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Responses of the macroalgae *Hypnea musciformis* after *in vitro* exposure to UV-B

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ABSTRACT

The *in vitro* effects of UVBR were investigated in apical segments of *Hypnea musciformis*. The plants were cultivated and exposed to photosynthetically active radiation (PAR) at 80 μmol photons m^{-2} s^{-1} and PAR + UVBR at 1.6 W m^{-2} at 3 h per day for 7 days. Toluidine Blue reaction showed metachromatic granulations in vacuole, and Periodic Acid Schiff stain showed a decrease in the number of floridean starch grains. UVBR also caused changes in the ultrastructure of cortical cells, which included increased thickness of the cell wall, reduced intracellular spaces, changes in the cell contour, destruction of chloroplast internal organization, and rough endoplasmic reticula increase. The algae cultivated under PAR-only showed growth rates of 9.7% day^{-1}, while algae exposed to PAR + UVBR grew only 3.2% day^{-1}. Furthermore, compared with control algae, phycobiliprotein contents (phycoerythrin, phycocyanin, and allophycocyanin) were observed to decrease after PAR + UVBR. However, chlorophyll *a* levels were not significantly different (ANOVA, $P = 0.52$) after exposure to PAR + UVBR. As a photoprotective adaptation strategy against UVB damage, an increase of 58.9% phenolic compounds and 3.6% of carotenoids was observed. Overall, these results lead to the conclusion that both ultrastructural damage and observable changes in metabolism occurred in *H. musciformis* after only 3 h of daily UVB exposure over a 7-d experimental period.

1. Introduction

The stratospheric ozone layer provides natural protection against ultraviolet radiation (UVR) exposure for all biological organisms (Madronich, 1992). It has been nearly three decades since the first reports about man-made changes in this protective barrier, which resulted from atmospheric pollutants, such as chlorofluorocarbons (CFCs), halocarbons, carbon dioxide (CO_2), and methyl chloroform (MCF). As a consequence of ozone layer depletion, ultraviolet B radiation (UVBR) (280–320 nm) is increasingly reaching the earth's surface (Mitchell et al., 1992; Hanelt and Roleda, 2009). UV energy induces photodamage in proteins, nucleic acids, and other compounds in biological tissues (Mitchell et al., 1992), as well as damage to cellular ultrastructure (Schmidt et al., 2009). Ultraviolet radiation affects all biological organisms, especially those in the aquatic ecosystem, provoking, for example, changes in macroalgae growth rates (Schmidt et al., 2009, 2010a,b).

One of the strategies used by macroalgae to survive when exposed to high levels of UVR is the synthesis and accumulation of photoprotective compounds, such as mycosporine-like amino acids (MAAs) and carotenoids, which directly or indirectly absorb UVR energy (Karsten and Wiencke, 1999). The phenolic compounds are also involved in protecting the thallus against direct exposure to solar light radiation, especially UVR, as observed in the brown alga *Ascophyllum nodosum* (Pavia et al., 1997). Several studies suggest that changes have occurred in the concentrations of chlorophyll *a* in *Mastocarpus stellatus* and *Chondrus crispus* (Roleda et al., 2004), as well as *Kappaphycus alvarezii* (Doty) Doty ex P. Silva (Schmidt et al., 2010a,b). Phycobiliprotein content has also been altered, as demonstrated in studies by Eswaran et al. (2001) and Schmidt et al. (2010a,b) reporting on *K. alvarezii*.

Changes in the ultrastructure of macroalgae exposed to UVBR have been reported in many studies. Some papers reported changes

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in carragenophytes subjected to UVBR, such as *K. alvarezii* (Schmidt et al., 2009, 2010a,b). These changes mainly occur in the chloroplasts, modifying the quantity, size, organization, as well as the number of thylakoids (Schmidt et al., 2009).

Hypnea is a source of *kappa* carrageenan and phycocolloids throughout the world, presenting significant economic importance (Reis et al., 2008). Among the many macroalgae found in the coastal systems, *Hypnea* J.V. Lamouroux is the biomarker of most probable consequence, owing to its worldwide distribution in the Atlantic, Indian and Pacific Oceans. *Hypnea musciformis* (Wulfen) J.V. Lamouroux is the best-known species in the genus *Hypnea* and has been reported to occur in many tropical and subtropical shores. This alga is known as a valuable resource for the production of *kappa* carrageenan (Reis et al., 2008). In Brazil, it is widespread along the Brazilian coast.

Despite its importance to ecology and the economy, the red macroalga *H. musciformis* has not been studied in the context of UVBR exposure. Thus, in this study, we investigated the *in vitro* effect of UVBR on this species, and we raise the following questions: (I) Do changes in cellular architecture and ultrastructure between the PAR-only (control samples) and PAR UVBR (treated plants) relate to *H. musciformis* UVRB sensitivity? (II) Is there a difference in the content of photosynthetic pigments, carotenoids and phenolic compounds and mitochondrial function after exposure to ultraviolet radiation-B?

2. Materials and methods

2.1. Algal material

H. musciformis samples were collected from Ponta das Canas Beach (27° 23′ 34″S and 48° 26′11″W) in February 2010 during the summer season. This species occurs in rocky intertidal beaches and is frequently epiphytic in *Sargassum cymosum*. The algal samples were collected from the rocks and transported at ambient temperature in dark containers to LAMAR-UFSC (Macroalgae Laboratory, Federal University of Santa Catarina, Florianopolis, Santa Catarina, Brazil). At noon on sunny days during the summertime, this region receives natural solar irradiation varying from 2.2 W m^{-2} to 3.5 W m^{-2} based on a daily UVB index that varies from 9 to 14 during a typical summer season.

To avoid contamination by the presence of epiphytes, the collected algae were meticulously cleaned with a brush and filtered seawater. The apical portions were maintained by immersing in seawater enriched with von Stosch medium. These segments were cultivated under the same laboratory conditions (detailed below) during 14 days (experimental acclimation period) before their utilization in the UVBR experiments.

2.2. Culture conditions

The apical thalli portions were selected (±1.0 g) from the *H. musciformis* samples and cultivated for 7 days in beakers with 500 mL natural sterilized seawater enriched with von Stosch medium at half strength (VSES/2) with ±34 practical salinity units (p.s.u.).

Culture room conditions were 24 °C, continuous aeration, illumination from above with fluorescent lights (Philips C-5 Super 84 16 W/840, Brazil), photosynthetically active radiation (PAR) at 60 μmol photons m^{-2} s^{-1} (Li-cor light meter 250,USA) and 12 h photocycle (starting at 8 h). UVBR was provided through a Vilber Lourmat lamp (VL-6LM, Marne La Vallée, France) with peak output at 312 nm. The intensity of UVB radiation was 1.6 W m^{-2} (Radiometer Model IL 1400A, International Light, Newburyport, MA, USA), and plants were exposed to PAR + UVBR from 12:30 to 15:30. To avoid exposure to UVC radiation, a cellulose diacetate foil having a thickness of 0.075 mm was utilized.

Apical thalli controls were evaluated using PAR-only, while exposed apical thalli were cultivated under PAR + UVBR. Samples for light and electron microscopy were fixed directly on day 7, the last day of experimentation, after the final exposure to UVB at 15:30 h. Twelve replicates were made for each experimental group.

2.3. Light microscope (LM)

Samples approximately 5 mm in length were fixed in 2.5% paraformaldehyde in 0.1 M (pH 7.2) phosphate buffer overnight. Subsequently, the samples were dehydrated in increasing series of ethanol aqueous solutions. After dehydration, the samples were infiltrated with Historesin (Leica Historesin, Heidelberg, Germany). Sections of 5 μm in length were stained with different cytochemical techniques and investigated with an Epifluorescent (Olympus BX 41) microscope equipped with Image Q Capture Pro 5.1 Software (Qimaging Corporation, Austin, TX, USA).

2.4. Cytochemical staining

LM sections were stained as follows: Periodic Acid-Schiff (PAS) used to identify neutral polysaccharides (Schmidt et al., 2009), Toluidine Blue (TB-O) 0.5%, pH 3.0 (Merck Darmstadt, Germany) used for acid polysaccharides through a metachromatic reaction (Schmidt et al., 2009), and Coomassie Brilliant Blue (CBB) 0.02% in Clarke's solution (Serva, Heidelberg, Germany) used for proteins (Schmidt et al., 2009,2010c). Controls consisted of applying solutions to sections without the staining component (*e.g.*, omission of periodic acid application in the PAS reaction). In order to reveal the floridean starch grains of polysaccharides, ultra-thin sections were treated with periodic acid and thiosemicarbazide silver proteinate (PA-TSC-SP) 1% (Electron Microscopy Sciences, Hatfield, PA, USA) according to Schmidt et al. (2009).

2.5. Confocal laser scanning microscopy (CLSM)

Algae samples were investigated by a laser scanning confocal microscope (Leica TCS SP-5, Wetzlar, Germany) and an Argon laser using 440, 488 and 514 nm excitation. A Leica HCX PLAPO lambda 63×/1.4–0.6 oil immersion objective was fitted on the inverted fluorescent microscope. The autofluorescence of the chlorophyll was used for visualization of the chloroplast structure. The LAS-AF Lite program (Leica) was also used for final processing of the confocal images.

2.6. Transmission electron microscope (TEM)

For observation under the transmission electron microscope (TEM), samples approximately 5 mm in length were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose overnight. The material was post-fixed with 1% osmium tetroxide for four 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. Four replicates were made for each experimental group; two samples per replication were then examined under TEM JEM 1011 (JEOL Ltd., Tokyo, Japan, at 80 kV). Similarities based on the comparison of individual treatments with replicates suggested that the ultrastructural analyses were reliable.

2.7. Growth rates (GRs)

Growth rates for treatment groups and control were calculated using the following equation: $GR\ [\%\ day^{-1}] = [(W_t/W_i) - 1] \times 100/t$,