

## Optimal conditions for tissue growth and branch induction of *Gracilariopsis persica*

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### Abstract

The species *Gracilariopsis persica* was first described by Bellorin et al. (2008). *G. persica* grows from late September to July and shows high growth rate from January to May in the Persian Gulf. Tissue growth and branch induction of red seaweed, *Gracilariopsis persica* from the Persian Gulf investigated under various culture levels of temperature, light intensity, photoperiod, salinity, initial length, propagule density and chemical preservatives. Optimal size of propagules used as seed was 2 cm and faster growth of tissue and branch induction obtained at lower density. The apical part of the *G. persica* showed as the starting point of growth. The *G. persica* showed optimal growth in PES medium at 24°C, 60 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity, 12L: 12D and salinity of 39‰. But maximum branch production occurred under condition of 24°C, 20 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity, photoperiod of 16L: 8D and salinity 39‰. Addition of chemical preservatives of p-hydroxybenzoic acid and potassium sorbate in culture medium showed marginal suppression on tissue growth and branch induction, that suitable for preparation of semi-axenic culture condition.

**Keywords:** Branch induction, *Gracilariopsis persica*, Preservative, Red seaweed, Tissue culture, Persian Gulf

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## Introduction

Red seaweed agarophytes are the main source of hydrocolloid agar production in their cell walls. Agarophytes are harvested commercially (Phillips and Williams, 2000) in some countries especially in the developing world, either from natural stocks and cultivated crop, and have considerable economic importance. The most important genera of commercially exploited agarophytes include *Gracilaria*, *Gracilariopsis* and *Gelidium*. Red seaweeds belong to the genus *Gracilaria* are very important as a food source for humans and marine animals, and as a source of industrial agars (Zemke-White and Ohno, 1999). *Gracilaria* is now the most important agarophyte producing about 60% of the agar in the world (FAO, 2010).

The genus *Gracilariopsis* of red seaweed is distributed throughout the tropical and warm-water regions of the world, and known as an agarophyte similar to the *Gracilaria*. It was segregated from *Gracilaria* by Dawson (1949) based on features of the cystocarps. Further compelling morphological evidence for considering *Gracilariopsis* as a distinct genus presented by Fredericq and Hommersand (1989a,b), based on detailed morphological and comprehensive studies of the genotype species of both genera (Bellorin et al., 2008). The species *Gracilariopsis persica* was first described by Bellorin et al. (2008) based on cellular and molecular analysis. *G. persica* grows from late September to July and shows high growth rate from January to May in the Persian Gulf (Salehia et al., 2010).

The overexploitation of the wild biomass of economically important agarophytes has led to the development of techniques for cultivation of seaweeds to meet increased demands (Raikar et al., 2001). If this fast growing species of *G. persica* can be used to produce in a mass scale by aquaculture, it will be also usable as an alternative agarophyte source in warm and high saline water of Persian Gulf. However, there are no data about the conditions of growth and reproduction of *G. persica* so far. Vegetative clones of *Gracilaria* and *Gracilariopsis* proved to grow indefinitely (Dawes, 1995; Hernandez et al., 2006).

Farming of *Gracilaria* and *Gracilariopsis* mainly based on the regeneration capacity of thallus fragments. It does not need sexual cycles or spore production process to propagate the seaweed crops. The initial fragment size and the original position of thalli along an axis are also parts of the few factors that the farmer can effectively handle in the field, improving production if dealt with properly (Santelices and Varela, 1995). Therefore, for tissue growth and branch induction of the *G. persica*, besides determining optimal culturing conditions of temperature, light intensity, light period and salinity, we compared propagule size, density, and growing point of branches as seeds. Chemical preservatives are also selected for bacteriostatic cultivation of the propagules.

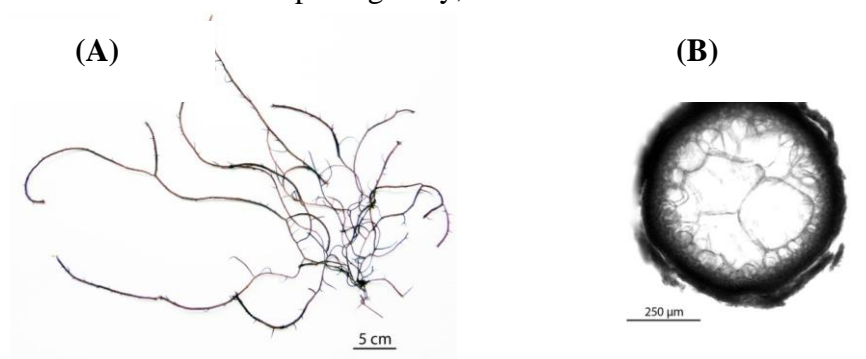
## Materials and methods

### *Tissue materials*

Samples of *Gracilariopsis persica* collected from the intertidal zone of Bandar-e-Abbas

(27°18'N, 56°30'E), Hormuzgan Province, Iran in February 2010. The plants transported to the laboratory, rinsed several times with filtered seawater to remove epiphytes and detritus, and kept in seawater tank for acclimatization. Morphologically,

the red seaweed *G. persica* seems to be the only cylindrical species with a slender and freely ramified thallus in the Persian Gulf, which makes its identification simple (Bellorin et al., 2008) (Fig. 1).



**Figure 1: Morphological shape (A) and cross section of main axis (B) of the seaweed sample collected in the intertidal zone of northern Persian Gulf**

#### *Culture condition*

Middle part of propagule used as primary materials for tissue culture and branch induction. Clean parts of seven samples cut in 5 cm fragments, to use as propagules. The propagules attached on rope and submerged in 500 mL beaker containing 200 mL of PES(Provasoli's enriched Seawater Medium) medium (Provasoli, 1968) for 10 days and experiments were repeated three times.

Standard culture condition adapted at 24°C, 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity (3000 Lux), 12L: 12D light cycle and 35‰ salinity. Light provided by white fluorescent lamps with programmable timer used to adjust photoperiod and intensity measured by luxmeter (Sekonic Lumimeter 246, Japan). A custom design culture table that capable to provide gradient of light intensity and various temperatures used to investigate light and temperature effects Different

salinities prepared by adding distilled water or solar salt (Daesang Co., Korea) to filter seawater to get the desired salinity of 24 to 42‰, which checked using Salinometer (Atago Master-S28M, Japan) (Raikar et al., 2001). Seawaters were autoclaved and enriched by PES medium.

After 10 days culture, wet weight of the tissues measured by removing extra water from tissues by centrifugation at 1000 x G for 5 min. Relative growth rate (RGR; %  $\text{day}^{-1}$ ) calculated using the formula:  $\text{RGR} = [(W_t - W_0)/W_0] \times 100 / 10$  days, where  $W_0$  is the initial weight,  $W_t$  is the final weight (Choi, Kim et al. 2006). Number of lateral branches was counted at the initial time and after 10 days culture. Statistical analyses carried out using SPSS v.16. A two-way ANOVA was used to test the effects of temperature and light on the relative growth rate and new branches induction. A one-way ANOVA was used to determine the

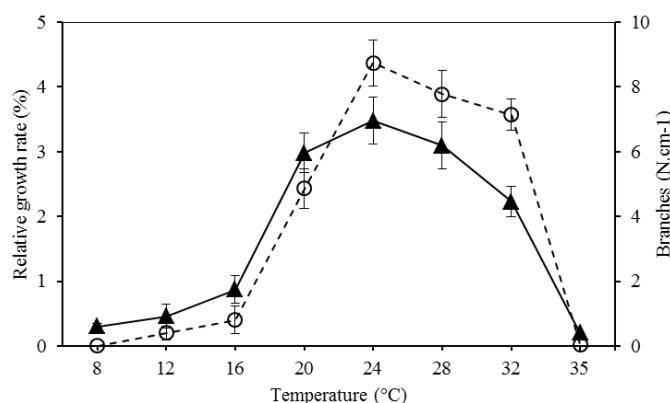
differences in weight, and branch number at various conditions.

### Chemical preservatives

To get better tissue growth and branch induction in a bacteria-free or bacteriostatic condition, several chemical preservatives using in food preservation were tested. Each preservative agent added to PES medium with a permissible amount as food additives (KFDA, 2004); such as 10 ppm menadione sodium bisulfate, 600 ppm sodium propionate, 270 ppm potassium sorbate, 230 ppm sodium dehydroacetate, 500 ppm sodium benzoate, 370 ppm sodium salicylate, 200 ppm chitosan, 100 ppm germanium oxide, and 250 ppm p-hydroxybenzoic acid.

## Results

### Temperature



**Figure 2.** Effects of different temperatures on tissue growth ( $\blacktriangle$ ) and branch induction ( $\circ$ ) of *G. persica* under standard culture condition. Values represent the mean  $\pm$  SE ( $n \geq 7$ ).

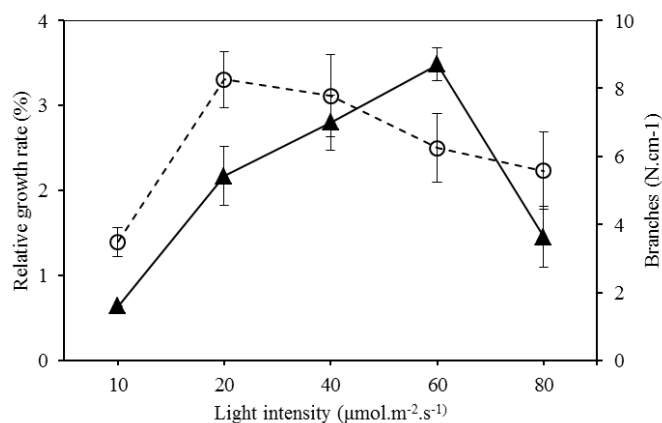
### Light intensity

Various light intensities of 10 to 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$  studied to determine the optimal range of required light intensity. It grew slowly at 20 and 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and maximum growth occurred at 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with the

To determine the optimal temperature for growth of the *G. persica*, tissues cultured at wide range of temperatures from 10 to 35 °C in PES medium under standard cultured condition. The *G. persica* showed optimal growth at 24°C with the average growth rate of 3.7% day<sup>-1</sup> (Fig. 2). It grew well in a range of 20 to 32°C, but tissues became completely discolored after 3 days at 35°C. Maximum new branches produced at 24°C with average branches of 8.5 per cm of tissues although branch production continued in 28 and 32°C. The results showed significant difference in growth rate and branch induction between temperatures below 20°C and 20-28°C ( $p < 0.01$ ). The growth rate and branch induction decrease at temperature above 32°C, and showed significant difference with temperature 20-28°C.

average growth rate of 3.5% day<sup>-1</sup> (Fig. 3), which shows significant difference with light intensities of 10 and 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Maximum number of new branches appeared at light intensity of 20  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and 8.3 branches/cm of tissue produced at this level of intensity. This level of light

intensity showed significant difference with but nearby similar to intensity of 40 light intensity of 10, 60 and 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

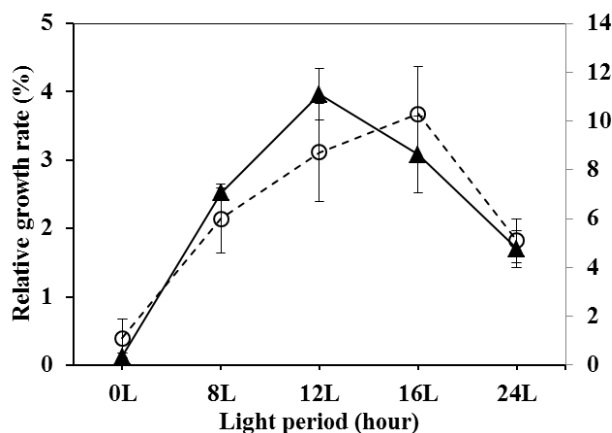


**Figure 3.** Effects of different light intensities on tissue growth (▲) and branch induction (○) of *G. persica* under standard culture condition. Values represent the mean  $\pm$  SE ( $n \geq 7$ ).

#### Photoperiod

*G. persica* showed the highest growth rates on a 12 h L: 12 h D cycle at 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (3.9%  $\text{day}^{-1}$ ) and showed significant

difference with 0L: 24D and 24L: 0D (Fig. 4), maximum branching occurred on a 16 h light: 8 h dark cycle with average branch numbers of 10.3 per cm of tissues.

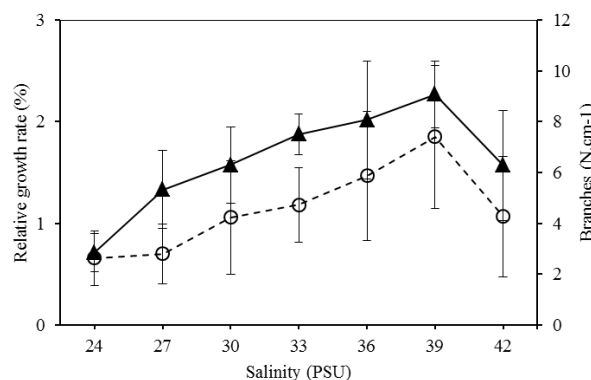


**Figure 4.** Effects of different light periods on tissue growth (▲) and branch induction (○) of *G. persica* under standard culture condition. Values represent the mean  $\pm$  SE ( $n \geq 7$ ).

### Salinity

Salinity experiments carried out at a range of salinities from 24 to 42 ‰ under standard culture condition. The tissues showed optimal growth at 39 ‰ with the average growth rate of 2.2% day<sup>-1</sup> (Fig. 5). It grew

2.0% day<sup>-1</sup> at normal seawater salinity of 39‰. Maximum new branches produced at 39 ‰ with average branch numbers of 7.4 per cm of tissues. Branch induction and growth rates showed significant difference at salinity of 24 and 39‰.

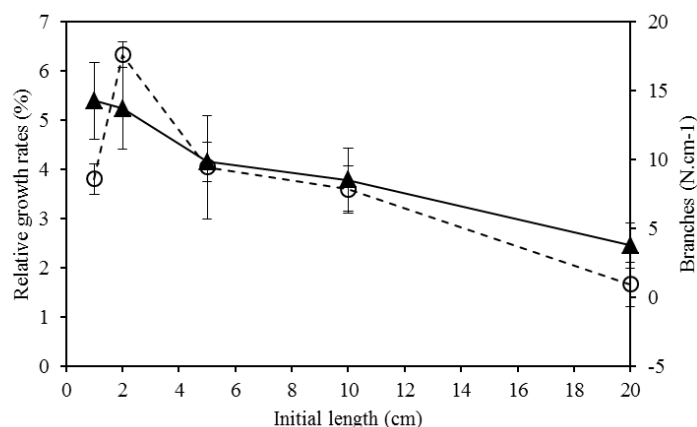


**Figure 5.** Effects of different salinities on tissue growth (▲) and branch induction (○) of *G. persica* under standard culture condition. Values represent the mean ± SE (n ≥ 7).

### Effects of initial length

To examine initial length of propagules on growth rates, different length fragments of 1 cm to 20 cm cultivated under standard cultural condition. The tissues of 1 to 2 cm showed the highest growth rate with the average growth rate of 5.4 or 5.2 % day<sup>-1</sup>

(Fig. 6). Maximum new branches were induced by 2 cm length of tissues with average branches of 17.6 per cm. Hence 2 cm length tissues can be used for seed maintenance or initiate in-door cultivation. Fragments of 2 cm length showed significant difference in production of new branches with other treatment ( $p < 0.0.1$ ).

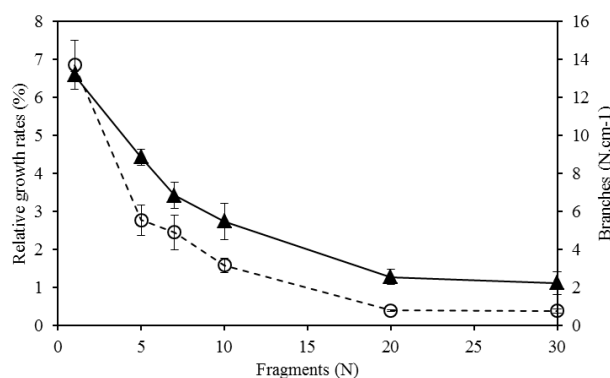


**Figure 6.** Effects of propagule size on tissue growth (▲) and branch induction (○) of *G. persica* under standard culture condition. Values represent the mean ± SE (n ≥ 7).

### Propagule density

Optimal propagule density of *G. persica* thalli in a closed culture system were investigated at different culture density. 1 to 30 pieces of 5 cm fragment size were cultured in 200 mL medium under standard culture condition. Less numbers of propagule appeared faster growth. When a single piece of propagule was cultured, it showed highest growth rate of  $6.6\% \text{ day}^{-1}$  that is

almost double comparing to 7 pieces-cultures at standard condition and showed significant difference (Fig. 7). Single piece culture produced more new branched of 13.7 per cm, and compare to 7 pieces culture, the induction was double. By increasing numbers of propagule, growth rates and branch induction were decreased to  $1.1\% \text{ day}^{-1}$  and 0.7 branches per cm, respectively, with 20 or more pieces of propagule.

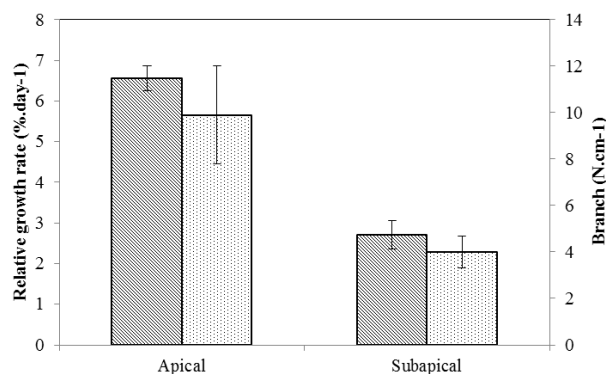


**Figure 7.** Effects of propagule densities on tissue growth (▲) and branch induction (○) of *G. persica* under standard culture condition. Values represent the mean  $\pm$  SE ( $n \geq 7$ ).

### Growing zone

Growing zone of *G. persica* thalli examined through culture of different parts of plant, such as tissues of apical fragment (5 cm) and subapical fragment (5 cm) under standard culture condition. Elongation of thalli occurred faster at

apical part with the average growth rate of  $6.6\% \text{ day}^{-1}$  (Fig. 8). New branches were induced more from the same apical part with average branches of 9.9 per cm. Thus, the apical part of *G. persica* appears as the growing point.

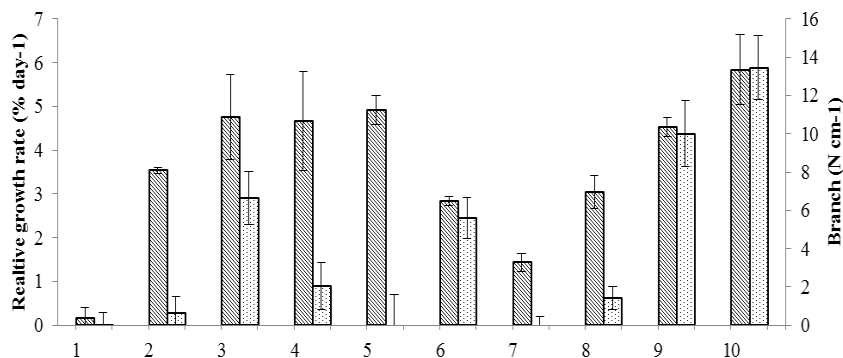


**Figure 8.** Comparison of apical and subapical parts on tissue growth (▨) and branch induction (▤) of *G. persica* under standard culture condition. Values represent the mean  $\pm$  SE ( $n \geq 7$ ).

### Treatment by chemical preservatives

For stock maintenance and propagule cultivation, it is necessary to culture propagules in a bacteria-free or bacteriostatic condition. Food chemical preservatives added to PES culture medium,

propagule tissues showed different growth rate and branching. Compare to control (without adding preservatives), potassium sorbate, sodium dehydroacetate, sodium benzoate and p-hydroxybenzoic acid had marginal negative effects on tissue growth (Fig. 9).



**Figure 9.** Effects of chemical preservatives on tissue growth (▨) and branch induction (▤) of *G. persica* under standard cultural condition. 1, menadione sodium bisulfate (10 ppm). 2, sodium propionate (600 ppm). 3, potassium sorbate (270 ppm). 4, sodium dehydroacetate (230 ppm). 5, sodium benzoate (500 ppm). 6, sodium salicylate (370 ppm). 7, chitosan (200 ppm). 8, germanium oxide (100 ppm). 9, p-hydroxybenzoic acid (250 ppm). 10, control. Values represent the mean  $\pm$  SE ( $n \geq 7$ ).

### Discussion

The fast growing and tall agarophyte species of *G. persica* is present in the restricted area of Persian Gulf coast. Collection site of the species is sand beach located in Bandar-e-Abbas city on the southern coast of Iran. *G. persica* grows fast after attaching to pebbles

or artificial substrates on sandy-loam or sandy bottoms, at the intertidal or sublittoral regions of coasts not exposed to strong wave actions or currents. The fronds are abundant from mid-October to May in this area. The plants are growing in cluster up to 250 cm long (Bellorin et al., 2008). The average sea



surface temperature ranges from 20 to 27°C throughout the growing period (Oct-May), and the salinity ranges from 37‰ in winter to 38‰ in summer (Bidokhti and Ezam, 2009). Hence in this research various culture condition to acquire the optimum results have been studied. Our results show the optimal growth of *G. persica* was occurred at 25°C, 60 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity and 39‰ salinity that are similar to the environmental condition of the natural growing site. The temperature, light and salinity are the most critical factors determining the growth and distribution of benthic marine algae (Raikar et al., 2001). *G. persica* tolerated relatively high temperature of 32°C and high salinity of 42‰. Those can be used as a thermo- and halo-tolerant gene resource. Branching more occurred in longer photo period (16h) and lower light intensity (20 μmol m<sup>-2</sup> s<sup>-1</sup>) compare to optimal growing light condition (Figs.3, 4). Primary growth initiate by producing more branches in sublittoral area during October, and secondary grow occurred by floating tall fronds in littoral zone in next months. Higher growth rate of apical part indicates the growing zone of the *G. persica*, located near the apical parts of frond, is similar to *G. chilensis* (Santelices and Varela, 1995) and *G. gracilis* (Smit and Bolton, 1999). To enhance biomass of the seaweed, branches may be induced at early culture period and set out to sea as main cultivation. More tissue growth and branch induction produced in high salinities (up to 39‰), which is indicating that the seaweed is adapted to high salinity condition and can be cultivated in closed seawater ponds. In case of *G. persica*, we found that dim light at 20 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity on a 16 h

light: 8 h dark cycle was optimum condition to induce more branches. To provide yearlong seed stock for seaweed cultivation, it is necessary to keep the seed stock in a condition of good viability and no bacterial contamination. This study showed Food preservatives additive can be used as a contamination inhibitor agents. When branch induction is also required during stock cultivation, p-hydroxybenzoic acid and potassium sorbate are recommended to be used. Sodium benzoate at 500 ppm produced no branches but main stems were elongated normally.

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