

Growth of Gametophytes and Sporophytes of *Grateloupia subpectinata* (Rhodophyta) in Culture

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Abstract – Comparison of growing thalli in alternating haploid and diploid phases of *Grateloupia subpectinata* (Rhodophyta) was studied. Fertile thalli from gametophyte and tetrasporophyte of *G. subpectinata* were collected from Yangyang, on the eastern coast of Korea. The size of the released tetraspores and carpospores was measured; the spores were then incubated at the temperature of 20°C, irradiance of 40 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ and photoperiod of 12L and 12D. Carpospores were also cultivated in the same conditions as the tetraspores culture. The crusts were subsequently transferred to a tank culture after six months. The specific growth rate (SGR) was measured by observing 50 crusts and 30 thalli. The released carpospores had a larger diameter (9.98 μm) than the tetraspores (9.38 μm). The crusts from the carpospores also show a higher specific growth rate (14.04% d^{-1}) than tetraspores (13.39% d^{-1}). After being transferred and cultured in a tank, the upright thalli grew slowly in May–June (13–15°C) and rapidly in July–September (17–22°C). The length of growing thalli of sporophyte from carpospores also revealed a higher specific growth rate (2.83% d^{-1}) than gametophytic thalli (2.38% d^{-1}). The specific growth rate of crusts and thalli developed from carpospores was higher than that of the crusts developed from tetraspores. This result suggests that the cultivation of sporophytes may be more profitable than gametophytes because harvesting can be done more efficiently.

Key words – *Grateloupia*, sporophyte, gametophyte, crust size, growth, culture

1. Introduction

Grateloupia is one of the most taxonomically complex genera of the Cryptonemiales and is known as the largest plant in the family of Halymeniaceae (Wilkes et al. 2005; Kraft 1977). Due to the similarities and variations in its

thallus structure (Kraft 1977; Baweja and Sahoo 2002), the species identification of *Grateloupia* has been a challenge. For example, *G. asiatica* has long been misidentified as *G. filicina* in Korea, Japan and China due to its gross morphological similarity. However, the molecular analysis of this alga suggested that *Grateloupia* has several distinct types of ampullae and undergoes post fertilization events that distinguish groups of species (Kawaguchi et al. 2001; Faye et al. 2004; De Clerck et al. 2005; Gargiulo 2013).

Grateloupia is an edible seaweed and a source of carrageenan. This algae has been used for seaweed salad in Korea (Kim and Park 2006; Adharini and Kim 2014). *Grateloupia subpectinata* Holmes was originally described on the basis of collected specimens from Japan. It is also reported as native to Korea and China, which was then introduced to Britain, France and Australia (Nelson et al. 2013). This species, however, has been treated as synonymous with *Grateloupia filicina* in Japan. Recently, Faye et al. (2004) reinstated *G. subpectinata* based on morphology and *rbc* sequence analysis. Although *G. subpectinata* is clearly distinguished from *G. asiatica* based on *rbcL* sequence phylogeny, both species are very difficult to identify in the field due to their morphological similarity.

The alternation of generations between the sporophyte and gametophyte stages is a mechanism for restoring the haploid number before the next generation of gametes and ensuring effective distribution and exchange of genetic material within the species (Thomas 2002). However, Hawkes (1990) stated that the production of carposporophytes can be defined as a mechanism of zygote amplification to increase reproductive potential. Basic information about reproductive biology and sporulation condition in early developmental patterns is

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important for spore-based cultivation methods (Michetti et al. 2013). Life history and seasonality affect the growth rate of alga (Kain and Destombe 1995).

A recent study found that in a natural environment, the density, length and width of *Grateloupia subpectinata*'s thalli significantly change monthly. This study also found that the length of tetrasporophytes and carposporophytes is significantly different (Adharini et al. 2016). Nevertheless, a study based on intensive and continuous monitoring in the laboratory and tank culture needs to be conducted to understand the complete life cycle. The purpose of this research is to study reproductive phenology, growth of spores and thalli in sporophyte and gametophyte stages. This information will be helpful in devising a strategy for the production of seedlings, and mass culturing of *G. subpectinata*.

2. Materials and Methods

Culture of tetraspores from wild plant

Mature tetrasporophytes were collected from Namae, Yangyang, Gangwon Province, which is located on the east coast of Korea in October, 2011 and were brought in an ice box to the laboratory of Gangneung-Wonju National University. A mature plant was selected and washed using sterile seawater and the epiphytes were removed using a brush.

The explant was then fragmented and immersed in 1% germanium dioxide for 10 minutes. The fragments were put into dishes and then incubated in sterile seawater under 20°C temp., 40 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ irradiance, and 12:12 L:D photoperiod. Released tetraspores were then cultured in the same culture conditions with PES medium. The size of the tetraspores and the germination of the tetraspores were observed under a light microscope, and the specific growth rate (SGR) of the crusts was measured after 35 days. The culture medium was changed every three days.

The crusts were then transferred to a tank in May 2012. The crusts were cultured until November 2012 when mature carposporophytes were produced. The thalli length was measured periodically, and the specific growth rate was measured until November 2012. Mature carposporophytes were brought to the laboratory to release the carpospores and to analyze the morphology of the carposporophytes.

Culture of carpospores

A mature carposporophyte from a tank culture was selected and cleaned (see above for the cleaning procedure). The

fragments were incubated under the same culture conditions as that of tetraspores as described above. The size of fifty carpospores and the development of carpospores were observed under a light microscope.

SGR of the crusts was measured using the same method described above. In June, 2013, the crusts from the carpospores were then transferred to a tank culture and cultured until mature. The thalli length was measured periodically, and the specific growth rate of sporophytes thalli was measured at the end of October 2013. Mature tetrasporophytes were brought to the laboratory in order to conduct an analysis on their morphology.

Gametophytes and sporophytes growth in tank culture

The incubated crusts at the laboratory were transferred into an indoor tank culture (big glass aquarium whose volume was 1 m³) with an irradiance of 30–70 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, and were equipped with a flowing water system. The tank was cleaned and the upright thalli were brushed every week. The thalli growth was measured in term of length every month.

The SGR of the crust size and the thalli length were calculated using the equation by Yong et al. (2013) as follows:

$$\text{SGR (\% day}^{-1}\text{)} \times 100\%$$

where L_t is the length of thalli at t time L_i is the length of thalli at the initial time and t is the number of culture days.

3. Results

Life history of *Grateloupia subpectinata*

Germlings of tetraspores and carpospores of *Grateloupia subpectinata* are attached firmly onto a glass or polystyrene dishes as discoid crusts. Each discoid crust produced several (5–10) upright thalli. The thalli became flattened-cylindrical, elongated and tapered at the tip. The main axis formed some branches arranged oppositely pinnately or irregularly pinnately. The branches in the middle portion were longer than those at the basal or upper portion of the main axis.

The carposporophytes had more branches and slightly rounded axis due to the formation of cystocarps. Meanwhile, the tetrasporophyte had a longer main axis, but fewer branches than those of the carposporophytes. The thalli of the tetrasporophytes were also more flattened than those of the carposporophytes. Healthy crusts and thalli were red brownish in color, but they became pale under high irradiance conditions, or become darkened when the temperature was too high.

The carposporophytes had denser medullary filaments than the tetrasporophytes. The carposporophytic cortex was composed of 6–7 cells, while those of the tetrasporophytes were only composed of 4–5 cell layers. The sporophytes had 4–5 cortex cell layers and less dense medullary filaments. Those cells were bigger and less dense than carposporophytic cortex cells. Therefore, the external morphology of the tetrasporophytes was more flattened than that of the carposporophytes.

Comparison of crust size and growth rate induced from carpospores and tetraspores of *G. subpectinata*

Spores germinated from fertile thalli developed to crusts which were cultured during 35 days. The diameter of spores from tetrasporophytes and carposporophytes on the time length of the culturing was compared. Released carpospores are spherical in shape and are longer in diameter size (9.98 μm) than those of tetraspores (9.38 μm) (Fig. 1). Crust size induced from carpospores and tetraspores were compared in the laboratory culture. Rapid growth was observed in the period between 2~5 weeks (0.2 mm to 1.2 mm).

In 35 days, the carpospore had higher growth than the tetraspore (Fig. 2). The crusts from the carpospores also

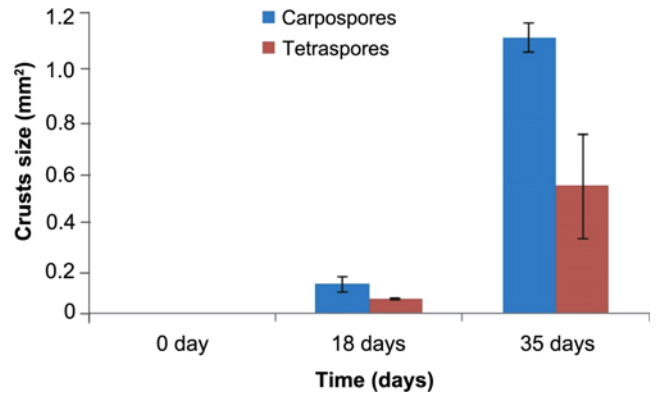


Fig. 2. Comparison of crust size induced from carpospores and tetraspores in laboratory culture. The crust size after 35 days of carpospores was bigger than tetraspores

showed a higher specific growth rate (14.04% day⁻¹) than those from the tetraspores (13.39% day⁻¹) (Fig. 3). The sporophytic thalli also showed a higher specific growth rate (2.83% day⁻¹) than those of gametophytes (2.38% day⁻¹). However, the sporophytic thalli had a longer main axis, but simple branches. On the other hand, the carposporophytic thalli had a shorter main axis than those of the sporophytes, and thus looked tuftier because of their longer first branches and some second branches.

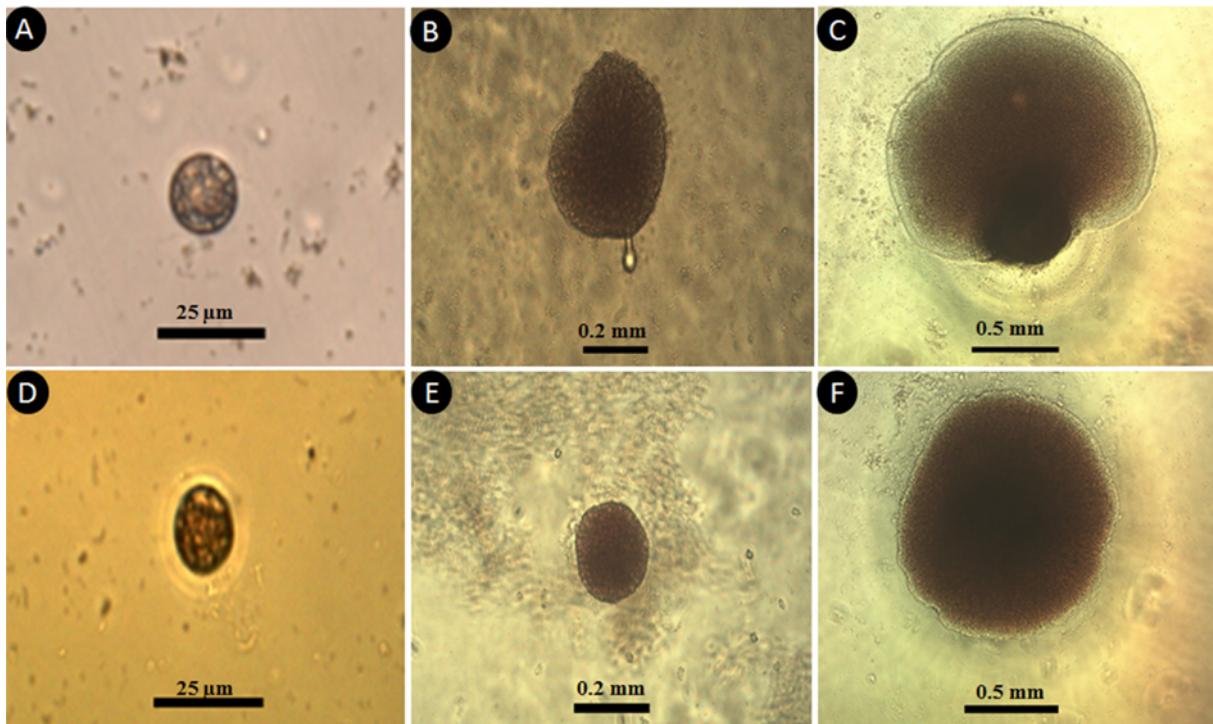


Fig. 1. Development of spores. (A) Released tetraspore; (B) Tetraspore development in 18 days; (C) Tetraspore development in 35 days; (D) Carpospore; (E) Carpospore development in 18 days; (F) Carpospore development in 35 days

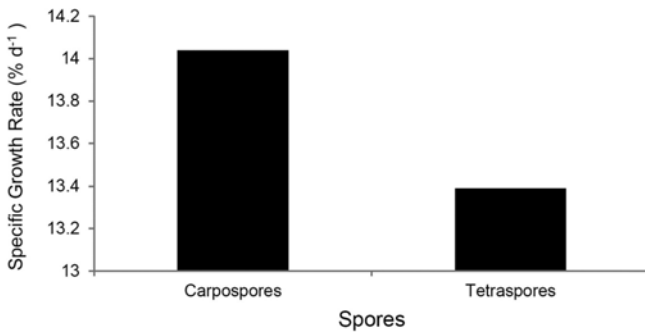


Fig. 3. Comparison of specific growth rate of crusts induced from carpospores and tetraspores

Growth of gametophytes and sporophytes thalli of *G. subpectinata* in tank culture

After 5 months of crust incubation in the laboratory, the discoid crusts were transferred to an indoor tank culture. The gametophytic thalli grew slowly in terms of length during May–June and then demonstrated a higher growth rate when observed in July. Those thalli grew rapidly from August to mid October (Fig. 4A–C). At the end of October, they barely

increased in length, but produced many primary and secondary branches (Fig. 4D). In November, the average length decreased because some of the thalli were fragmented at the tips (Fig. 4F). Meanwhile, the sporophytic thalli started to grow in July, and then grew rapidly until the end of October (Fig. 5A–F). Mature tetrasporophytes were noticed in October–November (Fig. 5G). Fragmentation also occurred in sporophytes after reaching maturity (Fig. 5H–I).

After 40 culture days in a tank culture, the crusts produced many upright thalli in a pattern of circular mounds. The morphology of male and female gametophytic thalli was similar. The upright thalli as the main axis grew slightly after 55 culture days. After 70 culture days, the upright thalli grew rapidly and started to form primary branches after 100 days, and in October, they reached the maximum length with the average length 5.2 cm. Subsequently, they produced many primary and secondary branches along the main axis. The thalli became mature after approximately 150 culturing days and formed many casposporangia spread on the branches and main axis, but not on the basal portion. The mature

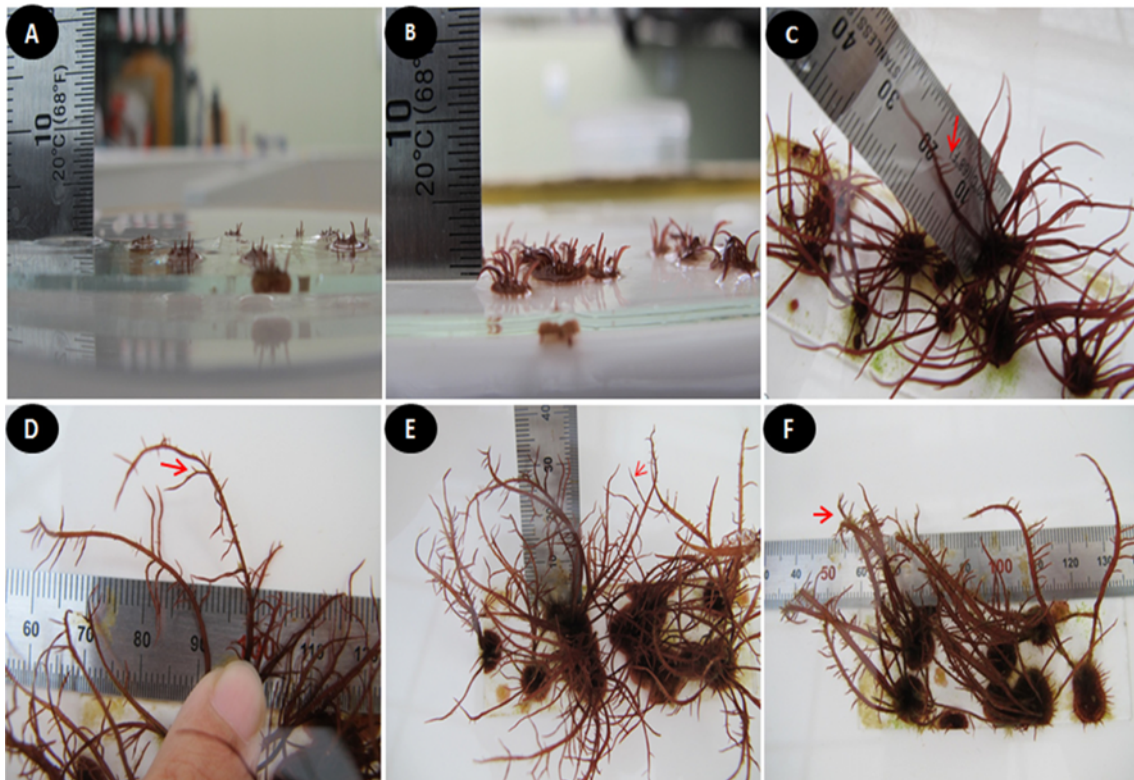


Fig. 4. Comparison of gametophyte thalli growth of *G. subpectinata* in tank culture. (A) Upright thalli after 40 culture days; (B) 55 culture days; (C) 100 culture days, upright thalli with primary branches (arrow); (D) 120 culture days, upright thalli with primary and secondary branches (arrow); (E) Carposporangia seen after 150 culture days (arrow); (F) Tips of thalli fragmented after maturation (arrow)

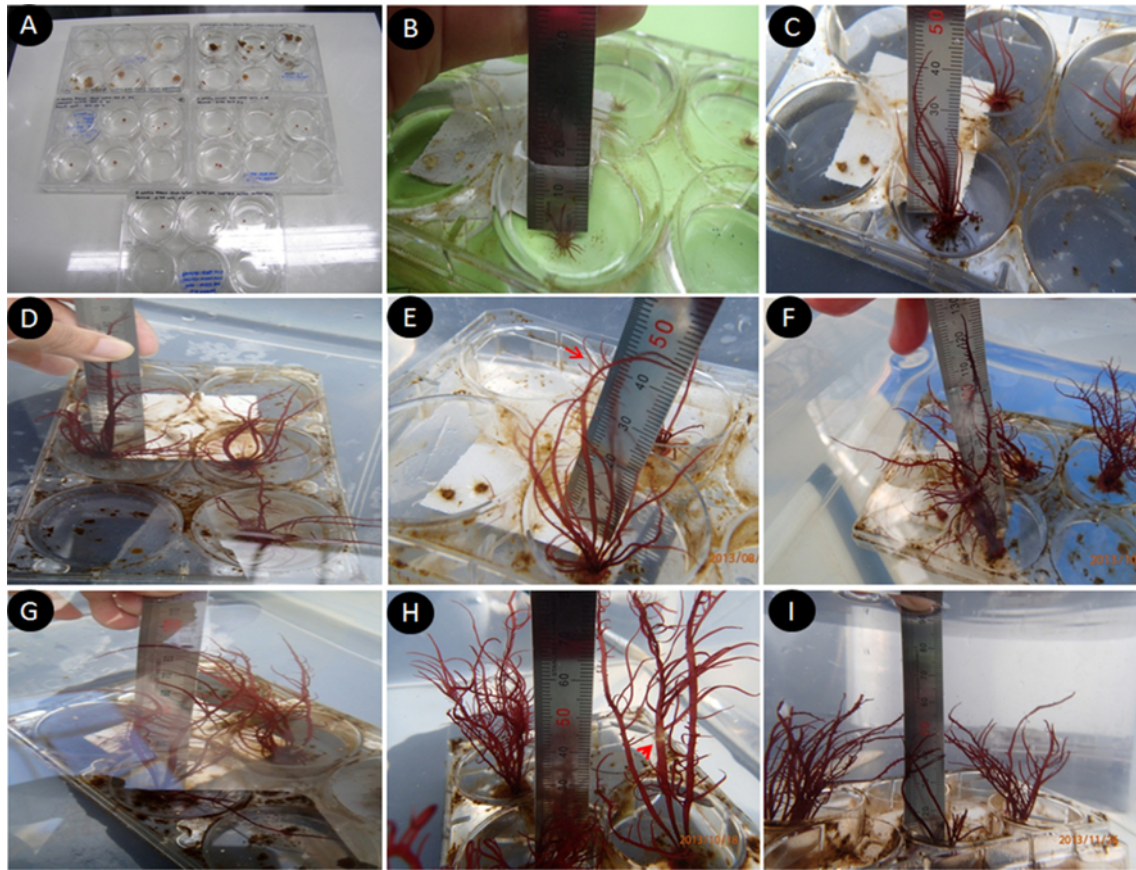


Fig. 5. Growth of sporophytes thalli of *G. subpectinata* in tank culture. (A) Crusts from carpospores before being transferred to tank culture; (B) Sporophytes thalli after 30 days; (C) 60 culture days; (D) 75 culture days, (E) Sporophytes thalli after 82 days, sporophytes thalli with many primary branches along main axis; (F) 115 culture days, the longest thalli; (G) 130 culture days, mature tetrasporophytes; (H) 160 culture days, upright thalli were fragmented after maturity; (I) Thalli were fragmented a lot after 198 days

carposporangia were visible with the naked eye and were noticed in white dots spread around the thalli (Fig. 4E).

The crusts from the carpospores generated upright thalli and became sporophytes. The thalli became softer, flatter and brighter in color than the gametophytic thalli. After more than 30 days in a tank culture, the sporophytes reached their maximum length with respect to their main axis. After 40 days, the branches started to grow along the primary branch.

However, unlike the gametophytes, sporophytes did not generate any second branches. The longest sporophytes reached 13 cm in length. However, after the sporophytes were became mature in October, the thalli became robust and infected by many epiphytes which caused the thalli to become fragmented (Fig. 5H–I). Comparison of growing thalli in two different life stages of *G. subpectinata* was summarized in Table 1.

Table 1. Comparison of gametophytes and tetrasporophytes of *G. subpectinata* in laboratory and tank culture

	Gametophyte (Tetraspores)	Sporophyte (Carpospores)
Diameter of spores	9.98 μm (n = 50)	9.38 μm^2 (n = 20)
SGR of crusts	13.39% d^{-1} (n = 50)	14.04% d^{-1} (n = 50)
SGR of thalli	2.38% d^{-1} (n = 20)	2.83% d^{-1} (n = 30)
No. of cortex cells	6–7 cells	4–5 cells
Width of thalli	2.24 (n = 10)	3.16 mm (n = 20)
First branch	+	+
Second branch	+	-

() indicated as number of samples measured

4. Discussion

Sporophytic and gametophytic thalli of *G. subpectinata* are isomorphic in their external morphology and color. In the present study, tetrasporophytic thalli in a tank culture had a longer main axis with simple branches, and carposporophytes had a shorter main axis but the length of their branches was longer. In a tank culture, carposporophytes also had second branches, while tetrasporophytes only had first ordered branches. Adharini et al. (2016) reported that the tetrasporophytes grew longer and had simple thalli whereas carposporophytes grew shorter and had tufted thalli due to elongation in wild population. *G. subpectinata* had flattened thalli with a mucilaginous to fleshy texture. Proliferations on axis were numerous and pinnate along the margins (Faye et al. 2004).

The carposporophytes have denser medullary filaments than the tetrasporophytes. The carposporophytes cortex is composed of 6–7 cells, while that of tetrasporophytes is composed of 4–5 cell layers. However, the cortex cells of the carposporophytes have slim and tightly composed cells. *Grateloupia turuturu* is distinguished by the anticlinal arrangement of its medullary filaments, a thinner cortex of roundish cells and an abrupt transition between cortex and medulla than *G. doryphora sensu stricto* (Gavio and Fredericq 2002). In *G. divaricata*, the thalli have more frequently branched thalli with a more rigid texture and thicker cortex (up to 20 cell layers) than *G. asiatica* (Kawaguchi et al. 2001). The cortex of *G. yangjiangensis* consists of five to seven outer layers and five inner layers of triangular or stellate cells (Wang et al. 2014).

Carpospores were bigger than tetraspores in size. The crusts developed from carpospores had a higher specific growth rate (SGR) than the crusts from tetraspores. Subsequently, the upright thalli (sporophytes) that originated from carpospores also had a higher SGR than the thalli developed from tetraspores. This may be because carpospores and sporophytic thalli have diploid cells, while tetraspores and gametophytic thalli have haploid cells. The same phenomenon was also reported in *G. gracilis* (Polifrone et al. 2006). The growth rate of *G. gracilis* was higher in the juvenile stage of tetrasporophytes than gametophytes. Avila et al. (2011) also reported that the tetrasporophytic thalli of *Chondracanthus chamissoi* had a higher growth rate than gametophytic thalli. Meanwhile, Destombe et al. (1993) reported that the survival of diploid *G. gracilis* in its juvenile stage was higher than in the haploid

stage. In the natural environment, sporophytic thalli of *G. subpectinata* at Yangyang were more abundant throughout the year than gametophytic thalli (Adharini et al. 2016). It has been suggested that sporophytes have a higher ability to survive and growth than gametophyte thalli.

After being transferred and cultured in a tank, the upright thalli grew very slow in May and June (13–15°C). This might be because they were still adapting to the change in conditions from the laboratory to the tank culture. From July to September, the upright thalli grew rapidly. This was probably because of the high water temperatures (17–22°C), causing rapid metabolism and spurring growth. Carposporophytes became mature and released carpospore in October and then developed to tetrasporophyte.

When the temperature decreased (below 15°C) in November, the average length decreased because some of the thalli were fragmented at the tips. This might also be because after the thalli released carpospores or tetraspores, they became weak and susceptible to epiphytes. The same phenomenon occurred in both gametophytes and sporophytes. (In natural environments, thalli also undergo fragmentation after maturation. The fragmentation of thalli may be used as part of this alga's dispersal strategy to colonize (Stiger and Payri 1999; Plouguerne et al. 2006; Prathep et al. 2007)).

This study revealed that sporophytes (carpospore) grow faster than gametophytes (tetraspore). The specific growth rate of crusts and thalli developed from carpospores was higher than that of the crusts developed from tetraspores. This result suggests that the cultivation of *G. subpectinata* sporophytes may be more profitable than gametophytes because the required time for sporophyte harvest is less than that of gametophytes, therefore reducing production costs.

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