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ARTICLE

Indoor culture scaling of *Gracilaria chilensis* (Florideophyceae, Rhodophyta): The effects of nutrients by means of different culture media

Escalada de cultivo en interiores de *Gracilaria chilensis* (Florideophyceae, Rhodophyta): Efectos de los nutrientes por diferentes medios de cultivo

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Resumen.- *Gracilaria chilensis* se distribuye en Nueva Zelanda (incluida la Isla Chatham) y Sudamérica. Esta especie tiene un ciclo de vida sexual de tres fases, así como una reproducción asexual y vegetativa, y es comercialmente importante, ya que es una valiosa fuente para producir agar. Sin embargo, los cultivos basados exclusivamente en la reproducción clonal y la propagación vegetativa han provocado una reducción de la diversidad genotípica, incrementando la susceptibilidad a la infección por epifitos y una disminución de la calidad. Por ello, es importante establecer cultivos de interior de *G. chilensis* a partir de talos, con un régimen reproductivo mixto (sexual y asexual), que mantenga la variabilidad genética de los cultivares. En este estudio, fue evaluada (como primera aproximación) la biomasa, la tasa de crecimiento y la productividad de los cultivos de interior de *G. chilensis* utilizando varios medios de cultivo [*i.e.*, medio von Stosch (VS), Basfoliar® Aktiv (BF), y mezclas de VS y BF, es decir, VS/BF-A y VS/BF-B]. El medio VS/BF-A mostró los mejores resultados en términos de biomasa (51,8 ± 3,7 g m^{-2}), tasa de crecimiento (4,55 ± 0,43 %d^{-1}) y productividad (14 g m^{-2} d^{-1}), incluyendo la ocurrencia de talos con cistocarpos. Los resultados positivos utilizando el medio VS/BF-A podrían atribuirse al hecho de que el BF proporciona diferentes elementos (K, Cu, Mo y Zn) que son cruciales, ya que están involucrados en numerosas funciones fisiológicas en las algas, y la razón N:P utilizada (1:1) ya que afecta positivamente al crecimiento y la productividad. Esta información proporciona un conocimiento actualizado fundamental sobre los cultivos de *G. chilensis* en condiciones controladas, promoviendo su cultivo exitoso con fines productivos.

Palabras clave: *Gracilaria chilensis*, cultivo en interior, crecimiento, productividad

Abstract.- *Gracilaria chilensis* is distributed in New Zealand (including Chatham Island) and South America. This species has a three-stage sexual life cycle, as well as asexual and vegetative reproduction, and is commercially important as it is a valuable source for producing agar. However, basing the crops exclusively on clonal reproduction and vegetative propagation has led to a reduction in their genotypic diversity, an increase in their susceptibility to infection by epiphytes, and a decrease in their quality. Thus, it is important to establish indoor cultures of *G. chilensis* from thalli with a mixed reproductive regime (sexual and asexual) that maintains the genetic variability of the cultivars. In this study, the biomass, growth rate, and productivity of indoor cultures of *G. chilensis* were evaluated (as a first approach) using various culture media [*i.e.,* von Stosch medium (VS), Basfoliar® Aktiv (BF), and mixtures of VS and BF, namely, VS/BF-A and VS/BF-B]. The VS/BF-A medium showed the best results in terms of biomass (51.8 ± 3.7 g m^{-2}), growth rate (4.55 ± 0.43 %d^{-1}), and productivity (14 g m^{-2} d^{-1}), including the occurrence of thalli with cystocarps. The positive results using the VS/BF-A medium could be attributed to the fact that BF provides different elements (K, Cu, Mo, and Zn) that are crucial, since they are involved in numerous physiological functions in the algae, and also to the N:P ratio utilized (1:1), which positively affects growth and productivity. This information provides pivotal updated knowledge regarding cultures of *G. chilensis* under controlled conditions, promoting its successful cultivation for productive purposes.

Key words: *Gracilaria chilensis*, indoor culture, growth, productivity

Introduction

In Chile, *Gracilaria chilensis* C.J. Bird, McLachlan, and E.C. Oliveira is distributed along the Chilean coast between Antofagasta (23°S) to Chiloé (43°S), finding establishments until Coyhaique (45°S) (Guillemin *et al*. 2008, Arakaki *et al*. 2015, Brito 2019). This species has a three-stage sexual life cycle characterized by an isomorphic alternation of generations, but its capacity for asexual and vegetative reproduction allows for its mass cultivation under diverse culture conditions (Yarish *et al*. 2012).

Seaweeds have been used as source of nutrients for human and animal consumption, and as commercialized compounds used for a variety of purposes in the pharmaceutical, cosmetics, and food industries (*e.g.,* Mišurcová *et al*. 2011, Wells *et al*. 2017, Latorre *et al*. 2019, Pinto *et al*. 2021). Particularly, *G. chilensis* is either used as a raw material for the extraction of agar or it is exported as dry alga. In some cases, this has led to the overharvesting of natural populations to meet commercial demands, which has triggered a decrease in the quantity and quality of the economically relevant features of the raw material (*e.g.,* agar content, Mac Monagail *et al.* 2017). Both wild and farmed populations of *G. chilensis* present relatively low genetic diversity in terms of expected heterozygosity (H_E lower than 0.4) and allele diversity (mean number of alleles lower than five) (Guillemin *et al*. 2008). This low genetic diversity can be explained mainly by a bottleneck in wild populations probably associated with the overexploitation of wild stands in the 1980s (*e.g.,* Vásquez & Westermeier 1993, Norambuena 1996, Guillemin *et al*. 2014). In addition, farming, mainly through vegetative propagation, has resulted in an alteration to its life cycle, enhancing asexual over sexual reproduction and the predominance of diploid individuals over the haploid phase (Guillemin *et al*. 2008). Genetic erosion of cultures has also occurred at the multilocus level with the domestication of *G. chilensis*. Farmed populations have retained only about one-third of the genotypic diversity observed in wild populations. Therefore, to maintain high-quality cultivars in terms of economically relevant features and high genetic diversity, it is important to include strains of *G. chilensis* with relatively high diversity (*i.e.*, in comparison to clonal farmed ones) as initial stock, and to include haploid and diploid thalli to develop a mixed reproductive (sexual and asexual) culture regime.

In particular, the cultivation of *Gracilaria* for production can be started from vegetative tips or from spores isolated from the fertile phases, by considering their haploid/diploid life cycle, and by maintaining controlled experimental conditions in order to obtain a viable biomass yield for the following cultivation stages (Alveal *et al*. 1997, Jayasankar & Varghese 2002, Yarish *et al*. 2012). Efficient indoor cultivation requires careful management of the essential factors involved to achieve a high biomass yield. A balance must be achieved among factors such as the type of culture medium, temperature regime, salinity, pH, nutrient concentration, and adequate irradiance (Titlyanov & Titlyanova 2010, Setthamongkol *et al*. 2015). For example, ammonia (NH_3), carbon dioxide (CO_2), and other nutrients and conditions in enriched culture medium ponds have a significant effect on the growth of *Gracilaria*, *Ulva,* and *Porphyra* species (Friedlander 2008). In the case of light intensity, an increase of this variable positively affects the growth of *Gracilaria gracilis*, as demonstrated by Freitas *et al*. (2019)[1], and with respect to the starting biomass used, it is necessary to start a culture considering the uptake of nutrients which depends on diverse physico-chemical factors (Roleda & Hurd 2019).

An important factor that must be considered is an adequate nitrogen/phosphorus (N:P) ratio, since this facilitates better nutrient assimilation by algae. In fact, it has been determined that an adequate N:P ratio promotes an increase in the specific growth rate of *Gracilaria lemaneiformis* (Yu & Yang 2008). Natural seawater provides many of the trace elements necessary for a successful culture, although its quantity may be insufficient. Indeed, most of the commercially available culture media contain macronutrients [nitrogen (N), phosphorus (P), potassium (K), etc.], essential micronutrients [iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), etc.], chelators, and vitamins (Yarish *et al*. 2012).

The use of salt mixtures and other components has been widely recommended in order to obtain an enriched culture medium (Andersen 2005, Yarish *et al*. 2012, Redmond *et al*. 2014). Indeed, many types of artificial media with different mixtures of components have been used, which stabilizes the seasonal fluctuations of macro- and micronutrients that seaweeds undergo in natural conditions (Berges *et al*. 2001, Andersen 2005). In fact, *Gracilaria* spp. have been successfully cultivated using modified enriched culture

[1]Freitas M, AP Correia, CR Pereira, JR Santos, TM Baptista, CN Afonso, MM Gil, A Pombo, C Tecelão, SL Mendes & TM Mouga. 2019. The influence of light and culture media on the growth of the red seaweed *Gracilaria gracilis* (Rhodophyta, Gracilariales) under laboratory conditions. IMMR'18 International Meeting on Marine Research, Peniche, Portugal, July 5-6, 2018. Frontiers in Marine Science Conference Abstract. <https://www.frontiersin.org/10.3389%2Fconf.FMARS.2018.06.00116/event_abstract>

media. For example, the cultivation of *Gracilaria verrucosa* under greenhouse conditions and using modified Johnson culture medium resulted in a relative growth rate between 0.5 and 6 %d^{-1} over a period of five months (Cirik *et al*. 2010). Using modified von Stosch medium, under optimized experimental conditions, and working with *G. verrucosa* as well, Mensi *et al*. (2011) showed that its cultivation had a growth rate of 3.2 %d^{-1}. In particular, these results showed an enhanced growth rate when the ammonium and nitrate concentrations increased from 0.01 mg L^{-1} to 2.5 and 2 mg L^{-1}, respectively. It is worth mentioning that the high cost of several of these media, particularly of those used for red seaweed cultivation, is such that their use is not a feasible option for the large-scale or mass cultivation of algae, which in most cases requires open flow culture systems.

In particular, von Stosch culture medium (VS, von Stosch 1964) is widely used because it contributes significant amounts of nitrogen (as nitrate), phosphorous (as monoacid phosphate), metals (such as Mn and Fe), and vitamins (B_{12}, B_7, and B_1). Indeed, it has been successfully used for the cultivation of the initial stages of red seaweeds such as *Palmaria palmata* (Titlyanov *et al*. 2006) and *Neopyropia yezoensis* (Ma *et al*. 2020). Another nutrient medium, Basfoliar® which has been used to promote the growth of algal species (Águila 2015, Ávila *et al*. 2018), consists of algal concentrate mixed with a series of macro- (N 3%, P_2O_5 27%, and K_2O 18%) and micronutrients (B 0.01%, Cu 0.02%, Fe 0.02%, Mn 0.01%, Mo 0.001%, and Zn 0.01%) [*i.e.,* Basfoliar® (BF) Aktiv; Hussain *et al*. 2019]. Águila (2015) used BF culture medium for the growth of *G. chilensis* thalli, stimulating its growth and obtaining a specific growth rate (SGR) of 7.86 %d^{-1} with a lower load of epiphytes versus the Provasoli culture medium (SGR, 7.60 %d^{-1}). Additionally, Ávila *et al.* (2018) utilized BF for *Chondracanthus chamissoi* growth in indoor cultures, with positive results obtained as well.

Due to the persistent necessity of exploring and deciding on the use of low cost, enriched media for indoor cultures of commercially important species, the aim of this study was to evaluate the *in vitro* effects of VS and BF media on the daily growth rate and productivity of *Gracilaria chilensis*, in order to use this knowledge for initial cultures of strains with valuable commercial characteristics.

Materials and methods

Sampling

The initial vegetative biomass was collected from a natural population at Dichato, Chile (36°32'2.44"S, 72°56'46.86"W), and transported in cool conditions using ice packs to Quintay Marine Research Center (CIMARQ), Andres Bello University, Chile. The collecting site was selected for its reported relatively high genotypic diversity in comparison to farmed populations (Guillemin *et al*. 2008), and the high quality of the biomass (personal observations). The biomass was washed using tap water and immediately acclimatized for one week in filtered seawater (1 µm). Cultivation conditions were maintained at 32.3 of salinity, a 12:12 photoperiod (L:D) with a photon flux of 150-230 µmol $m^{-2}s^{-1}$, and 24 h aeration, in outdoor tanks.

Experimental set-up

Culture media

Four culture media were utilized, each with a different composition: von Stosch (VS), Basfoliar® Aktiv (BF), and two mixtures with different proportions of VS and BF, namely VS/BF-A and VS/BF-B (Table 1). For the preparation of the enriched medium with VS (Table 1), 8 mL of the solution was added to 1 L of 0.22 µm filtered seawater. For the enriched medium with BF (Table 1), 500 µL

Table 1. Composition of the culture media used for indoor cultivation of *Gracilaria chilensis* / Composición de los medios de cultivo utilizados para el cultivo interior de *Gracilaria chilensis*

von Stosch (VS)	Basfoliar® Aktiv (BF)
$NaNO_3$ (0.0875 g N 100 mL^{-1})	Total nitrogen (4.17 g 100 mL^{-1})
$Na_2HPO_4 \cdot 12H_2O$ (0.01164 g P 100 mL^{-1})	P_2O_5 (37.5 g 100 mL^{-1}) (16.4 g P 100 mL^{-1})
Vitamin B_1	K_2O (25 g 100 mL^{-1})
Vitamin B_{12}	B (0.014 g 100 mL^{-1})
Vitamin B_7-B_8	Cu [Cu(II)-EDTA] (0.028 g 100 mL^{-1})
$FeSO_4 \cdot 7H_2O$ (0.00869 g 100 mL^{-1})	Fe [Fe(II)-EDTA] (0.07 g 100 mL^{-1})
$MnCl_2 \cdot 4H_2O$ (0.00124 g 100 mL^{-1})	Mn [Mn(II)-EDTA] (0.014 g 100 mL^{-1})
EDTA (0.0465 g 100 mL^{-1})	Mo (0.0012 g 100 mL^{-1})
	Zinc (0.014 g 100 mL^{-1})
Mass ratio N/P in the culture medium	
von Stosch (VS)	7.5:1
Basfoliar® Aktiv (BF)	1:4
VS/BF-A	1:1
VS/BF-B	1:3.5

of BF was added to 1 L of 0.22 µm filtered seawater. Two additional mixtures were made as follows (Table 1): for the VS/BF-A medium, 6 mL of VS and 42 µL of BF were added to 1 L of seawater, and for the VS/BF-B medium, 4 mL of VS and 250 µL of BF were added to 1 L of seawater. To each medium, 4 mL of GeO_2 (1 mg mL^{-1}) and 2 mL of a mixture of antibiotics (penicillin 2.5 mg mL^{-1}, ampicillin 2.5 mg mL^{-1}, and streptomycin 2.5 mg mL^{-1}) were added. Finally, a culture medium using only filtered seawater was used as control.

Preparation of biomass and scaling

Initially, 100-150 thalli from 20 different individuals in a vegetative state were randomly selected from the acclimatization culture. The material was brushed and then washed with 1.0, 0.45, and 0.22 µm of filtered seawater; then, thalli were washed with distilled water for 60 s to remove epiphytes (Fig. 1A-C). Briefly, lateral tips of approximately 0.5-1.0 cm from clean biomass were cutted and collected in a Petri dish under a laminar flow chamber and cleaned using agar plates (Fig. 1D-F). Approximately 600-800 tips were obtained.

Each culture medium (treatment) consisted of three replicates, each with 40-50 tips. Cultures were grown and maintained in 250 mL Erlenmeyer flasks at 20 ºC, with a 12:12 photoperiod (L:D) and an initial light intensity of 15-20 µmol $m^{-2}s^{-1}$ (cold-white light), with a horizontal orientation to the biomass, and without aeration for the first three weeks (Table 2, Fig. 2A).

From the third week of cultivation, an aeration system was installed to supply carbon dioxide to Erlenmeyer flasks (Table 2, Fig. 2B). Culture scaling consisted of changing culture medium, increasing light intensity, and doubling the volume of the medium each week to transfer the tips into subsequently larger glass containers according to the volume achieved (1, 2, 5, and 25 L (with 15 L of medium) bottles) (Fig. 2C-F) without GeO_2 and antibiotics. Once a total volume of 70 L (Fig. 2G) was reached, the biomass was transferred to in a single aquarium tank (Fig. 2H). The cultures were kept for eight weeks, and the mass of algal tissue for each change of medium was recorded weekly.

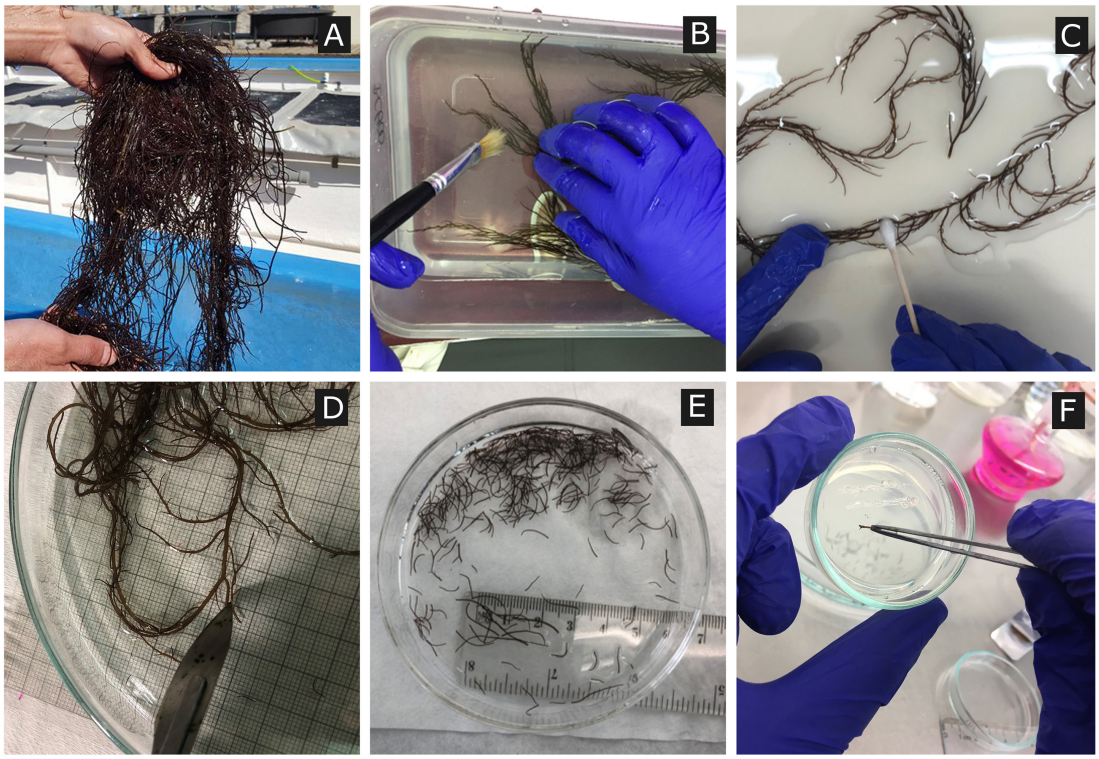

Figure 1. Separation, cleaning, and cutting of *Gracilaria chilensis*: A) selection of the biomass; B) cleaning with distilled water and filtered seawater at 1.0, 0.45 and 0.22 µm; C) cleaning with cotton wool to remove epiphytes; D) selection of the tips for cutting; E) cutting of the tips under a laminar flow chamber; F) dragging of the tips in agar to remove impurities / Separación, limpieza y corte de *Gracilaria chilensis*: A) selección de la biomasa; B) limpieza con agua destilada y agua de mar filtrada a 1,0, 0,45 y 0,22 µm; C) limpieza con algodón para remover epífitos; D) selección de los ápices para corte; E) corte de los ápices bajo cámara de flujo laminar; F) arrastre de los ápices en agar para eliminar las impurezas

Table 2. Experimental conditions for indoor cultivation of *Gracilaria chilensis* / Condiciones experimentales para el cultivo interior de *Gracilaria chilensis*

Time (week)	Volume of enriched medium (mL, Erlenmeyer flask)	Light intensity (µmol $m^{-2}s^{-1}$)	Air flow
0	250	15-20	No
1	250	15-20	No
2	250	15-20	No
3	250	40	Yes
4	250	40	Yes
5	500	40	Yes
6	500	80	Yes
7	500	80	Yes
8	500	80	Yes

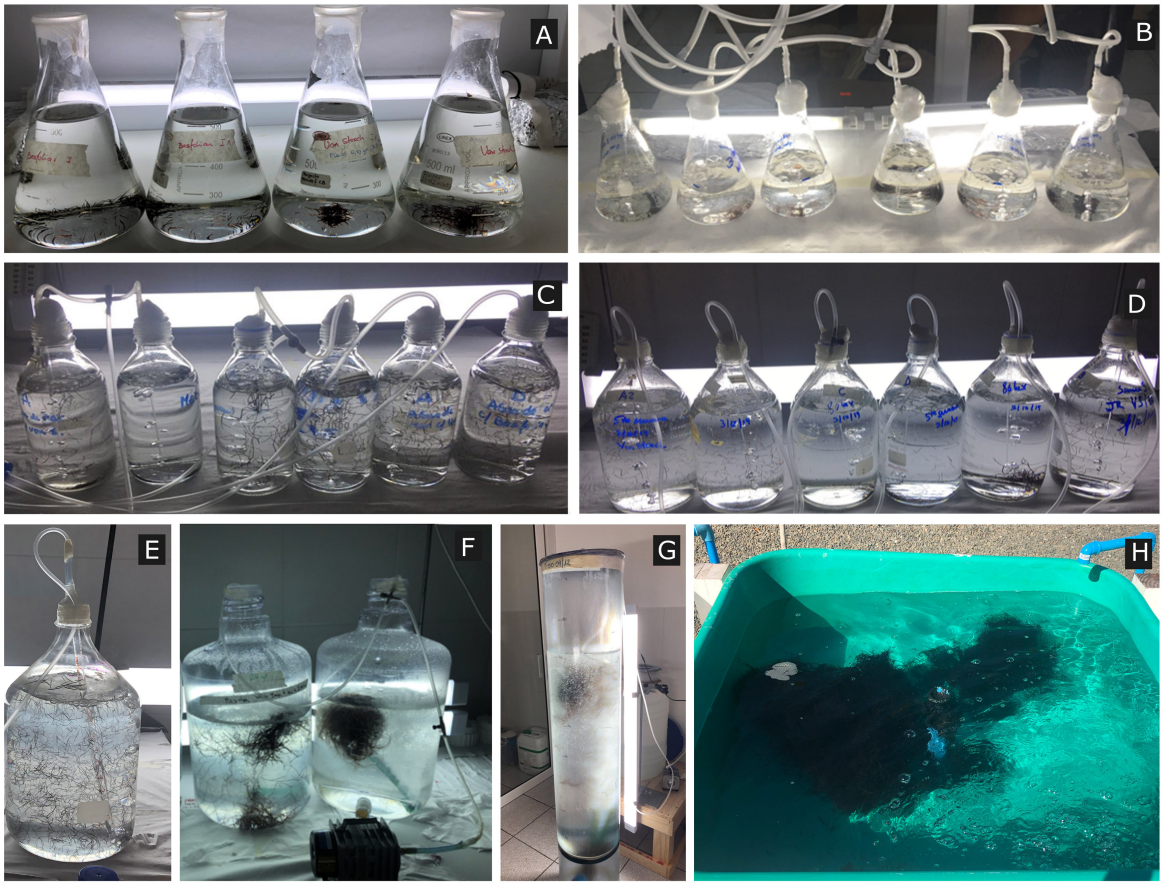

Figure 2. Scaling of *Gracilaria chilensis* cultures under 12:12 (L:D) conditions at 20 °C. A) maintenance of the initial cultures in 250 mL Erlenmeyer flasks for 14 days without aeration, 15-20 µmol $m^{-2}s^{-1}$; B) day 21, bubble aeration was incorporated, 15-20 µmol $m^{-2}s^{-1}$; C) day 28, transfer to 1 L bottles, 40 µmol $m^{-2}s^{-1}$; D) day 35, transfer to 2 L bottles, 40 µmol $m^{-2}s^{-1}$; E) day 42, transfer to 5 L bottles, 80 µmol $m^{-2}s^{-1}$; F) day 49, transfer to 25 L bottles (with 10 L medium), 160 µmol $m^{-2}s^{-1}$; G) day 56, transfer to a vertical 70 L aquarium, 320 µmol $m^{-2}s^{-1}$; H) transfer to 250 L pond covered with Raschel mesh, 160-240 µmol $m^{-2}s^{-1}$ / Escalado de los cultivos de *Gracilaria chilensis* en condiciones 12:12 (L:D) a 20 °C. A) mantención de los cultivos iniciales en matraces Erlenmeyer de 250 mL durante 14 días sin aireación, 15-20 µmol $m^{-2}s^{-1}$; B) día 21, se incorporó aireación por burbujeo y la intensidad de la luz se mantuvo en 15-20 µmol $m^{-2}s^{-1}$; C) día 28, transferencia a botellas de 1 L, 40 µmol $m^{-2}s^{-1}$; D) día 35, transferencia a botellas de 2 L, 40 µmol $m^{-2}s^{-1}$; E) día 42, transferencia a botellas de 5 L, 80 µmol $m^{-2}s^{-1}$; F) día 49, transferencia a botellas de 25 L (con 10 L de medio), 160 µmol $m^{-2}s^{-1}$; G) día 56, transferencia a un acuario vertical de 70 L, 320 µmol $m^{-2}s^{-1}$; H) transferencia a un estanque de 250 L cubierto con malla Raschel, 160-240 µmol $m^{-2}s^{-1}$

Biological and productive parameters

The length of thalli during the initial time of culture was determined by using a 60 cm ruler, and photographed using a portable camera (iPhone 4®, Apple, Cupertino, CA).

The biomass (g m^{-2}) was registered every seven days using a digital analytical balance (Kern, Germany), and was standardized with respect to the area of the container by considering the bottom and the lateral sections.

The daily growth rate (DGR, $\%d^{-1}$) was determined by the following equation:

$$DGR(\%d^{-1}) = \left[\left(\frac{w_t}{w_{t-1}}\right)^{\frac{1}{t}}\right]100\%$$

Where, w_t is the mass after seven days of culture, w_{t-1} is the mass at the previous week, and t is the time expressed in days (Yong *et al*. 2013).

Productivity (g m^{-2} d^{-1}) was calculated using only the culture medium with the highest results in terms of biomass and DGR.

Statistical analysis

Quantitative analyses were performed for the effect of factors of interest [*i.e.,* culture medium (VS, BF, VS/BF-A and VS/BF-B) and time (weeks of culture)] on the biomass and DGR for each culture medium (VS, BF, VS/BF-A, and VS/BF-B). To test the assumptions of normality and the homogeneity of variances, the Shapiro-Wilk and Levene tests were applied. To evaluate if there was a significant interaction between culture medium and time on biomass or DGR, a two-way repeated measures ANOVA was performed on biomass and DGR data, separately. This was followed by a Tukey's test, which was used to determine in which specific treatments such differences exist. In this case, pairwise comparisons among the treatments were made on a week-by-week basis. All statistical analyses were performed in the statistical environment R package (R Development Core Team 2020), and significances were set at $P < 0.05$.

Results

Length and biomass of *Gracilaria chilensis*

An increase in the average length of the thalli was observed (100-200% longer) after 14 days of cultivation (Fig. 3A) by considering the central axis of each thallus. The growth of small branches was registered, which increased with greater growth of the ramifications (Fig. 3B-D), and in some tissues (15-20%), reproductive structures (cystocarps) were distinguished (Fig. 3E).

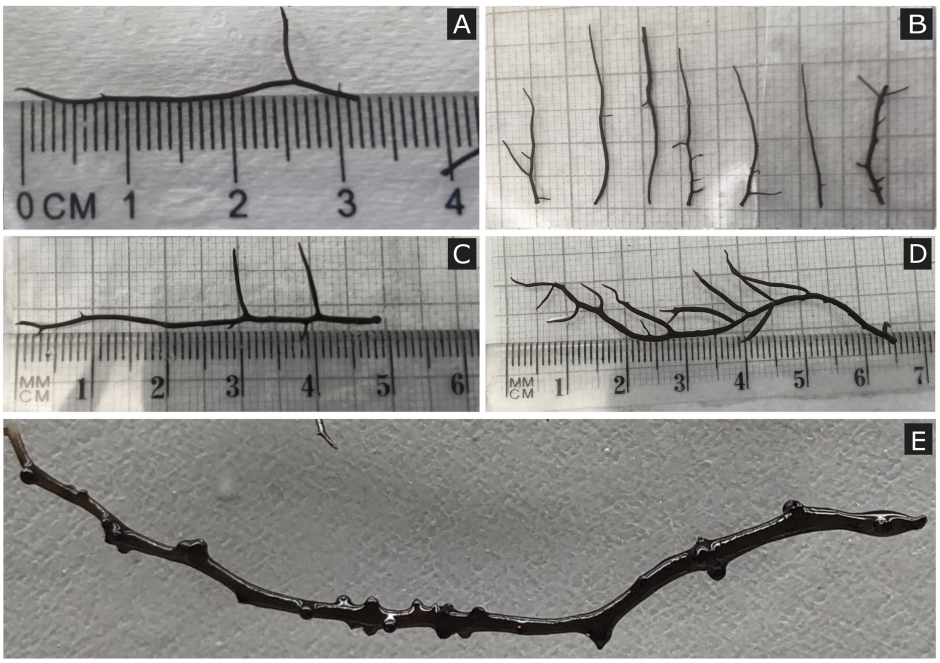

Figure 3. Thalli growth of *Gracilaria chilensis* in VS/BF-A medium. A) growth of the apices at 14 days of culture, 3 cm in length; B) days 29-35, formation of new branches, 3 to 3.2 cm in length; C) day 42, tips 5 cm in length; D) day 49, development of the new branches, thalli reached between 7 and 8 cm in length; E) days 42-49, the presence of reproductive structures (cystocarps) / Crecimiento de talos de *Gracilaria chilensis* en medio VS/BF-A. A) crecimiento de los ápices a los 14 días, longitud 3 cm; B) día 29-35, ápices presentan formación de nuevas ramas, 3 a 3,2 cm de largo; C) día 42, los ápices con 5 cm de longitud; D) día 49, desarrollo de las nuevas ramas, talos entre 7 y 8 cm de longitud; E) día 42-49, presencia de estructuras reproductivas (cistocarpos)

The biomass of *G. chilensis* increased in all the culture media tested (Fig. 4). However, the greatest increase in biomass was recorded using the VS/BF-A medium, reaching an average value of 51.8 ± 3.7 g m^{-2} at seven weeks of cultivation (Fig. 4). The biomass obtained with VS/BF-A medium was eight times greater than that obtained with control treatment (filtered seawater at 0.22 μm), and four times greater than that obtained with VS/BF-B medium. Under VS treatment, the second highest biomass value was recorded, which was 26.2 ± 1.0 g m^{-2} at seven weeks of cultivation (Fig. 4). Comparing the biomass in VS medium with that in control treatment, the former was four times higher.

According to the ANOVA, there were statistically significant two-way interactions between the culture medium (treatment) and week (time) on the biomass of *G. chilensis*, $F_{(28, 56)}$= 28.8, $P < 0.0001$ (Table 3). Independently, both factors (time and treatment) showed statistically significant main effects on the biomass as well (Table 3). According to the Tukey's test, media did not show differences during the first week of culture (Fig. 4, $P > 0.05$). However, from the fourth week, VS/BF-A medium was significantly different from the rest of the media ($P < 0.05$), and always showed the highest biomass of *G. chilensis*. The growth of the biomass in VS/BF-A medium during the seven weeks of cultivation had an exponential trend (R^2= 0.94) (Fig. 4 and Table S1). A similar trend was observed for VS medium, where a relatively and significant increase in the biomass was observed (Fig. 4, Table S1).

Table 3. Two-way repeated measures ANOVA for biomass of *Gracilaria chilensis* when grown in different culture media and over the course of seven weeks. DFn and DFd indicate the degrees of freedom in the numerator and the denominator, respectively; F indicates that an F-distribution (F-test) was used; *P* specifies the *P*-value (*P* < 0.05); ges is the generalized effect size (amount of variability due to the within-subjects factors) / ANDEVA de dos vías de medidas repetidas para la biomasa de *Gracilaria chilensis* cultivada bajo distintos medios y en el transcurso de siete semanas. DFn and DFd indican los grados de libertad en el numerador y el denominador, respectivamente; F indica que una distribución de F (F-test) fue utilizada; *P* especifica el valor de *P* (< 0,05); y ges es el tamaño de efecto generalizado (cantidad de variabilidad debida a los factores intra-sujetos)

Effect	DFn	DFd	F	*P*	ges
Medium	4	8	59.626	5.4 e-06 *	0.906
Week	7	14	70.573	7.7 e-10 *	0.852
Medium:Week	28	56	28.826	4.2 e-24 *	0.861

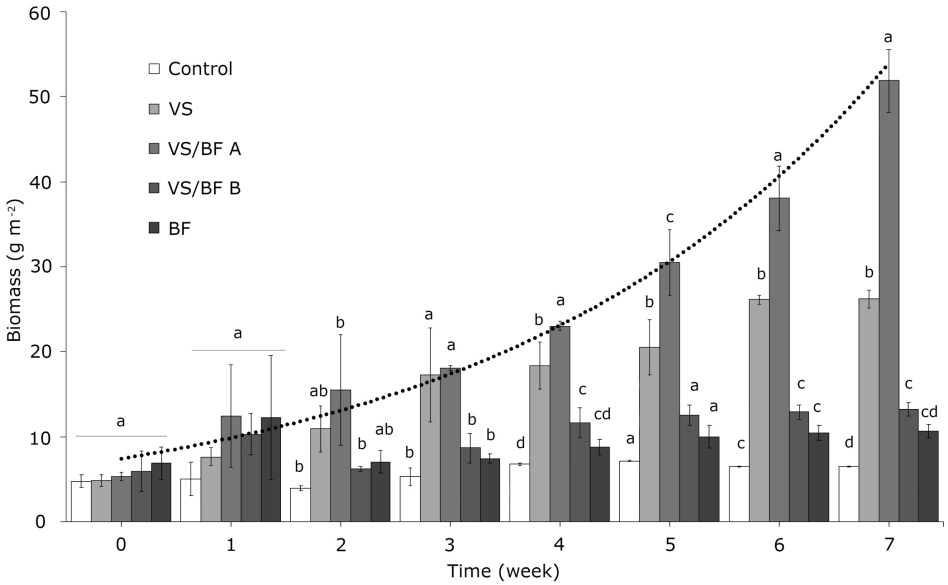

Figure 4. Net biomass (fresh weight, g m^{-2}) of *Gracilaria chilensis* in indoor cultivation for 7 weeks. Bars represent mean ± SD (n= 3). The same letter indicates no significant differences (*P* > 0.05) / Biomasa neta (peso fresco, g m^{-2}) de *Gracilaria chilensis* en cultivo interior durante 7 semanas. Las barras representan la media ± DE (n= 3). Las letras iguales indican que no hay diferencias significativas (*P* > 0,05)

Daily growth rate (DGR)

In the first week of cultivation, the DGR values were high for all media, except for the control condition; however, no significant differences were determined between cultures ($P > 0.05$) (Fig. 5). For control, BF, and VS/BF-B media, negative DGR values were obtained principally during the second week of cultivation. Some thalli presented necrotic tissues, and therefore they were discarded and not considered in total algal biomass measurements. It is important to mention that VS medium showed a constant trend in DGR but, from the fourth week, decreased considerably (Fig. 5). In addition, for BF and VS/BF-B media, although the DGR values were positive from the third week of cultivation, they also decreased as the experimental time progressed (Fig. 5).

There were statistically significant two-way interactions between the culture medium (treatment) and week (time) for DGR of *G. chilensis*, $F_{(24, 48)} = 2.4$, $P < 0.01$ (Table 4). Independently, both factors (time and treatment) showed statistically significant main effects on DGR as well (Table 4). Nonetheless, according to Tukey's test, only at 6 weeks of cultivation there were positive values for DGR obtained in all media, but with significant differences between VS/BF-A and the rest of the culture media (in all cases $P < 0.05$) (Fig. 5). VS/BF-A medium reached a value of 4.55 ± 0.43 % d^{-1} at seven weeks (Fig. 5, Table S2).

Table 4. Two-way repeated measures ANOVA for daily growth rate (DGR, %d^{-1}) of *Gracilaria chilensis* when grown in different culture media and over the course of seven weeks. DFn and DFd indicate the degrees of freedom in the numerator and the denominator, respectively; F indicates that an F-distribution (F-test) was used; *P* specifies the *P*-value (*P* < 0.05); ges is the generalized effect size (amount of variability due to the within-subjects factors) / ANOVA de dos vías de medidas repetidas para la tasa de crecimiento diaria (DGR, %d^{-1}) de *Gracilaria chilensis* cultivada bajo distintos medios de cultivo y en el transcurso de siete semanas. DFn and DFd indican los grados de libertad en el numerador y el denominador, respectivamente; F indica que una distribución de F (F-test) fue utilizada; *P* especifica el valor de *P* (< 0,05); y ges es el tamaño de efecto generalizado (cantidad de variabilidad debida a los factores intra-sujetos)

Effect	DFn	DFd	F	*P*	ges
Medium	4	8	18.225	4.0 e-04 *	0.235
Week	6	12	4.416	1.4 e-02 *	0.462
Medium:Week	24	48	2.432	4.0 e-03 *	0.408

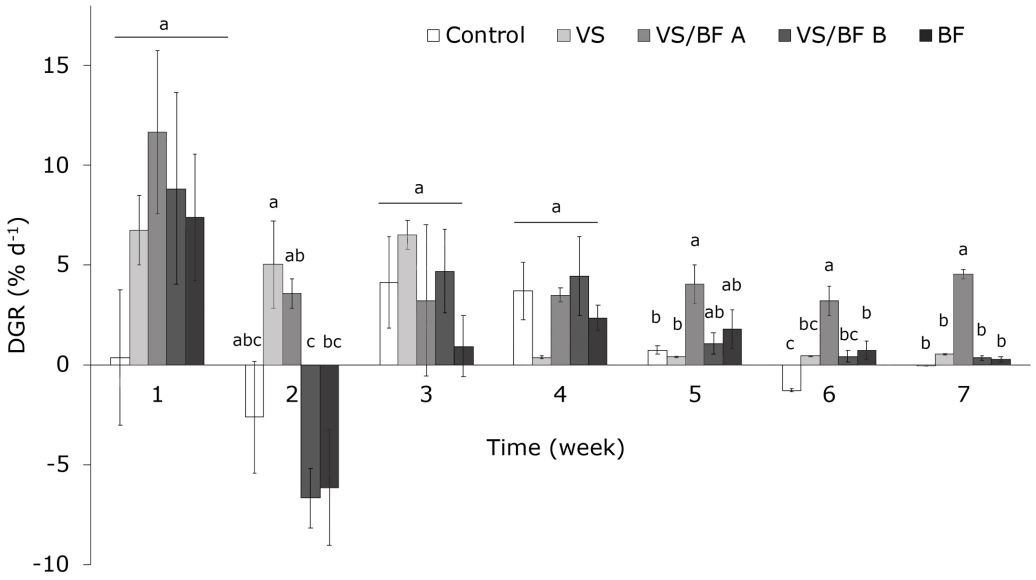

Figure 5. Daily growth rate (DGR, %d^{-1}) of *Gracilaria chilensis* in indoor cultivation using different culture media [control (seawater), VS, BF, VS/BF-A, and VS/BF-B]. Bars represent mean ± SD (n= 3). The same letter indicates no significant differences (*P* > 0.05) / Tasa de crecimiento diaria (DGR, %d^{-1}) de *Gracilaria chilensis* en cultivo de interior utilizando diferentes medios de cultivo [control (agua de mar), VS, BF, VS/BF-A y VS/BF-B]. Las barras representan la media ± DE (n= 3). Las letras iguales indican que no hay diferencias significativas (*P* > 0,05)

Productivity

Gracilaria chilensis exposed to VS/BF-A medium showed a positive increase with an exponential trend ($R^2 = 0.91$), registering a maximum value of 14 g m^{-2} d^{-1} by eight weeks of cultivation (Fig. 6). It is important to note that productivity increased 18 times between the first and eighth week of cultivation.

Discussion

This study provides a first approach for an indoor culture of *Gracilaria chilensis* thalli using different enriched media during seven weeks of cultivation. Both factors (time and medium) showed statistically significant main effects on both the biomass and DGR of *G. chilensis* and a significant two-way interaction between them. The highest biomass increase was observed for VS/BF-A medium from the fourth week of culture onwards, whereas the second highest biomass increase was registered for VS medium. On the other hand, while the biomass increase in *G. chilensis* was positively correlated with increasing time, the highest DGR increase was observed in the first week for VS/BF-A and in the first three weeks for VS medium. Whereas for the other media DGR decreased almost to zero in week 7, DGR using VS/BF-A medium was nearly 5% in week 7. Therefore, the methodology developed in this study has been proved to be successful, since it was possible to obtain a higher biomass, increased growth rate and higher productivity using enriched seawater medium VS/BF-A as compared with the other media. Moreover, the growth rate of *G. chilensis* thalli using VS/BF-A medium was higher and quicker (after 1 week), remained relatively high (5% in week 7), and allowed attainment of higher productivity than the other media tested.

Different sources and combinations of nutrients were analyzed in order to find an ideal relationship between growth potential and price of the medium. Among these was Basfoliar® (Basfoliar Aktiv, BF), which is a biostimulant medium rich in different macro- and micronutrients (Lötze & Hoffman 2016, Rengasamy *et al*. 2016, Hussain *et al*. 2019), and which furthermore has a low cost (USD 6.8 per L). In addition, the successful use of BF has been previously registered for *C. chamissoi* (Ávila *et al*. 2018), and *G. chilensis* (Águila 2015), where thalli showed greater growth (in terms of length) as compared to filtered seawater or Provasoli-enriched medium. Von Stosch (VS) is also a common medium used in the development and growth

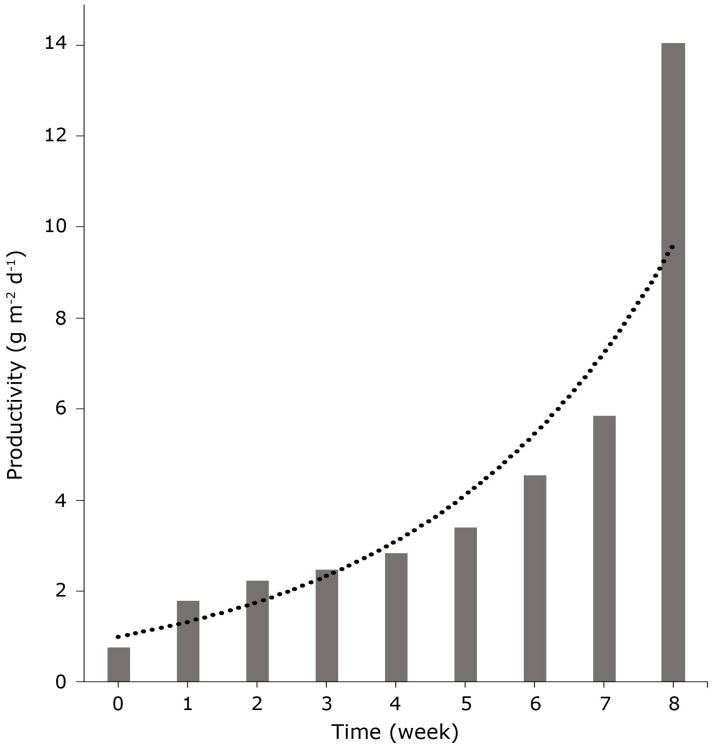

Figure 6. Productivity (g m^{-2} d^{-1}) of *Gracilaria chilensis* with the chosen culture medium VS/BF-A during 8 weeks (56 days) of culture / Productividad (g m^{-2} d^{-1}) de *Gracilaria chilensis* con el medio de cultivo elegido VS/BF-A durante 8 semanas (56 días) de cultivo

of early stages of various red seaweeds species, such as *Neopyropia yezoensis* (Ma *et al*. 2020) and *Kappaphycus alvarezii* (Pedra *et al*. 2017). However, the use of this medium increases the operating expenses, which could render the whole culture unviable. In fact, 1 L of VS (in Chile) currently costs USD 22.30, a comparatively high price. Moreover, although a relatively high biomass of *G. chilensis* was obtained using VS medium, the achieved results were better with VS/BF-A medium, which showed the highest biomass, DGR and productivity. It is postulated that VS/BF-A medium is economically viable for biomass cultivation of *G. chilensis* due to the overall low cost of this culture medium (USD 1.30 per 10 L).

Additionally, VS/BF-A medium showed a higher biomass and growth rate than VS and BF media (Figs. 4 and 5). A possible explanation for this could be that, to simulate algae growth, a higher ratio of nitrogen to phosphorus is needed. Yu & Yang (2008) found that growth rates of *G. lemaneiformis* were accelerated when N:P ratio was increased from 50:3.13 µmol L^{-1} to 400:25 µmol L^{-1} but decreased significantly when the ratio was increased further. In another study with *G. lemaneiformis,* the optimal N:P ratio was 500:0.5 µmol L^{-1} (Duan *et al.* 2019). In the present work, the proportion of nutrients (N:P) in VS/BF-A medium was optimal (1:1) under operational conditions used for *G. chilensis* culture. On the contrary, for VS/BF-B medium, the lower growth of *G. chilensis* could be explained because of excess P with respect to N in this medium (Table 1), resulting in negative growth rates (Fig. 5). The experimental conditions (time, temperature, light, and aeration) were identical and constant for the different groups during the experimental period, except for the amount of nutrients and their effects on the biomass and DGR of *G. chilensis* which varied between the culture media utilized (Table 1). Therefore, this strongly suggests that the proportion of nutrients and their bioavailability must be considered for an adequate scaling of *G. chilensis* biomass. Micronutrients are necessary for various metabolic processes in algae (Andersen 2005), and their presence influences algal growth. Therefore, culture media (BF, VS/BF-A and VS / BF-B) having greater quantities of these could have had a positive effect on biomass and DGR, but N:P ratio of VS / BF-A had a greater impact on biomass and DGR (Figs. 4 and 5), even more than VS/BF-B (containing more micronutrients than VS / BF-A).

VS/BF-A medium in the present study displayed a high growth rate, despite it having a low 1:1 mass ratio of N and P, which is contrary to the results of other studies (*e.g*., Harrison & Hurd 2001, Yu & Yang 2008, Duan *et al*. 2019). This medium contains BF, which contributes with other nutrients (*e.g.,* K, Mn, Fe, Zn and Cu, Hussain *et al*. 2019) which probably positively affected the growth of *G. chilensis*. A similar pattern has been observed in *Bostrychia radicans* and *Caloglossa leprieurii* by supplementing the medium with K (Yarish *et al*. 1980), which is indeed present in BF medium; furthermore, in *Gracilaria tenuistipitata* and *Gracilaria perplexa,* apical growth and callus formation have been stimulated by supplementing them with auxins and cytokinin (Yokoya *et al*. 2004), which are also present in BF (Hussain *et al*. 2019).

Gracilaria requires significant light intensities for normal growth, and some species can acclimatize their photosynthetic conditions according to irradiance conditions (Yang *et al*. 2015). In the scaling stage carried out for productivity assessment of *G. chilensis*, there was a successive increase in volume of nutrients, air flow, and light intensity. This methodology was successful because the productivity registered in *G. chilensis* was 14 g m^{-2} d^{-1} by the eighth week (56 days) of cultivation, taking into consideration that the cultures were started from short thalli. Unfortunately, we were unable to maintain the number of replicates as the productivity experiment progressed, due to limited space and capacity in the laboratory at the time of the experimental work, but the obtained results strongly suggest that rapid growth of *G. chilensis* occurred under the selected conditions. Further studies are required in order to define the optimal light conditions under which the productivity of *G. chilensis* is higher when using VS/BF-A medium. For example, Kim & Yarish (2014) compared productivity of *G. tikvahiae* exposed to different culture media, and the highest productivity was obtained with the VS medium, with a value of 0.24 g L^{-1} d^{-1} recorded under optimal light intensities of 100 and 250 µmol $m^{-2}s^{-1}$. In this way, the effect of light intensity and nutrients can affect carbon/nitrogen ratio and, consequently, growth of the algae (Cronin & Hay 1996). In addition, it is suggested that air flow must be controlled, since this is the source of CO_2, which generates the movement of the water, and allows for better diffusion of the nutrients (Msuya & Neori 2008). In this regard, incorporating filtered air into the media positively affected *G. chilensis* culture's ability to reach higher productivity in a short time. However, based on the results, it is not recommended to incorporate air flow during the first weeks of cultivation, due (mainly) to the fact is a potential source of contamination during the initial stage of cultivation.

In summary, this study provides pivotal updated information regarding cultures of *G. chilensis* from thalli under controlled conditions that use a mixture of oligoelements, which stimulate growth and even formation of reproductive structures at a lower overall cost. Further major analyses of the reproduction context of this species under controlled conditions are required.

Acknowledgments

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Supplementary material

Table S1. Assignment of culture medium treatments to groups with means that were significantly different from each other (at $P < 0.05$) for the dependent variable biomass of *Gracilaria chilensis* according to post hoc Tukey's test. Pairwise comparisons between medium treatments were made on a week-by-week basis / Asignación de los tratamientos con distintos medios de cultivo a grupos que muestran medias significativamente diferentes entre ellas (con un valor de $P < 0,05$) para la variable dependiente biomasa de *Gracilaria chilensis* de acuerdo al test *a posteriori* de Tukey. Las comparaciones pareadas fueron hechas semana a semana

Week	Medium	Mean biomass	Group	Mean squared error	Degree of freedom	Mean all treatments	Coefficient of variation
Zero	BF	0.591	a	0.015	10	0.477	25.910
Zero	VS-BF-B	0.509	a	0.015	10	0.477	25.910
Zero	VS-BF-A	0.457	a	0.015	10	0.477	25.910
Zero	VS	0.416	a	0.015	10	0.477	25.910
Zero	Control	0.409	a	0.015	10	0.477	25.910
One	VS-BF-A	1.064	a	0.146	10	0.817	46.850
One	BF	1.050	a	0.146	10	0.817	46.850
One	VS-BF-B	0.881	a	0.146	10	0.817	46.850
One	VS	0.655	a	0.146	10	0.817	46.850
One	Control	0.433	a	0.146	10	0.817	46.850
Two	VS-BF-A	1.328	b	0.075	10	0.749	36.509
Two	VS	0.936	ab	0.075	10	0.749	36.509
Two	BF	0.606	ab	0.075	10	0.749	36.509
Two	VS-BF-B	0.533	b	0.075	10	0.749	36.509
Two	Control	0.342	b	0.075	10	0.749	36.509
Three	VS-BF-A	1.548	a	0.051	10	0.973	23.277
Three	VS	1.476	a	0.051	10	0.973	23.277
Three	VS-BF-B	0.742	b	0.051	10	0.973	23.277
Three	BF	0.640	b	0.051	10	0.973	23.277
Three	Control	0.457	b	0.051	10	0.973	23.277
Four	VS-BF-A	1.969	a	0.017	10	1.175	11.227
Four	VS	1.570	b	0.017	10	1.175	11.227
Four	VS-BF-B	1.000	c	0.017	10	1.175	11.227
Four	BF	0.754	cd	0.017	10	1.175	11.227
Four	Control	0.582	d	0.017	10	1.175	11.227
Five	VS-BF-A	2.610	c	0.042	10	1.381	14.806
Five	VS	1.755	b	0.042	10	1.381	14.806
Five	VS-BF-B	1.073	a	0.042	10	1.381	14.806
Five	BF	0.856	a	0.042	10	1.381	14.806
Five	Control	0.613	a	0.042	10	1.381	14.806
Six	VS-BF-A	3.252	a	0.024	10	1.610	9.584
Six	VS	2.234	b	0.024	10	1.610	9.584
Six	VS-BF-B	1.104	c	0.024	10	1.610	9.584
Six	BF	0.898	c	0.024	10	1.610	9.584
Six	Control	0.560	c	0.024	10	1.610	9.584
Seven	VS-BF-A	4.432	a	0.023	10	1.856	8.217
Seven	VS	2.241	b	0.023	10	1.856	8.217
Seven	VS-BF-B	1.132	c	0.023	10	1.856	8.217
Seven	BF	0.915	cd	0.023	10	1.856	8.217
Seven	Control	0.560	d	0.023	10	1.856	8.217

Table S2. Assignment of culture medium treatments to groups with means that were significantly different from each other (at $P < 0.05$) for the dependent variable daily growth rate (DGR, $\%d^{-1}$) of *Gracilaria chilensis* according to post hoc Tukey's test. Pairwise comparisons between medium treatments were made on a week-by-week basis / Asignación de los tratamientos con distintos medios de cultivo a grupos que muestran medias significativamente diferentes entre ellas (con un valor de $P < 0,05$) para la variable dependiente tasa de crecimiento diaria (DGR, $\%d^{-1}$) de *Gracilaria chilensis* de acuerdo al test *a posteriori* de Tukey. Las comparaciones pareadas fueron hechas semana a semana

Week	Medium	Mean DGR	Group	Mean squared error	Degrees of freedom	Mean all treatments / week	Coefficient of variation
One	VS-BF-A	11.679	a	38.523	10	7.007	88.578
One	VS-BF-B	8.832	a	38.523	10	7.007	88.578
One	BF	7.395	a	38.523	10	7.007	88.578
One	VS	6.757	a	38.523	10	7.007	88.578
One	Control	0.373	a	38.523	10	7.007	88.578
Two	VS	5.033	a	14.291	10	-1.367	-276.466
Two	VS-BF-A	3.575	ab	14.291	10	-1.367	-276.466
Two	Control	-2.616	abc	14.291	10	-1.367	-276.466
Two	BF	-6.157	bc	14.291	10	-1.367	-276.466
Two	VS-BF-B	-6.673	c	14.291	10	-1.367	-276.466
Three	VS	6.522	a	16.073	10	3.907	102.625
Three	VS-BF-B	4.707	a	16.073	10	3.907	102.625
Three	Control	4.130	a	16.073	10	3.907	102.625
Three	VS-BF-A	3.234	a	16.073	10	3.907	102.625
Three	BF	0.939	a	16.073	10	3.907	102.625
Four	VS-BF-B	4.459	a	3.946	10	2.876	69.072
Four	Control	3.700	a	3.946	10	2.876	69.072
Four	VS-BF-A	3.495	a	3.946	10	2.876	69.072
Four	BF	2.356	a	3.946	10	2.876	69.072
Four	VS	0.369	a	3.946	10	2.876	69.072
Five	VS-BF-A	4.039	a	1.297	10	1.614	70.553
Five	BF	1.800	ab	1.297	10	1.614	70.553
Five	VS-BF-B	1.080	ab	1.297	10	1.614	70.553
Five	Control	0.758	b	1.297	10	1.614	70.553
Five	VS	0.393	b	1.297	10	1.614	70.553
Six	VS-BF-A	3.228	a	0.512	10	0.711	100.546
Six	BF	0.736	b	0.512	10	0.712	100.546
Six	VS	0.439	bc	0.512	10	0.713	100.546
Six	VS-BF-B	0.435	bc	0.512	10	0.714	100.546
Six	Control	-1.281	c	0.512	10	0.715	100.546
Seven	VS-BF-A	4.547	a	0.053	10	0.716	19.991
Seven	VS	0.559	b	0.053	10	0.717	19.991
Seven	VS-BF-B	0.361	b	0.053	10	0.718	19.991
Seven	BF	0.277	b	0.053	10	0.719	19.991
Seven	Control	0.007	b	0.053	10	0.720	19.991