Effects of Environmental Factors and Plant Hormones on Sexual Reproduction of Agarophyte Seaweed, *Gracilaria fisheri* (Rhodophyceae)

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ABSTRACT

Stimulation of sexual reproduction was conducted for strain improvement of the agarophyte, *Gracilaria fisheri* under cultivation. Thirty sporelings from tetraspores with initial height of 2 cm were cultured in 1 L aeration tube flasks under different levels of salinity: 10, 20 and 30 ‰; light intensity: 20, 40 and 60 µmol photons·m^{-2}·s^{-1}; carbon dioxide: 0 and 1 %; and concentrations of two plant hormones, 6-benzyladenine and auxin: 0.00, 0.05, 0.10 and 0.50 mg·L^{-1}, separately tested. Formation of reproductive cells was examined from the external surface of the seedlings every week. The results showed that cystocarps were recognized at all levels of salinity and light intensity; the fastest development was at the week 2nd at salinity 20 ‰ and light intensity of 60 µmol photons·m^{-2}·s^{-1}. Cross sections of thalli exhibited completely male structures and cystocarps. Under supplemention with carbon dioxide, 6-benzyladenine and auxin, the formation of sexual reproductive cells in the seaweed was not found. This study suggests that salinity and light intensity could be used for stimulation of sexual reproduction of *G. fisheri*, while carbon dioxide and the two plant hormones used might inhibit the formation of sexual reproductive cells in this algal species.

Keywords: Agarophyte, Environmental factor, *Gracilaria fisheri*, Hormone, Sexual reproduction

INTRODUCTION

Gracilaria is one genus of red algae which has more than 150 species found throughout the world (Tseng and Xia, 1999). The alga is used as a type of marine food for humans (Zemke-White and Ohno, 1999) and it is an important raw material to produce agar, for which 60-80 % of the total production comes from cultivation. The demand for the material is growing quickly due to increasing population and emerging infectious diseases, leading to greater use of agar (Kain and Destombe, 1995; Yeong *et al*., 2014). Therefore, *Gracilaria* is widely cultivated in many countries such as China, Chile, Indonesia, Malaysia and Thailand to support the need for raw material (Bixler and Porse, 2010).

Gracilaria fisheri is a gracilaroid species used for the extraction of agar in Thailand and other nearby countries (Chirapart *et al*., 2006). Previous studies have mainly focused on eco-physiological characterization, agar quality, and pharmaceutical chemistry of *G. fisheri* (Chirapart *et al*., 2006; Khreauthong *et al*., 2018). *Gracilaria* cultivation will continue to be an approach to efficiently producing this species (Ryder *et al*., 2004; Bezerra and Marinho-Soriano, 2010). However, seedlings produced by asexual reproduction suffer a growth

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rate decrease after 2-3 years and are easily attacked by disease (Buschmann *et al*., 1995; Glenn *et al*., 1996; Mantri *et al*., 2009). Therefore, it is necessary to find techniques to promote the growth of the crop or to develop new strains that can provide high growth as well as be resistant to environmental change (Mantri *et al*., 2009). Sexual reproduction in the alga can provide new generations in natural populations, but it is rare to find in the commercially cultivated crop (McLachland and Bird, 1986). The influence of various factors on sexual reproduction and other parts of the life cycle must be known to successfully maintain algal strains (Kain and Destombe, 1995). In this study, the influences of salinity, light intensity, carbon dioxide and plant hormones on the formation of female reproductive cells of *G. fisheri* in the laboratory were investigated. The first three factors are known as main influences on growth of algae, while the last factor has been reported to promote growth and reproductive stimulation in various algae (Lobban and Harrison, 1994). It is hoped that the study will result in a reduction of the shortage of seedlings which are required in *G. fisheri* cultivation.

MATERIALS AND METHODS

Seedling preparation of Gracilaria fisheri

Tetrasporophyte mother plants of *Gracilaria fisheri* were collected from earthen ponds in Ranot District, Songkhla Province, southern Thailand. The thalli were cleaned using a brush and washed in sterilized seawater several times to remove contaminants, and forceps were used to remove visible epiphytes. The cleaned samples were cultured in a 0.5 tonne tank for 1 week prior to use as mother plants. The stimulation for tetraspore release was done by placing honeycomb plastic plates at the bottom of the cleaned tank, filled with filtered seawater (20 ‰). The culture tank was then set up in laboratory conditions of 25 ºC with 12:12 h light:dark photoperiod, and 1 $mL \cdot L^{-1}$ modified von Stosch (Grund) medium (MGM) was added for stimulation of spore release (Andersen *et al*., 2005; Yu *et al*., 2013). After two weeks, the mother thalli were taken out, and spores in the plastic plates were further cultured until the young thalli grew to 2 cm long (within 6 weeks). The sporelings were scraped from the plastic plates and separated into small clusters. The sporelings were weighed and counted before being used.

Salinity effects on reproduction of Gracilaria fisheri seedlings

The seedlings of *G. fisheri* were used for culture under different salinity levels (10, 20 and 30 ‰) with the initial density of 0.7 $g \cdot L^{-1}$ (following Yu *et al*., 2013) in 1 L round-bottom flasks with a tube for supplying air from a pump. The experiment was done in three replications at 25 ºC under 60 µmol photons$\cdot m^{-2} \cdot s^{-1}$ light intensity and 12:12 h light:dark period. Water enriched with MGM was renewed every week. Fresh weight and length of the samples (n=30) were measured every 7 days for a total of 7 weeks. The formation of a reproductive organ on the thalli surface was observed by naked eye and by using stereomicroscope in swollen parts. Data related to reproductive formation were recorded and maturation was confirmed by cross section of the reproductive organ to check carpospores contained within during the last week under compound microscope.

Light intensity effects on reproduction of Gracilaria fisheri seedlings

For the light intensity assessment, seedlings were cultured under different light intensities: 20, 40 and 60 µmol photons$\cdot m^{-2} \cdot s^{-1}$ (following Yu *et al*., 2013) with the initial density of 0.7 $g \cdot L^{-1}$. Growth of the algal samples was determined in 1 L round-bottom flasks with an arm supplying air from a pump; the seawater used was adjusted to optimum salinity of 20 ‰ (based on the previous experiment for reproductive organ formation). The experiment was done (n=3) under a cycle of light:dark 12:12 h at 25 ºC and using culture media with MGM enrichment. Fresh weight and length of the samples (n=30) were measured every 7 days for 7 weeks. The formation of reproductive organs was monitored as in the previous experiment, and confirmed by cross section at the end of the experiment.

Carbon dioxide effects on reproduction of Gracilaria fisheri seedlings in optimal growth conditions

In this experiment, the sporelings were cultured in 10 L round plastic tanks of 15 cm in diameter and 40 cm high in three replications. Carbon dioxide was supplied into the cultured tanks at two levels, 0 and 1% (v/v), at a flow rate of 1 $L \cdot min^{-1}$ (using pure CO_2 mixed with air before feeding, following Mercado *et al*., 1999). The air inlet was placed at the bottom. The culture tanks were filled with 10 L of sterilized seawater at optimal salinity (20 ‰) based on the prior experiment, and using MGM-enriched culture medium. The algal samples were cultured at the initial density of 0.7 $g \cdot L^{-1}$ under optimal light intensity (60 µmol photons$\cdot m^{-2} \cdot s^{-1}$) based on the prior experiment, under a light:dark period of 12:12 h at 25 ºC. The length of the seedlings (n=30) was measured every 7 days for 7 weeks. The formation of reproductive organs was monitored as in the previous experiments, and confirmed by cross section at the end of the experiment.

Plant hormone effects on reproduction of Gracilaria fisheri seedlings

Two plant hormones, namely 6-benzyladenine and auxin, were applied in four concentrations (following Yokoya *et al*., 2004; Yong *et al*., 2014): 0.00, 0.05, 0.10 and 0.50 $mg \cdot L^{-1}$. The experiment was performed in three replications in 1 L round-bottom flasks with air supplied by pump. The algal samples were cultured at the initial density of 0.7 $g \cdot L^{-1}$ under light:dark period of 12:12 h at 25 ºC, and using seawater with MGM culture medium at the optimum salinity of 20 ‰. The length of the seedlings (n=30) was measured every 7 days for 7 weeks. The formation of reproductive organs was monitored as in the previous experiments, and confirmed by cross section at the end of the trial.

Calculation of relative growth rate

The relative growth rate (RGR) of the seaweed was calculated according to the following formula (Lobban and Harrison, 1994): RGR for weight (%$\cdot day^{-1}$)=100 (ln Wt - ln Wo)$\cdot t^{-1}$ (in days), where Wt=the final weight (g), Wo=the initial weight, and t=the interval time (days). Likewise, RGR for length was calculated in the same way.

Photosynthesis of Gracilaria fisheri seedling under different conditions

Samples of *G. fisheri* from each treatment (salinity: 10, 20, 30 ‰; light intensity: 20, 40, 60 µmol photons$\cdot m^{-2} \cdot s^{-1}$; carbon dioxide: 0, 1%; plant hormone: 0.05, 0.10, 0.50 $mg \cdot L^{-1}$) were taken at the end of each trial to measure photosynthesis at PSII maximum quantum efficiency of photochemistry (F_V/F_m) with chlorophyll fluorometer by dark-adaptation for 30 min before F_V/F_m measurement using Junior PAM model the Walz Teaching-PAM fluorometer (PAM-200), Heinz Walz GmbH, Germany.

Data analysis

Data of growth from each experiment are reported as mean±standard deviation ($\bar{x}$ ±SD). One-way ANOVA was used for data analysis and means were compared using Tukey's test, with a critical probability value at 0.05.

RESULTS AND DISCUSSION

Salinity effects on sexual reproduction of seedlings

Growth and maturation of sporelings of *Gracilaria fisheri* were found at all levels of salinity. The highest growth was observed at salinity 30 ‰ after week 4 of culture, with the sporeling size reaching 17.9±4.6 cm at week 7, at the end of the trial. Cystocarps became mature at salinity of 30 ‰ at week 2, when sporelings had a height of 5.6±1.5 cm. At salinity 10 and 30 ‰, the sporelings formed cystocarps a week later than those cultured at salinity of 20 ‰ (Figure 1a). The RGR of 4.50± 0.55 %$\cdot day^{-1}$ was highest at salinity 30 ‰, and was significantly different ($p<0.05$) from sporelings cultured at salinity 20 ‰ (4.26±0.63 %$\cdot day^{-1}$) and 10 ‰ (3.51±0.71 %$\cdot day^{-1}$) (Figure 1b). Biomass of the alga at the end of week 7 in 10, 20 and 30 ‰ seawater was 14.97±0.57, 17.89±1.14 and 20.17± 1.38 $g \cdot L^{-1}$, respectively (Figure 1c) and RGR of biomass was 6.25±0.10, 6.61±0.13 and 6.91±0.14 %$\cdot day^{-1}$, respectively (Figure 1d). The result of

the salinity was similar to Zhou *et al*. (2013), who studied the effect of salinity on the development and release of carpospores of *G. lemaneiformis*, and found this occurred most readily at 30–35 ‰ but was possible at a wide range of salinity. Furthermore, the present study disagreed with Choi *et al*. (2006), who studied the effect of salinity on growth of *G. verrucosa* and *G. chorda* from Korea, and found that both species grew in a wide range of salinity ranging from 5–35 ‰, with the optimum range of 15–30 ‰. Bird and McLachlan (1986) reported that *Gracilaria* spp. became pale in color and died when salinity was less than 15 ‰, while Kumar *et al*. (2010) reported that *Gracilaria corticata* in salinity below 15 ‰ expressed weakened thalli.

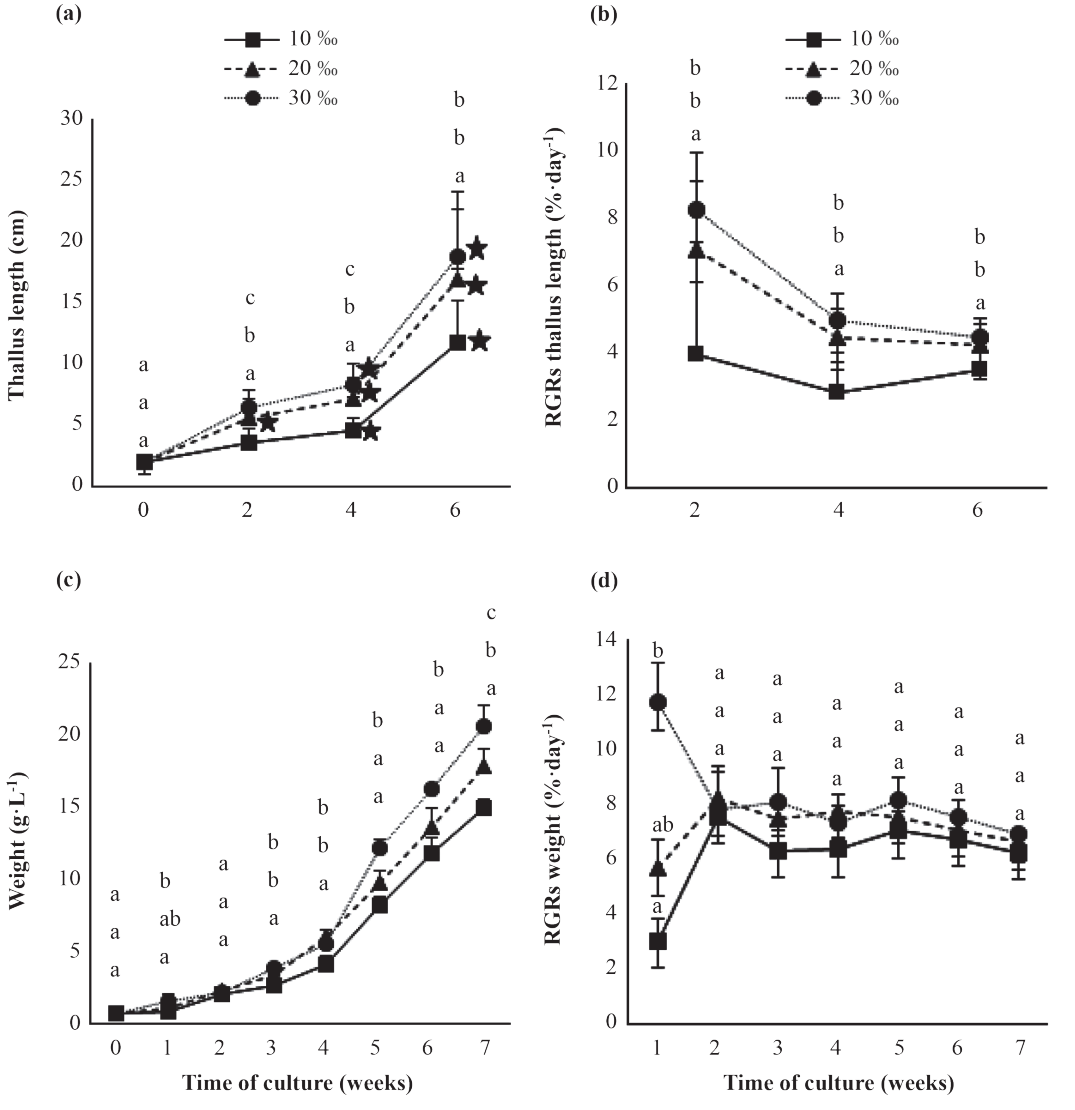

Figure 1. Growth of *Gracilaria fisheri* cultured under different salinity levels with 60 µmol photons·m^{-2}·s^{-1} light intensity, 12:12 h light:dark period at 25 °C for 7 weeks: (a) thallus length, (b) RGR for thallus length, (c) thallus weight, and (d) RGR for weight. Stars indicate cystocarp formation. Different letters at a given time indicate significant differences ($p<0.05$).

Light intensity effects on sexual reproduction of seedlings

Cystocarp formation under different light intensities was first observed, along with the best growth, at 60 µmol photons·m^{-2}·s^{-1} at week 2, with an average thallus length of 4.9±1.2 cm, and was significantly higher ($p<0.05$) than growth under 20 and 40 µmol photons·m^{-2}·s^{-1}. The formation of reproductive cells in 20 and 40 µmol photons·m^{-2}·s^{-1} treatments occurred one week later than the treatment with 60 µmol photons·m^{-2}·s^{-1} (Figure 2a). Average RGR by length ranged from 3.86±0.55 to 4.60±0.73 %·day^{-1} (Figure 2b), while weight ranged from 15.63±1.24 to 21.11±1.1 g·L^{-1} (Figure 2c). The relative growth rates for weight for the 7-week trial were 6.33±0.17, 6.72±0.1 and 6.95±0.1 %·day^{-1} (Figure 2d). The growth of the seaweed in the present study is in line with Zhou *et al*. (2013), who found that light intensity showed an optimal range of 15–45 µmol photons·m^{-2}·s^{-1}. Light is one of the most important factors in the growth of algae, along salinity (Bird and Mclachlan, 1986) and temperature (Pakker *et al*., 1995). Our results on the growth of *G. fisheri* were similar to reports showing that optimum salinity of seaweed in Atlantic and Pacific oceans ranged from 15-30 ‰ (Bird and Mclachlan, 1986) and the optimum temperature in Caribbean tropical seaweeds ranged from 25-30 ºC (Pakker *et al*., 1995). In addition, *Gracilaria* spp. isolated from Japan, Malaysia and Indonesia showed an increased growth rate when light intensity increased from 10 to 60 µmol photons·m^{-2}·s^{-1} (Beer and Levy, 1983; Raikar *et al*., 2001; Reddy *et al*., 2003).

Carbon dioxide effects on sexual reproduction of seedlings

The addition of 1% carbon dioxide in *G. fisheri* culture inhibited growth and formation of reproductive tissue compared to the control. The thalli height at week 2 when adding 1% CO_2 was significantly lower than the control (5.4±1.0 cm, Figure 3a) and lower than the average RGR for height in the control (12.82±4.4 %·day^{-1} , Figure 3b). The weight (Figure 3c) and RGR for weight (Figure 3d) of *G. fisheri* cultured in 1% CO_2 and the control showed similar trends to height and RGR for height. The thalli of the alga in 1% CO_2 changed to green color and the thalli seemed weakened; long periods of carbon dioxide feeding will degrade chlorophyll, even at low concentration. In the present study, we used only 1% CO_2, which is a low concentration, but our results disagreed with Chen *et al*. (2018), who reported that 1% carbon dioxide increased the growth of algae, while Xu *et al*. (2008; 2010) and Zou and Gao (2009) also reported that increasing carbon dioxide provided benefits in raising the alga *G. lemaneiformis*. These previous reports used short periods of CO_2 feeding and showed enhanced growth of the algae, but this was not observed in the present study, as a longer period of CO_2 feeding inhibited the algal growth.

Plant hormone effects on sexual reproduction of seedlings

This study found no effects on cystocarp formation in the samples cultured in MGM medium with the addition of plant hormones 6-benzyladenine and auxin. The experiment was terminated after culturing for three weeks due to thalli weakness and loss of color at all concentrations of the hormone used. However, another study reported that auxin and cytokinin promoted growth of the red alga *Kappaphycus alvarezii* (Hayashi *et al*., 2008). It also stimulated cell division in *Gracilaria tenuistipitata* and *G. perlexa* when adding auxin: cytokinin at ratio of 1:10 µmol (Yokoya *et al*., 2004). Our study indicated that the plant hormones were not suitable for improving sexual reproduction and growth of *G. fisheri*.

Effects of environmental factors on photosynthesis

The ratio that indicates the quantum efficiency of photosystem II (F_v/F_m) of the final biomass in the experiments on salinity, light intensity, carbon dioxide and hormone were in the ranges of 0.54±0.09-0.63±0.14, 0.46±0.11-0.56±0.18, 0.49±0.09-0.65±0.07 and 0.50±0.15-0.60±0.09, respectively (Figure 4). The range of F_v/F_m was similar to *Gracilaria lemaneiformis* under the addition to selenite at concentrations of 0, 200, 500 and 800 mg·L^{-1} which provided F_v/F_m in the

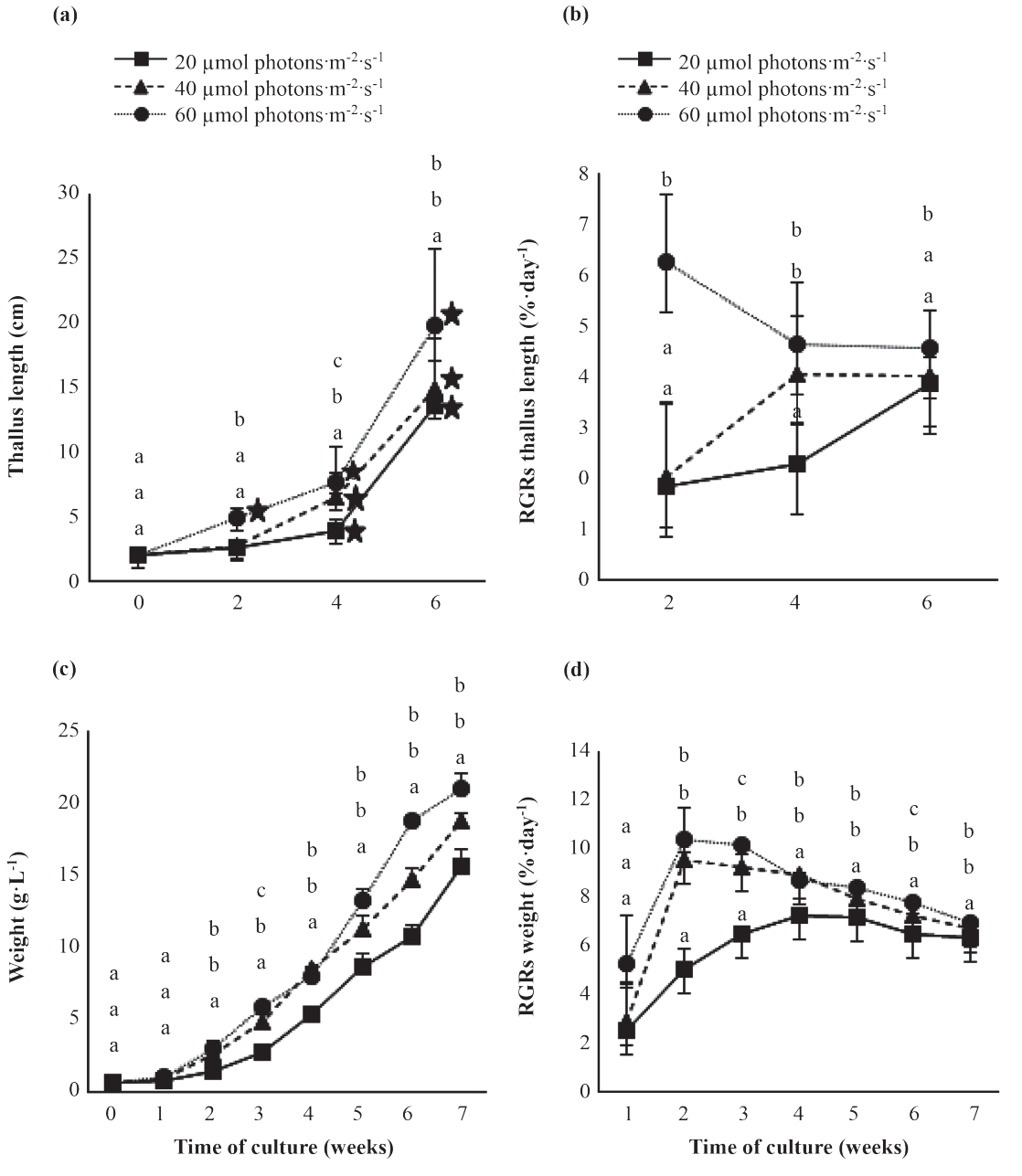

Figure 2. Growth of *Gracilaria fisheri* cultured under different light intensities in 20 ‰ seawater at 12:12 h light:dark period, at 25 °C for 7 weeks: (a) thallus length, (b) RGR for thallus length, (c) thallus weight, and (d) RGR by weight. Stars indicate cystocarp formation. Different letters at a given time indicate significant differences ($p<0.05$).

range of 0.4-0.5 (Liu *et al*., 2018) because selenite can promote the photosynthesis of the alga. The present study was also in agreement with Chen *et al*. (2015), who found that adding carbon dioxide to the red alga, *Gracilaria lemaneiformis* caused biomasses to increase and photosynthesis rates decease when compare to those grown in untreated water.

Characteristics of reproductive organ formation

Maturation of cystocarps occurred under the wide range of salinity and light intensity used in the treatments. Cystocarps could be observed in the thalli that had a minimum length of 4.60± 0.73 cm; development of the cystocarp is shown

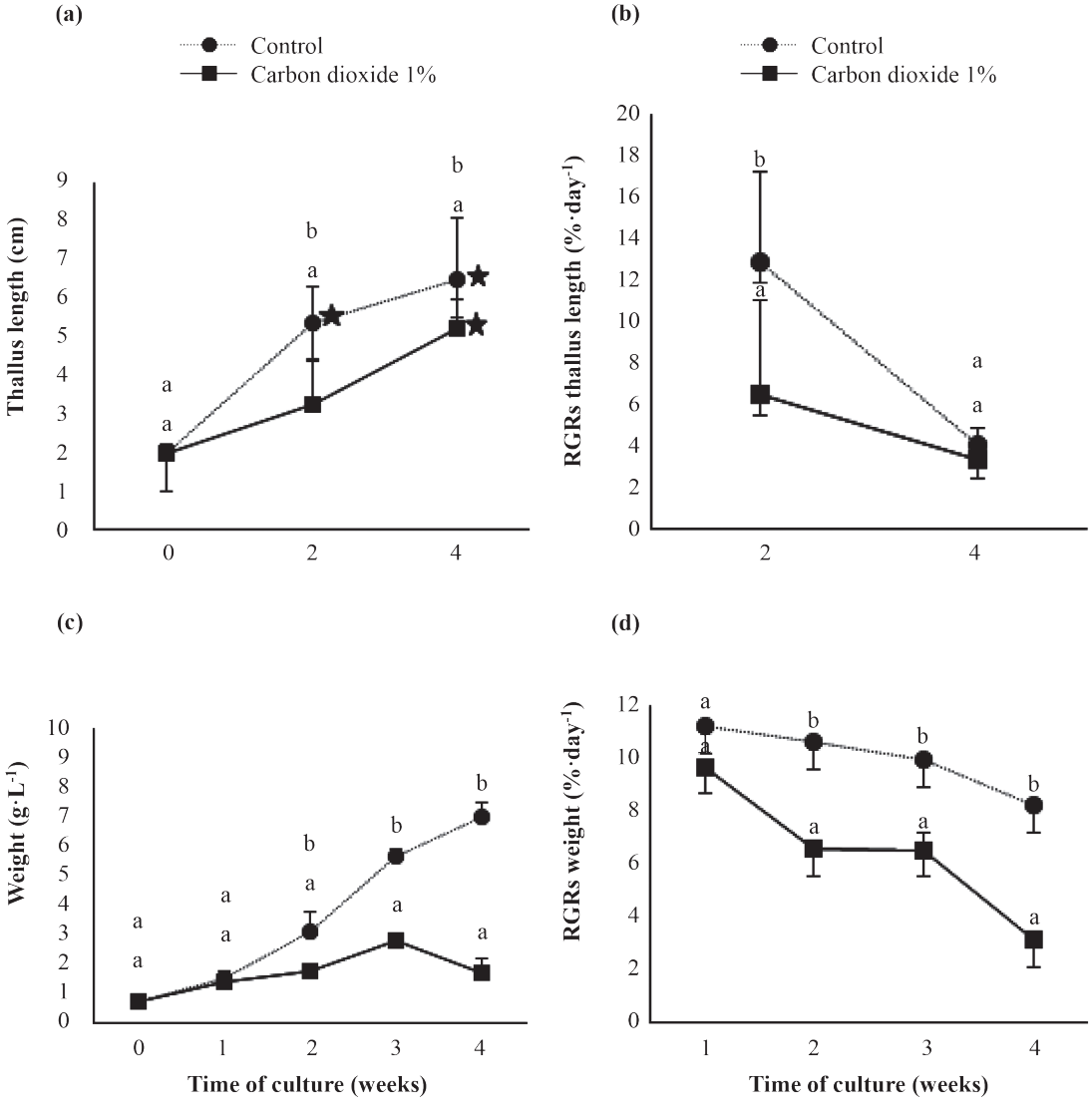

Figure 3. Growth of *Gracilaria fisheri* cultured under different carbon dioxide levels at salinity 20 ‰, 60 µmol photons·m^{-2}·s^{-1} light intensity, 25 °C and a 12:12 h light:dark period for 7 weeks: (a) thallus length, (b) RGR for thallus length, (c) thallus weight, and (d) RGR for biomass weight. Stars indicate cystocarp formation. Different letters for a given time indicate significant differences ($p<0.05$).

in Figure 5. The young cystocarp was initially observed protruding from the thallus surface in week 2 with size of 0.2±0.1 mm (Figure 5b). The cystocarp structure was completely developed and forming carpospores in week 6 (Figure 5c), then in week 7 the cystocarp of 0.5 cm height was found releasing carpospores on the bottom of the culture flask (Figure 5d). Summaries of growth and reproduction characteristics are shown in Table 1 and Figure 6.

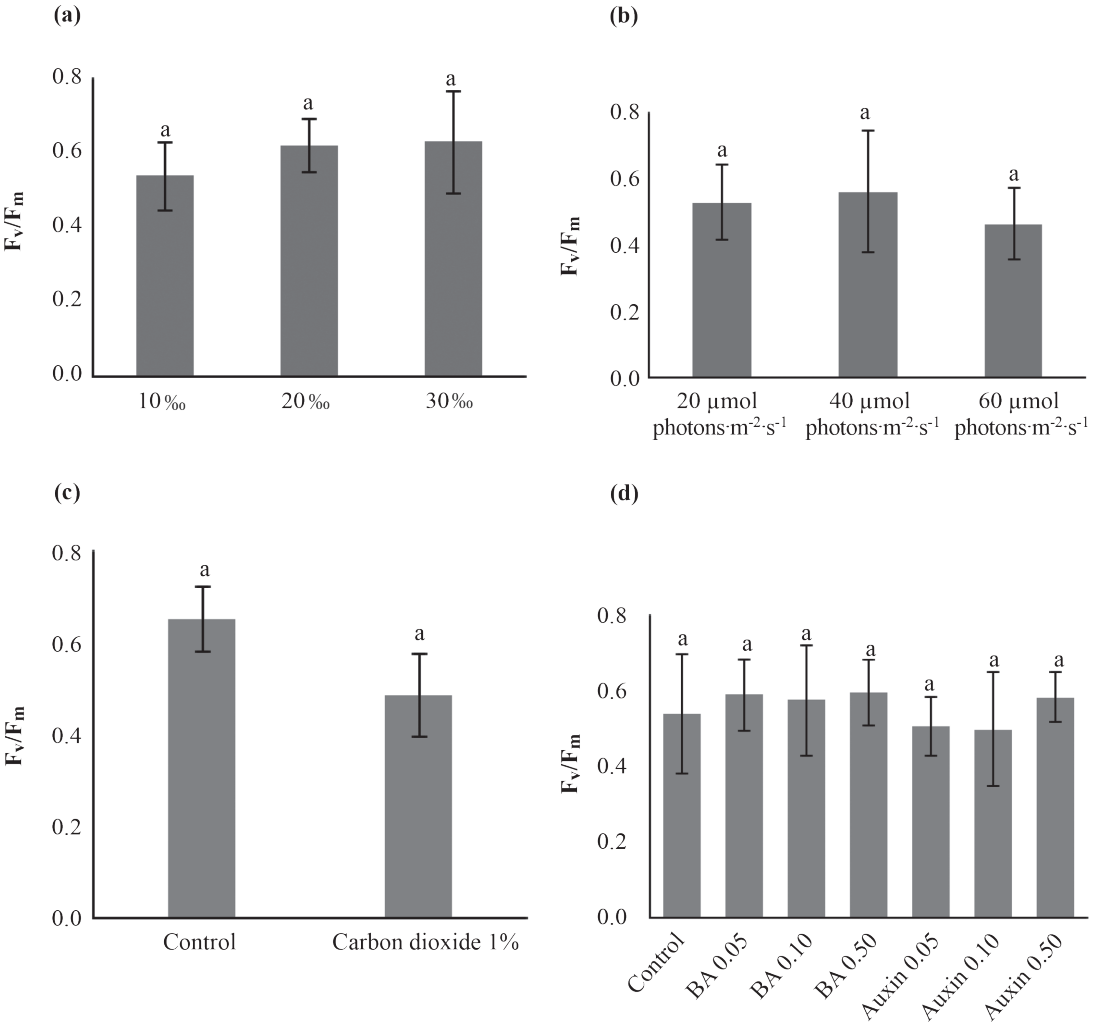

Figure 4. F_v/F_m of *Gracilaria fisheri* under various conditions (a) salinity, (b) light intensity, (c) carbon dioxide, and (d) plant hormones. Different letters above the bars indicate significant differences ($p<0.05$).

Table 1. Examination of the reproductive organ formation of *Gracilaria fisheri* under different of salinity levels, light intensities and carbon dioxide concentrations.

Experiment order	Salinity (‰)	Light Intensity (µmol photons•m^{-2}•s^{-1})	Carbon dioxide (%)	Earliest cystocarp (weeks)	Completed cystocarp (weeks)	Thallus length (cm)
1	10	20	0	3	6	4.6±1.1
	20	20	0	2	6	5.6±1.6
	30	20	0	3	6	8.3±1.7
2	20	20	0	3	6	3.9±0.9
	20	40	0	3	6	6.5±2.0
	20	60	0	2	6	5.2±0.9
3	20	60	0	2	4	5.4±1.9
	20	60	1	-	-	3.3±1.7

Note: - Not found

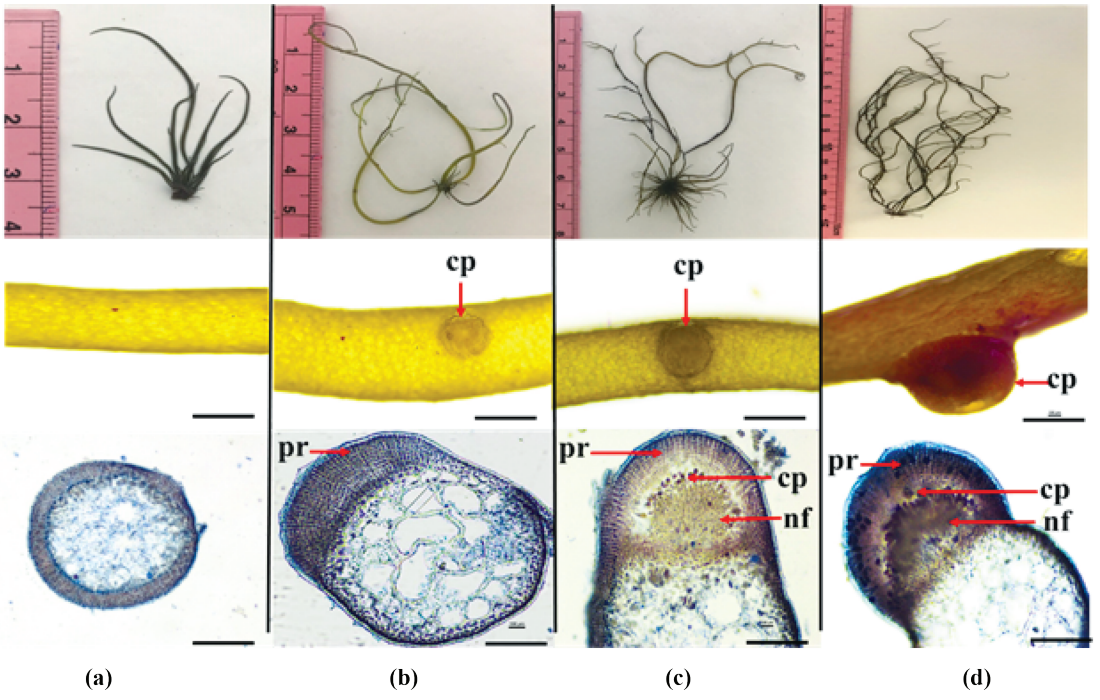

Figure 5. Development of cystocarp of *Gracilaria fisheri* at various stages and magnifications, and in cross-section. (above = whole thallus, middle = thallus fragment and below = cross section): (a) week 0, (b) week 2, (c) week 6, and (d) week 7 (pr = pericarp, cp = carpospore, nf = nutritive filament; scale bar in middle = 100 µm and below = 50 µm)

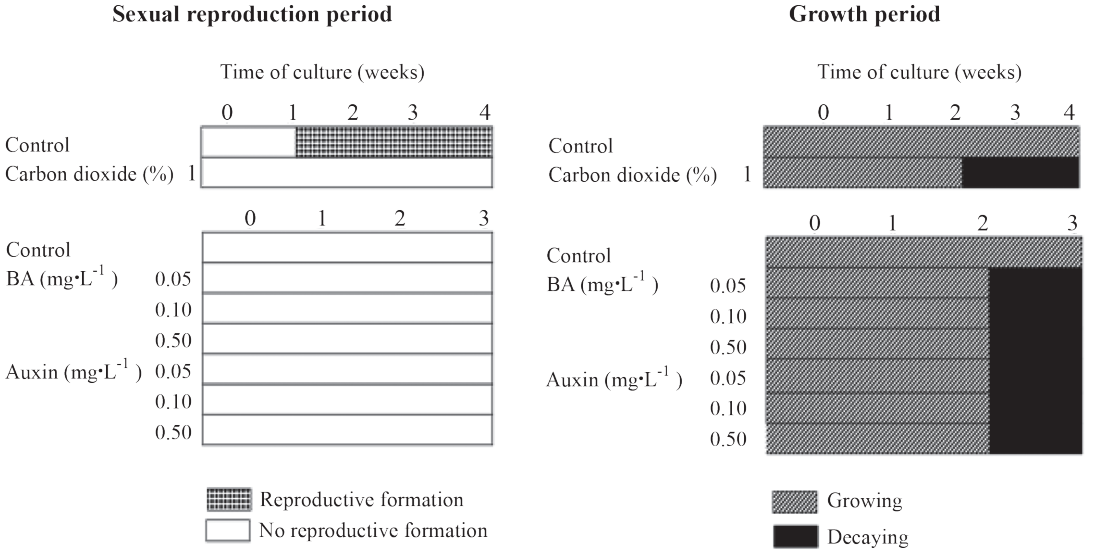

Figure 6. Periods of sexual reproduction and growth of *Gracilaria fisheri* under different carbon dioxide concentrations and under different concentrations of two plant growth hormones.

CONCLUSION

This study found that *Gracilaria fisheri* produced female reproductive cells across the range of environmental factors tested, including salinity and light intensity. Under 20 ‰ salinity and 60 µmol photons·m^{-2}·s^{-1}, *G. fisheri* was able to produce the young sexual reproductive organ in week 2. Complete cystocarps were found at week 6. The addition of chemical factors and plant hormones, including 1% carbon dioxide and addition of 6-benzyladenine and auxin seemed to inhibit cystocarp formation. Therefore, to stimulate *G. fisheri* to produce female reproductive cells, appropriate salinity and light intensity should be used.

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