in order to identify possible structural and ultrastructural changes that may result.

2. Materials and methods

2.1. Algal material

Carposporophytic specimens of *N. helminthoides* (Figures 1A and 1B) were collected from Ponta das Canas Beach (27° 23′ 34″ S and 48° 26′ 11″ W), Florianópolis, Santa Catarina, Brazil, during the winter season of 2010 during low tide in the morning. The algal samples were collected from the rocks and transported at ambient temperature in dark containers to the Plant Cell Biology Laboratory (Federal University of Santa Catarina, Florianópolis, Santa Catarina, Brazil).

2.2. Culture conditions

The samples were incubated in Petri dishes with slides on the bottom and containing natural seawater, ± 34 practical salinity units, and enriched with von Stosch medium. These were kept in the dark at 24 °C, awaiting the release of carpospores. Twelve hours after spore release, the branches were removed, and the slides were exposed to illumination from above with fluorescent lights (Philips C-5 Super

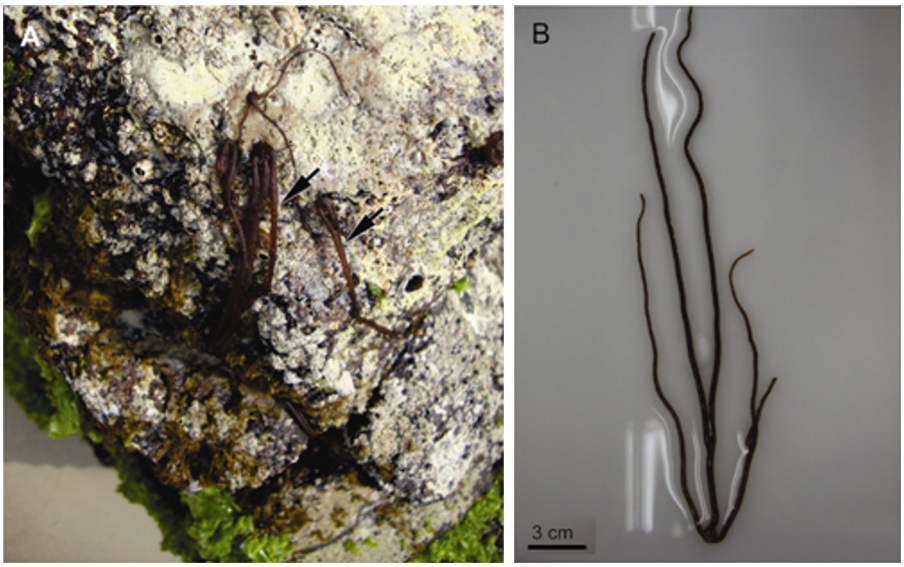

Figure 1. The carposporophytic specimens of *Nemalion helminthoides* (arrows) collected from Ponta das Canas Beach, Florianópolis, Brazil. (A) Note the *N. helminthoides* attached on rock. (B) Detail of *N. helminthoides*.

84 16W/840, Brazil) to recreate photosynthetically active radiation (PAR) at 60 μmol photons m^{-2} s^{-1} (LI-COR light meter 250, USA) with a 12-hour photocycle (starting at 8 hours), as described previously [27].

Two experimental groups were evaluated: (1) control carpospores under PAR only; and (2) exposed carpospores under PAR + UV-B radiation. UV-B radiation was provided through a Vilber Lourmat lamp (VL-6LM; Marne La Vallée, France) with peak output at 312 nm. The intensity of UV-B radiation was 0.35 W/m^2 (Radiometer Model IL 1400A; International Light, Newburyport, MA, USA). To avoid exposure to UV-C radiation, a cellulose diacetate foil with a thickness of 0.075 mm was utilized. Different intensities of UV-B radiation treatment were achieved based on daily measurements under natural environmental conditions.

The samples were treated over the course of 12 days with PAR only and PAR + UV-B radiation. Following 2 hours of exposure to PAR + UV-B radiation beginning at 12:00 hours, samples utilized for transmission electron microscopy (days) were prepared every 3 days (3 days, 6 days, 9 days and 12 days) from three cells and fixed on slides. Medium was changed every 3 days. Four replicates were made for each experimental group.

2.3. Confocal laser scanning microscopy

Algae samples were investigated by confocal laser scanning microscopy (Leica TCS SP-5; Wetzlar, Germany). Control and treated samples were attached on slides and sealed using colorless nail polish. A Leica HCX PLAPO lambda 63x/1.4-0.6 oil immersion objective was fitted on the inverted fluorescent microscope [32]. Chloroplast autofluorescence was observed at 488 nm laser wavelength excitation with emission spectrum from 639 nm to 701 nm [33]. The autofluorescence intensity of chlorophyll *a* in cells of control and treated *N. helminthoides* samples was calculated by measuring two cells per individual ($n = 30$), where the intensity of each pixel was measured using the LAS-AF Lite program (Leica). The LAS-AF Lite program (Leica) was also used for final processing of the confocal images.

2.4. Transmission electron microscopy (TEM

For observation under transmission electron microscopy (TEM), samples were fixed overnight with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose [34]. The material was postfixed with 1% osmium tetroxide for 4 hours, dehydrated in a graded acetone series, and embedded in Spurr's resin. Thin sections were stained with aqueous uranyl acetate followed by lead citrate, as described previously [35]. Four replicates were made for each experimental group; two samples per replication were then examined under TEM at 80 kV (JEM 1011; JEOL, Tokyo, Japan). Similarities based on the comparison of individual treatments with replicates suggested that the ultrastructural analyses were reliable.

2.5. Data analysis

Data were analyzed by unifactorial analysis of variance and Tukey's *a posteriori* test. Statistical analyses were performed using the Statistica software package (release 10.0), considering $p \leq 0.05$ as significant.

3. Results

3.1. Observations under confocal microscopy

The autofluorescent intensity of *N. helminthoides* germlings is shown in Figure 2. During 3 days and 6 days in culture, *N. helminthoides* germlings showed no differences between the control and treated samples (Figures 3A–3D). However, at 9 days and 12 days, the autofluorescent

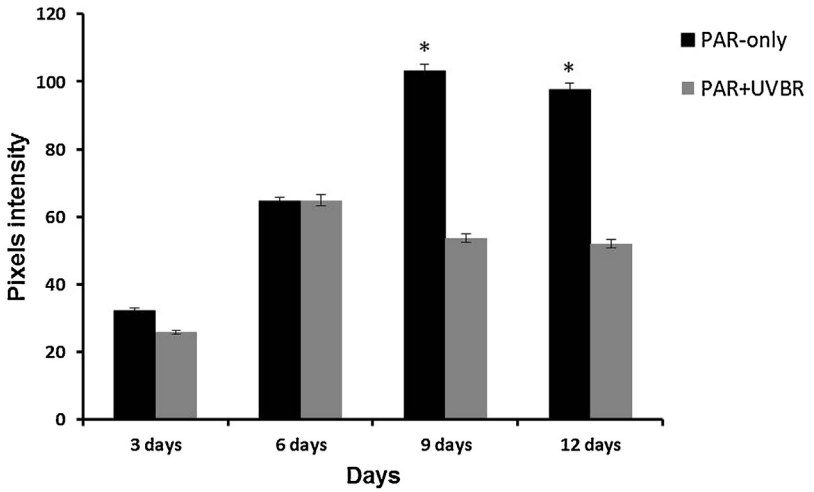

Figure 2. The autofluorescence intensity (pixels per μm^2) of *Nemalion helminthoides* germlings exposed to PAR only and PAR + UV-B radiation 2 hours per day over a period of 12 days.
Data are means of quadruplicates. Mean ± standard deviation, $n = 50$.
* Indicate significant differences according to Tukey's post hoc test $p \leq 0.05$.
PAR = photosynthetically active radiation.

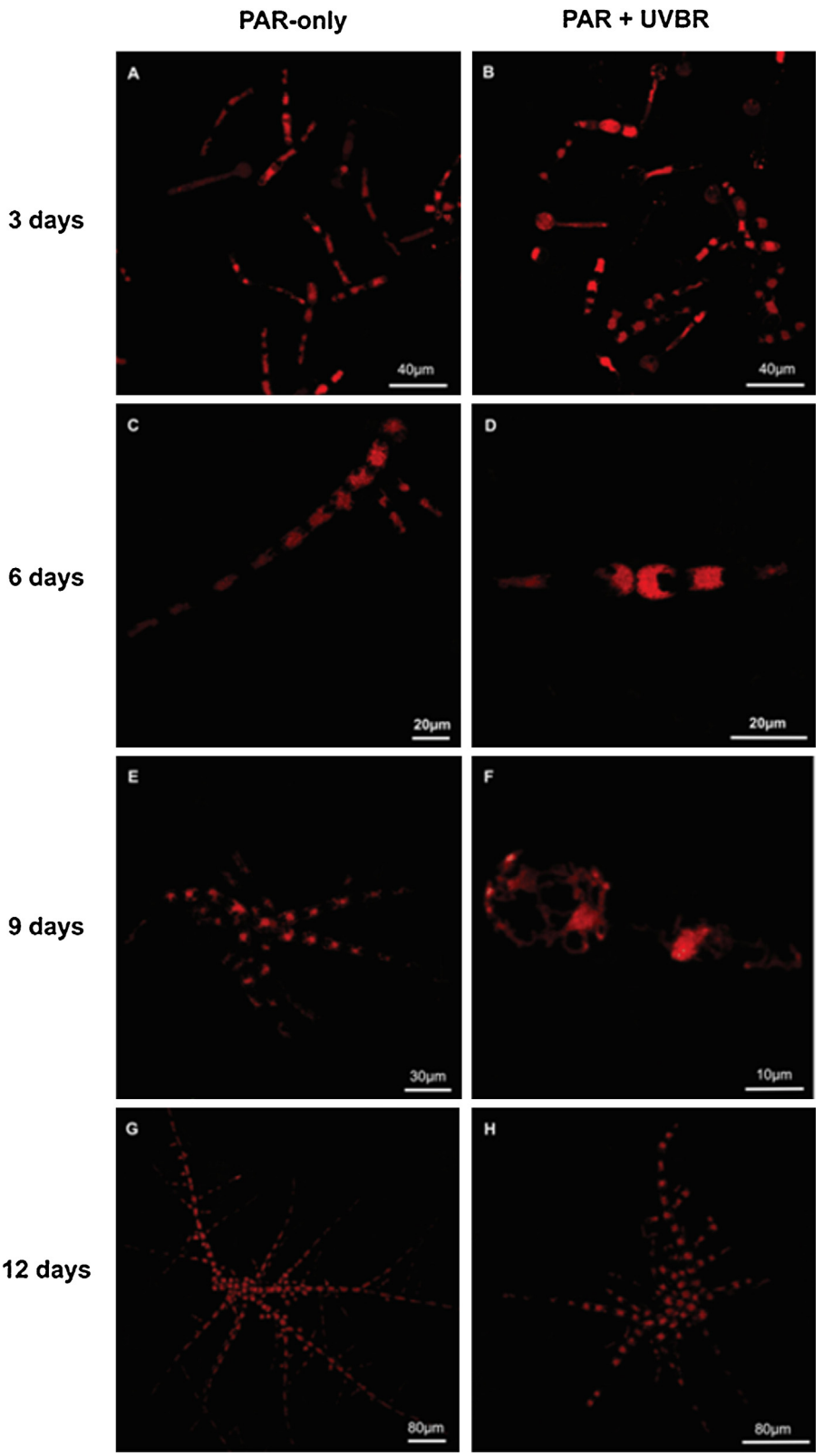

Figure 3. Confocal microscopy of *Nemalion helminthoides* germlings exposed to PAR only and PAR + UV-B radiation 2 hours per day over 12 days. PAR = photosynthetically active radiation.

intensity decreased after exposure to PAR + UV-B radiation (Figures 3E–3H), when compared to control samples.

3.2. Observations under TEM

Control *N. helminthoides* germlings at 3 days and 6 days were observed to be surrounded by a thin cell wall (Figure 4A). These germlings had a single, large, stellate chloroplast with a single pyrenoid (Figures 4A and 4B) and numerous starch grains (Figure 4A). The chloroplast contained parallel thylakoids and numerous plastoglobuli (Figure 4B). At this stage, small mitochondria and hypertrophic Golgi bodies were observed (Figure 4C). The presence of chloroplastidial ribosomes was observed (Figure 4D). The lobes of the chloroplast enclosed the eccentric nucleus with an electron-dense nucleolus (Figure 4E). In addition, cells were joined through pit connections (Figure 4F). The pit plug filled the pit connection with slightly granular, electron-dense material. This plug was covered by two cap layers (Figure 4F) and was composed of protein that filled the channel between the daughter cells, resulting in partial cytokinesis. At 9 days and 12 days (Figures 5A–5F), control *N. helminthoides* germlings showed an increase in starch grains (Figures 5A and 5B). The nucleus showed an electron-dense nucleolus (Figure 5C) and chloroplast with plastoglobuli (Figure 5D).

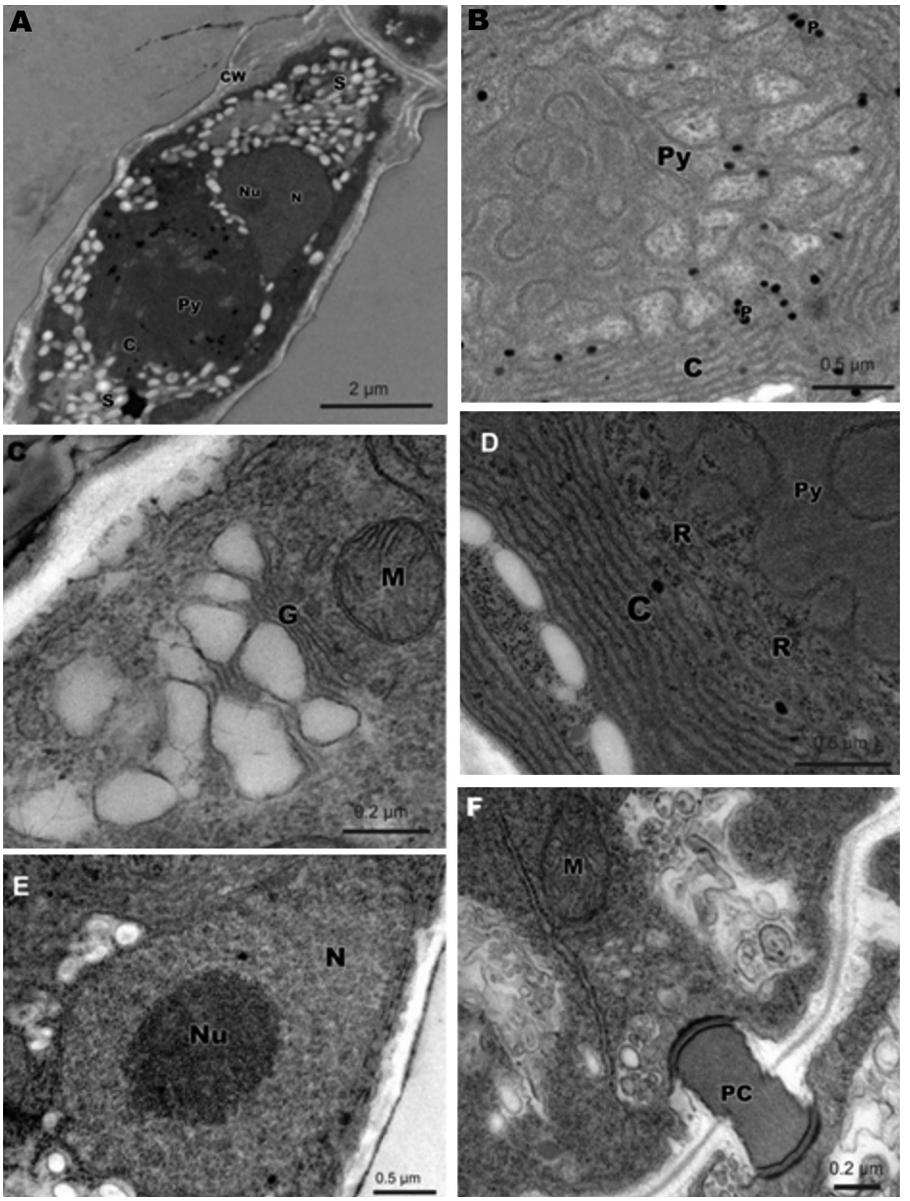

Figure 4. Transmission electron micrographic images of *Nemalion helminthoides* germlings exposed to photosynthetically active radiation only during 3 days and 6 days. (A) Note the cell with stellate chloroplast (C), pyrenoid (Py), starch grains (S), and thin cell wall (CW). Observe the presence of nucleus (N) and nucleolus (Nu). (B) Magnification of previous figure showing detail of pyrenoid and plastoglobuli (P). (C) Hypertrophic Golgi body (G) and mitochondria (M). (D) Note the chloroplastidial ribosomes (R). (E) Nucleus (N) with electron-dense nucleolus (Nu). Note pit connection (PC) between the cells.

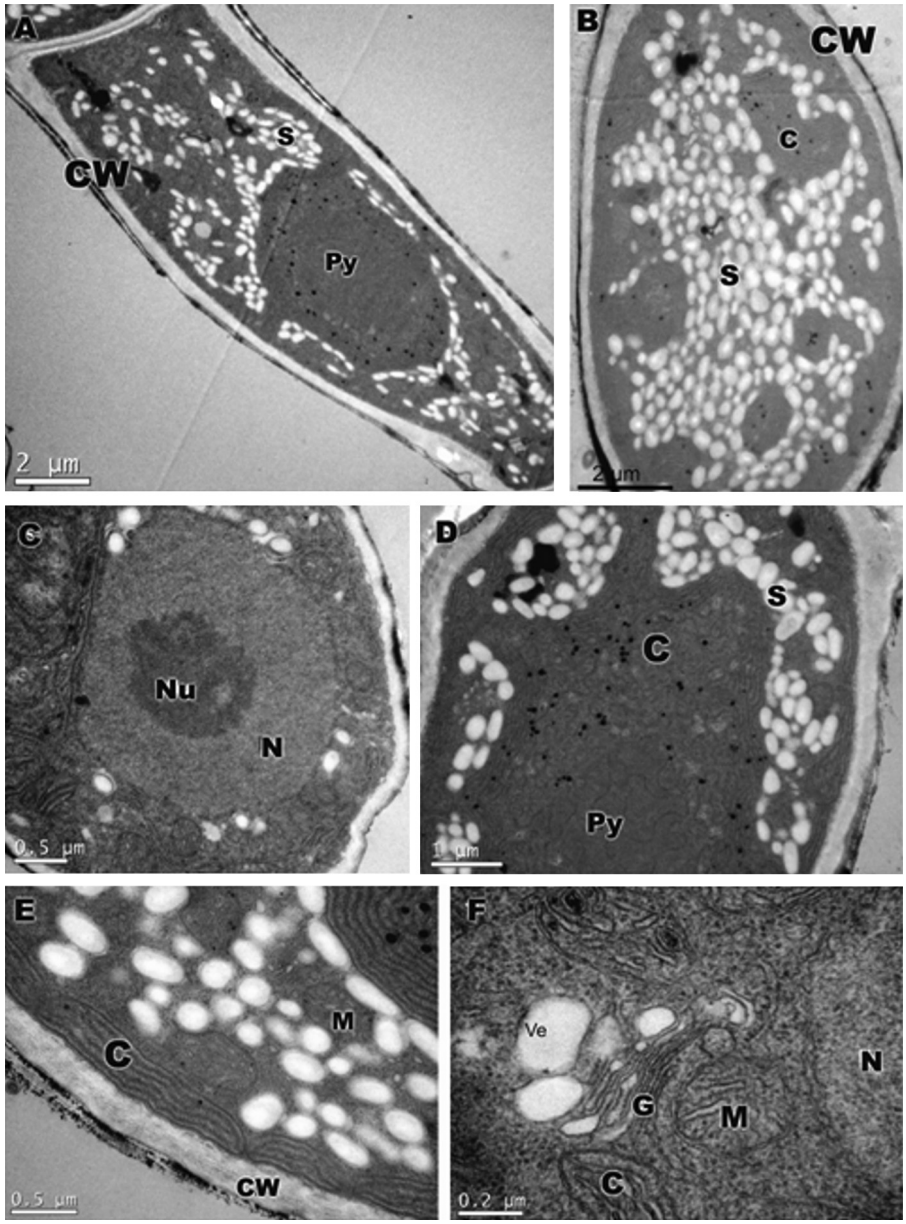

Figure 5. Transmission electron micrographic images of *Nemalion helminthoides* germlings exposed to photosynthetically active radiation only during 9 days and 12 days. (A, B) Observe the large quantity of starch grains (S). (C) Nucleus (N) with electron-dense nucleolus (Nu). (D) Chloroplast (C) with associated starch grains. (E) Chloroplast and mitochondria (M). (F) Note the association of Golgi bodies (G), mitochondria, chloroplast, and nucleus.

Chloroplasts were observed to be associated with mitochondria, nuclei and Golgi bodies (Figures 5E and 5F).

However, after germlings of *N. helminthoides* were exposed to PAR + UV-B radiation for 2 hours per day during a 3-day period, some ultrastructural changes were noted (Figures 6A–6F). Specifically, the treated cells showed an organization similar to that observed in control cells, namely, stellate chloroplast with single pyrenoid (Figures 6B and 6C), together with intact mitochondria and Golgi bodies (Figure 6D). However, the thylakoids appeared to be somewhat dilated (Figures 6E and 6F). More strikingly, after 6 days of exposure to PAR + UV-B radiation, the germlings cells of *N. helminthoides* (Figures 7A–7D) showed an increase in cell wall thickness (Figure 7A) and a large quantity of starch grains (Figures 7A and 7B). The thylakoids were disrupted, and the number of plastoglobuli had increased in the chloroplasts (Figures 7A–7C). The mitochondria appeared swollen (Figure 7D). Finally, after 9 days and 12 days of exposure to PAR + UV-B radiation (Figures 8A–8D), the germling cells of *N. helminthoides* showed a reduced number of starch grains, increased cell wall thickness, and vacuolated cytoplasm (Figures 8A and 8B). The chloroplasts exhibited an irregular, disrupted morphology (Figure 8C). The mitochondria appeared swollen (Figure 8D), and the Golgi bodies were atrophied (Figure 8D).

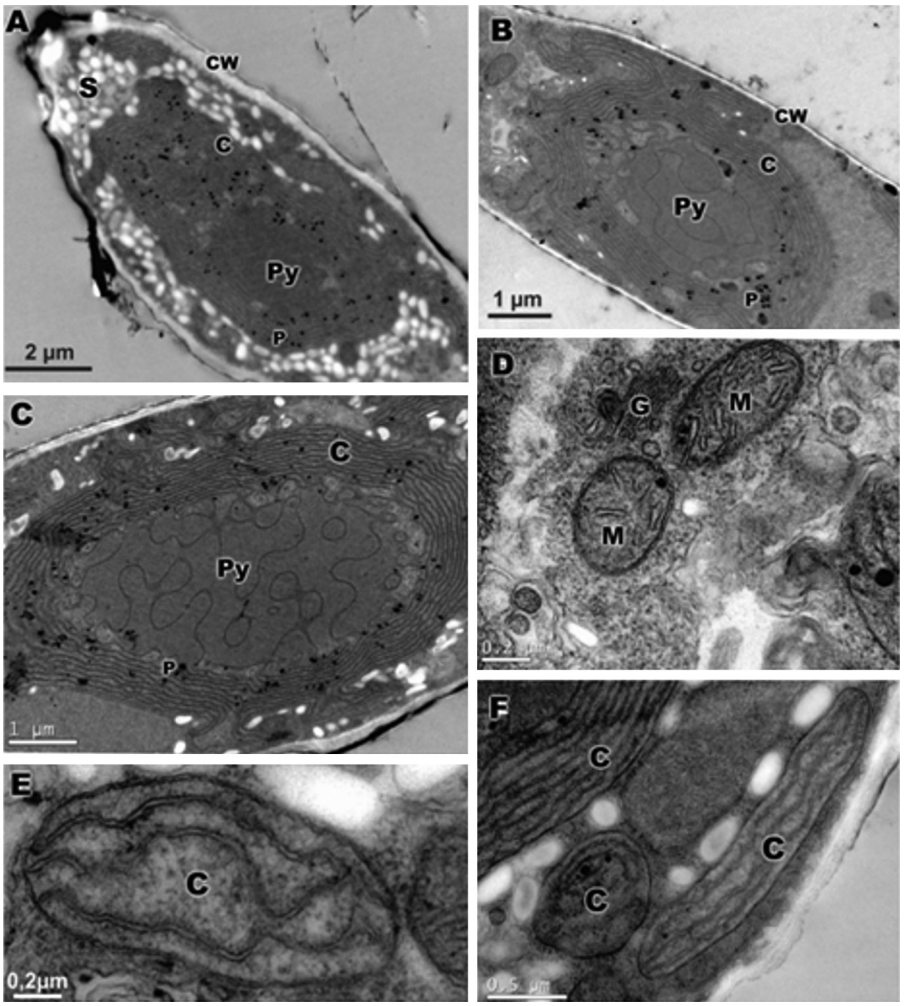

Figure 6. Transmission electron micrographic images of *Nemalion helminthoides* germlings exposed to photosynthetically active radiation + UV-B radiation during 3 days. (A–C) Note the cell with stellate chloroplast (C), pyrenoid (Py), starch grains (S), plastoglobuli (P) and thin cell wall (CW). (D) Note the association of Golgi bodies (G) and mitochondria (M). (E, F) Note the chloroplast with irregular morphology.

4. Discussion

The present study demonstrated that ultrastructure of *N. helminthoides* germlings is affected by exposure to PAR + UV-B radiation during 2 hours per day over the course of 12 days. At the early stage of development, these results showed more sensitivity to UV-B radiation because this period is typically characterized by intense cell proliferation. As observed under TEM, these newly proliferating cells presented alterations in cellular organization and morphology, as well as disruption of chloroplast and mitochondria. Decreased intensity of autofluorescence was also observed under confocal microscopy. Studies on the effects of UV radiation on marine macroalgae have largely been restricted to mature stages of life. However, several reports indicate that young tissue may be more susceptible to these perturbations [5–7,9–13,36,37].

As observed under TEM, the chloroplasts of control *N. helminthoides* germlings showed a typical structure of red algae with a single, large, stellate chloroplast with a single pyrenoid. By contrast, the chloroplasts of samples exposed to UV-B radiation showed significant structural changes, including modification in the size and organization of thylakoids. Other studies with red macroalgae exposed to UV radiation have also reported ultrastructural changes in the chloroplasts with irregular morphology and disruption, including vegetative thallus of *Condracanthus teedei* exposed to UV-B intensity at 1.6 W/m^2 during 7 days, 3 hours per day [8]; thalli of *Porphyra acanthophora* var. *brasiliensis* exposed to UV-B intensity at 0.35 W/m^2 during 21 days, 3 hours per day [32]; vegetative thallus of *Laurencia catarinenses* and *Palisada flagellifera* treated with UV-B intensity at 0.35 W/m^2 during 7 days, 3 hour per day [38]; carpospores of *Iridaea cordata* exposed to three different intensities of UV-B (0.17 W/m^2, 0.50 W/m^2, and 0.83 W/m^2) during 3 days, 3 hour per day [15]; and tetraspores and early development of *Gelidium floridanum* exposed to UV-B intensity at 0.12 W/m^2 during 15 days, 2 hour per day [2]. Changes in chloroplast morphology led to reduction in photosynthetic activity [39,40].

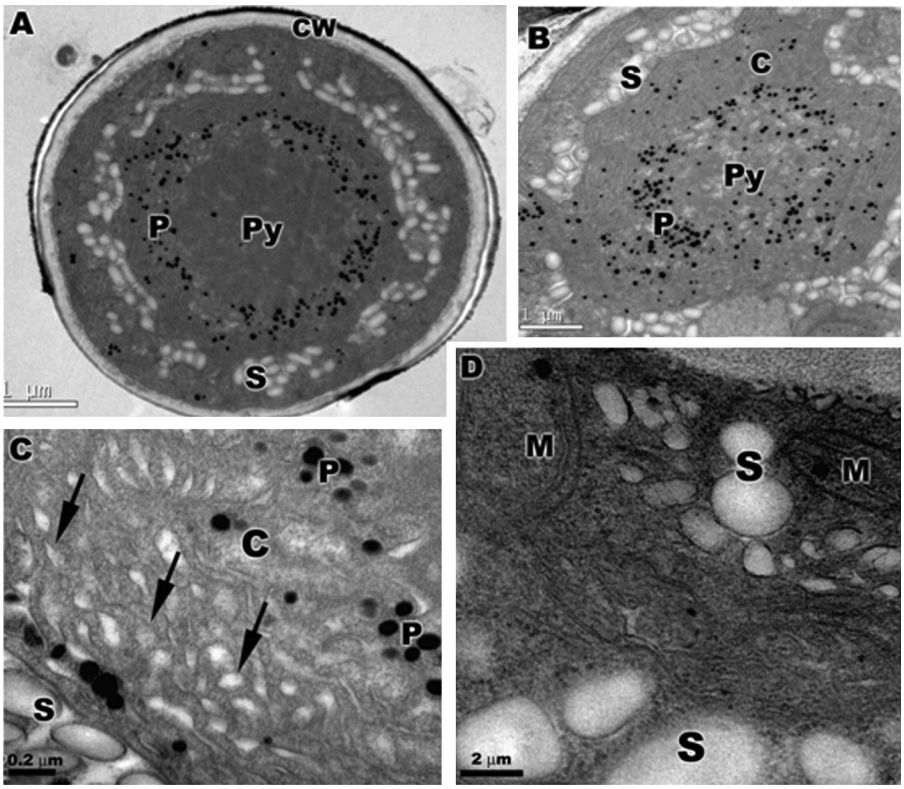

Figure 7. Transmission electron micrographic images of *Nemalion helminthoides* germlings exposed to photosynthetically active radiation + UV-B radiation during 6 days. (A, B) Observe the cell wall thickness (CW) and increase in plastoglobuli volume (P). Note the presence of pyrenoid (Py). (C) Magnification showed thylakoid disruption (arrows). Note the swollen mitochondria (M).

The cells of *N. helminthoides* present a central pyrenoid, an evolutionarily primitive structure. This structure is surrounded by the chloroplast, and it functions as a reserve for proteins and enzymes involved in photosynthesis, including RuBisCO (ribulose-1,5-bisphosphate carboxylase oxygenase) [41,42]. After the 6th day of UV-B radiation exposure, the pyrenoid begins to break down, probably as a result of thylakoid destabilization. This dramatic change may be associated with low photosynthetic efficiency since it is directly linked to the CO_2 assimilation and photorespiratory pathways [43,44].

Apart from morphological changes in chloroplasts and pyrenoid, confocal microscopy revealed a significant reduction in the autofluorescence of chlorophyll *a* from the 9th day of exposure to UV-B radiation. Although this is a new technique, it represents a scientific breakthrough in order to allow analysis of the photosynthetic activity within individual chloroplasts in intact and living cells [45].

Even though many studies show that UV-B radiation has a negative impact on algae photosynthesis [5,7,9,46], other studies show that other factors are associated with exposure to UV-B radiation, and that these factors can lead to different physiological responses of algae to stress. For example, Gordillo et al [47] observed that two algal species had a higher growth rate and increased rate of photosynthesis after exposure to UV-B radiation with an increase of CO_2 to the environment. Moreover, in a study of the red algae *Gracilariopsis tenuifrons*, the best physiological stress responses were obtained from algae exposed to UV-B radiation concurrent with growth medium supplemented with nitrogen [48].

The electron-dense lipid droplets described in the chloroplast of *N. helminthoides* tetrasporophytes are plastoglobuli and are interpreted as lipid material with a reserve role. In the present study, samples exposed to PAR + UV-B radiation, as analyzed by TEM, revealed an increase in the number of plastoglobuli in the chloroplast. When algae are subjected to stress, nitrogen limitation and lipid synthesis are observed [12,49]. These phenomena occur because the pathways to form protein-containing cell structures are suppressed. Studies show a relationship between chloroplasts and plastoglobuli, indicating that maximal plastoglobuli enrichment was strongly correlated with minimal thylakoid development [50,51]. Similar results were reported with the formation of plastoglobuli in vegetative thallus of *Kappaphycus alvarezii* [34] after exposure to UV-B radiation, and in juvenile gametophytes of *Laminaria digitata* and *Saccharina latissima* exposed to UV radiation [50]. This increase in the number of lipids can be considered as a change in metabolism, which, in turn, results in the reduction of cell proliferation. The mitochondria of treated *N. helminthoides* germlings also showed ultrastructural changes. The mitochondria appeared swollen, and the inner mitochondrial membrane was organized in sacculi. Other studies report ultrastructural changes in the mitochondria of *Palmaria palmata* and

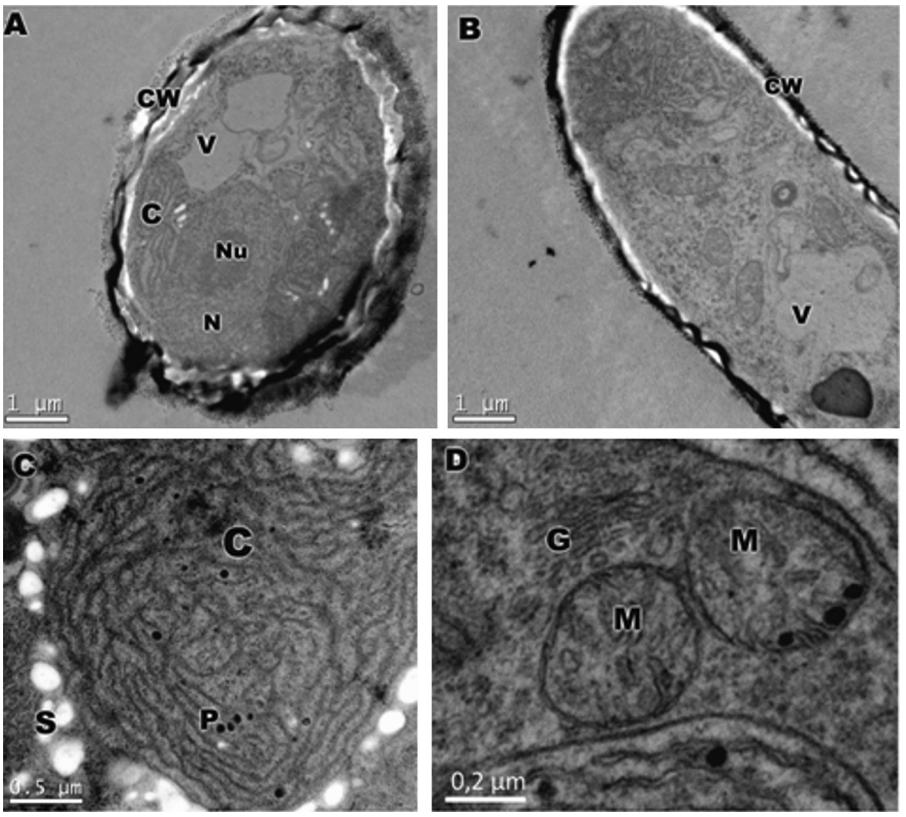

Figure 8. Transmission electron micrographic images of *Nemalion helminthoides* germlings exposed to photosynthetically active radiation + UV-B radiation during 9 days and 12 days. (A, B) Note the cell wall thickness (CW) and formation of small vacuole (V). Intact nucleus (N) and nucleolus (Nu) (C) Irregular thylakoid membranes. (D) Swollen mitochondria (M) and atrophied Golgi bodies (G).

Palmaria decipiens. In *P. palmata*, these changes were manifested by an apparent swelling and by changes in the inner mitochondrial membrane from a tubuli- to sacculi-type structure when exposed to UV-A + UV-B radiation [52]. Changes in mitochondria also alter the metabolism of algae, reaching cellular respiration and energy supply, as well as other metabolic pathways [53], leaving, in turn, a much-weakened alga. In our study, we noted that UV-B radiation caused cellular damage to *N. helminthoides* at the germling stage, in particular, damage to the intracellular architecture. Additionally, changes in developmental patterns were observed, including loss of polarity in the first divisions of carpospores and abnormal stem ramification, indicating that the initial stages of development were more sensitive to UV-B radiation.

Conflicts of interest

The authors declare that they have no conflict of interest.

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