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Pyropia acanthophora var. *brasiliensis* E. C. Oliveira and Coll (Rhodophyta: Bangiales) cultivated in seawater under laboratory conditions favors the production of economically important secondary metabolites

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Abstract. *Pyropia* represents most domesticated seaweed in the world. Therefore, we aimed to determine if cultivation of the red macroalga *Pyropia acanthophora* E. C. Oliveira and Coll (Rhodophyta: Bangiales) under laboratory conditions would affect the presence and concentration of secondary metabolites. To accomplish this, experiments were performed with ambient sample and acclimated sample (laboratory conditions). The conditions of the culture room were as follows: 24 °C, salinity of 35‰, constant aeration, irradiance of 80 µmol.photons.m^{-2}.s^{-1} and photoperiod of 12 h, for 7 days. Ambient sample showed lower concentrations of allophycocyanin and phycoerythrin when compared to the acclimated sample, but phycocyanin concentration was higher in the ambient sample. Carotenoids showed higher concentrations in the acclimated sample when compared to the ambient sample. Total phenolics were insignificant, while total flavonoids were higher in the ambient sample. No pattern in the production of these secondary metabolites could be identified. On the other hand, the acclimated samples showed a greater inhibition of the free radical DPPH, indicating a higher antioxidant activity. Acclimatization under laboratory conditions, in which *P. acanthophora* is submerged in seawater, favors the production of economically important secondary metabolites as a result of submersion stress and changes in cultivation patterns, such as irradiance and photoperiod.

Keywords: Carotenoids; Flavonoids; Phenolics; Phycobiliproteins.

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Introduction

Pyropia is the most domesticated seaweed in the world (Schweikert et al., 2011). It is known as Nori, the seaweed of sushi, and used in food all over the world (Zhang et al., 2003; Zhou et al., 2012). In the environment, it is located in the interdial zone where it is submerged during high tide and exposed at low tide (Chan et al., 2012).

The main secondary antioxidant metabolites present in red macroalgae are carotenoids, phenolics and flavonoids (Aple and Hirt, 2004; Ganesan et al., 2008; Borges et al., 2011). In addition, phycobiliproteins, photosynthetic pigments, also have an antioxidant function (Chamorro et al., 2002; Romay et al., 2003), as they absorb radiation (Munier et al., 2014), acting as a protector of chlorophyll *a*, the principal pigment related to photosynthesis.

Phycobiliproteins, allophycocyanin (APC), phycocyanin (PC) and phycoerythrin (PE), are organized into a complex antenna on the chloroplast thylakoid membrane where APC is at the center above chlorophyll, followed by PC and PE at the terminal (Parmar et al., 2013).

Carotenoids have two cellular functions. The first is the pigment function, acting to absorb light for photosynthesis (Christaki et al., 2012), and the second is photoprotection, eliminating reactive oxygen species (ROS) (Uenojo et al., 2007; Ramlov, 2010). Carotenoids are used as food additives in the form of dyes and vitamins and also in the cosmetics and pharmaceutical industries because of their protection against free radicals (Fré et al., 2014).

Flavonoids are the largest class within phenolics, and they act in defense against solar radiation, especially ultraviolet radiation, and against aggression by pathogens, predators or parasites (Dai and Mumper, 2010). Phenols and flavonoids have antioxidant properties because they are reducing agents, hydrogen donors, and singlet oxygen suppressors (Rice-Evans et al., 1995; Papp et al., 2004). In addition, they also act as a metal chelator (Rice-Evans et al., 1995; Papp et al., 2004). Flavonoids are compounds that can be used for their antimicrobial (Seenivasan et al., 2012) and antitumor activities (Lavakumar et al., 2012). Phenolic compounds provide antiproliferation (Murugan et al., 2014) and anti-inflammatory activity (Guimarães, 2014).

This work aims to analyze the presence and concentration of secondary metabolites of the red macroalga *Pyropia acanthophora var. brasiliensis* E. C. Oliveira and Coll (Rhodophyta: Bangiales) cultivated under laboratory conditions to determine any resultant changes in the quantity of these metabolites.

Materials and methods

Collection and processing of biological material

The gametophytes of *P. acanthophora var. brasiliensis* were collected in the upper portion of the interdial zone of the rocky shore of Sambaqui Beach, Florianópolis, Santa Catarina State, Brazil, in August and September 2015. The environmental

conditions of the water were 19 °C, salinity 35‰, and photoperiod of 10 h clear and 14 h dark. Algal thalli were transported to the Laboratory of Plant Cell Biology, which is located at the Federal University of Santa Catarina (Florianópolis), in plastic containers containing seawater. In the laboratory, thalli were washed with filtered and sterilized seawater and screened for the removal of epiphytes.

Some healthy portions were stored in liquid nitrogen for extraction of the secondary metabolites; these were denominated ambient samples. Other healthy portions were cultivated in the laboratory (n = 4) with sterilized seawater from the collection site, plus von Stosch's medium (100%, Edwards 1972), for 7 days under controlled conditions; these were denominated acclimated samples.

The conditions of the culture room were as follows: temperature of 24 °C ± 2 °C, salinity of 35‰, constant aeration, irradiance of 80 ± 10 μmol.photons.m^{-2}.s^{-1} (florescent lamps, Philips C-5 Super 84 16 W/840, Brazil), and photoperiod of 12 h. At the beginning of the fourth day of experiment, seawater was changed.

Phycobiliproteins

The extraction of phycobiliproteins was accomplished through the grinding of 0.5 g (fresh mass) in liquid nitrogen. The macerate was suspended in 3 mL of 50 mM phosphate buffer, pH 5.5 and 4 °C. The solution was centrifuged for 20 min (4,000 rpm at 4 °C), and the supernatant was removed and kept in the dark until reading at 498, 615 and 651 nm in a microplate reader (Infinite M200, Tecan Multileiter). The formulas described by Kursar et al. (1983) were used. The analyses were performed with n = 4, and the results were expressed in μg of pigments per g of dry mass.

Extraction and dosing of total carotenoids

Samples of 0.5 g (fresh mass) were ground in liquid nitrogen, macerated in 10 mL of pure methanol, and kept at rest (1 h) in a darkroom. Then, the reading was carried out at 450 nm in a microplate reader (Aman et al., 2005). The quantification of the total carotenoids was made from the standard curve of β-carotene (6.25 to 50 μg.mL^{-1} – r^2 = 0.99; y = 1.624x). The analyses were performed with n = 4, and the results were expressed in μg per g of dry mass.

Extraction and dosing of total phenolics

Samples of 0.5 g (fresh mass) were ground in liquid nitrogen, macerated in 10 mL of 80% methanol, and kept at rest (1 h) in a darkroom. The analysis was performed using the Folin-Ciocalteau spectrophotometric method based on Waterman and Mole (1994). 50 μL aliquots from the supernatant of the extracts were added to a concentrated solution of 180 μL of distilled water, 10 μL of the Folin-Ciocalteau (Vetec) reagent and 30 μL of 20% sodium carbonate (Na_2CO_3, w/v) and incubated for 1 h at room temperature. Thereafter, the reading was carried out at 750 nm on a microplate reader. The quantification of the total phenolic compounds was done from the standard curve of gallic acid (1 to 50 μg.mL^{-1} - r^2 = 0.99; y = 0.0004x). The analyses were performed with n = 4, and the results were expressed in μg of gallic acid per g of dry mass.

Extraction and dosing of total flavonoids

The extract was prepared as described above for total phenolics. An aliquot of 0.5 mL of each extract was used to determine the total flavonoid content in the presence of 0.5 mL of aluminum chloride solution (2% in

methanol) and 2.5 mL of ethanol, according to Zacarias et al. (2007), with modifications. After standing for 1 h, the reading was carried out at 420 nm on a microplate reader. The quantification of flavonoids was made from the standard quercetin curve (1 to 20 $\mu g.mL^{-1}$ - $r^2 = 0.99$; $y = 0.009x$). The analyses were performed with n = 4, and the results were expressed in µg of quercetin per g of dry mass.

Inhibition of Radical DPPH (1,1-diphenyl-2-picrylhydrazyl)

An aliquot of 250 µL of DPPH (Sigma, 0.1 mM in 80% methanol) methanolic solution was added to 50 µL of the extract prepared as described above for total phenolics. The decrease in absorbance at 530 nm was measured on a microplate reader after 30 min incubation of the sample, according to Kim et al. (2002). The analyses were performed with n = 4, and the results were expressed as porcentage of free radical sequestration DPPH.

Statistical analysis

The data were submitted to the *t*-test using Statistica v. 10.0 software, considering the significance of the test at 5%.

Results and discussion

Secondary metabolites are compounds that have no direct function on the growth and development of organisms, but they do play a key role in their interaction with the environment (Verpoorte, 2000). Most secondary metabolites acting as antioxidants and scavengers of free radicals calculated as porcentage inhibition were verified in the acclimated samples. In nature, *P. acanthophora var. brasiliensis* occurs in the interdial zone and is submerged only during high tides. However, under laboratory conditions, the alga is commonly submerged 24 h a day, which can be stressful, resulting in an imbalance in metabolite production and causing, in turn, the formation of ROS such that the production of secondary metabolites serves to repair damage caused by stress.

The thalli of ambient samples is greenish and about 16 cm wide (Figure 1a), while the acclimated thalli is fragmented and presents a brownish coloration (Figure 1b), indicating that the stem is forming fertile cells, a possible form of dispersion of the species in an unfavorable environment, such as submersion.

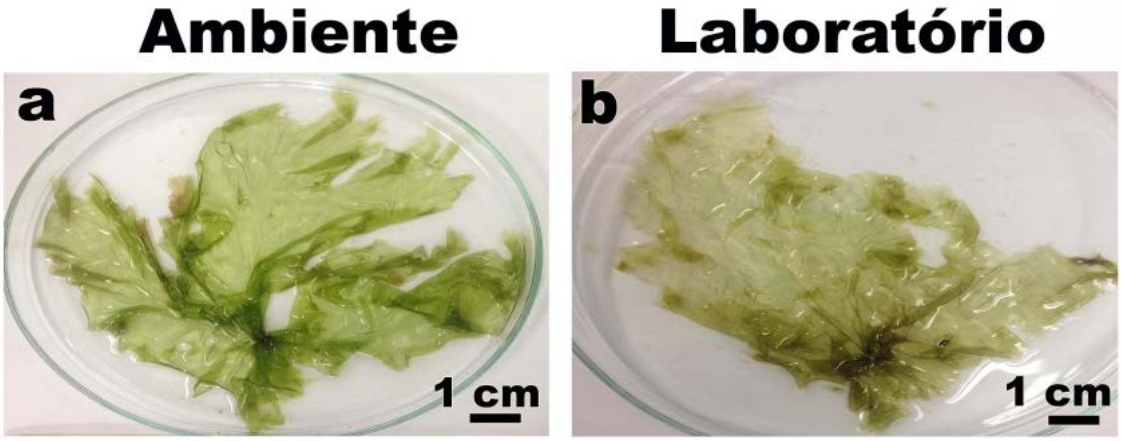

Figure 1. External morphology of the thalli of the red macroalga *Pyropia acanthophora* var. *brasiliensis* from **a.** ambient and **b.** acclimated in laboratory.

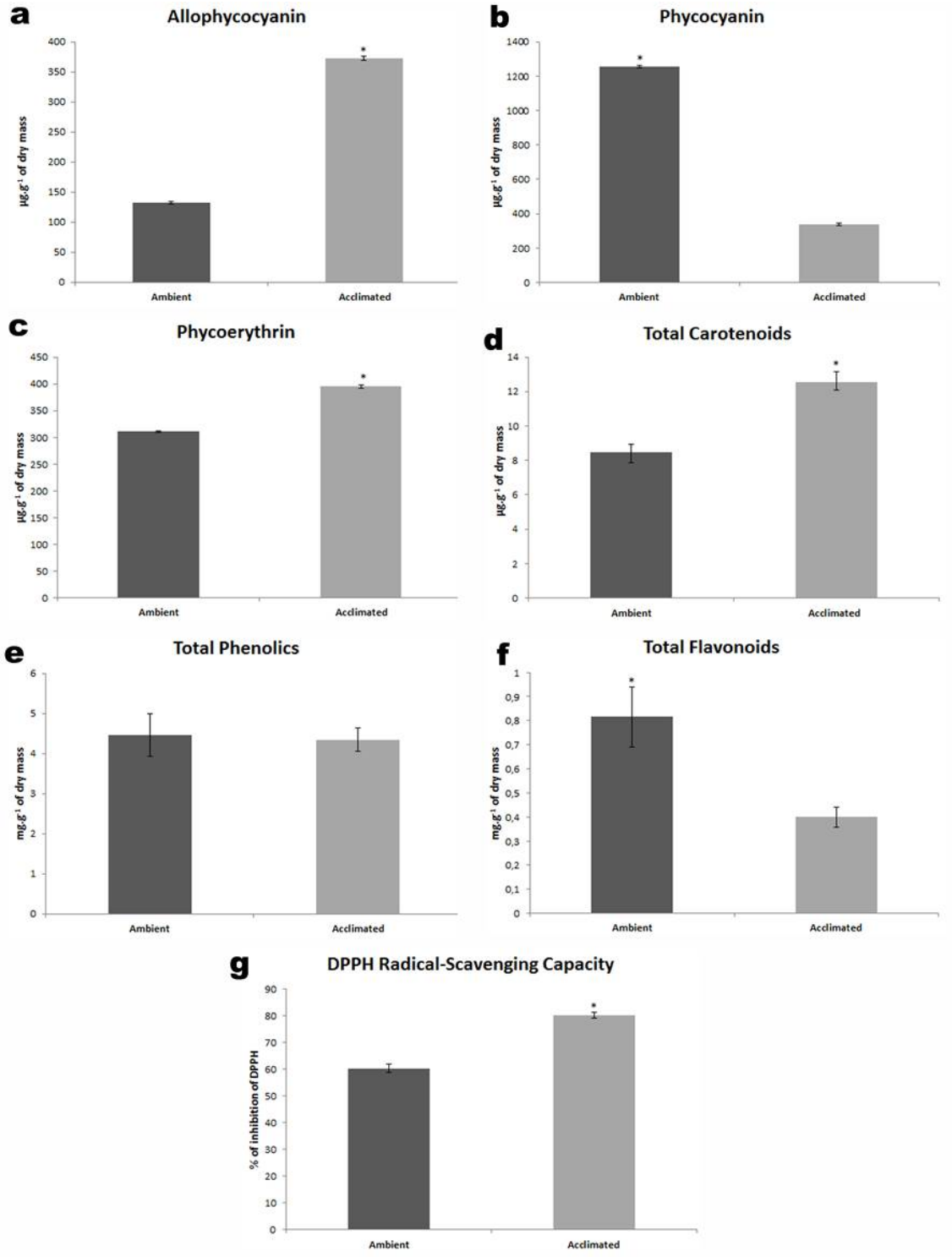

Figure 2. Content of **a.** allophycocyanin (µg.g^{-1} of dry mass), **b.** phycocyanin (µg.g^{-1} of dry mass), **c.** phycoerythrin (µg.g^{-1} of dry mass), **d.** carotenoids (µg.g^{-1} of dry mass), **e.** phenolic (mg.g^{-1} of dry mass), **f.** flavonoid (mg.g^{-1} of dry mass), **g.** inhibition of DPPH (%) of ambient and acclimated samples of *Pyropia acanthophora var. brasiliensis* (n = 4, mean ± SD). Asterisks indicate significant differences according to t test ($p \leq 0.05$).

The concentration of phycobiliproteins underwent changes after acclimatization. The ambient sample showed significantly lower concentrations of allophycocyanin (APC) [$t_{(4)} = 56.56$, $p < 0.0001$] (Figure 2a) and phycoerythrin (PE) [$t_{(4)} = -64.32$, $p < 0.0001$] when compared to the acclimated sample (Figure 2c). On the other hand, the concentration of phycocyanin (PC) [$t_{(4)} = 180.00$, $p < 0.0001$] was higher in the ambient sample compared to the acclimated sample (Figure 2b). It is suggested that the highest concentrations of phycobiliproteins occurred in acclimated samples from exposure to direct light for 12 h, a greater exposure than that found in the field of only 10 h. The increase of phycobiliproteins was also observed in *Aglaothamnion uruguayense* (W. R. Taylor) N. E. Aponte, D. L. Ballantine and J. N. Norris exposed to ultraviolet-B radiation (Ouriques et al., 2017) and *Gracilaria tenuistipitata* C. F. Chang and B. M. Xia exposed to hypoxic and hypersaline conditions (Israel et al., 1999). In addition to the function of accessory pigments and photoprotection of chlorophyll *a*, the phycobiliproteins can act as oxidation-reduction (REDOX) components in order to eliminate ROS (Kumar et al., 2010; Rodriguez-Sanchez et al., 2012).

Carotenoids, phenolics and flavonoids are the main antioxidant agents in red algae (Aple and Hirt, 2004; Ganesan et al., 2008; Borges et al., 2011) under stress conditions. The concentration of total carotenoids was higher in the acclimated sample [$t_{(4)} = -9.26$, $p < 0.001$] when compared to the ambient sample (Figure 2d). The total phenolic concentration was not significantly affected by acclimation compared to the ambient sample [$t_{(4)} = 5.21$, $p < 0.01$] (Figure 2e). However, the concentration of total flavonoids was higher in the ambient sample [$t (4) = 5.21$, $p < 0.01$] (Figure 2f). No pattern in the production of these secondary metabolites was observed. On the other hand, the acclimated samples showed a greater inhibition of the free radical DPPH [$t_{(4)} = -15.04$, $p < 0.001$] (Figure 2g), indicating a higher antioxidant activity in these samples and suggesting, in turn, that the acclimatized condition becomes a stress factor that accounts for the high concentration of carotenoids in the acclimated sample. Meanwhile, the lower concentrations of carotenoids compared to phycobiliproteins shows that carotenoids act to protect the photosynthetic apparatus of algae (Ramlov et al., 2011).

Many of these compounds have biological activity of interest to the pharmaceutical and food industries. It follows that the isolation and chemical characterization of secondary metabolities produced in greater quantities by acclimated algae submerged 24 h a day would have economic significance.

Conclusion

P. acanthophora var. brasiliensis cultivated in seawater under laboratory conditions favors the production of economically important secondary metabolites, such as phycobiliproteins and carotenoids, owing to submersion stress and changes in cultivation patterns, such as irradiance and photoperiod.

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Conflict of interests

The authors declare that there are no conflicts of interest.

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