



An orthogonal design for optimization of growth conditions for all life history stages of *Gracilariopsis lemaneiformis* (Rhodophyta)

Wei Zhou, Zhenghong Sui ^{*}, Jinguo Wang, Lianpeng Chang

Key Laboratory of Marine Genetics and Breeding Ministry of Education, Ocean University of China, Qingdao 266003, People's Republic of China

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ABSTRACT

Gracilariopsis lemaneiformis is an important economic macroalgae worldwide. At present it is mainly cultured by vegetative propagation, which is labor intensive, bulky, and requires large amounts of propagating material. To identify the optimal conditions for spore release, spore development, and fertilization success of *Gp. lemaneiformis*, four levels of each of four factors (light, temperature, salinity, and photoperiod) were tested using an orthogonal experimental design. Variations in temperature and salinity had significant effects on carpospore yield, and the optimal conditions for carpospore release were 20 °C, 35‰, 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and 8/16–14/10 h L/D. The main factors that influenced tetraspore release were temperature, light, and salinity, with the optimal combination being 25 °C, 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 35‰, and 8/16–14/10 h L/D. Temperature variation also had an effect on spore development, whereas the effects of the other factors were not significant. The optimal conditions for tetraspore development were 25 °C, 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 12/12 h L/D, and 25‰, and for carpospore development they were 25 °C, 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 30‰, and 12/12 h L/D. Temperature significantly affected fertilization success of male and female gametophytes, whereas salinity, photoperiod, and light intensity had no significant effect ($P > 0.05$). The optimal combination of factors for fertilization success was 25 °C, 30‰, 12/12 h L/D, and 15–45 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The culture time of sporeling growth was reduced by about 3–4 months in the study, the results of which can also provide valuable information for genetic breeding, large-scale culture of spores, and algal cultivation.

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1. Introduction

Gracilariopsis is a genus newly separated from the genus *Gracilaria* in the family Gracilariaceae Nageli (Rhodophyta) (Bird et al., 1992, 1994; Fredericq and Hommersand, 1989; Gurgel, 2002). *Gracilariopsis* spp. are widely distributed around the world and are important economic macroalgae, as they are utilized for agar extraction and potentially may play an important role in bioremediation (Freile-Pelegrin and Murano, 2005; Lapointe and Ryther, 1978; Marinho-Soriano, 2001; Marinho-Soriano and Bourret, 2003; Zhou et al., 2006). Zhang et al. (2009) also suggested that many Gracilariaceae species may prove to be ideal material for genetic research.

Large-scale raft cultivation of *Gracilariopsis lemaneiformis* dates back to the 1950s (Tseng, 2001; Wu, 1998; Zou et al., 2004). Since the successful transplantation of the northern wild-type strain to southern China in the 1990s, *Gp. lemaneiformis* has become the third largest cultivated algae species in China, and related industries also have developed (Fei, 2004; Fei et al., 1998; Liu, 2001; McHugh,

1991; Yang et al., 2006). Currently, *Gp. lemaneiformis* is cultivated mainly by vegetative propagation, which does not only grow fast but is also harvested easily from the seawater (Yang et al., 2006). Especially, the single rope floating raft cultivation based on vegetative propagation has been reported to be more productive than other methods, such as cage, bottom-stocking and net culture (Critchley, 1993; Subba Rao and Mantri, 2006). Furthermore, thalli from vegetative propagation have a high genetic stability. However, methods of vegetative propagation are labor intensive, bulky, and requires large amounts of propagating material. Hurtado-Ponce et al. (1992) reported that 20–30% of the harvest may be used as seed material for subsequent cultivation.

Gracilariopsis species show a typical polysiphonia-type life history with three phases consisting of morphologically identical tetrasporophyte (2n) and gametophyte (n) phases and an additional carposporophyte (2n) phase (Kain and Destombe, 1995). It takes nearly 12 months to complete the life history in lab culture (Plastino and Oliveira, 1988). Through meiosis, mature tetrasporophytes give rise to tetraspores (n), which subsequently develop into male and female gametophytes (Engel et al., 2001). Fertilization occurs on the female gametophyte and the zygote grows into the carposporophyte. Carposporophytes release thousands of carpospores (2n), which then develop into tetrasporophytes (Yamamoto and Sasaki, 1988).

^{*} Corresponding author at: College of Marine Life Sciences, Ocean University of China, Qingdao 266003, People's Republic of China. Tel.: +86 532 82031128, +86 13854283869(mobile); fax: +86 532 82031647.

E-mail address: suizhengh@ouc.edu.cn (Z. Sui).

Spore culture is an alternative cultivation technique, and it has proven suitable for mass production of many seaweeds (Le Gall et al., 2004; Levy et al., 1990; Lin et al., 1979). Spore (carpospores or tetraspores) culture has been demonstrated to be technically feasible for *Gracilaria* species (Glenn et al., 1996; Polifrone et al., 2006; van der Meer, 1981). The major advantage of the spore propagating method is that only a small amount of reproductive material is required, and it can provide a large number of unified seedlings of desired ploidy (Mantri et al., 2009). More productive cultivation methods are needed in order to mass produce *Gp. lemaneiformis*. Thus, an extensive spore culture method for this species should be established as an alternative to vegetative propagation (Glenn et al., 1998; Guzmán-Urriostegui and Robledo, 1999; Levy et al., 1990). The following problems may occur in this method: (1) there is a relatively long lag period for sporeling growth; (2) genetic differentiation may occur and produce sporelings with undesirable characteristics, such as non-tolerance to high temperature and low growth rate, which might be negative for a production. However, these problems can be overcome through the application of growth condition optimum to promote the growth of spores and the choice of the parents before the fertilization, where both sides should be with expected traits.

Normally, the life history of *Gp. lemaneiformis* can be completed in the laboratory within about 1 year, but this time interval is too long for genetic breeding in the lab where a fast life cycle is required. Multiple factors influence spore release, spore development, and fertilization success of male and female gametophytes, but detailed knowledge about these factors is lacking. Herein, the effects of temperature, light, salinity, and photoperiod on spore release, spore development, and the success of fertilization success of *Gp. lemaneiformis* were examined using an orthogonal experimental design. The aims of this study were to identify the optimal culture conditions for each growth period of this species in order to accelerate the completion of its life history and to utilize sexual reproduction instead of vegetative propagation for field cultivation of *Gp. lemaneiformis*.

2. Materials and methods

Fertile tetrasporophytes and cystocarps of *Gp. lemaneiformis* used in this study were collected in November 2009 from the intertidal zone of Zhanshan Bay, Qingdao (36° 0'N, 120° 3'E), China. After washing the specimens in seawater to remove epiphytes and sand, the thalli were rinsed with sterilized seawater twice and soaked in 1% sodium hypochlorite for 2 min. The thalli were treated in antibiotic seawater containing 0.1 g L⁻¹ kanamycin, 0.2 g L⁻¹ nystatin, 0.3 g L⁻¹ penicillin, 1.0 g L⁻¹ streptomycin sulfate, 0.02 g L⁻¹ cefotaxime, and 0.1 g ml⁻¹ GeO₂ for about 6 h. The organisms then were cultured continuously for a week in sterilized seawater containing PES culture medium (Provasoli, 1968) under the following conditions: temperature of 20 °C, photon flux density of 30 μmol m⁻² s⁻¹, salinity of 30‰, and photoperiod of 12/12 h light/dark (L/D). These algae then were used in the following experiments.

2.1. Distinguishing tetrasporophytes and gametophytes of *Gp. lemaneiformis*

Gp. lemaneiformis showed a typical isomorphic alternation of generations, so there was no difference in morphology between immature haploid and diploid individuals. However, mature tetrasporophytic thalli could be identified by the presence of tetrasporangia scattered over the whole thallus to form reddish marbling. Mature female gametophytes had cystocarps that were macroscopic hemispherical swellings present on the surface of female branches, and male gametophytes possessed superficial cortical crypts and slight elevation of the thallus surface (Fredericq and Hommersand, 1989; Xu et al., 2008). Specimens of *Gp. lemaneiformis* had multiple branches that consisted of first (Fig. 1, arrow 1), second (Fig. 1, arrow 2), and third generation



Fig. 1. Mature *Gp. lemaneiformis* collected in Nov. 2009 from the intertidal zone of Zhanshan bay, Qingdao (36° 0'N, 120° 3'E), China. The different branches are shown by the black arrow. 1. The first generation branching; 2. The second generation branching; 3. The third generation branching. Scale bars = 2 cm.

branches (Fig. 1, arrow 3) based on the branching sequence from the main stem.

2.2. Experimental design and statistical analysis

In order to identify the optimum experimental conditions with the fewest experiments, the experiments were designed following the orthogonal method (Taguchi, 1987). An orthogonal design allows the effects of each individual factor tested to be separated (Abud-Archila et al., 2008). Four levels of each of the four selected factors (light, temperature, salinity, and photoperiod) were examined in 16 experimental runs [orthogonal array L₁₆(4⁵)]. Table 1 shows the four factors (coded as A, B, C, and D) and their levels (coded as 1, 2, 3 and 4). The values chosen for each level were based on the appropriate range found in the literature (Bird et al., 2009; Li et al., 1984; Wang et al., 2010; Whyte et al., 2009; Yang et al., 2006; Ye et al., 2006).

The results of the 16 different combinations were evaluated using range and variance analysis. K₁, K₂, K₃, and K₄ were the average values of Level 1, Level 2, Level 3, and Level 4 for each factor, respectively. Optimal conditions were determined by the highest value of K (K_{max}) of the various factors (Cornejo-Mazon et al., 2008; Venkata Mohan and Chandra Mouli, 2008). The range (R) was the difference between the maximum and minimum average (K_{max} – K_{min}) for each factor, which was used to rank the four factors with respect to variability in magnitude. Analysis of variance (ANOVA) was also used to determine how much of the variation was contributed by each factor and the significant effect (Snedecor and Cochran, 1989). ANOVA was based on the sum of squares of deviations from the overall mean (SS), the

Table 1
Levels and factors of the L₁₆(4⁵) orthogonal experimental design.

Factor	A Temperature (°C)	B Light (μmol·m ⁻² ·s ⁻¹)	C Salinity (‰)	D Photoperiod (h L/D)
Level 1	30	30	30	14/10
Level 2	20	15	20	10/14
Level 3	25	45	35	12/12
Level 4	15	60	25	8/16

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