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New Insights on the Sporulation, Germination, and Nutritional Profile of *Gracilaria gracilis* (Rhodophyta) Grown under Controlled Conditions

Marta V. Freitas, Teresa Mouga *, Ana Patrícia Correia, Clélia Afonso and Teresa Baptista

MARE—Marine and Environmental Sciences Centre, Instituto Politécnico de Leiria, Edifício Cetemares, Av. Porto de Pesca, 2520-641 Peniche, Portugal; marta.freitas@ipleiria.pt (M.V.F.); ana.c.correia@ipleiria.pt (A.P.C.); clelia@ipleiria.pt (C.A.); teresa.baptista@ipleiria.pt (T.B.)

* Correspondence: mougat@ipleiria.pt; Tel.: +351-262-783-607

Abstract: The red seaweed *Gracilaria gracilis* is a widely cultivated species known for its high agar content. It is also an important source of proteins, minerals, and vitamins. The chemical profile of seaweed depends on the cultivation methods used and the growing conditions to which they are exposed. Thus, two independent methods of sporulation and germination were tested upon *Gracilaria gracilis* grown in controlled conditions. During the tests, different substrates, culture media and incubation times were tested to induce cystocarp maturation. The results showed that cystocarp maturation and spore release were successful, with a visible volume increase and format change in the protruding cystocarps. Furthermore, the process of maturation to germination was accomplished, fulfilling the complete life cycle. In parallel, the nutritional profile of the biomass obtained was evaluated and compared with the nutritional values of biomass collected from the environment. Results showed no significant differences between wild specimens and cultivated ones in organic matter, ash content, lipid content, carbohydrates, or phycocolloid content. The present work, therefore, presents two simple alternative methods with potential applications in start-ups aimed at the cultivation of seaweed. Through these methods, it is possible to obtain biomass with nutritional characteristics similar to those obtained in the wild.

Keywords: Rhodophyta; sporulation; germination; life cycle; biochemical profile; seaweed culture

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1. Introduction

Gracilaria sp. is a highly sought after agarophyte with great economic potential that has been explored as raw material by worldwide agar industries. These industries heavily depend on global landings of the red seaweed *Gelidium*, a Rhodophyta with agar quality and yield far superior to that of any other seaweed species. Nevertheless, this species cannot be economically cultivated, which forces industry to rely solely upon natural resources. Thus, the global industry of bacteriological and technical agar suffers constraints due to the collapse of *Gelidium* landings and restrictive export quotas, increasing dependence on resource management [1] or exploration of alternative agarophyte species such as *Gracilaria* sp.

Additionally, *Gracilaria gracilis* holds ample nutritional and nutraceutical value, shown by research published worldwide throughout the last decade [2–9]. This further accounts for the attention and subsequent exploration efforts centered on this particular seaweed. Specifically, *Gracilaria gracilis* has a high protein content (values as high as 45% dw) and a low lipid content, coupled with a low ω-6/ω-3 ratio and high content of arachidonic acid (PUFA ω-6) [3]. *Gracilaria gracilis* is also a valued source of the red pigment R-phycoerythrin [3,8] which, upon extraction and purification, is applied as a natural colorant and fluorescent probe with numerous applications in the food, cosmetic, and pharmaceutical industries [10]. *Gracilaria gracilis* extracts have shown a high phenol and flavonoid

content, exhibiting antioxidant activity [6] and considerable antibacterial activity against a number of infectious agents, e.g., strains from the genus *Vibrio* [2] and the species *Bacillus subtilis* [5]. It has also been shown to improve growth, health, and bacterial resistance in aquaculture fishes [11].

Native populations of *Gracilaria* sp. present high growth rates, but are no match for the extensive overharvesting they have suffered due to ever-growing market demands. This is a situation shared by *Gelidium* [12,13]. However, *Gelidium* is a challenging species to cultivate, with just a number of studies found in the literature, e.g., the experimental trials of *Gelidium corneum* (as *G. sesquipedale*) culture in chemostats [14,15]. In contrast, *Gracilaria* species can be easily cultivated, which has led to an increasing investment in development of improved targeted culture techniques [16,17]. Indonesia, China, and Chile have adopted this solution and currently lead the industry in agar manufacturing, a feat they have achieved through in- and offshore cultivation of gracilarioids [18].

Portugal has a long history of exploration of raw seaweed material for their agar industries. Recently, species such as *Gracilaria* sp. have been locally explored in domestic seaweed industries and researched as a potential natural source of food, feed, and bioactive compounds. However, as far as our knowledge goes, *Gracilaria* sp. is not yet exported by Portugal as raw material for the agar industry.

The high regeneration ability of *Gracilaria* sp. is widely exploited in culture to fruitfully implement vegetative propagation [19,20]. New biomass is obtained from the tips of single fronds and is thus genetically identical to the parent frond. Artificial vegetative propagation may be useful when the goal is to achieve crop consistency to obtain large amounts of biomass with a specific trait [21]. However, it is ineffective, as it is labor-intensive and requires substantial amounts of biomass [20,22]. By repeatedly using the same stock, growth rates and productivity tend to decline, ultimately leading to a drop in the production of *Gracilaria* sp. commercial cultures after 2 to 3 years due to thalli aging or intensive harvest [19,20]. In opposition, spore-based culture methods require simple equipment and lesser amounts of starting material, allowing start-up culture from spore seeding and sustainably production of substantial amounts of biomass [22–24]. They also allow growers to rejuvenate cultures and recover productivity [25]. Additionally, possibly due to the result of different genotypes, thalli originated from spores have the benefit of presenting higher polymorphism than vegetative thalli—thus generating higher genetic variability and an increased ability to adapt to environmental fluctuations [23].

Furthermore, there is a general agreement that the understanding of the early developmental pattern of spores is crucial in determining the success of spore-based cultivation methods [17,24]. To perform spore-based culture, the knowledge of the complex *Gracilaria* life cycle is mandatory. *Gracilaria* sp. has a *Polysiphonia*-type three-phasic life history, presenting isomorphic alternation of diploid tetrasporophyte and haploid gametophyte generations. Upon fertilization of the female gametophyte thallus, the zygote develops a diploid carposporophyte structure that produces and releases diploid carpospores. These carpospores then develop into diploid tetrasporophytes which, in turn, undergo meiotic division and produce haploid tetraspores [26].

In light of this, the present work aimed to study two different sporulation and germination methodologies targeted to *Gracilaria gracilis* (Stackhouse) Steentoft, L.M. Irvine & Farnham 1995 (Rhodophyta, Florideophyceae, Gracilariales) from Buarcos, Portugal, thus offering baseline information on potential applications in sporeling nurseries within seaweed farms and an alternative method to vegetative propagation. A nutritional profile was assessed for the cultivated biomass, to be discussed with present findings regarding the nutritional value obtained from wild *Gracilaria gracilis* populations from Buarcos and Bom Sucesso, Portugal. The data obtained were also compared with those already published for wild populations [3,4], which are known for favorable biochemical profiles that may meet nutritional and nutraceutical demands.

2. Materials and Methods

2.1. Sampling and Acclimatization

Fronds of *Gracilaria gracilis* were harvested from Figueira da Foz: Buarcos (FFBC, 40°09′57″ N, 8°53′05″ W) (water temperature: 17 °C, salinity: 39 psu) and Lagoa de Óbidos: Braço do Bom Sucesso (LOBS, 39°24′1″ N, 9°13′11″ W) (water temperature: 15 °C, salinity: 38 psu) in Portugal during low tide and transported to the laboratory in dark, cooled boxes. In the laboratory, the collected biomass was thoroughly washed with filtered seawater and carefully cleaned afterwards by removing epiphytes, debris, and necrotic parts. Each thallus was then washed with running seawater and cleaned thoroughly to remove debris, necrotic parts, epiphytes, and other lingering organisms.

Selected samples collected from FFBC were acclimatized to laboratory conditions by exposing them to artificial light (13 ± 1 μmol m^{-2} s^{-1} white cool light, OSRAM Lumilux Skywhite) and constantly aerated seawater (35 ± 0.5 psu, 20 ± 1 °C) in 60 L plastic, open, dark containers. This acclimated biomass was prepared for the procedures and trials described in Sections 2.2–2.4. The remaining harvested biomass was stored at −20 °C to be applied in nutritional profile assessments as described in Section 2.5.

2.2. Preparation of Biological Material

After the acclimatization and tip selection step, healthy thalli (2–3 cm each) were carefully selected, isolated, and cleaned according to Yarish et al. [27]. Briefly, healthy thalli were rinsed in a series of three vessels holding sterilized seawater, followed by a quick rinse in sterilized distilled water to induce osmotic shock and remove any remaining contaminant organisms. Fragments were cut from the thalli and individually cleaned with sterilized cotton-tipped swabs. Under sterile conditions, each fragment was dragged through agar (1.0% bacteriological agar, VWR, Radnor, in 1:1 distilled water/seawater ratio) to clean out remaining contaminants. All the glassware and water used during this step and onwards were sterilized by autoclave (121 °C, 15 min), and glassware was acid washed in a hydrochloric acid solution (HCl, 15%) and rinsed with distilled water prior to autoclaving.

2.3. Assay in Petri Dishes (Assay A)

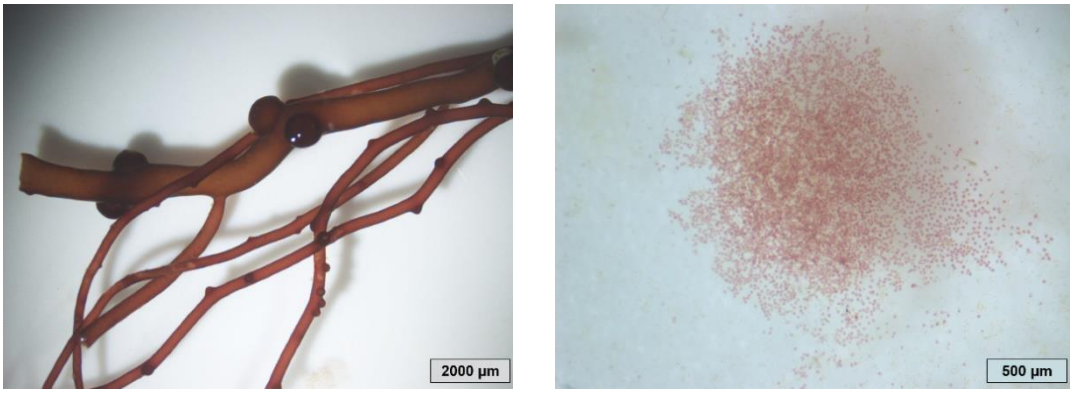
Cleaned female gametophyte fragments were randomly distributed by 20 mL Petri dishes, aiming at a count of 20 cystocarps (ca. 1.0 mm when mature), per dish, in a total of four dishes per treatment. Five treatments were tested to induce cystocarp maturation (Table 1), all of them performed in the dark in a climatic chamber set at 5 °C. Treatments A and B were adapted from Yarish et al. [27] for *Gracilaria tikvahiae*. In these treatments, the fragments were wrapped in damp paper towels and stored in plastic bags for 6 (Treatment A) and 12 (Treatment B) h. Treatments C, D, and E are all adaptations from Abreu et al. [28] for *Agarophyton vermiculophyllum* (as *Gracilaria vermiculophylla*), where thalli portions were placed in Petri dishes containing full-strength Von Stosch Enriched (VSE) culture media, adapted for red seaweed use [29], and germanium dioxide (GeO_2) (1 mL L^{-1}) to prevent diatom proliferation, for 2 (Treatment C), 6 (Treatment D), and 12 (Treatment E) h.

Table 1. Treatments adopted to induce cystocarp maturation and spore release.

Treatment	Substrate	Container	Duration
A	Moist paper	Plastic bag	6 h
B	Moist paper	Plastic bag	12 h
C	VSE + GeO_2	Petri dish	2 h
D	VSE + GeO_2	Petri dish	6 h
E	VSE + GeO_2	Petri dish	12 h

Cystocarp maturation was seen by visible volume increase and format change in the protruding cystocarps (Figure 1a). After cystocarp maturation, all samples were transferred to a climatic room set at 20 ± 1 °C, under a Grolux and daylight combo of

20 ± 0.5 µmol photons m^{-2} s^{-1} with a photoperiod set at 16:8 L:D, to induce spore release. Cystocarps in plastic bags (A and B) were transferred to Petri dishes containing seawater supplemented with VSE. After one week, the number of spore agglomerates were counted on a stereomicroscope (Stemi 2000-C, Zeiss, Oberkochen, Germany), as the abundance of spores made direct count impossible. The criteria adopted to quantify agglomerates defined an agglomerate as any group of spores large enough to be easily identified by the naked eye, corresponding to at least 100 spores. Each of these was marked as an agglomerate (Figure 1b). Quadruplicates were performed for each assay.



(**a**) (**b**)

Figure 1. (**a**) Detail of *Gracilaria gracilis* female gametophyte showing mature (large and protruding) and immature (smaller) cystocarps, scattered across the thalli, and (**b**) example of a gathering of *Gracilaria gracilis* carpospores shed by a matured cystocarp, referred to as agglomerate in the present work.

2.4. Assay in Erlenmeyers (Assay B)

The current assay was aimed at testing whether it was possible to induce maturation, sporulation, and germination in a simple, all-in-one procedure. After acclimatization and tip selection, healthy thalli (2–3 cm each) were carefully selected, isolated, and cleaned according to Yarish et al. [27], as described above. Ten fragments were randomly selected and cut, bearing either one cystocarp each (corresponding to female gametophytes) or bearing no cystocarps. The latter did not present any reproductive structures, corresponding to either male or immature female gametophytes or immature tetrasporophytes, all of which are isomorphic. Afterwards, tips were individually placed in previously sterilized Erlenmeyer flasks filled with seawater (75 mL, 35 psu) with the modified VSE nutrient media for red seaweeds [29]. GeO_2 (Germanium dioxide) was added to the medium (1 mL L^{-1}) to prevent the growth of epiphytic diatoms. Flasks were placed on a bench orbital (VWR) at 100 rpm at room temperature (20 ± 2 °C), then placed under indirect natural sunlight. A net was placed above the system to protect the material from strong radiation. Light intensity was measured at various times of day, with values shifting from 3.45 µmol m^{-2} s^{-1} (measured in the first hours in the morning under a heavily clouded sky) to 40.71 µmol m^{-2} s^{-1} (measured at mid-day with a clear sky) (Illuminance Meter T-10, Konica Minolta, Tokyo, Japan). Fragments were examined daily to check for any signs of stress, which would be marked by the loss of red color, hard consistency, or visible contaminations. After one month, all setups were observed under a stereomicroscope (Stemi 2000-C, Zeiss, Oberkochen, Germany) to check for released and germinated spores. Assay was considered successful whenever visible spore germination was seen, and these were transferred to renewed culture media. Thalli showing small reddish spots were identified as tetrasporophytes carrying tetraspores, and the whole thalli were also transferred to new Erlenmeyers. Contaminated thalli, or thalli with neither cystocarps nor tetraspores, were discarded. Culture media was renewed monthly until the new germlings from successful germinations reached 3 to 5 mm length. From this point onwards, these

thalli were transferred and scaled-up to flat-bottom flasks (from 250 mL up to 5000 mL) with constant aeration, to perform the scale-up and to set up a culture system for *Gracilaria gracilis* indoors for future analyses and assays.

2.5. Nutritional Profile of Cultured Biomass

Fresh amounts of biomass—randomly harvested from the ongoing *Gracilaria gracilis* culture systems—were tested in order to build a simple nutritional profile for these exclusively laboratory-grown algae, along with the wild samples collected from LOBS and FFBC. Biomass was applied either fresh (for organic matter, ash, and vitamin C content) or dried at 25 °C for 48 h (FD115 Binder, Tuttlingen, Germany) and ground to powder (for all other analyses). All biochemical assessments were performed in triplicate.

2.5.1. Moisture, Organic Matter, and Ash Determination

Moisture, organic matter, and ash determination followed AOAC methods [30]. Fresh biomass was dried in an oven (Binder, FD115) at 105 °C for 48 h, and then left to cool and dry in a desiccator until a constant weight was achieved. Ash content was determined by heating the biomass sample in a muffle furnace (Nabertherm, B170) at 525 °C for 5 h, before being allowed to cool in a desiccator until a constant weight was achieved.

2.5.2. Determination of Crude Protein

Nitrogen determination followed the Kjeldahl method [31]. Samples were processed in digestor (Digestor2006, Foss, Hillerød, Denmark) and distilling (Kjeltec2100, Foss, Hillerød, Denmark) units. The crude protein content was estimated by multiplying the values of organic nitrogen content by a conversion factor of 4.59 [32]. Protein content was calculated in % of dry weight (% dw).

2.5.3. Lipid Content

Lipid extraction and quantification was performed according to Folch [33]. Briefly, 1 g of dried biomass was homogenized for 5 min in 10 mL of chloroform and methanol solution (2:1 v/v). The mixture was then cleaned with a NaCl solution (0.8 %) and the chloroform phase filtered through a separatory funnel with sodium sulphate. Extraction of the water phase was performed one more time with chloroform, and both chloroform phases were combined after filtration. The solvent was then removed on a rotary evaporator, and the lipidic content was weighted. Lipid content was calculated in % of dry weight (% dw).

2.5.4. Carbohydrate Content

The total carbohydrate determination followed the phenol/sulphuric acid method of Dubois [34]. Extraction was performed with 5 mL of hydrochloric acid solution (HCl, 2.5 N) added to 100 mg of dried seaweed and incubated at 100 °C for 3 h. After cooling and neutralization with sodium carbonate, distilled water was added until a total volume of 100 mL was reached, followed by centrifugation at 3500 rpm for 8 min. To the supernatant, 1 mL of deionized water was added until a total volume of 1 mL was reached, followed by the addition of 1 mL of phenol solution (5%) and 5 mL of sulfuric acid (H_2SO_4, 96%). Each sample tube was then vortexed and left to rest for 10 min before being incubated in a water bath at 25–30° C for 20 min. The absorbance was then read at 490 nm with a UV-visible spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA, USA). A solution of glucose (0.1 mg mL^{-1}) was used as standard to calculate carbohydrate content, expressed in % of dry weight (% dw).

2.5.5. Vitamin C Quantification

Vitamin C determination was performed by titrimetry, as described in AOAC methods [30], in the presence of metaphosphoric acid, which is a fundamental stabilizing agent that minimizes oxidation rates. A solution of ascorbic acid (1 mg mL^{-1}) was used as

standard. Vitamin C content was calculated in milligrams of ascorbic acid per gram of seaweed dry weight (mg g^{-1} dw).

2.5.6. Phycocolloid Extraction

Methods of aqueous extraction of phycocolloids followed Pereira et al. [35] and Pereira [36]. Briefly, 1 g of dry sample was rehydrated with 1.5 mL distilled water to eliminate the hydro/soluble fraction and then immersed in 150 mL distilled water at 80 °C for 4 h (aqueous extraction). Hot filtration under suction was then performed twice, followed by phycocolloid precipitation through three cycles of freeze/thawing processes, with removal of the water fraction between cycles. The phycocolloid precipitate was dried at 60 °C for 12 h and then weighed. Phycocolloid content was calculated in % of dry weight (% dw).

2.6. Data Analyses

The maturation assays were performed considering $n = 4$. A one-way analysis of variance (ANOVA) was performed upon treatments successful in inducing spore release, heralded by homogeneity of variances validation using IBM SPSS Statistics 25. ANOVA was also performed upon the nutritional profile data, preceded by homogeneity of variances validation; whenever this validation was not fulfilled, the non-parametric test of Kruskal–Wallis was performed instead. Differences were considered significant at p-value < 0.05. Data were expressed as mean ± standard deviation. These statistical analyses were performed in IBM SPSS Statistics 27.

3. Results

3.1. Assay in Petri Dishes (Assay A)

In the present study, carpospore maturation was performed in Petri dishes in the dark, and successful carposporangia maturation was visually confirmed in all treatments. Agglomerates formed by spore release, however, were only observed in the dishes exposed in the dark from 6 to 12 h; after two hours exposure in the dark, no spores were seen. Thus, regardless of the positive maturation in all assays, only Treatment A, D, and E successfully achieved spore release, with no statistical differences found between agglomerate counts after one week (Kruskal-Wallis, $p > 0.05$). Treatment D had the highest agglomerate count (56.50 ± 18.48), followed by treatment A (49.50 ± 33.91). Treatment E had the lowest agglomerate count (22.25 ± 9.54). Treatment D (6 h in the dark, with VSE medium, at 5 °C) had the highest agglomerate count (56.50 ± 18.48), and was seemingly the most promising, not only for the higher number of released spores (although not statistically significative), but also because the methodology was simpler, involving maturation in VSE culture media. That method does not require any additional steps after maturation. Moreover, successful maturation in only six hours would be useful if the intention were to quickly obtain a high number of spores. Treatment A, on the other hand, requires further handling and transfer of matured cystocarps from the moist paper into the Petri dishes for spore release, and such manipulation may accidentally damage the thalli or cause contamination in the already stressed individuals. During the current assay, germination was achieved in Petri dishes that remained undisturbed for at least 17 days after counting the agglomerates. However, only a few numbers of tetrasporophyte germlings were obtained, which prompted the need to refine the follow-up methodology and germination conditions to increase these numbers.

Generally, during sporulation methodologies, authors work with readily matured cystocarps [16,17,24], which may prevent the prospect of working from spores at earlier stages of development, especially when fertilized female gametophytes might not be available in nature all year round. Sporulation in marine seaweeds is affected by external condition changes such as irradiation, photoperiod, salinity, and temperature, which trigger specific acclimation and adaptation responses by algae, including vegetative and reproductive events [37]. Thus, we hypothesize that placing the cystocarps in the dark

and lowering the temperature accelerated the maturing process may have triggered cell communication events, leading towards maturation.

Treatments B and C yielded no spore release after one week and were therefore ineffective methods to induce sporulation of *Gracilaria gracilis*. Reasons for this failure may lie in the methodology adopted for each treatment. Treatment B lasted 12 h in moist paper, which was not the ideal substrate in which to keep the seaweed. Meanwhile, Treatment C lasted for only two hours, which may not have been enough time in the dark to induce a complete cystocarp maturation.

As mentioned, although spore agglomerates were quantified by the seventh day, the first spore discharges were observed between two and three days for all the successful assays (Treatments A, D, and E) (Figure 2). Considering the high number of agglomerates compared to cystocarps, and the fact that Petri dishes were stirred once per day, it can be assumed that each cystocarp had more than one spore release episode. There does not seem to exist a general agreement regarding carpospore outputs within the *Gracilaria* genus.

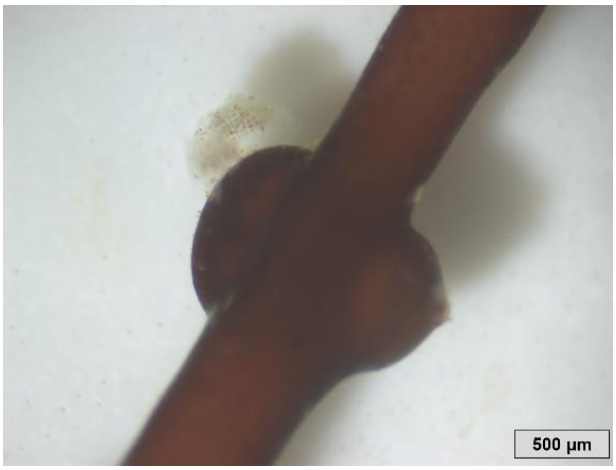


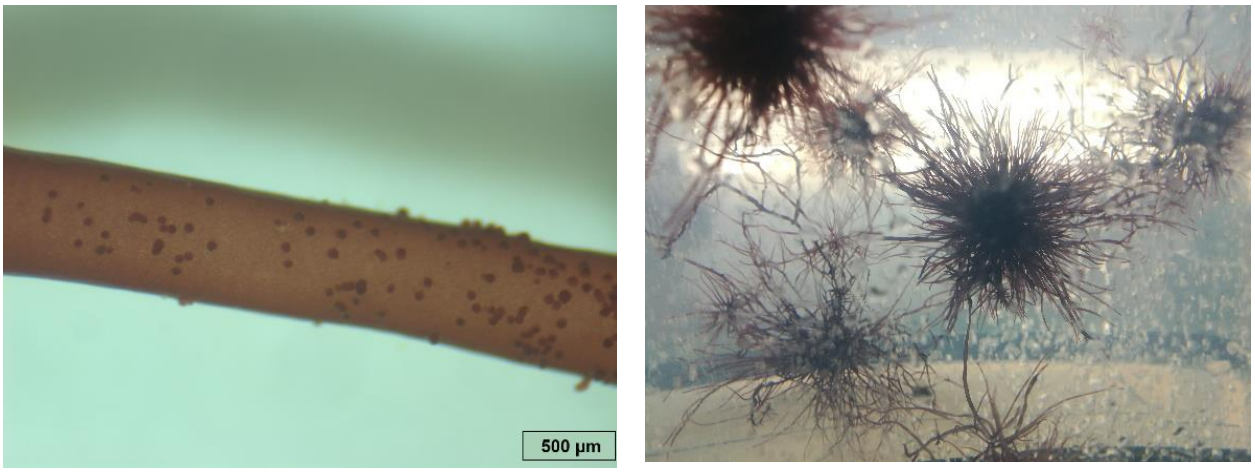
Figure 2. First stage of *Gracilaria gracilis* sporulation event. Spores are being released from the left cystocarp.

3.2. Assay in Erlenmeyers (Assay B)

After 44 days in the Erlenmeyer setups, a series of red spots were detected in the selected tetrasporophyte thalli of three Erlenmeyers. These were visually confirmed as tetrasporangia on the surface of thalli (Figure 3a). Throughout the following months, a few of these sporelings remained attached to the surface of the parental tetrasporophytes, growing as gametophytic thalli on a single diploid thallus (Figure 3b). This phenomenon was also detected in *Gracilaria tikvahiae* [38], *G. debilis* [39], and *G. gracilis* [40].

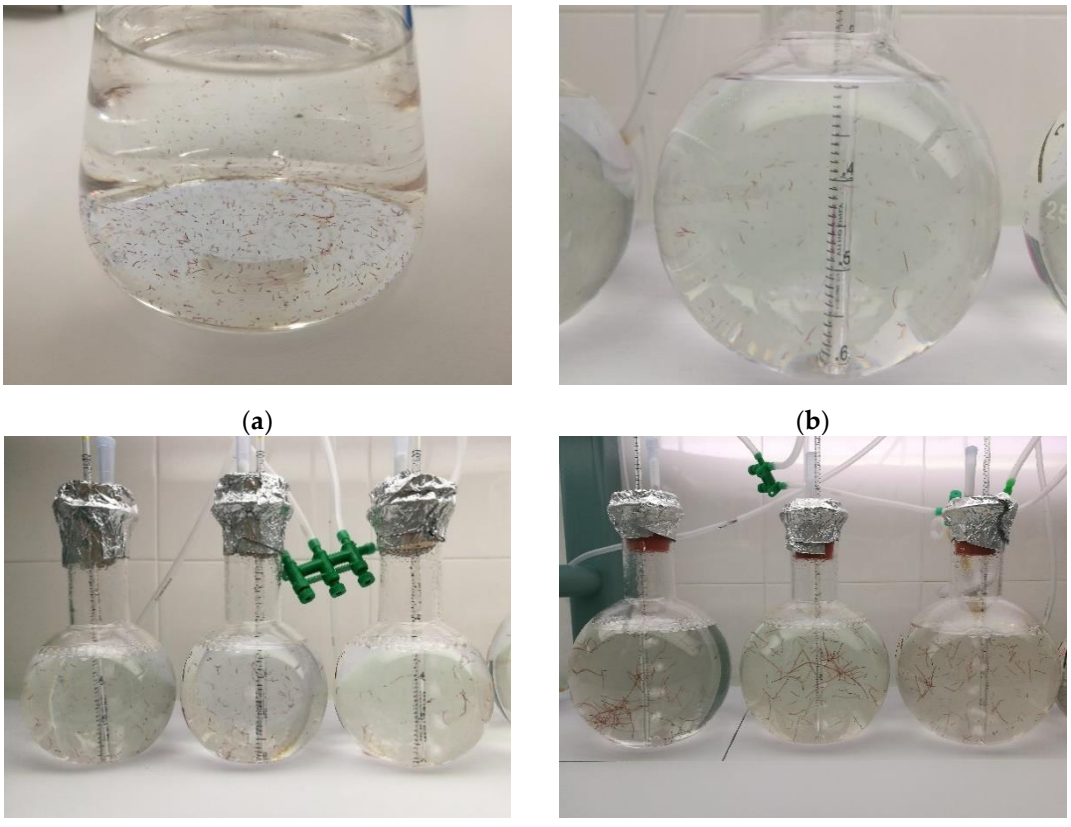
In all Erlenmeyer systems carrying one thallus bearing one cystocarp each, spore release was observed. On the four remaining Erlenmeyer setups, no spores or spots were observed, possibly because of young tetrasporophyte thalli that did not undergo meiotic division or male gametophytes.

The germlings obtained in the present work—either carpospore-derived tetrasporophytes or tetraspore-derived gametophytes—were used to implement start-up indoor cultures of *Gracilaria gracilis* with progressive scale-ups following growth (Figure 4).



(a) (b)

Figure 3. (**a**) Tetraspores distributed throughout a parental tetrasporophyte and (**b**) sporelings growth and development, attached to parental tetrasporophyte thalli after seven months in culture.



(a) (b)

(c) (d)

Figure 4. *Cont.*

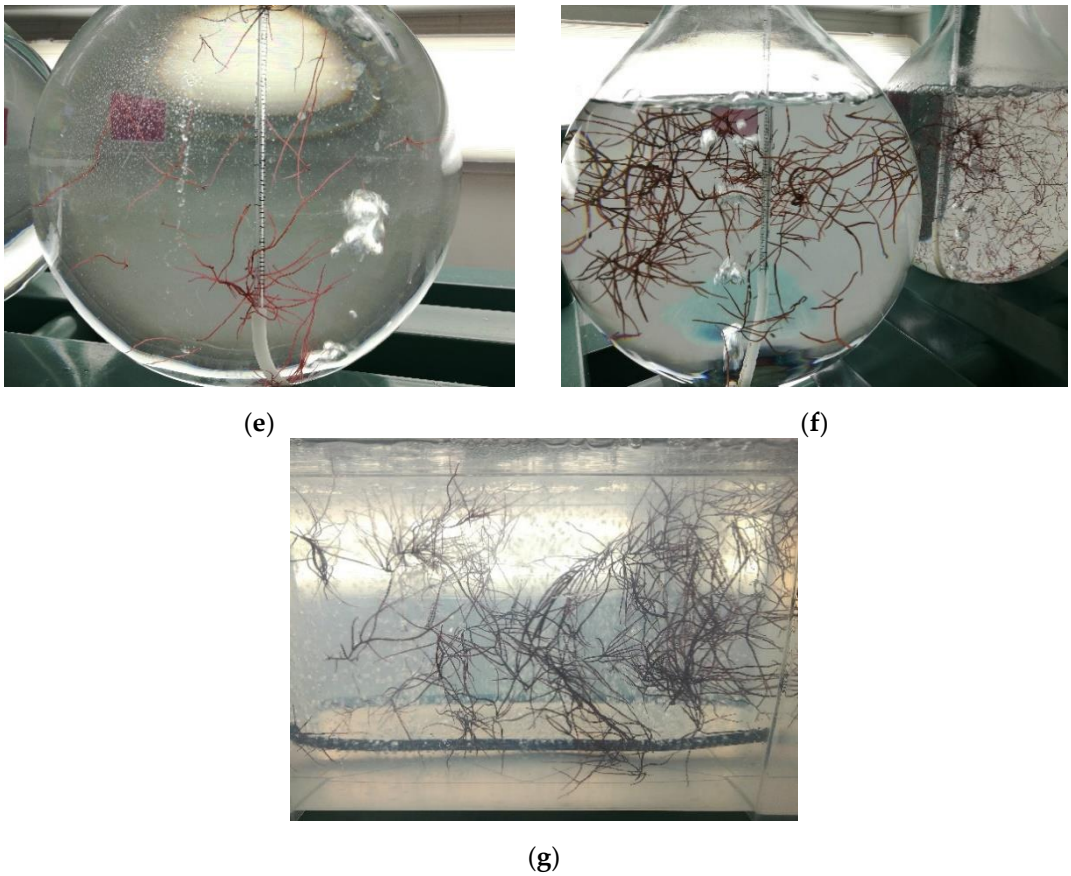


Figure 4. Growth and development of *Gracilaria gracilis* sporelings throughout six months in culture, showing biomass with (**a**) one month, (**b**) two months, (**c**) three months, (**d**) four months, (**e**) five months, and six months in (**f**) flat-bottom flasks (100 mL, 250 mL, 500 mL, 1 L, 2 L and 5 L, respectively), and (**g**) tank (50 L).

3.3. Nutritional Profile of Cultured Biomass

Table 2 displays the nutritional profile of indoor-grown *Gracilaria gracilis*, assessed 12 months after its germination, and from the wild *G. gracilis* populations, harvested from FFBC and LOBS.

Table 2. Nutritional profile of indoor grown *Gracilaria gracilis* 12 months after germination, and from natural populations harvested from Figueira da Foz: Buarcos (FFBC) and Lagoa de Óbidos: Bom Sucesso (LOBS). Results are expressed as mean ± standard deviation (n = 3). ^{a,b} and ^{c} stand for statistically significant differences between samples.

Parameter	Mean ± SD		
	Culture	FFBC	LOBS
Moisture (% fw)	82.04 ± 1.14 ^{a}	77.81 ± 1.59 ^{b}	72.93 ± 1.71 ^{c}
Organic matter (% dw)	75.66 ± 0.33 ^{ab}	81.04 ± 2.58 ^{a}	72.85 ± 3.69 ^{b}
Ash (% dw)	24.34 ± 0.33 ^{ab}	18.96 ± 2.58 ^{a}	27.15 ± 3.69 ^{b}
Total protein (% dw)	21.58 ± 0.10 ^{a}	11.80 ± 0.36 ^{b}	14.20 ± 0.98 ^{ab}
Lipid content (% dw)	1.21 ± 0.02	1.38 ± 0.05	1.40 ± 0.31
Carbohydrates (% dw)	38.35 ± 0.74	40.72 ± 2.69	44.12 ± 5.32
Vitamin C (mg g^{-1} dw)	0.306 ± 0.04	0.18 ± 0.21 ^{a}	0.27 ± 0.38
Phycocolloids (% dw)	18.51 ± 8.10	20.11 ± 3.76	17.62 ± 2.77

Regarding the moisture content, values ranged from 72.02 ± 1.49% fw to 77.81 ± 1.59% fw for wild populations, while a slightly higher value was observed for cultured samples

(82.04 ± 1.14% fw). Significant differences were found between all considered samples (ANOVA, $p < 0.05$), namely between the wild populations from FFBC and LOBS (ANOVA, Tukey, $p = 0.004$), and between the cultured *Gracilaria gracilis* and its wild counterpart from FFBC (ANOVA, Tukey, $p = 0.02$) and LOBS (ANOVA, Tukey, $p < 0.05$).

Concerning the organic matter, the value obtained for the cultivated *Gracilaria gracilis* (75.66 ± 0.33% dw) was lower than that observed for FFBC populations (81.04 ± 2.58% dw), but higher than that observed for LOBS populations (72.85 ± 3.69% dw). Naturally then, the ash content follows the same pattern, but inverted. For both organic matter and ash content values, significant differences were found between both wild populations of FFBC and LOBS (ANOVA, Tukey, $p = 0.10$), but not between each wild population with the cultured biomass (ANOVA, Tukey, $p > 0.05$).

The protein values obtained for cultured *Gracilaria gracilis* (21.58 ± 0.10% dw) were higher than those obtained from wild sources, differing significantly from FFBC wild counterparts (11.80 ± 0.36% dw) (Kruskal-Wallis, $p = 0.02$). Interestingly, the protein content of wild populations of LOBS (14.20 ± 0.98% dw) did not differ significantly from that of cultivated *G. gracilis* nor from that of FFBC (Kruskal-Wallis, $p > 0.05$).

Regarding lipid content, values among all samples considered were low, ranging from 1.21 ± 0.02% dw from cultured biomass, to 1.40 ± 0.31% dw from LOBS wild populations. Although the lipid content from cultured *Gracilaria gracilis* was slightly lower than its wild counterparts from both FFBC and LOBS, significant differences were not found between them (ANOVA, $p > 0.05$).

As for carbohydrates, cultivated *Gracilaria gracilis* presented a value of 38.35% dw, while the wild populations from FFBC and LOBS presented higher values (40.72 ± 2.69% dw and 44.12 ± 5.32% dw, respectively). However, significant differences were not found in the carbohydrate content between cultured and wild populations (Kruskal-Wallis, $p > 0.05$).

Cultivated *Gracilaria gracilis* was found to have a higher vitamin C content (0.31 ± 0.04 mg g^{-1} dw), than those of the wild populations from FFBC and LOBS (0.18 ± 0.21 mg g^{-1} dw and 0.27 ± 0.38 mg g^{-1} dw, respectively), with significant differences found between all samples considered (ANOVA, $p = 0.04$).

Finally, the phycocolloid content from cultivated *Gracilaria gracilis* was 18.51% dw, while the wild populations from FFBC and LOBS were 20.11 ± 3.76% dw and 17.61 ± 2.77% dw, respectively. No significant differences were found for any of the samples considered (ANOVA, $p > 0.05$).

4. Discussion

Regarding sporulation, all treatments presented spore maturation, but only those with 6 h of darkness and 12 h of darkness in Petri dishes (Treatment A, D, and E) had obtainable spore discharge. Still, very heterogeneous counts were achieved for spore agglomerates, which likely suggest that an even higher number of replicates must be considered in the future, in order to pinpoint the best cystocarp maturation procedure with certainty. Continuous darkness also promoted higher spore discharges in *Gracilaria foliifera* [41], *Gracilaria corticata* [42], and *Gracilariopsis lemaneiformis* [43]. Santelices [44] mentioned that the influence of light and darkness upon spore release remains unclear, although low light seems to increase spore release. Kain and Destombe [26] stated that different gracilarioid species show peak discharges at separate times.

A few authors reported that, for the majority of *Gracilaria* species studied, maximum carpospores discharge occurs within the first three to four days, and afterwards quickly declines; this was observed in *Gracilaria corticata* [45], *Crassiphycus corneus* (formerly *Gracilaria cornea* [46], *Gracilaria pacifica* [47], and *Gracilaria dura* [24]. For *Gracilaria gracilis*, Lefebvre et al. [48] saw rhythmic release for about a month, but Michetti et al. [16] reported a maximum number of carpospores released by day 7, with 70% of carpospores released within the first two weeks. All these inconsistencies in spore discharge patterns can be explained by species and population origins [46], phenological and physiological differences among species, and the diversity of methods employed to induce sporulation [24].

As to the life cycle of *Gracilaria gracilis*, all steps were achieved in the laboratory, in seven to eight months. It was possible to identify a few fronds with thalli bearing tetrasporangia, as well as thalli corresponding to female gametophytes with cystocarps along the thalli. Nevertheless, it was a slow process, with sporelings taking months to reach lengths on the order of mm. This was in agreement with other works, e.g., Michetti et al. [16], who also obtained sporelings reaching around 1.5 mm after two months of culturing. Other authors have also completed *Gracilaria* sp. life cycle in culture, namely *Gracilariopsis longissima* (as *Gracilaria verrucosa*) [49,50], and *Agarophyton vermiculophyllum* [28] within variable, yet similar, timeframes. This method was achieved using basic laboratory tools and equipment, a lean approach to germination methods that was partially or exclusively designed for outdoor spaces [25,51,52]. It required large-scale tanks right from the start, supplementary material, and occasionally, readily-available coastal areas.

Considering the nutritional profile of the cultivated biomass of *Gracilaria gracilis* the moisture, organic matter and ash content were similar to those found in the literature for wild biomass. The ash value obtained in the present study closely matched those obtained for other gracilarioid species (namely *Gracilaria dominguensis* and *Gracilaria birdiae* [53]). The ash value was also substantially higher than that obtained by Rasyid et al. [7], who reported values of 6.78% dw for Indonesian *Gracilaria gracilis* populations, and by Rosemary et al. [54] for Indian *Gracilaria corticata*, and *Gracilaria edulis* (8.10 and 7.36% dw, respectively). The ash proportion present in food and feed sources may contain essential microelements for human and animal health [55], and the presently-cultivated *Gracilaria gracilis* may be no exception to this. Literature also refers to an organic matter content of 67.21% dw and an ash content of 24.8% dw for wild *Gracilaria gracilis* collected from FFBC [4].

The protein values obtained for cultured *Gracilaria gracilis* were appreciable and comparable to those found in the literature, namely for wild *G. gracilis* from FFBC (20.2% dw) [4], worldwide [3,7], and red seaweeds in general [4]. Rosemary et al. [54] obtained similar values of protein from *G. edulis* and *G. corticata* (25.29 and 22.84% dw, respectively). Rasyid et al. [7] obtained substantially lower values for wild *Gracilaria gracilis* collected from Indonesia (10.86% dw), and Debbarma et al. [56] reported a 14.26% dw of protein content for *G. edulis* from India. Noticeably higher protein values were found in *Porphyra tenera* [57] and *Palmaria palmata* [58], whereas noticeably lower values were reported on occasion for several other red seaweed species [59]. It is known that environmental conditions heavily shape protein content in seaweeds [60]. Still, despite variation across taxa, the generally high protein content—and multiple associated bioactivities [61]—gives seaweeds relevance as health promoters, nutraceutical agents, and as a healthy and nutritious gastronomic ingredient [62].

The low lipid content obtained from the cultivated *Gracilaria gracilis* (1.20% dw) matched the published values in the literature, e.g., for wild *G. gracilis* from FFBC (0.60% dw) [4], worldwide [3,7] and seaweeds in general [4,56,61,63,64]. Exceptions to this rule, however, can be also found across published works—for example regarding *Gracilaria corticata* and *Gracilaria edulis* [54]. Nonetheless, as pointed by the authors, fat content in *Gracilaria* can diverge across species and source. Generally, according to Lordan et al. [65], seaweeds are poor energy providers, due to the particularly low lipid content observed across species.

Cultured samples presented lower values of carbohydrates than those reported from previous authors, such as those obtained for FFBC wild *Gracilaria gracilis* populations (46.6% dw) [4]. Values obtained were also substantially different than other examples found in the literature, where reports of a carbohydrate content as high as 63.13% dw for wild *G. gracilis* [7] or as low as 4.71% dw for *Gracilaria edulis* [54] were found. These shifts might be explained by factors such as salinity, sunlight intensity, and temperature, which shape geographic locations in different ways [66].

The vitamin C content in the cultured *Gracilaria gracilis* samples was found to be remarkably lower than what was obtained by Rosemary et al. [54] for *Gracilaria corticata* and *Gracilaria edulis* (14.66 and 13.41 mg g^{-1}, respectively). Vitamin content in seaweeds

varies considerably throughout the literature, which can be explained by a myriad of factors, e.g., algal species and growth stage [67], geographical region, environmental factors such as temperature, salinity, seasonality, and light availability [54,67,68], and differences in the preservation and processing methods adopted by each author [54,68]. Nevertheless, seaweeds are a good source of vitamins, regarded highly for their biochemical functions, antioxidant activity, and other health benefits. In particular, the water-soluble vitamin C in seaweed plays a role in decreasing blood pressure and reducing the risk of cancer [67].

Finally, the phycocolloid content from cultivated *Gracilaria gracilis* was found to be markedly higher than that obtained by Pereira [36] from FFBC populations of *G. gracilis* and *Gelidium pulchellum*, (11%) (respectively 8 and 11% dw, both also extracted by aqueous approach), but lower than *Gracilaria multipartita* (37% dw, aqueous extraction). By comparison, cultivated *Gracilaria gracilis* seemed to offer an appreciable phycocolloid content. Present results additionally showed that cultivated *G. gracilis* had a higher content than its wild counterpart, although this could hypothetically change according to the harvest season for the latter.

Considering the few differences found in nutritional assessments performed from wild *Gracilaria gracilis* from different geographical locations, care must be given when comparing such results to those obtained from cultured *G. gracilis* in the present study. We postulate that geographic location and harvest season significantly account for the variations found in the literature. It also must be stressed that the harvesting period and season are important points to be taken into careful consideration when comparing wild to cultured *Gracilaria gracilis* harvested from the same geographical location. This gracilarioid presents high plasticity to environmental conditions, with resulting shifts in its nutritional composition.

5. Conclusions

The present work offers two methods that are essential steps to develop *Gracilaria gracilis* cultures from spores: one method to induce cystocarp sporulation and the other to induce spore germination. We believe the two distinct methods have potential applications to the expanding commercial aquaculture of seaweed due to the simplicity of the set-up and the methodology applied. Each can be further developed and adapted according to each commercial seaweed farm's demands. Regarding sporulation, the proposed methodology to induce cystocarp maturation prior to sporulation allows the user to consider working with immature cystocarps right away instead of waiting for their natural development, thus speeding up the process (to up to three days from spore maturation induction to spore release). This will increase the number of spores obtained within any given timeframe, and consequently, will improve biomass earnings and overall profit on a commercial perspective. The fact that germination was achieved under natural light and room temperature allows for energy savings in the long term. If the proposed methodologies were adopted as routine in seaweed aquaculture, the extra costs of climate chambers and artificial lights could be subtracted from the production expenses. It is also possible to adopt this method to grow either haploid or diploid individuals, or a combination of both.

Finally, the biomass obtained from the exclusively laboratory-grown *Gracilaria gracilis* had a similar nutritional profile to that obtained from wild populations. Thus, it is hereby proposed that the present methodologies should be adapted to commercial set-ups. In this way, valuable nutritional biomass can be cultivated instead of harvested, ensuring ecological sustainability by preserving natural populations. The laboratory-grown *Gracilaria gracilis* offers a nutritional profile that recommends its consideration as a functional food; it is packed with high protein and carbohydrate contents, coupled with low lipid values. This is a combination that can also be found in conventional food sources that have been universally endorsed as functional foods that boost human health and well-being.

Our findings, thus, highlight cultivation methods in a small-scaled laboratorial setting, as opposed to large-scale set-ups performed outdoors. However, the scale of the methods described herein do not preclude its adaptation into large outdoor setups with mass

cultivation of high-quality seaweed in mind. From a functional food perspective, next steps should include the enrichment of our seedstock through manipulation of culture conditions, such as light source and intensity, temperature, and nutrient media, in order to enhance its nutritional profile.

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