

Growth and accumulation of carotenoids and nitrogen compounds in *Gracilaria domingensis* (Kütz.) Sonder ex Dickie (Gracilariales, Rhodophyta) cultured under different irradiance and nutrient levels

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Article

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Abstract: Effects of the interaction of irradiance and nutrient levels on growth and contents of photosynthetic pigments, carotenoids and proteins in *Gracilaria domingensis* (Kütz.) Sonder ex Dickie (Gracilariales, Rhodophyta) were investigated experimentally. Nutrient availability provided by dilutions of the nutrient solution of von Stosch (25 and 50%, which corresponded to nitrate concentrations of 125 and 250 μmol , respectively) and two photon flux densities [low PFD (50 ± 5) and high PFD (100 ± 5) $\mu\text{mol photons m}^{-2} \text{s}^{-1}$] were tested. Growth rates of *G. domingensis* were stimulated by high PFD. The interaction between high nutrient availability (50% VSES) and high PFD stimulated the accumulation of total soluble protein. Phycobiliprotein concentrations (phycocerythrin, phycocyanin, and allophycocyanin) and carotenoid contents were influenced by irradiance levels. Phycobiliprotein concentrations were higher at low PFD and high irradiances stimulated carotenoid accumulation. These results reflect the function of these pigments in photoprotection and the acclimation of *G. domingensis* to changes in irradiance levels. Our results indicate that light is a limiting factor for *G. domingensis* growth, that variations in phycobiliprotein contents under different irradiance levels are related to photoacclimation process, and that higher carotenoid contents at high irradiances are due to a photoprotection mechanism.

Keywords:

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Introduction

Gracilaria domingensis (Kütz.) Sonder ex Dickie (Gracilariales, Rhodophyta) is a common species on the northeastern Brazilian coast, where it has been collected and exported to supply the Japanese food market (Plastino et al., 1999). In Brazil, several studies have been conducted with *G. domingensis* because of its colour polymorphism, which includes green, red and brown forms (Guimarães et al., 2003). The colour phenotype of *G. domingensis* obeys a simple Mendelian inheritance, with two nuclear co-dominant alleles (red and green) at one locus, while heterozygous tetrasporophytes have a brownish phenotype (Plastino et al., 1999). Moreover, the life history of this species is of the Polysiphonia-type (Guimarães et al., 1999). Since its cultivation is feasible on the northeastern and southern Brazilian coast, there is a growing

interest in the study of its secondary metabolites as potential biologically active molecules. In addition, *G. domingensis* is also a potential source of dietary proteins, amino acids, lipids and essential fatty acids for humans and animals (Gressler et al., 2010).

Light and nutrient availability are factors that affect seaweed growth and these effects have also been reported for *Gracilaria* species (Wilson & Critchley, 1997; Ursi & Plastino, 2001; Kakita & Kamishima, 2006; Ferreira et al., 2006). However, few reports have described the effect of these factors on the accumulation of pigments and proteins (Andria et al., 1999; Zou & Gao, 2009). Denault et al. (2000) observed that concentrations of chlorophyll a and carotenoids increased with nitrogen concentration in *Gracilaria tikvahiae* McLachlan. Concentrations of carotenoids and phycobiliproteins are influenced by irradiances in *Gracilaria* species (Carnicas et al., 1999; Anderson

et al., 2006) as photoprotection and photoacclimation responses.

The aims of the present work were to determine the effects of photon flux density and nutrient levels on growth rates and accumulation of proteins, photosynthetic pigments and carotenoids in *Gracilaria domingensis*.

Materials and Methods

Fertile female gametophytes of *Gracilaria domingensis* were collected from the intertidal region of Lagoinha Beach, Santa Catarina state, southern Brazil (27°35'S and 48°33'W). Voucher specimens were deposited in the SP Herbarium at the Institute of Botany, São Paulo State, Brazil, under the accession number SP 400837. Unialgal cultures of tetrasporophytes were started from carpospore germination and cultured in sterilized seawater (salinity of 32±2 PSU) enriched with 25% of von Stosch's solution (VSES medium), according to Edwards (1970), with a reduction of 50% in the concentration of vitamins. During the first month of carposporeling culture, germanium dioxide (1 mg L⁻¹) was added to the medium. Cultures were incubated at 22±2 °C under a photon flux density (PFD) of 75±5 μmol photons m⁻² s⁻¹, provided by cool-white fluorescent lamps with a 14:10 h light:dark cycle, without aeration. PFD was measured with a quantum photometer (LI-185, Li-Cor Inc., USA) equipped with an underwater quantum sensor (LI-193 SA, Li-Cor Inc., USA). Medium renewal was carried out every week.

Experiments of irradiance and nutrient levels

Apical segments of tetrasporophytes of *Gracilaria domingensis* isolated from unialgal cultures were used in the experiments. Two dilutions of the nutrient solution of von Stosch (25 and 50%, which corresponded to nitrate concentrations of 125 and 250 μmol, respectively) were tested under two photon flux densities [low irradiance (50±5) μmol photons m⁻² s⁻¹ and high irradiance (100±5) μmol photons m⁻² s⁻¹]. Each treatment was performed in six replicates of six apical segments (2 cm) in each. These explants were cultured in Erlenmeyer flasks with 300 mL of culture medium. Other experimental conditions were the same as described for unialgal cultures.

Fresh biomass was recorded weekly at the same intervals as medium renewal. Growth rates were calculated as $[\ln(B_f/B_0)/T_f] \times 100\%$ (Brinkhuis, 1985), where B_0 is the initial fresh biomass, B_f is the final fresh biomass and T_f corresponds to the experimental period (28 days).

Carotenoid analyses

Carotenoids were extracted from samples (1.0 g fresh mass, n=3) using hexane:acetone (1:1, v/v) containing 100 mg L⁻¹ *tert*-butyl hydroxytoluene (BHT). Solutions were filtered through a cellulose membrane to remove particles and the organosolvent extract was evaporated under a N₂ flux. The residue was dissolved in hexane (3 mL). Prior to chromatographic analysis, in 1 mL of the organosolvent extract was added 10% KOH in methanol (100 μL/mL) in order to obtain complete carotenoid saponification, which allowed better identification of each compound by HPLC. This solution was incubated (3 h in the dark at room temperature), followed by washing with distilled-deionized water (three times). The de-esterified extract was collected, concentrated under a N₂ flux and resolubilized in hexane:acetone:BHT (100 μL) for further chromatographic analysis, as previously described (Kuhnen et al., 2009). A concentrated sample (10 μL, n=3) was injected onto the liquid chromatograph (Shimadzu LC-10A) equipped with a C₁₈ reverse-phase column (Vydac 218TP54; 250 mm x 4.6 mm Ø, 5 μm, 30 °C), protected by a 5 μm C₁₈ reverse-phase guard column (Vydac 218GK54), and an UV-visible detector (450 nm). Elution was performed with MeOH:CH₃CN (90:10, v/v) at a flow rate of 1 mL.min⁻¹. Carotenoid identification (α -carotene, β -carotene, lutein, zeaxanthin, and β -cryptoxanthin) was performed using retention times and co-chromatography of standard compounds (Sigma-Aldrich, St. Louis, MO, USA), as well as by analogy with other reports of carotenoid analysis by RP-HPLC-UV-visible under similar conditions (Scott & Eldridge, 2005; Hulshof et al., 2007). Carotenoid quantification was based on standard curves, employing the lutein standard curve (0.5 - 45 μg mL⁻¹; $y = 7044x$; $r^2 = 0.999$) for lutein, zeaxanthin and β -cryptoxanthin quantification and the β -carotene standard curve (0.01-12 μg mL⁻¹; $y = 1019x$; $r^2 = 0.998$) for α - and β -carotene quantification.

Analyses of photosynthetic pigments and total soluble proteins

Pigment extractions were carried out at 4 °C and samples (75 mg of fresh mass, n=3) were ground to a powder with liquid nitrogen and mixed with 50 mM phosphate buffer (pH 5.5). The homogenates were centrifuged at 14000 rpm for 20 min for separation of the phycobiliproteins present in the supernatants. Chlorophyll *a* was extracted after dissolving the pellet in 90% acetone and centrifuging at 10000 rpm for 15 min. Pigments were quantified by spectrophotometry (Shimadzu-UV 1800) and concentrations were calculated according to Kursar et al. (1983) for phycobiliproteins and Jeffrey & Humphrey (1975) for chlorophyll *a*.

For total soluble protein analyses, the algal biomass (75 mg, n=3) was ground with liquid nitrogen, and extractions were carried out at 4 °C using 0.2 M phosphate buffer (pH 8.0) containing 5 mM EDTA and 1 mM DTT. Buffer was added in the ratio of 10 ml g⁻¹ fresh biomass and the homogenates were centrifuged at 12000 rpm for 15 min. Total soluble protein contents were determined according to Bradford (1976), using a Bio-Rad protein assay kit and BSA as standard.

Data analysis

Data were analyzed by bifactorial Analysis of Variance (ANOVA) and the Student-Newman-Keuls' test (Zar, 1999). All statistical analyses were performed using the Statistica software package (Release 6.0), considering $p \leq 0.05$. Homogeneity of the variance was tested using Levene's test.

Results

Effects of the interaction of PFD and VSES concentration on the growth rates of tetrasporophytes of *Gracilaria domingensis* were not significant, while high PFD stimulated the growth (Figure 1, Table 1).

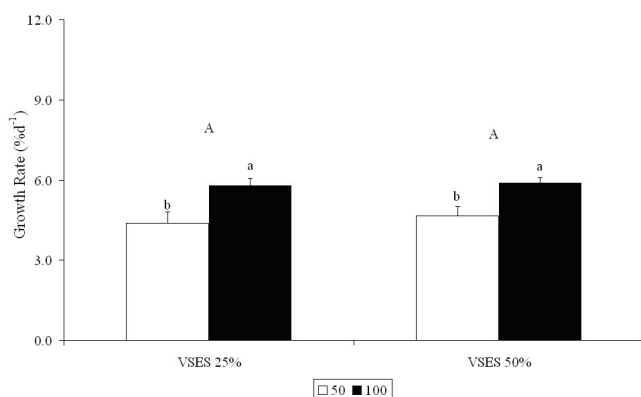


Figure 1. Growth rates (% d⁻¹) of apical segments of tetrasporophytes of *Gracilaria domingensis* cultured in von Stosch medium (25% and 50% VSES) under 50 (white bars) and 100 (black bars) $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for four weeks. Values are averages \pm SD (n= 6), with six segments of 2 cm per replicate. Different letters indicate significant differences according to the Student-Newman-Keuls' test ($p \leq 0.05$). Capital letters indicate significant differences between VSES concentrations and lower case letters indicate differences between irradiance levels.

An interaction between PDF and VSES concentration stimulated protein accumulation and tetrasporophytes cultured in 50% VSES under high PFD showed the highest protein concentration (Figure 2, Table 2).

Table 1. Two-away ANOVA of percentages of growth rates of tetrasporophytes of *Gracilaria domingensis* cultured in von Stosch medium (25 and 50% VSES) under photon flux densities (PFD) of 50 and 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for four weeks.

Variable	df	F	p
VSES	1	1.604	0.219952
PFD	1	99.347	0.000000
VSES x PFD	1	0.469	0.501380

Phycobiliprotein contents were not influenced by the interaction between PFD and VSES concentration (Table 3). However, PFD stimulated phycobiliprotein accumulation, with the highest concentrations of phycoerythrin, phycocyanin and allophycocyanin being present at low PFD (Figure 3, Table 3). The chlorophyll concentration was not affected by PFD and VSES concentration (Figure 3, Table 3).

Carotenoid composition (lutein, zeaxanthin, β -cryptoxanthin and trans- β -carotene) showed a variation, which was influenced by the culture conditions of the tetrasporophytes. Figure 4 shows a

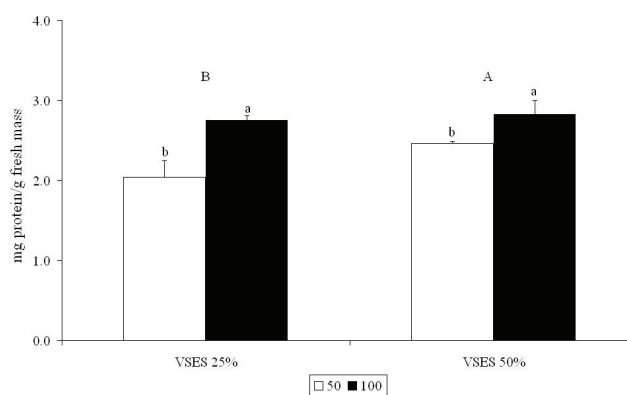


Figure 2. Total soluble protein content in apical segments of tetrasporophytes of *Gracilaria domingensis* cultured in von Stosch medium (25% and 50% VSES) under 50 (white bars) and 100 (black bars) $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for four weeks. Values are averages \pm SD (n=3), with six segments of 2 cm per replicate. Different letters indicate significant differences according to the Student-Newman-Keuls' test ($p \leq 0.05$). Capital letters indicate significant differences between VSES concentrations and lower case letters indicate differences between irradiance levels.

Table 2. Two-away ANOVA of total soluble protein content of tetrasporophytes of *Gracilaria domingensis* cultured in von Stosch medium (25 and 50% VSES) under photon flux densities (PFD) of 50 and 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for four weeks.

Variable	df	F	p
VSES	1	9.543	0.014
PFD	1	44.622	0.0001
VSES x PFD	1	4.684	0.062

typical HPLC profile for the carotenoids.

The interaction between PFD and VSES concentration did not affect lutein and *trans*- β -carotene concentrations (Table 4). Concentrations of both compounds were only influenced by PFD, with the highest concentrations detected at high photon flux density (Figure 5A, E, Table 4). Free zeaxanthin and β -cryptoxanthin concentrations were influenced by the interaction between PFD and VSES concentration (Table 4), while treatment with low nutrient concentrations

(25% VSES) under high PFD stimulated the highest accumulation of free zeaxanthin (Figure 5B) and high nutrient concentrations (50% VSES) under high PFD stimulated the highest β -cryptoxanthin concentration (Figure 5D). For tetrasporophytes cultured under low PFD, the areas of the esterified zeaxanthin peaks were too small to allow quantification. At high PFD, the esterified zeaxanthin concentrations were the highest at low nutrient concentrations (25% VSES, Figure 5C).

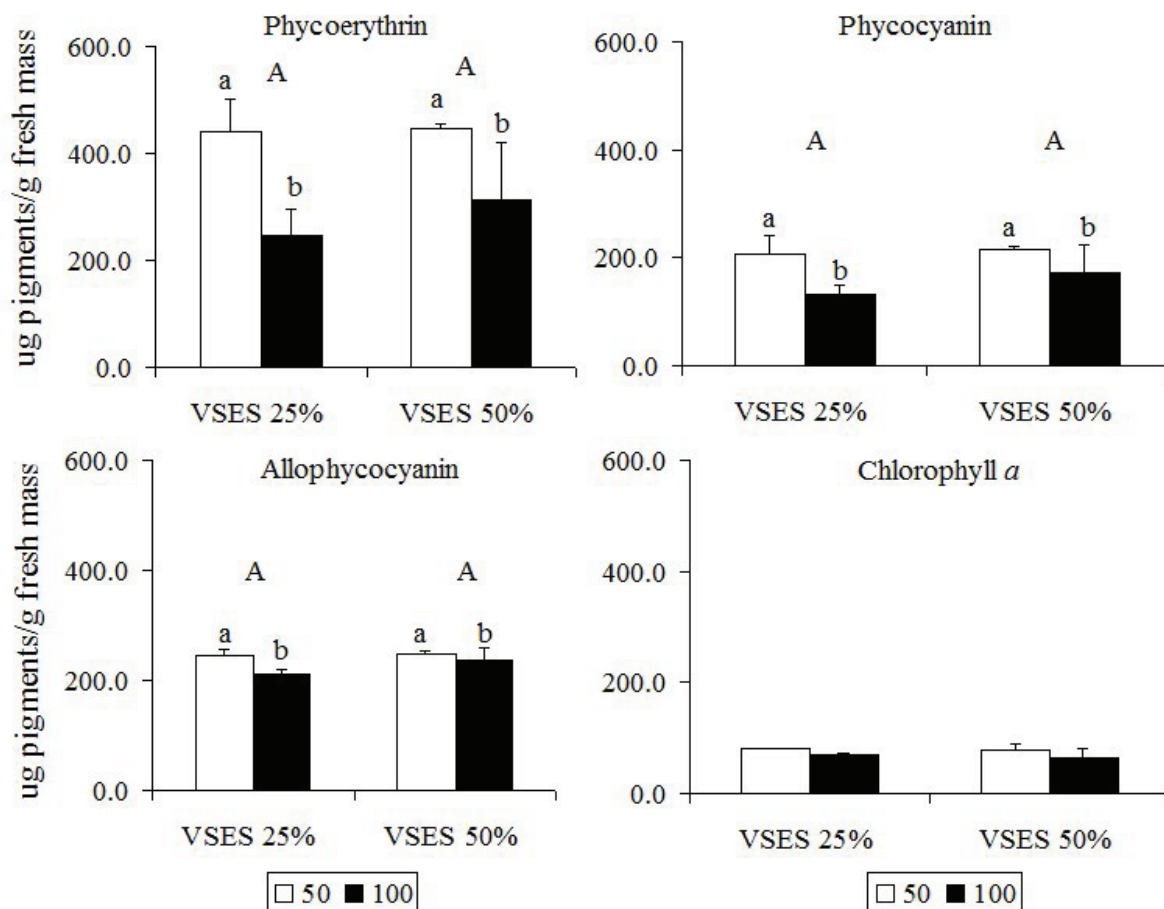


Figure 3. Photosynthetic pigment concentrations in apical segments of tetrasporophytes of *Gracilaria domingensis* cultured in von Stosch medium (25% and 50% VSES) under 50 (white bars) and 100 (black bars) $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for four weeks. Values are averages \pm SD (n=3), with six segments of 2 cm per replicate. Different letters indicate significant differences according to the Student-Newman-Keuls' test ($p < 0.05$). Capital letters indicate significant differences between VSES concentrations and lower case letters indicate differences between irradiance levels.

Table 3. Two-way ANOVA of photosynthetic pigment concentrations of tetrasporophytes of *Gracilaria domingensis* cultured in von Stosch medium (25 and 50% VSES) under photon flux densities (PFD) of 50 and 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for four weeks.

Variable	df	Phycoerythrin		Phycocyanin		Allophycocyanin		Chlorophyll a	
		F	p	F	p	F	p	F	p
VSES	1	0.905	0.369	1.699	0.228	2.993	0.121	0.201	0.665
PFD	1	18.196	0.002	10.214	0.012	8.038	0.021	4.921	0.0573
VSES x PFD	1	0.684	0.431	0.769	0.405	1.792	0.217	0.057	0.816

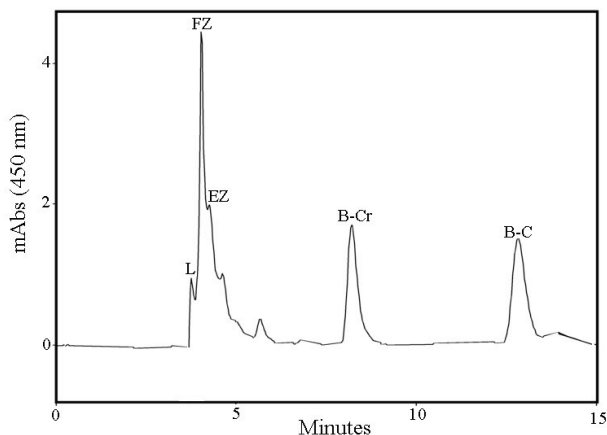


Figure 4. HPLC chromatogram for carotenoids detected in tetrasporophytes of *Gracilaria domingensis* cultured in 25% VSES under 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Detection wavelength 450 nm. L, lutein; FZ, free zeaxanthin; EZ, esterified zeaxanthin; β -Cr, β -cryptoxanthin; β -C, trans- β -carotene.

Discussion

Irradiance was a limiting factor for growth of *G. domingensis* and the highest growth rate was observed at high PFD. *G. domingensis* is found in the intertidal region and it is exposed to high irradiances. Moreover, growth of *G. domingensis* was not influenced by nutrient concentrations. Similarly, growth rates of *G. foliifera* var. *angustissima* (Harv.) Taylor (Lapointe, 1981) were not influenced significantly by an increase in nitrate concentrations.

Light (PFD) influenced phycobiliprotein concentrations in *Gracilaria domingensis*. The highest concentrations of phycoerythrin, phycocyanin and allophycocyanin were observed in tetrasporophytes cultured at low PFD. Similar results have also been reported for other *Gracilaria* species (Zou & Gao, 2009) and could be related to a photoacclimation process at low irradiance. Concentrations of chlorophyll a in *G. domingensis* were not influenced by irradiance and nutrient levels and were lower than the phycobiliprotein contents.

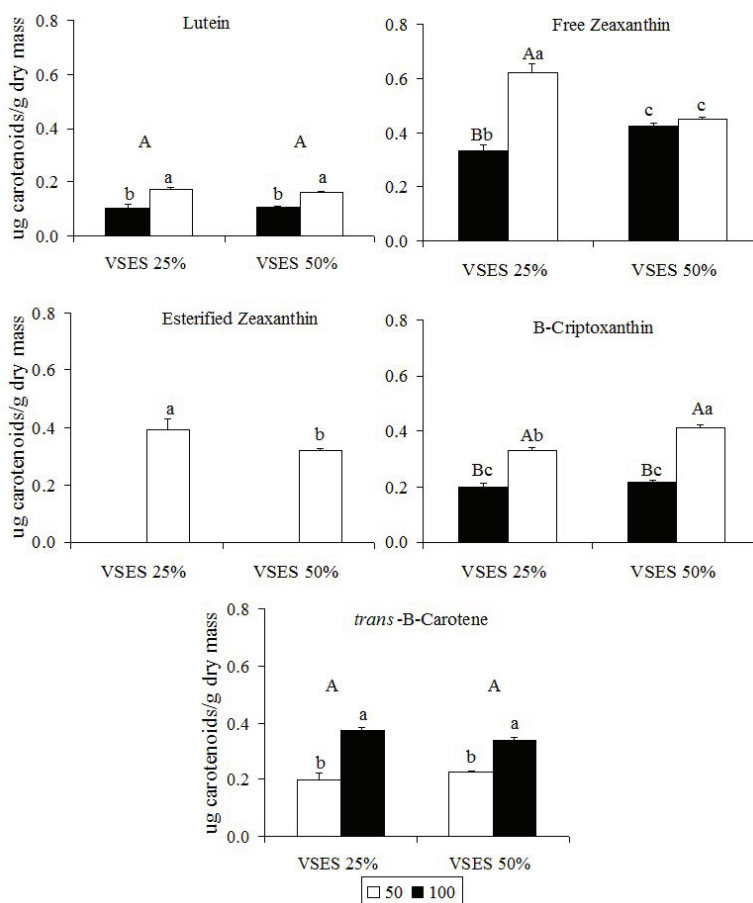


Figure 5. Carotenoid concentrations in apical segments of tetrasporophytes of *Gracilaria domingensis* cultured in von Stosch medium (25% and 50% VSES) under 50 (white bars) and 100 (black bars) $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for four weeks. Values are averages \pm SD ($n=6$), with six segments of 2 cm per replicate. Different letters indicate significant differences according to the Student-Newman-Keuls' test ($p \leq 0.05$). Capital letters indicate significant differences between VSES concentrations, and lower case letters indicate differences between irradiance levels.

Table 4. Two-way ANOVA of carotenoid concentrations of tetrasporophytes of *Gracilaria domingensis* cultured in von Stosch medium (25 and 50% VSES) under photon flux densities (PFD) of 50 and 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for four weeks.

Variable	df	Lutein		Free zeaxanthin		β -cryptoxanthin		β -carotene	
		F	p	F	p	F	p	F	p
VSES	1	2.742	0.136	8.565	0.022	62.623	0.000047	0.343	0.574
PFD	1	327.370	0.000	135.584	0.000008	643.148	0.000	357.044	0.000
VSES x PFD	1	2.379	0.161	95.728	0.000025	23.570	0.001	14.722	0.225

The relationship between pigment content and nutrient availability has been investigated in several studies (Jones et al., 1996; Godillo et al., 2006), indicating that *Gracilaria* species are able to assimilate nitrogen and, when present in excess, nitrogen is stored as proteins or pigments (Kosovel & Talarico, 1979). However, this is not the only factor determining the pigment concentrations. Light plays a key role and exerts an effect opposite to that of nitrogen (Talarico & Maranzana, 2000), as observed in our results. According to Lapointe (1981), the interaction between these two factors affects the pigment concentrations and increases or decreases in these concentrations are related to the photosynthetic capacity.

Accumulation of total soluble protein was influenced by the interaction between PFD and nutrient availability. The highest protein concentration was observed at high nutrient concentration (50% VSES) under high PFD. As discussed previously, *Gracilaria* species have the capacity to store nitrogen during periods of high nitrogen availability. Under optimal conditions for photosynthesis, there is no need to store nitrogen as phycobiliproteins and *G. domingensis* tetrasporophytes then store the excess nitrogen preferentially in the form of proteins. Andria et al. (1999) observed that the total soluble protein content of *Gracilaria* sp. decreased when the species was cultured under conditions of limited availability of nitrogen.

High levels of irradiance stimulated carotenoid accumulation in *G. domingensis* tetrasporophytes. Carotenoid concentrations were lower than phycobiliprotein concentrations, reflecting the carotenoid function of protecting the photosynthetic apparatus. Our results show that the β -carotene pathway was more active, since the concentrations of zeaxanthin, β -cryptoxanthin and β -carotene were higher than that of lutein. According to Demming-Adams (1990), zeaxanthin is a key pigment involved in the photoprotective response to the stress caused by high irradiance, because xanthophyll is more efficient at dissipating the excess energy (Frank et al., 2001). The high content of zeaxanthin during the acclimation to high irradiances suggests a response to the stress caused by excess light in *G. domingensis*. In a similar experiment, Carnicas et al. (1999) also observed an increase in zeaxanthin concentrations with increasing irradiance in *G. tenuistipitata* var. *liui* Zhang & B.M. Xia.

The decrease in concentration of free zeaxanthin in *G. domingensis* cultured under conditions of low nutrient availability (25% VSES) and low PFD and the reduction in β -cryptoxanthin concentration under low nutrient availability (25% VSES) and high PFD could be a photoacclimation response to low nutrient availability.

In conclusion, our results indicate that light is a limiting factor for *G. domingensis* growth, that variations in phycobiliprotein contents under different irradiance levels are related to photoacclimation process, and that higher carotenoid contents at high irradiances are due to photoprotection mechanism.

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