



Mass cultivation of economically important red alga *Gracilariopsis lemaneiformis* (Gracilariaceae, Rhodophyta) from tetraspores and carpospores

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ABSTRACT

Gracilariopsis lemaneiformis is an economically important red alga worldwide, and is currently cultured by vegetative propagation. However, information related to the practice of spore culturing in red alga, especially for this species, is limited. Here, we describe a successful pilot study that used tetraspores and carpospores for the industrial cultivation of *G. lemaneiformis* in Jiaozhou Bay, Qingdao, China. Morphological observation and culture studies of *G. lemaneiformis* spores were carried out in the laboratory and field. Related spore parameters of daily output, germination rate, survival number, and growth rate were determined. The results showed that the maximum daily output occurred on the 6th day for tetraspores, with a value of $5.71 \pm 0.68 \times 10^5 \text{ g}^{-1}$ fresh weight, and on the 4th day for carpospores, with the value of 1876 ± 153 carpospores per cystocarp. During early culturing, the development and growth of tetraspores and carpospores were very similar. Inoculation density variation had significant effects on germination rate, survival number, and growth rate, the highest values of which were recorded at medium density, high density, and medium density inoculation, respectively. After 3 weeks in the field, the tetrasporelings and carposporelings were ~3 cm in length. Different heights below sea level also had a notable effect on sporeling growth, and the optimal condition was 0.5 m below sea level. After further culturing, the sporelings showed fast adaptation to the sea with average growth rates of $6.21 \pm 0.48\% \text{ day}^{-1}$ for tetrasporophytes and $4.21 \pm 0.36\% \text{ day}^{-1}$ for gametophytes. This study has demonstrated that the mass cultivation of *G. lemaneiformis* using tetraspores and carpospores under controlled culture conditions is feasible, and has also provided valuable information for improving the only available culture method (vegetative propagation) for *G. lemaneiformis* in the field.

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1. Introduction

Gracilariopsis lemaneiformis (Bory) Dawson, Acleto et Foldvik is widely distributed in temperate zones around the world and is an important economic macroalgae (Wu and Pang, 1998). Compared to other species, *G. lemaneiformis* has a higher growth rate and produces a high-quality gel; hence its thalli are mainly used for agar extraction (Chang et al., 2014) and as human food or abalone feed (Qi et al., 2010). *Gracilariopsis* also serve as efficient bio-filtration systems, removing excess nitrogen, phosphorus, and heavy metals in seawater, and thus play a major role in bioremediation and the prevention of seawater eutrophication (Fei, 2004; Yang et al., 2006).

Wild thalli of *G. lemaneiformis* have multiple branches, are normally reddish purple, and grow out from a discoid holdfast, which is covered

by sand on gravel in the lower intertidal zone (Wang et al., 2010). Over the past few decades, natural stocks of *G. lemaneiformis* were available all year round along the coast of China, but they have gradually become scarce because of human disturbance and excessive sampling (Zhou et al., 2014). Historically, most of the wild stocks were harvested and used as starting materials for marine aquaculture (Ye et al., 2006). However, it has been difficult to meet the increasing demand of the agar industry (Fei et al., 1998). Thus, greater attention has been given to the development of algae cultivation in many countries, such as Chile, America, and China (Buschmann et al., 2001; Glenn et al., 1996; Li et al., 1984). Not until successful transplantation of the northern wild-type strain to the southern region and the introduction of improved variety 981 did large-scale cultivation of *G. lemaneiformis* become successful in China (Fei et al., 1998). According to official statistics, *G. lemaneiformis* has become the second largest cultivated macroalgal species, and is broadly cultivated along the coast of Guangdong, Fujian, Zhejiang, Shandong, and Liaoning Provinces in China (Chinese Ministry of Agriculture, 2014).

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At present, the culture of *G. lemaneiformis* is mainly based on vegetative propagation and relies on fragments of thalli, rather than spores, including carpospores and tetraspores from mature thalli, as the propagating starting material for producing biomass (Fei et al., 1998; Li et al., 1984; Yang et al., 2006; Zhou et al., 2013a; Zhou et al., 2013b). This farming method is similar to that of most of the *Gracilaria* species. There are a number of techniques, such as bottom-stocking, rope, cage, net, and floating raft culture, etc. (Critchley, 1993), that lead to higher productivity, easier harvesting and higher genetic stability, especially the single rope floating raft technique (Subba Rao and Mantri, 2006). However, during long-term cultivation, the single rope floating raft technique method also has many disadvantages: firstly, the method is generally labor intensive and bulky, because it requires a lot of manual labor during the hand-planting process (Mantri et al., 2009); and secondly, it is not very efficient because a great deal of material is needed to start the cultivation (Glenn et al., 1996). Normally, 30% of the harvest is used as seed materials for subsequent cropping (Hurtado-Ponce et al., 1992). Last, but not the least, seed aging easily occurs, which can lead to declining growth rates due to repeated and continuous farming from the same stock (Oliveira et al., 2000).

The spore culture method is an alternative option for reducing the disadvantages listed above, and has attracted the attention of many macroalgae operations (Martín et al., 2011). Under natural conditions, spore (carpospore or tetraspore) culturing has been successfully applied to many species of *Gracilaria*, such as *Gracilaria parvispora* (Glenn et al., 1996; Glenn et al., 1998), *Gracilaria chilensis* (Alveal et al., 1997; Halling et al., 2005), *Gracilaria dura* (Mantri et al., 2009), and *Gracilaria gracilis* (Hughes et al., 2014; Michetti et al., 2013), but the number of these successful applications is rare in natural seawater. *G. lemaneiformis*, with its typical polysiphonia-type life history, which is similar to that of *Gracilaria* spp., can develop to maturity and release surprisingly large numbers of tetraspores or carpospores from a small thallus biomass (Zhou et al., 2013a). According to Ye et al. (2006); Wang et al. (2010), and Zhou et al. (2013a), the use of these spores as seeds allows large amounts of biomass to be produced under laboratory conditions, which further implies that the spore culture method is potentially feasible for *G. lemaneiformis* in natural waters.

Information related to the practice of red alga spore culturing, especially *Gracilariopsis*, is limited. Here, we describe a successful pilot study that used tetraspores and carpospores to industrially cultivate *G. lemaneiformis* in Jiaozhou Bay, Qingdao, China, and the future aim is to scale up the spore culture method (sexual propagation) and improve the only field culture method for *G. lemaneiformis* (vegetative propagation).

2. Materials and methods

2.1. Location of experiments and treatment of reproductive thalli

Culture experiments were carried out from June to December, 2012 and from June to December, 2013 in Jiaozhou Bay (36°02' N, 120°17' E), Qingdao, on the east coast of Shandong Province, China. Wild-type gametophytes and tetrasporophytes of *G. lemaneiformis* from the *Gracilaria* and *Gracilariopsis* germplasm banks at the Key Laboratory of Marine Genetics and Breeding of Ministry of Education, Ocean University of China, were transferred to Jiaozhou Bay and cultured to maturity. Subsequently, reproductive tetrasporic and cystocarpic thalli of *G. lemaneiformis* were collected in July and transported to the laboratory using labeled plastic bags at low temperature. The thalli were washed with seawater in order to remove sand, mud, and visible epiphytes, and then rinsed in antibiotic seawater for ~6 h to reduce the bacterial load (Ye et al., 2006). Mature cystocarpic and tetrasporic thalli could be readily identified with a light microscope (Olympus CX31, Olympus, Tokyo, Japan) based on the surface morphology (Xu et al., 2008). After that, the two groups were individually kept in plastic containers containing Provasoli enriched seawater culture medium culture medium

(Provasoli, 1968) at 25 °C, 15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 35 psu, and a 12:12 LD cycle for tetrasporophytes, and at 20 °C, 35 psu, 15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and a 12:12 LD cycle for carpospores (Zhou et al., 2013a). These algae were then used in the following experiments.

2.2. Inoculation of net curtains, and early culture of microsporelings in tanks

As shown in Fig. 1, the inoculated system was mainly composed of net curtains, a submersible pump, ropes stocked with thalli, translucent plastic wrap, a square plastic box, and seawater. The net curtain (20 cm long $\times$ 15 cm wide) was used for spore attachment and as an inoculated substrate, which was made by adding epoxy to cotton rope (2 mm diameter). The submersible pump was adjusted so that the seawater in the system was kept moving in a stable direction, thereby ensuring a uniform distribution of the spores. Ropes with attached thalli were horizontally tied to the upper side of the box and the depth was kept constant at 5 cm below the seawater surface. Plastic wrap covered the upper surface of the plastic box and was used to prevent water loss and reduce contamination of the system. Both the square plastic box and the seawater (salinity, 35 psu) were sterilized to avoid contamination.

Mature tetrasporic and cystocarpic thalli (50 g each), that were ready to release spores, were separately put into different 20 L sterilized seawater systems containing ten net curtains, all of which were placed in the greenhouse under the following conditions: for tetraspore release: 25 °C, 15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a 12:12 LD cycle; and for carpospore release: 20 °C, 15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and a 12:12 LD cycle (Zhou et al., 2013a). Normally, spore release would start within a week and was checked using an inverted microscope (CKX41, Olympus, Japan). The average density of spore attachment on each net curtain was quantified by counting the average number of spores in 30 microscopic fields at 10×10 magnification. Three different inoculation densities were set and the net curtains were divided into three groups, i.e., a high density of >100 spores field^{-1} , a low density of <10 spores field^{-1} , and a medium density of 30–60 spores field^{-1} . The total number of spores released per day (daily output of spores) was also counted, and the count was continued until the tetrasporic or cystocarpic thalli stopped shedding spores.

The inoculated net curtains were then transferred to a 50 L tank with natural seawater that had been enriched with PES culture medium. The tetraspore and carpospore curtains were continuously aerated and kept at 25 °C, 45 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and under a 12:12 LD cycle and 25 psu, for tetraspores, and at 25 °C, 45 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 30 psu and a 12:12 LD cycle for carpospores (Zhou et al., 2013a). The early spore culturing stage lasted for 5 weeks until upright individuals formed. During this time the culture medium was replaced once a week.

2.3. Morphological changes, germination, survival, and growth of microsporelings on net curtains at different inoculation densities

During early culturing, the developmental process of the spores on the net curtains at different inoculation densities was observed and

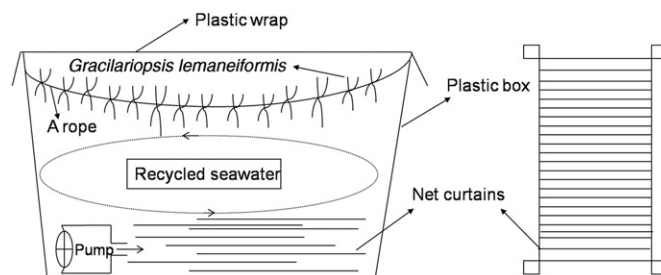


Fig. 1. Schematic diagram of the inoculation system.

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