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Growing the reproductive cells (carpospores) of the seaweed, *Kappaphycus striatum*, in the laboratory until outplanting in the field and maturation to tetrasporophyte

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Abstract Carposporophytes of the seaweed, *Kappaphycus striatum*, from the wild were made to shed spores in the laboratory and grown in multi-step culture method until they reached maturity. For each succeeding transfer onto increasingly bigger culture vessels, there was a marked increase in the growth of carposporelings. When plantlets were ready for outdoor culture, they were placed in aquaria and concrete tanks and later moved to the sea in net cage and long-line for grow-out culture. Successfully growing sporelings from carposporophytes in the laboratory until they reach market size seems to depend on the stage of sporelings and environmental factors such as photoperiod and temperature. In this study, carpospore progenies (diploids) also matured into tetrasporophytes and haploid progenies showed resistance to higher temperature.

Keywords Carposporophyte · Temperature · Tetrasporophyte · Grow-out · Growth

Introduction

The bulk of Philippine seaweed production is *Kappaphycus* which are mostly produced by marginal fisherfolk. *Kappaphycus* is farmed in shallow areas using the fixed-bottom method and in deeper portions using the raft method. Low capitalization of seaweed farming, good economic returns, and high demand of carrageenan in the world market have led to the expansion of *Kappaphycus* farming areas in the Philippines. Although *Kappaphycus alvarezii* is the preferred species for farming because of its high carrageenan content, it grows well only in certain specific areas. Thus, seaweed farmers in many parts of the country turn to other eucheumoid species like *Kappaphycus striatum* and *Eucheuma denticulatum* for farming.

For some time now, seaweed farmers in the Philippines have complained of their stocks growing slowly when compared with stocks several years ago. One factor that may have contributed to this and also the deterioration in the quality of carrageenan is the common practice of farmers to use cuttings from their present stocks as plantlets for the succeeding crops. This continuous transplantation from a single parent stock might have eroded some important genetic traits that resulted in the lesser quality of the present stocks. Periodic infusion of new stocks as source of plantlets is necessary to prevent the deterioration of the genetic properties of seedlings. This calls for new methods other than vegetative cuttings to produce new plantlets for the seaweed industry.

There are already attempts to produce seedlings from reproductive or somatic source. For instance, mature carposporophytes of *K. alvarezii* shed spores in vitro and viable spores were produced (Azanza-Corrales et al. 1992; Azanza and Aliaza 1999). Successful culture of micropropagules of *K. alvarezii* was already done in the laboratory (Dawes and Koch 1991; Dawes et al. 1993, 1994). Smaller sections of seaweeds were used for tissue culture and showed some promise (Muñoz et al., 2006; Hurtado and Biter 2007; Hurtado et al. 2009; Hayashi et al. 2008). Tetrasporophyte progenies have been shed in the laboratory and grown outdoors (Bulboa et al. 2007, 2008; Bulboa and De Paula 2005). These studies, however, were all done in the laboratory and the succeeding stages, the nursery and grow-out phases, have hardly been worked out.

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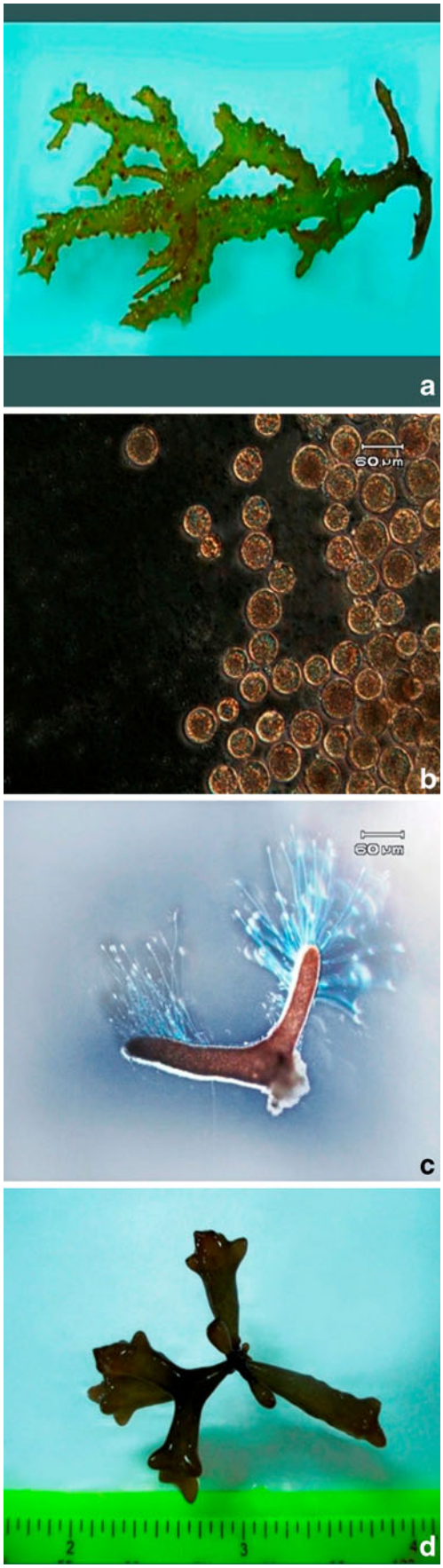

Fig. 1 *Kappaphycus striatum*. **a** Plant with cystocarps; **b** carpospores; **c** sporeling on slide; **d** sporeling in a 2-L bottle

The nursery phase is as important as the laboratory phase since we can test the quality of the seedlings at this stage prior to dissemination to the farmers for grow-out culture. In this study, we attempted to grow *K. striatum* continuously from the laboratory until it is grown outdoors in real farm conditions. Carpospores were shed in vitro, grown into plantlets in the laboratory and in tanks, and into market size in sea cages and grow-out long-line systems.

Materials and methods

Collection of samples, spore shedding, and measurement

Specimens of *K. striatum* bearing cystocarps (Fig. 1a) were collected in Sabang, Guimaras, Philippines (122°40.8′ E/ 10°29.6 N). The specimens were transported the same day to the SEAFDEC (Southeast Asian Fisheries Development Center) Algal Production Laboratory in Tigbauan, Iloilo, Philippines. The seaweeds were placed in a white basin with sterile seawater (SSW). These were cleaned using a soft brush and rinsed repeatedly to remove adhering debris. Portions with cystocarps were sectioned and placed on a glass slide in a petri dish with 20 mL SSW covered with a black cloth overnight. The black cloth was removed the following day when the spores where shed. The size of the spores was measured.

Culture in the laboratory

A multi-step method was used, where sporelings were transferred to increasingly bigger culture vessels as their size increased. Seawater was filtered using a 0.45 µm Whatman sterile membrane filter and sterilized at 121 psi for 30 min. Autoclavable materials were wrapped in plastic and sterilized in an autoclave. Glassware covered with aluminum foil was sterilized in 100°C oven for 5 h. Other material that cannot be sterilized in an autoclave was placed under a UV light for 1 h in the laminar flow.

Unialgal culture was maintained in the laboratory. The first stage of the laboratory culture was growing the sporelings on glass slides. Upon settlement of spores on the glass slides, the slides were placed inside a wide-mouthed capped bottle (50 mL capacity) filled with sterile seawater; enriched with modified Grund medium (MGM) (McLachlan 1973) at 1 mL L^{-1}; and kept in incubator with fluorescent lighting at an irradiance of 50 µmol photons m^{-2} s, 13L:11D photoperiod, at 25°C. The slides were oriented at 45°. Daily water change was done and MGM

was replenished every water change. Growing sporelings on the slides were measured using a vernier caliper. The length of the sporeling was the measurement of the slide with the sporeling minus the height of the slide. Sporelings with approximately 3 mm length were scraped and transferred to a 250-mL flask. The sporelings (n=55) were transferred to 250-mL bottles after 154 days.

Sporelings were weighed together and average weight was computed to obtain the initial weight of the sporelings in the 250-mL bottle. The content of the culture bottle (250 mL and 2 L) was poured into a funnel with a plankton net (150 μm mesh size). The plankton net with sporelings was blotted on a folded paper towel for 5 s and repeated once. Individual weighing of plantlets was done on day 7 in a 250-mL bottle.

The flasks were moderately aerated and kept under the same condition. When sporelings reached approximately 10 mm, these were transferred to a 2-L culture flask and grown under the same conditions. Plantlets (n=51) were transferred after 63 days to five 2-L bottles. Weight was measured every 7 days. Plantlets were transferred to the aquarium after 28 days.

Outdoor cultures

In 10-L aquaria

Plantlets (n=42) generated from the 2-L culture were transferred to a 10-L aquarium in the wet laboratory. The roofing in the wet lab is made of fiberglass. Ultra-violet treated seawater was used to grow the plantlets in the aquarium. The water in the aquarium was moderately aerated. Water change was done twice a week and MGM (1 mL L^{-1}) was replenished after every water change. Irradiance (Licor LI-250-A), water salinity (Atago refractometer), and temperature were measured daily. Weights of seaweeds were measured every 7 days. Plantlets were grown for 28 days in the aquarium.

In 1-ton concrete tank

Plantlets (n=10) from the 10-L aquaria were further grown in a 1.6 m^3 concrete tank. 1-m-long ropes, tied to a sinker at one end and to a buoy at the other end, were placed inside the tank. The water depth was 1 m. The sporelings were tied at the middle of each 1-m rope. The tank was provided with a flow-through water system at a flow rate of 10 L min^{-1} and moderately aerated. MGM (technical grade) was used to fertilize the medium. The seaweeds were weighed every 7 days. Fertilization was done after weighing the seaweeds. Static condition was allowed overnight after the application of fertilizer. Light, temperature, and salinity were monitored daily.

The plantlets were transferred to the net cage after 91 days in the tank.

Cage culture

A floating net cage (17 mm mesh size, 5 m W×5 m L×2 m H) was installed at the SEAFDEC Igang Marine Station in Guimaras Island (122°31′E/10°30′ N). The cage is made of bamboo and surrounded by floaters. Sinkers were provided at the bottom to hold the nets underwater. A 5 m long monofilament line was placed inside the net cage. The plantlets were tied to these lines using soft straw at 50 cm interval and 50 cm below the water surface. Fronds were cleaned daily to remove adhering debris. Maturity of Ks1-CP (n=10) was inspected monthly under a microscope. Cross-sections of the thallus were made using a thin blade. Sections were placed on a depression slide and viewed under a compound microscope.

Water quality in the cage and the grow-out was monitored every month. Colorimetric determination of total ammonia-nitrogen (TAN), nitrite-nitrogen (NO_2^--N) and phosphate-phosphorous (PO_4^{-3}-P) was based on the method of Strickland and Parsons (1972). Nitrate-nitrogen (NO_3^--N) was determined using the flow injection analyzer. pH, temperature, and salinity were monitored. Light was measured every weight monitoring in the cage and grow-out farm.

Grow-out culture

The seaweeds harvested after the cage experiment were transferred to the grow-out farm in Panubolon Island, Guimaras, Philippines (122°35.5 E /10°25.5′ N). The farm is located in an intertidal flat in a cove with moderate wave action. In the farm, the lines were arranged parallel to the waves and were pegged at both ends with bamboo 1 m long. The polyethylene line was 10 m long and the sporelings were tied using a soft straw at 25-cm intervals. Sporeling growth was monitored for 135 days.

Temperature experiment

Temperature tolerance was studied to determine the growth rate of *K. striatum* at different temperatures. Haploid *K. striatum* (92 days old) sporelings (Ks1-TP) used in this experiment were progenies of the tetrasporophytes (Ks1-CP) that matured in the cage. Tetrasporelings were grown in T-flasks for 42 days at 15°C, 25°C, 35°C with three replicates for each treatment and two individuals per replicate. This was conducted in a temperature gradient incubator with 50 μm photons m^{-2} s^{-1} irradiance and salinity of 30. Photoperiod was 13L:11D. Water was changed every day and nutrients were replenished every

water change. The lengths of plantlets were measured every 7 days. Data were subjected to one-way analysis of variance to test if there is a difference in the growth rates at different temperatures and the Duncan Multiple Range Test was applied to determine at which temperature the algae could grow best.

Growth (μ) was expressed as specific growth rate (SGR)

$$\mu = \frac{\ln \text{ final weight} - \ln \text{ initial weight}}{\text{day}_{\text{final}} - \text{day}_{\text{initial}}} \times 100$$

Where $\ln_{\text{final weight}}$ is the natural logarithm of the final weight and $\ln_{\text{initial weight}}$ is the natural logarithm of the initial weight.

Growth measurement was done in every step. The initial weight in each step was the final weight of the previous step.

Results

The batch of carpospores that survived in this study was given a code of Ks1-CP (Fig. 1a). The mean diameter of carpospores was 40.82±1.52 µm (n=50; Fig. 1b). The carpospores settled after 3 days. Hyaline hairs appeared on day 8 and were visible until the sporelings attained a dome shape (Fig. 1c).

Figure 2 shows the growth of Ks1-CP in the laboratory and outdoor cultures. The average weight of sporelings after 63 days in the 250-mL bottles was 0.04±0.05 g with SGR of 4.94% day^{-1}. The average weight of 51 individuals (Fig. 1d) in five 2-L flasks and grown for 28 days was 0.60±0.14 g with SGR of 9.63% day^{-1}.

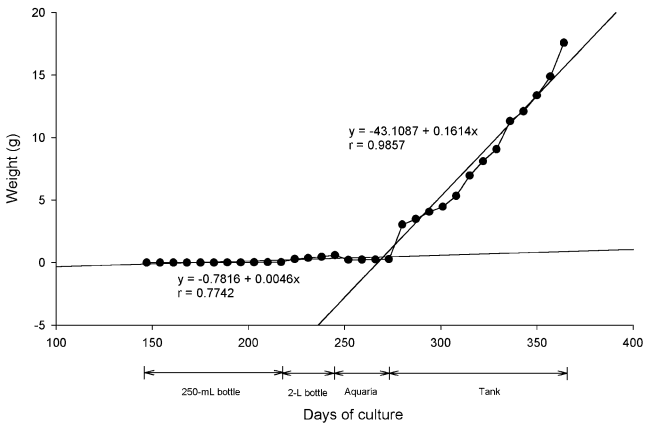

Fig. 2 Growth of *K. striatum* (Ks1-CP) in the laboratory and outdoor tank

The average weight of the sporelings (n=42) in the aquarium after 28 days was 0.28±0.01 g with SGR of −2.74% day^{-1}. Ten (10) individuals measuring approximately 30 mm long had an average weight of 17.57±2.80 g and growth rate of 4.55% day^{-1} after 91 days in the tank. The peak SGR was observed on day 280 (34.19% day^{-1}) in the tank; after which, the SGR decreased from day 315 to day 364 (7.7–4.6% day^{-1}).

Figure 3 shows the growth of the Ks1-CP in net cage culture and grow-out farm. The plantlets (n=10) from the concrete tank were transferred to a net cage in SEAFDEC's Igang Marine Station (IMS) and grown for 120 days. From an initial average weight of 17.57±2.80 g, they grew to an average weight of 495±2.71 g after 120 days. SGR is 2.78% day^{-1}. From the net cage culture, the seaweeds were further grown for 135 days in real grow-out farm conditions in Panobulon Island, Guimaras (1.5 h from IMS). The 10 individuals in the grow-out farm had an average weight of 530±1.84 g and SGR of 0.05% day^{-1} after 135 days in the grow-out farm. Slow growth was observed in the grow-out phase of Ks1-CP than in the outdoor culture. During the cage and grow-out culture, nitrogen in the water column was monitored and levels were very low, almost undetectable as shown in Table 1. Other parameters were within tolerable and narrow range which is normal in the tropical waters where *Kappaphycus* are grown. Irradiance (Table 2) was higher in the aquarium (up to 173 µmol photons m^{-2} s^{-1}) than in the incubator (50 µmol photons m^{-2} s^{-1}) and the water temperature was 2–3.5°C higher in the aquarium than in the incubator. Irradiance in the natural condition, net cage, and farm, were expected to be higher than the tank and aquarium.

Specimens bearing tetrasporangia were collected among Ks1-CP grown in the cage after 60 days. The tetrasporophytes were brought to the laboratory to shed spores. Tetrasporelings (Ks1-TP) are now being grown in the tank from the laboratory.

The temperature experiment (Fig. 4) showed that Ks1-TP sporelings died in the second week at 15°C and survived in temperatures 25°C and 35°C. However, there is no significant difference in the growth of Ks1-TP grown in 25°C and 35°C.

Discussion

We were able to collect mature carposporophyte of *K. striatum* from the wild and spores were shed just after 1 day in the laboratory. This enabled us to document the development of the spores into plantlet in the laboratory and followed its development and growth using a series of culture conditions until finally transplanting them in real

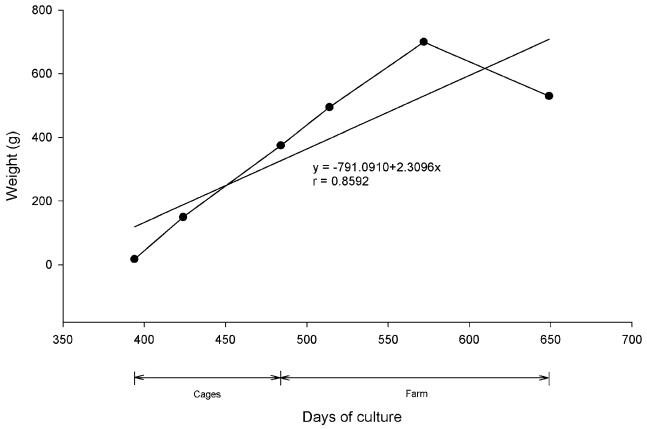

Fig. 3 Growth of *K. striatum* (Ks1-CP) in the net cage and grow-out

grow-out farm conditions in the natural environment. This is the first report that describes the development and growth of *K. striatum* from carpospores in the laboratory and in grow-out conditions in the wild until they reach market size and maturity.

The settlement of the spores on glass slides was a very convenient method because of the ease of cleaning the sporelings and of changing the water. Diploids (this study) and haploids (Bulboa et al. 2007) showed difference in the growth rate in the laboratory. Diploids in this study weighed 3.05 g (n=10) in approximately 13 months while haploids weighed 1 g (n=50) after 5 months in the laboratory before the grow-out culture. The plantlets in this study were transferred to the net cage at 17 g, while the study in Brazil started growing *K. striatum* in natural conditions at 1 g. Although weights were not presented in the Brazilian study, the SGR in the grow-out in Brazil (6.4% to 4.3% day^{-1}) was higher than the result in this study (2.78% to 0.05% day^{-1}).

Generally, a marked increase in growth when sporelings transferred from a smaller to a bigger culture container as was observed when sporelings were transferred from the 250-mL to 2-L bottle, tank to net cage, and net cage to farm, except during transfer from 2-L bottle to 10-L aquarium where growth decreased. The additional extra space and nutrient availability in larger spaces explains the increase in growth at each change. In tank conditions, the flow-through water system brings constant water movement that could have simulated the natural condition and brought extra nutrients (aside from the weekly enrichment supplied) to favor good growth. In net cage and farm conditions, the daily tidal exchange of water is enough to provide the nutrients for growth.

The decrease in growth from the 2-L to the 10-L volumes may be due to irradiance and temperature in the aquarium. Water in the 2-L bottles was changed everyday whereas the water in the aquaria was static and changed only twice in a week. Under these conditions, sporelings in the aquaria developed “ice-ice” disease after 5 days in the aquaria. They recovered only after water was changed every day for 12 days.

The increase in the SGR on day 280 could be due to a shift from a static (aquarium) to a flow-through condition (tank) and bigger area. The observed slow down in the SGR on the succeeding days in the tank led us to think that probably, outplanting could have been done a month earlier when SGR was high or is still increasing. Outplanting at the higher SGR would ensure that fast-growing young cells are present that will eventually result to fast growth of the plant.

As mentioned earlier, just like in grow-out culture, not all species of *Kappaphycus* thrive well in tanks. However, the tank culture phase is essential if a land-based nursery is to be built to provide continuous supply of seedlings for outplanting by the farmers especially during conditions where there is shortage of seedlings and during mortalities. Moreover, the tank phase in this study was a transition stage to the grow-out condition. In a country like the Philippines where several typhoons occur in a year, a land-based nursery is advantageous over a sea-based nursery since mortalities due to typhoons can be avoided. In many instances, destruction of seaweed stocks in grow-out farms after a typhoon had been observed in the country. It is expensive to maintain land-based nurseries, but this may be undertaken by institutions with existing facilities.

In the net cage, there was adequate nutrient exchange due to tidal currents since the cage was located in the open sea. Furthermore, the cages were installed alongside fish cages. Low nitrogen levels suggest that nutrients as a result of fish excretion and excess uneaten feeds in the fish cages

Table 1 Water chemical parameters during the grow-out culture

	pH	Ammonia-nitrogen (mM)	Nitrate-nitrogen (mM)	Nitrite-nitrogen (mM)	Phosphate-phosphorous (mM)
Farm	7.79 ±0.02	0.003±0.000	Not detected	Not detected	Not detected
Cage	7.95 ±0.12	0.000±0.000	Not detected	Not detected	0.00 ±0.00

Table 2 Water physical parameters during the culture

	Temperature (°C)	Salinity	Irradiance* (μmol photons $m^{-2}s^{-1}$)
Aquarium	27.0–28.5	28.5–31.0	106.3–173.3
Tank	27.0–29.0	29.0–31.0	141.8–408.6
Net cage	27.5–29.0	30.0–31.0	600.6–1987.0
Farm	28.0–29.0	30.0–31.0	268.7–1672.0

*Light was measured at noon

are low. But Ks1-CP was growing in the net cage even at lower concentration of nutrient in the water. During cage culture, however, cleaning or changing the nets has to be done regularly to ensure continuous water exchange and prevent fouling to occur. The mesh size of the nets should also be considered so that fish fry and juveniles that nibble on seaweeds are kept out.

The slow growth in the grow-out phase may be due to some environmental factors like temperature. In the present study, air temperature during April to May 2008 was high, up to 35.5°C based on the records of the Philippine Atmospheric, Geographical and Astronomic Authority (PAGASA). During this time, the seawater temperature reached 29°C. Although Ks1-CP survived this very high temperature, this could have affected its growth. In Brazil and Japan where conditions are subtropical, growth of *K. striatum* from spores was higher compared to this study (Bulboa et al. 2007; Gerang and Ohno 1997).

An interesting observation for Ks1-CP in this study was its unusually high tolerance to temperature. In the field, when the seawater temperature reached 29°C, the other *K. striatum* var. Green Sacol existing in the area decolorized and died, whereas Ks1-CP survived. The variant, *K. striatum* var. Green Sacol had been planted as a crop in the intertidal zone. Because the area is shallow, the rise in water temperature was quick and this has extended the exposure of the seaweed to high air temperature (35.5°C in April and May, 2008) during low tides and resulted to its death. Thus, because of unpredictable environmental conditions due to climate change, i.e., increase in the seawater temperature, there is a need for species that could tolerate high temperatures for mariculture.

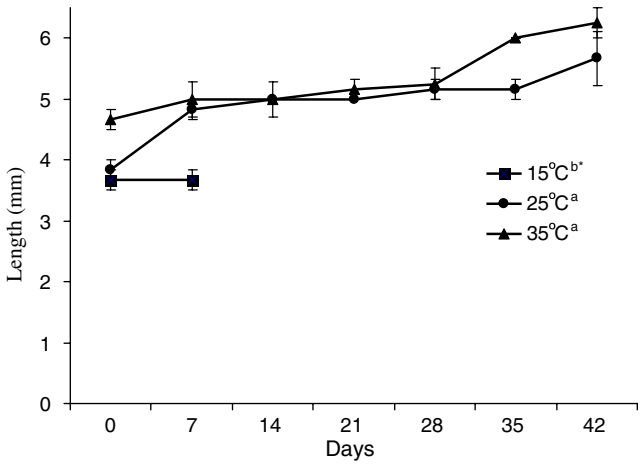

Fig. 4 Growth of *K. striatum* (Ks1-CP) germlings at different temperatures. *Temperatures with the same letters are not significantly different at $p=0.05$

Another factor that may have contributed to the slow growth of Ks1-CP during grow-out culture in this study compared with other studies is photoperiod. In Brazil and Japan, *K. striatum* was grown under longer days in summer, 14L:10D, and good growth were observed. In the Philippines where there is an equal length of day and night, almost year-round, growth was slower compared to that in Japan and Brazil. In the present study, tetrasporophyte was collected among the Ks1-CP cultured in net cage and sporelings are now being cultured in the laboratory. In Brazil and Japan, where there is longer day in the summer and shorter during cold months, maturation was not obtained among the *K. striatum* cultures. The mature tetrasporophyte samples used in Brazil was collected from the Philippines and no mature carposporophyte was reported after several years of growing in Brazil.

Occurrence of epiphytes and 'ice-ice' disease were also encountered during the culture. Epiphytes observed by Hurtado et al. (2006) and later verified as *Neosiphonia* by Vairappan (2006) were also present in Ks1-CP. Epiphytes were observed during the hot months in April and May. The rainy and typhoon season starts in June until November. The weight of Ks1-CP increased in the grow-out farm during these months until June. The decrease in the growth of Ks1-CP in the grow-out farm was observed after harsh water conditions brought about by the typhoon Frank in Iloilo in June 2008. Wounds left by detached branches developed into ice-ice, but Ks1-CP recovered from the mechanical damage caused by strong wave action during the typhoon.

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