Short term effects of irradiance on the growth of *Pterocladiella capillacea* (Gelidiales, Rhodophyta)

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Pterocladiella capillacea has been economically exploited for agar extraction in the Azores for many years. Harvesting dropped to a full stop in the early 1990s due to a population collapse, but restarted in 2013. Since then it has been intensively harvested and overexploitation must be prevented, with both sustainable harvesting and effective cultivation practices. This study represents the first attempt to determine optimal conditions for *P. capillacea* production in the Azores, and evaluates its vegetative growth in two experiments using von Stosch's medium designed to test entire thallus and tips portions response to different irradiances (30, 70 and 150 µmol photons m^{-2} s^{-1}). The best relative growth rate (RGR) was recorded at 150 µmol photons m^{-2} s^{-1} for the entire thalli and tips after two-weeks and three-weeks, respectively, indicating that an acclimation period is necessary to assure the growth of this alga under experimental conditions. Higher RGR was obtained at higher irradiance (3.98 ± 2.10% fm day^{-1}), but overall, growth rates were low or negative. Epiphytes were a serious problem towards the end of the entire thallus experiments, where *Feldmannia irregularis* proliferate at all irradiances. Future cultivation approaches complemented with other relevant environmental factors (e.g. pH, photoperiod, salinity), are recommended.

Key words: epiphytes, growth, *in vitro* cultivation, light intensity, *Pterocladiella capillacea*

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INTRODUCTION

Pterocladiella capillacea (S.G. Gmelin) Santelices & Hommersand is a well-known red alga, recognized to produce a very high quality bacteriological agar and agarose (Bixler & Porse 2010; Boo et al. 2010; Rhein-Knudsen et al. 2015; Lahaye & Rochas 1991). The demand for agar producing seaweeds is high; nonetheless, all raw materials are obtained from harvests of wild plants, resulting in limited or overexploited natural seaweed stocks (Bixler & Porse 2010; Callaway 2015; Marinho-Soriano 2017). Wild plants of *P. capillacea* are relatively small (up to 17cm), and

mostly inhabit tropical and subtropical waters, although a few extend into temperate latitudes (Lawson & John 1982; Santelices 1998; Patwary et al. 1998; Santelices & Hommersand 1997; Neto 2000a, Neto 2000b; Boo et al. 2010). The growth in length of its axes and branches, as in all genera presently recognized in the Gelidiaceae, depends on the activity of a dome-shaped apical cell (Rodríguez & Santelices 1987; Abbott 1997, Lee 2008). Its erect fronds can persist for up to 7 years although the average is only 2-3 years (Dixon & Irvine 1977). *P. capillacea* growth is site specific and influenced by irradiance, temperature and water movement (e.g. Macler & Zupan 1991; Santelices 1991; Oliveira & Berchez 1993; Friedlander 2008).

Epilithic in the lower intertidal and shallow subtidal (down to 15m), this species is widely distributed in the Azores (Neto 2000b), where it was considerably harvested between 1950-80 t, reaching an annual biomass between 1000-2000 t, (Fralick & Andrade 1981; Santos & Duarte 1991; Melo 2002; Santos & Melo 2018), dried and then exported for agar extraction (Neto et al. 2005). Due to a population collapse in the early 1990s, harvesting dropped to a full stop, but restarted in 2013 and, at the present, *P. capillacea* is being harvested in six of the nine Islands of the Archipelago (Terceira, São Miguel, Graciosa, Faial, São Jorge and Pico Islands; LOTAÇOR 2019) and sold almost exclusively to IBERAGAR S.A. and TAE Lda. companies (AOnline 2016; unpublished observations). In 2016, 450 wet weight (ww) tonnes of *P. capillacea* were harvested, representing a total value of 450000 € (GaCS 2017), corresponding grossly to 1 € Kg^{-1} ww. Due to this recent resurge of the exploitation, there is an urgent need to implement good management practices for the harvest of the wild resource and to promote the species cultivation (Patarra et al. 2014; Rebours et al. 2014; Netalgae 2012; Patarra 2018).

Today there is not any known commercial cultivation of this Gelidiales (Melo 1998; Friedlander 2008; Callaway 2015: Marinho-Soriano 2017; Santos & Melo 2018), mostly due to its slow growth rates (Friedlander & Lipkin 1982; Friedlander & Zelikovitch 1984; Macler & Zupan 1991; Yokoya & Oliveira 1992; Oliveira & Berchez 1993; Felicini et al. 2002; Fujimoto et al. 2014). However, land base studies on *P. capillacea* in Israel, revealed promising growth rates per week (28.3% in average) during winter time (Gal-Or & Israel 2004). According to a revision of the Gelidiales cultviation made by Friedlander (2008) distinct types of seaweed material, namely different sized portions from differents parts of the thallus can have different growth. This was earlier observed by Rodríguez (1996) who reported that the vegetative growth in length and branch proliferation of *Gelidium sclerophyllum* was favored by medial fragments, whereas the rhizoidal propagation was favoured by apices.

To our best knowledge, a single study on the physiological responses of Azorean Gelidiales was performed (Fralick et al. 1990), and authors indicated that optimal photosynthesis activity occurred at 177 µmol m^{-2} s^{-1}, between 15 and 25ºC. To move to an effective culture of this species, a clarification of the most relevant factors and constraints for its production is needed. The present work is a first attempt to understand how different irradiances affect the vegetative growth of distinct portions of the thallus (entire thallus and tips) of *P. capillacea* from the Azores.

MATERIAL AND METHODS

Experimental setup

Sterilized natural seawater (SNW) was prepared by filtering the water with 0.2 µm pore size filter and autoclaved. Von Stosch's enrichment culture medium (VSE) was prepared according to Guiry & Cunningham (1984). All the material used during media preparation and in the experiments was sterilized either by autoclave (2 atm, 120ºC, 20 min) or by using a drying oven (150ºC, 2 hr).

Fronds were collected by hand, between December 2013 and February 2014, from the low intertidal zone of a rocky shore of the south coast of São Miguel Island (37° 50′ N, 25° 30′ W; Azores archipelago), where stable populations of *P. capillacea* are known to occur (Neto 2000b, Neto 2000a, Neto 2001, Wallenstein et al. 2008). Fronds were transported to the laboratory within 4 hr of the collection and kept in natural seawater tanks (5 L). At each collection time, voucher

specimens were made and deposited in the Herbarium Ruy Telles Palhinha (AZB) of the Biology Department, University of the Azores (*P. capillacea*, AZB- SMG-13-90, AZB-SMG-14-01, AZB-SMG-14-07).

One week before the experiments, algae were incubated in 1 L Erlenmeyer flasks containing 500 ml of SNW enriched with VSE and kept in constant movement through bottom aeration.

A preliminary experiment was conducted to evaluate the effect of different pre-cleaning solutions (sodium hypochlorite 1%; betadine 10% and SNW) on the growth of epiphytes and endophytes of *P. capillacea* (Table 1). The pre-cleaning solution Bleach promoted the growth of the red endophytes *Anotrichium* spp. and *Epicladia* spp. (unpublished data). Based on these preliminary results, the pre-cleaning solution Betadine 10% was used in all subsequent experiments. For this, fronds of *P. capillacea* were rinsed under tap water and visible epibionts and surface sediments were removed by hand and gently brushed if necessary. Then, algae were checked under a dissecting microscope and the healthy ones retained for the cultivation essays. Entire thalli (of 2-3cm long) and tips portions (1cm long) were cleaned in SNW, submerged for 30s in the pre-cleaning solution of Betadine 10% and cleaned again in SNW; then they were randomly assigned to experimental units, 1 L Erlenmeyer flasks each containing eight individual entire thallus or tips portions (depending on the subsequent experiment) and 500 ml of SNW enriched with VSE. The growth of excised fragments was evaluated in two experiments designed to test if distinct algae material: i) entire thallus (ET) and ii) tips portions (TP) respond differently under different photon flux densities (PFD, 30, 70 and 150 μmol photons m^{-2} s^{-1}).

The experiments were carried in cultivation chambers (Sanyo MLR-351; Japan) using a photoperiod of 12L:12D and 18ºC temperature as fixed factors and lasted 5 weeks. The temperature of 18ºC was used to match natural conditions of the collecting site and the average annual sea surface temperature (SST) in the Azores archipelago (DETRA 2013, Patarra *et al.* 2017). The 12L:12D photoperiod chosen in the experiments relies on the fact that there is no consensus on the best L:D combination for *P. capillacea* growth (e.g. 10:14 LD, Fralick et al. 1990, 12:12 L:D; Nasr et al. 1966, 16.8 LD; Yokoya & Oliveira 1992, 12:12 LD; Felicini et al. 2002). The irradiance was measured with a LI-250 Light Meter (LI-COR, USA) inside the culture chambers and whenever the algae were collected in the field. When the culture medium was changed, the algal material was blotted dry and weighted as fresh mass (fm) using a digital balance (Kern ALJ220-5DNM, Germany; ±0.0001 g precision). In each of these occasions, algae were placed on plasticized graph paper and photographed (Sony α230 with an objective of 18-55mm, Japan) for posterior length analyses. The pH was monitored (three measurements per replicate) with a pH sensor (Hanna Instruments, HI98127 USA).

Growth responses

Once a week, fresh biomass (g) and length (cm) were registered, and relative growth rate was calculated (RGR; % fm day^{-1}) for each replicate (n=4, SE) in all treatments. Length of individual thallus/portions was obtained from the above mentioned images using the AxioVision LE Digital Imaging Software (SE 64 Rel. 4.9.1., Germany) and by measuring each individual thallus/portion from its base to the top. At each sampling time the RGR was calculated for each replicate flask according to the following formula (Abreu et al. 2011):

$$RGR = \frac{[\text{Ln (final fm)} - \text{Ln (initial fm)}]}{\text{time (days)}} \times 100$$

These calculations were based on the sum value of the eight individual thallus/portions in each flask. For the analysis (see section 2.3), the mean value of weekly growth rates measured throughout the experiment was used to account for temporal growth rates variation.

Data analysis

For all analysis, we used flasks as sampling units. One-way analysis of variance (ANOVA, e.g. Underwood 1997; PFD as a fixed factor with 3 levels, 4 replicates each) was used to test for differences in RGR in the different treatments.

Prior to the analysis, Cochran's test was used to test for homogeneity of variances and transformations were applied whenever necessary. When this was not possible, analyses were run on the untransformed data, since ANOVA is robust to departures from this assumption when designs are balanced and replication is high (Underwood 1997). However, a more conservative p-value ($\alpha = 0.01$) was used. The *a posteriori* Student–Newman-Keuls (SNK) test was used to investigate differences among levels within significant terms. All statistical analyses were performed using the software GMAV v5.

RESULTS

The abiotic factors (temperature, irradiance) monitored during the experiment were kept in the pre-defined range. Carbon availability was always in optimal levels, as determined by the pH recorded values, ranging between 7.00 ± 001 and 7.93 ± 0.02 in ET experiment and 7.02 ± 0.05 and 7.90 ± 0.02 in TP experiment.

Table 1. List of epiphytes and endophytes identified at the end of the preliminary experiment. SNW - sterilized natural seawater.

Taxa	Authorities	Occurrence	Pre-cleaning solutions		
			SNW	Betadine 10%	Bleach
RHODOPHYTA					
Anotrichium tenue	Nägeli	endophyte			x
Bangia spp.	Lyngbye	epiphyte			x
Ceramium rubrum	C. Agardh	epiphyte			x
CLOROPHYTA					
Cladophora spp.	Kützing	epiphyte		x	
Enteromorpha multiramosa	Bliding	epiphyte	x		
Epicladia spp.	Reinke	endophyte			x
Ulva compressa	Linnaeus	epiphyte	x		x
Ulva rigida	C. Agardh	epiphyte		x	x
HETEROKONTOPHYTA					
Feldmannia irregularis	(Kützing) Hamel	epiphyte	x	x	

In the ET experiment there was no biomass increase in any of the treatments for the first 3 weeks (Figure 1A, B). However, pronounced weight and length increments were recorded after the third week for the 150 µmol photons m^{-2} s^{-1} treatment (Figure 1A), and the same pattern occurred from the fourth to the fifth week at 70 µmol photons m^{-2} s^{-1} (Figure 1A). However, in both situations an increase in the epiphytes (mostly *Feldmannia irregularis*) growth (from the base to the top of the thalli) was recorded, as well as an accumulation of a layer of died cells over the thalli surface. Due to the amount of epiphytes growing at 150 µmol photons m^{-2} s^{-1} at the fifth week, no measurements were taken from that moment forward and for RGR statistical analysis only 30 and 70 µmol photons m^{-2} s^{-1} treatments were used (Table 2). The lower RGR was observed at 30 µmol photons m^{-2} s^{-1} (-2.02 ± 1.25% fm day^{-1}) indicating loss of tissue (Figure 1C, Table 2). Overall, the best entire thalli RGR was recorded at 150 µmol photons m^{-2} s^{-1} (3.98 ± 2.10% fm day^{-1}, Figure 1C).

As for the TP experiment, there was a similar growth pattern for the 70 and 150 µmol photons m^{-2} s^{-1} treatments, with slight mass increments

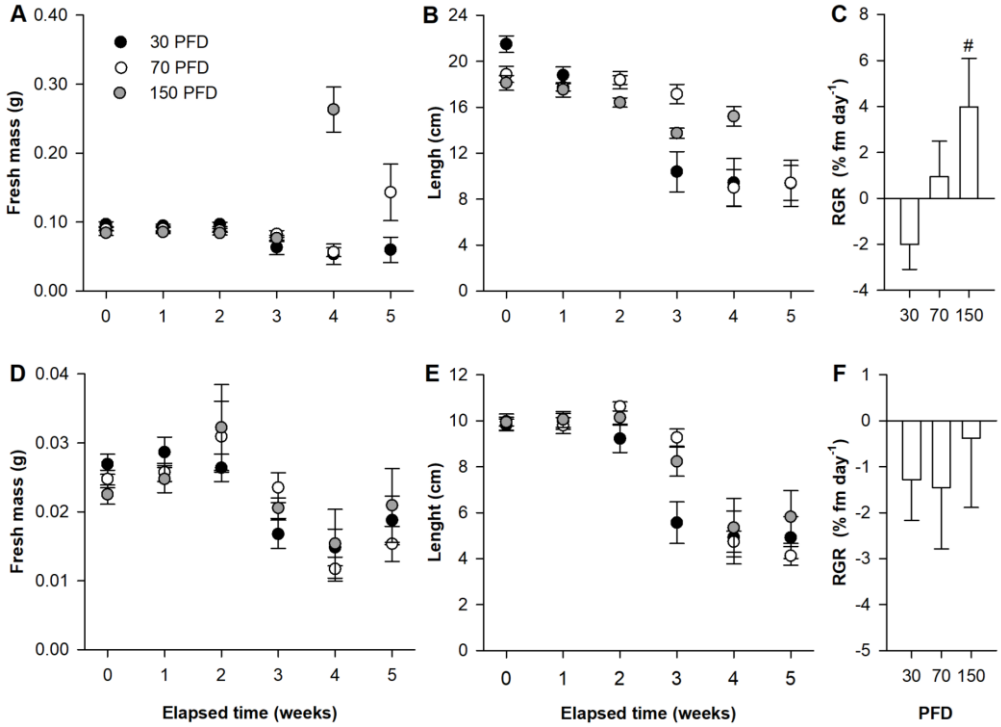

Fig. 1. Effect of photon flux density (PFD) on the growth of *P. capillacea* entire thallus (ET; panels A, B and C) and tips portions (TP; panels D, E and F) cultivated at three PFD (30, 70 and 150 µmol photons m^{-2} s^{-1}), 18 ºC and 12:12 L:D photoperiod, after five weeks in culture. Fresh mass and length (mean ± SE, n=4); relative growth rate (RGR, mean ± SE, n=20; $p < 0.05$). # mean RGR of four weeks, not used in the statistical analysis.

after two weeks in culture (Figure 1D, E). No recovery was observed but the growth was lower at the higher irradiance (150 µmol photons m^{-2} s^{-1}) (Figure 1E, Table 2) and no epiphytes were recorded. Nevertheless, RGR was negative at all PFD (Figure 1F). As for the ET, the best growth was obtained for the higher PFD used. Experiments lasted 5 weeks, since then algae were mushy and colorless (some of them even white).

Table 2. Univariate analysis of variance (ANOVA) examining the effect of photon flux density (two levels for entire thallus experiment, ET; three levels for tips portions experiment, TP; fixed) on the relative growth rate of *P. capillacea* cultivated at 18 ºC, and 12:12 L:D photoperiod, after 5 weeks in culture.

	ET				**TP**			
Source	df	MS	*F*	*P*	df	MS	*F*	*P*
PFD	1	86.44	2.42	0.1280	2	6.70	0.21	0.8135
Residual	38	35.71			57	32.36		
Cochran's test		*C* = 0.67 ns				*C* = 0.47 ns		
Transformation		none				none		

PFD = photon flux density; ET = entire thalus experiment; TP = tips portions experiment; n.s., not significan

DISCUSSION

The best growth performance observed at high PFD for both, the entire thallus and the tip portions, submitted to an longer acclimation period (seen as necessary to assure the growth of *P. capillacea* under experimental conditions) is in agreement with reports for this species by other researchers (e.g. Nasr et al. 1966; Correa et al. 1999; Felicini et al. 2002). However, the unexpected decrease in length observed for both entire thallus and plant tips is in disagreement with reports by other authors, which reported that, although *P. capillacea* exhibited low growth rates, it maintained a viable growth for several days or weeks in artificial conditions (e.g. Stewart 1984; Macler & Zupan 1991; Yokoya & Oliveira 1992, Oliveira & Berchez 1993, Felicini et al. 2002; Friedlander 2008; Fujimoto et al. 2014). This may be related with the collection time of the algae material used to run laboratory experiments. In fact, Stewart (1984) reported a best initial growth and survival for cultures initiated with Californian of *P. capillacea* collected between October and March, corresponding to local late autumn/winter period. Similar results were reported by Gal-Or & Israel (2004) for Israeli material. In the present study all the laboratory trials were run at 18 ºC (the mean annual SST in the Azores Archipelago, DETRA 2013, Patarra et al. 2017), but the algae were collected from December to early Spring when SST varies between 16 and 17 ºC (DETRA 2013). It is likely that the observed negative growth could be related to higher temperature used. In fact, a poor growth performance of *P. capillacea* at high temperatures has been reported by several authors (Fralick et al. 1990; Yokoya & Oliveira 1992; Gal-Or & Israel 2004; Fujimoto et al. 2014). It is, however, worth considering, that *P. capillacea* in the Azores is distributed from low intertidal to shallow subtidal zones (Neto 2000a, Neto 2000b, Neto 2001: Wallenstein et al. 2008), being adapted to a wide range of irradiances and water temperatures, although the temperature range in shallow subtidal zones does not vary greatly (HI 2000). Furthermore, Fralick & Andrade (1981) observed that wild Azorean plants exhibited optimal growth at 20-22 ºC, coinciding to the period between June and October when the irradiance level is higher in the Azores.

Regarding irradiance, the obtained mean growth rates were negative/low at all PFD, and, even in the higher irradiances, their was loss of tissue, and the thallus was mushy, colorless or even white at the end of the experiments. This is in disagreement with reports by other researchers for Gelidiales: Macler & West (1987) kept vegetative cultures of *Gelidium coulteri* for more than a year with an exponential growth rate less than 10% day^{-1} at 150 µmol photons m^{-2} s^{-1}; Fralick et al. (1990) in a research on the physiological responses of *Pterocladia* and *Gelidium* (Gelidiales, Rhodophyta) from the Azores reported optimal photosynthesis for *P. capillacea* at 177 µmol photons m^{-2} s^{-1} with oxygen production values remaining high until 320 µmol photons m^{-2} s^{-1}; Rico (1991) reported maximum growth rates (10.0% day^{-1}) for *Gelidium pulchellum* at 130-µmol photons m^{-2} s^{-1}; Sousa-Pinto et al. (1999) observed that the growth of *Gelidium pulchellum* pieces increased with irradiance up to 130 µmol photons m^{-2} s^{-1}; Gal-Or & Israel (2004) recorded maximal growth rates for *P. capillacea*, using a gradient table, at 100 µmol photons m^{-2} s^{-1} with winter plants; Harb et al. (2018) reported a higher growth rate for *P. capillacea* at 300 µmol photons m^{-2} s^{-1}, as long as increasing photosynthetic pigment and protein contents.

Worth considering, however, that Stewart (1984) reported that higher levels of irradiance favored contaminants and were correlated with larger numbers of bleached *P. capillacea* thalli that did not exhibit measurable growth. This is similar to what we observed in the present study, in which the growth of the epiphyte *Feldmannia irregularis* at 150 µmol photons m^{-2} s^{-1} after 4 weeks in culture was a serious problem (growing from the base to the top of the thalli). According to Friedlander (2008) one of the biological factors that most affects *Gelidium* cultivation is indeed the growth of epiphytes. Sousa-Pinto et al. (1999) in their study on the effect of light on the growth of *Gelidium pulchellum* reported that the algae were heavily epiphytized (with cyanobacteria, fungi and algae) at the higher irradiances (240-430 µmol photons m^{-2} s^{-1}). Fungi and cyanobacteria

proliferation were inhibited with antibiotics, but green algae persisted and were only marginally contained after washing the thalli with distilled water in-between medium changes. Buschmann et al. (1997) also reported problems with Ceramialean epiphytes on *Gracilaria* and observed that their abundance increased significantly from the apical (new tissues) to the central parts of the thalli (older tissues). Probably the lower load of epiphytes observed on the apical tips portions of *P. capillacea* in the present study is related to the new age of this tissue, but could be also related with the adopted cleaning procedures. From the known cleaning methods (e.g.: i) cleaning the algae with a soft brush; ii) wash them with distilled water; iii) using pre-cleaning solutions, such as sodium hypochlorite, Betadine 10%, antibiotics) used by several authors (see Salinas 1991; Sousa-Pinto et al. 1999; Redmond et al. 2014), the one adopted in the present study (pre cleaning followed by the use of Betadine 10%) seemed to be effective for some epiphytes identified in the preliminary experiments with the tip portions (TP).

The obtained values for the culture medium pH in all experiments suggest the algae were not CO_2 limited (in accordance to Lignell & Pedersén 1989; Hargreaves 1998; Mercado et al. 2001).

Altogether, the adopted combination of irradiance, temperature, nutrients and water aeration in the present study was not the best option for the growth of *P. capillacea* from the Azores. Nonetheless, small studies, as the present ones, are an important tool for the collection of data on the biology of the species, that could be used in future long-term experiments. Considering the economic importance of the species in the region, future cultivation approaches using different culture combinations, complemented with relevant environmental factors (e.g. pH, photoperiod, salinity) and a delicate selection of fast growing ecotypes are recommended.

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