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Differences in physiological responses between winter and summer *Gracilaria tenuistipitata* (Gigartinales, Rhodophyta) to varying temperature

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Abstract. *Gracilaria tenuistipitata* var. *liui* Zhang *et* Xia, which is farmed in brackish water ponds of southern Taiwan as a major food of sea abalone, produces poor yields in winter. This study investigated the effects of year, location (pond), and season on the physiological responses of *G. tenuistipitata* to varying temperature. Neither year nor pond affected the physiological parameters. Season significantly affected the specific growth rate, the recovery specific growth rate, and the levels of free proline. The specific growth rate was different between winter (January in 1996, 1997 and 1998) and summer (August in 1996, 1997 and 1998) plants, which grew in a range of 14.1–32.3°C and 11.8–37.3°C with the maximum at 23 and 24.5°C, respectively. The recovery specific growth rate after transferal to 25°C showed a similar trend. Free proline accumulated only in summer plants at both 20 and 35°C. Thus, the growth and free proline levels at varying temperature are season-dependent events in *G. tenuistipitata*.

Keywords: *Gracilaria tenuistipitata*; Growth; Proline; Season.

Introduction

Species of the red algal genus *Gracilaria*, typically found on the intertidal regions of tropical and subtropical areas, have been studied worldwide because of their increasing market value as agarophytes (Armisen, 1995). Their growth depends mainly on temperature (Gressner, 1970; Hurtado-Ponce and Umezaki, 1987), light (Beer and Israel, 1983), and salinity (Yu and Pedersen, 1990). In Taiwan, several *G.* species are found (Yang and Chiang, 1982), and the species *G. tenuistipitata* var. *liue* Zhang *et* Xia is now extensively farmed in the brackish water ponds mostly located in Anping, Tainan and is now used as a food source for the cultivation of sea abalone, *Haliotis diversiolor supertexta* Lischke (Chiang, 1981). The production of *G. tenuistipitata* is poorer in winter than in summer, possibly due to seasonal changes and overgrowing of competing seaweeds, mainly *Enteromorpha* and *Chaetomorpha* (Chiang, 1981).

Macroalgae in many habitats have various physiological mechanisms for responding to environmental changes, and the ability to tolerate environmental disturbances often contributes to their success in marine communities. In addition to genetic adaptation (Kuebler et al., 1991), the survival of a certain species is often determined by its ability to acclimate to environmental changes. Acclimation is known to be a widespread phenomenon in nature, and long-term responses can be observed in the course of a season. A variety of reports show the occurrence of seasonal changes in the photosynthesis and respiration of intertidal and subtidal algae (Duarte and Ferreira, 1995; Kuppers and Weidner, 1980; Newell and Pye, 1968). It is widely accepted that acclimation allows seaweed to optimize photosynthetic ability and respiration as well, and hence growth, in response to seasonal changes in water temperature (Davison, 1987; Davison and Davison, 1987; Egan et al., 1989). As reported by Friedlander et al. (1987), *G. conferta* in Israel shows a growth reduction in winter (December-March) and a peak in summer (August), mainly due to extreme seawater temperatures during these seasons: 13°C and 31°C, respectively.

This study was conducted to compare physiological differences between winter- (January in 1996 and 1997) and summer-grown (August in 1996 and 1997) *G. tenuistipitata* by determining their responses to various temperatures ranging from 10–40°C in laboratory for 5 days. This work was carried out in 1996, 1997, and 1998. The quantitative changes in physiological parameters were examined, including specific growth rate, recovery specific growth rate (i.e. repairing ability), net photosynthesis, pigments (chlorophyll a and phycobiliprotein), and soluble nitrogen compounds (soluble protein and free amino acid). Free proline in algae accumulates under several stresses such as salinity (Bartels and Nelson, 1994; Csonka, 1989) and the presence of heavy metals (Wu et al., 1995a,b). The effects of seasonal acclimation on levels of free proline in response to varying temperature were also determined in *G. tenuistipitata*.

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Materials and Methods

Material Sampling and Temperature Treatment

Thalli of *Gracilaria tenuistipitata* var. *liue* Zhang *et* Xia were randomly collected in either January (winter plants) or August (summer plants) 1996–1997 from three independent but closely adjacent brackish water ponds located at Anping, Tainan, Taiwan. Air and water temperatures at these ponds were determined twice every day, once at 12:00 AM and again at 10:00 PM. Air temperature was measured at 1 m above water level while water temperature was measured at 60 cm below water level (Figure 1). Salinity was measured with a Horiba water quality checker (U-10, Horiba Ltd., Kyoto, Japan) and ranged from 14–26‰. Following collection, a branch tip of about 6 cm and of fresh weight ranging from 1.8–2.2 g was cultured 5 days in a polycarbonate vessel (Magenta GA-7 vessel, Sigma, St. Louis, MO., USA) containing 300 mL seawater (30‰ salinity, pH 7.8) enriched with full strength Provasoli nutrient solution (Provasoli, 1968) and 3 mM $NaHCO_3$ as inorganic carbon source. The nutrient-enriched seawater was changed everyday, and the photoperiod was 12 h : 12 h with photon irradiance (400–700 nm) ranging from 80–120 μmol photon m^{-2} s^{-1} in the absence of algae, achieved by a mixture of six 40W cool-fluorescent (FL40D, China Electric Apparatus Ltd., Taiwan, ROC) and two 60W incandescent (I60, China Electric Apparatus Ltd., Taiwan, ROC) lamps. Exposure to various temperatures was achieved by incubating plants at 10, 15, 20, 25, 30, 35 or 40°C for 5 days and then transferring them to 25°C for another 5 days to evaluate their repairing ability by determining their recovery specific growth rate between Day 5 and Day 10. There were three replicates for each treatment.

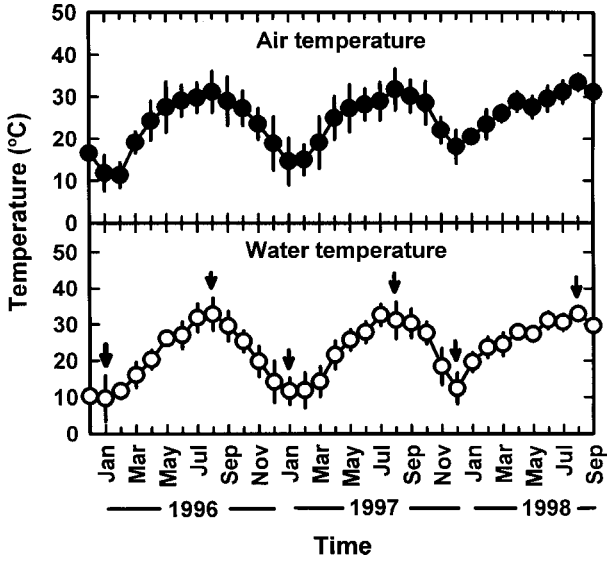

Figure 1. Changes of air and water temperature in *Gracilaria* ponds from December, 1995 to September, 1998. Arrows indicate the sampling time, and bars indicate the standard deviation (n=3).

Determination of Net Photosynthesis

After exposure to various temperatures for 5 days, net photosynthesis was determined by measuring oxygen production using a Clark-type oxygen electrode fitted with a DW3 chamber (Hansatech, Kings Lynn, Norfolk, England) and thermostated at 25°C by a temperature controller (ANCER AR-1 model, Chin-Chi Trading Co., LTD., Kaohsiung, Taiwan) connected to the water jacket of the electrode. Thalli of 50 mg fresh weight (about 2.5 cm long) were held with a nylon hook in a 20 mL square section reaction vessel filled with 10 mL artificial seawater (pH 7.8) and incubated in darkness until there was a steady O_2 evolution. Photosynthetic O_2 evolution was started by illumination. The artificial seawater was composed of 0.5 M NaCl, 30 mM $MgSO_4$, 10 mM KCl, 10 mM $CaCl_2$, and 30 mM Tris-HCl (pH 7.8) (Einav et al., 1995) and was enriched with 6 mM $NaHCO_3$ as the inorganic carbon source. Six mM of $NaHCO_3$ was determined on preliminary experiments to be the optimal concentration for obtaining the maximal net photosynthetic O_2 evolution rate (data not shown). The photon irradiance was 300 μmol photon m^{-2} s^{-1} provided by low voltage Tungsten halogen lamps (12V, 50W, Sylvania, Japan). Light measurement was carried out with a LI-188B integrating radiometer using a spherical quantum sensor LI-193SA (LI-COR, Nebraska, USA). The O_2 electrode system was calibrated daily with sodium dithionite-treated artificial seawater, which is referred to an N_2 line, and O_2-saturated artificial seawater. After measurement of net photosynthesis, thalli were immediately lyophilized at -60°C. Their dry weights were determined, and they were then stored at -70°C in a freezer for further analysis.

Determination of Specific Growth Rate and Recovery Specific Growth Rate

Growth was monitored via fresh weight changes over the five-day experiments. Specific growth rate (% d^{-1}) was calculated from the equation $(\ln (W_5/W_o)/5)\times100$ (Patway and van der Meer, 1984). W_o and W_5 were the initial and final fresh weights of the thalli, respectively. The recovery specific growth rate was measured to compare the repairing ability of the plants after exposure to various temperatures. It was calculated after keeping the plants at 25°C for 5 days from the following equation: $(\ln (W_{10}/W_5)/5)\times100$.

Determination of Pigments, Free Proline, Free Amino Acid, and Soluble Protein

Lyophilized thalli were ground to powder in liquid nitrogen, and fifty volumes of extraction buffer (50 mM sodium phosphate buffer, pH 7.0) were added as sample homogenate. Before centrifugation, chlorophyll was extracted from 40 μl of sample homogenate for 6 h with 960 μl of 100% ethanol (HPLC grade, Merck, Darmstadt, Germany) and determined according to the method of Wintermans and De Mots (1965) after centrifugation for 10 min at 15,000 *g* under 4°C. For the determination of phycobiliprotein, free proline, free amino acid, and

soluble protein; the sample homogenate was centrifuged for 10 min at 12,500 *g* to obtain the supernatant. Phycobiliproteins in the supernatant were measured in a spectrophotometer (Model U-2001, Hitachi, Tokyo, Japan) (Kursar et al., 1983). Free proline was determined according to the method of Bates et al. (1973) using L-proline as standard. Free amino acids were determined by the photometric ninhydrin method (Moore and Stein, 1948) using L-leucine as standard. Soluble protein was estimated by the method of Bradford (1976) using bovine serum albumin as the standard protein.

Statistics

A three-way ANOVA (SAS for DOS, Version 6.03, SAS Ltd., NC, USA) was used to analyze the effects of year, pond, and season on the physiological parameters. The comparison of changes of free proline levels in response to temperature treatment was performed by Duncan's multiple-range test (SAS for DOS, Version 6.03, SAS Ltd., NC, USA), and the comparison of the responses of other physiological parameters to temperature treatment was analyzed by covariance (ANCOVA) on regression of data (SAS for DOS, Version 6.03, SAS Ltd.,

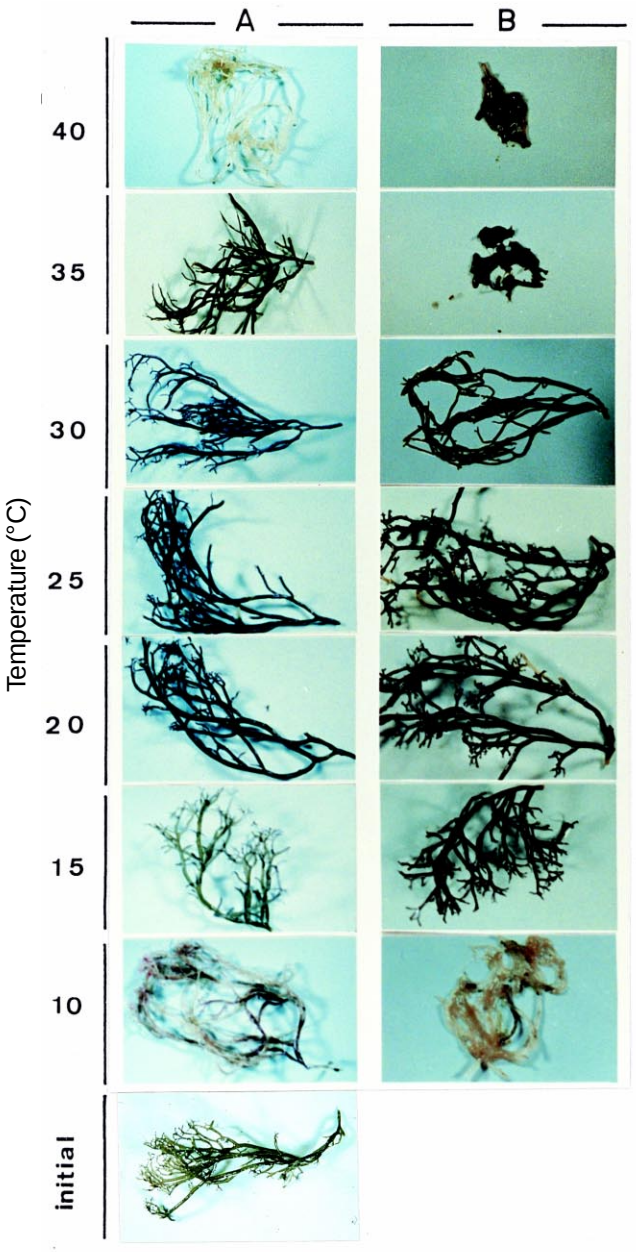

Figure 2. Appearance of winter (January, 1997) *Gracilaria tenuistipitata* after exposure to various temperatures for 5 days (A) and after changing the temperature to 25°C keeping the plants under this temperature for 5 days (B). Thallus before temperature treatment is shown at the bottom of figure.

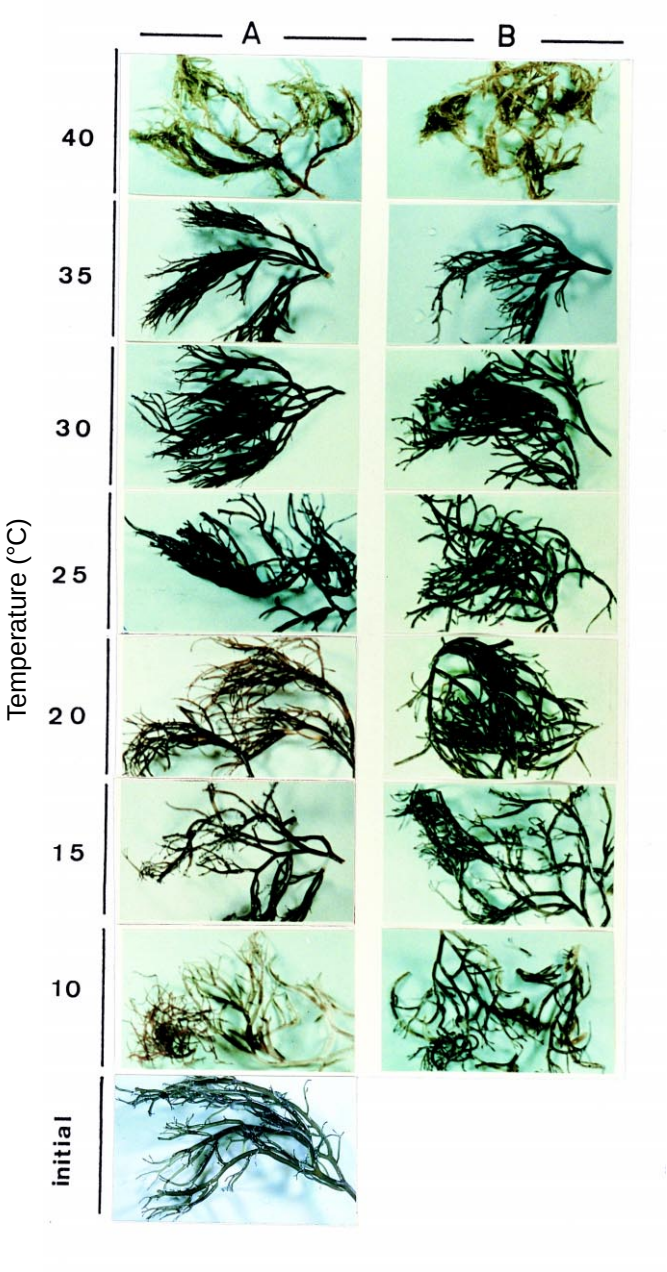

Figure 3. Appearance of summer (August, 1997) *Gracilaria tenuistipitata* exposed to various temperatures for 5 days (A) and after changing temperature to 25°C keeping the plants under this temperature for 5 days (B). Thallus before temperature treatment is shown at the bottom of figure.

NC, USA). Results were considered to have reached statistical significance when $p<0.05$.

Results

Appearance

Because changes in appearance were uniform in the experiments of 1996, 1997 and 1998, only the photograph obtained from the 1997 experiment is shown in Figures 2 and 3. After a five-day incubation, the winter plants appeared healthy at 20, 25 and 30°C, but became green at 15°C and bleached at both 10 and 40°C (Figure 2A). Thalli exposed to 15°C gained their normal color after recovery under 25°C for 5 days (Figure 2B). Although winter plants had a healthy appearance after 5 days at 35°C (Figure 2A), when the temperature was changed to 25°C, they corrupted completely, with a dark brown covering the broken down thalli (Figure 2B). Summer plants bleached at both 10 and 40°C but appeared healthy at other temperatures (Figure 3A). After recovery to 25°C for 5 days, both 15°C- and 35°C-treated summer plants were disjointed at the tips or intervals (Figure 3B).

Specific Growth Rate and Recovery Specific Growth Rate

Based a three-way ANOVA analysis, neither the specific growth rate nor the recovery specific growth rate were significantly affected by year ($p=0.7270$ and $p=0.6091$, respectively) or pond ($p=0.7426$ and $p=0.3672$, respectively), but they were significantly affected by season ($p=0.0410$ and $p=0.0284$, respectively).

The specific growth rate was significantly different in response to varying temperature (ANCOVA, $p=0.0395$). Winter plants showed a positive growth response over a range of 14.1–32.3°C with the maximum at 23.2°C while summer plants grew over a wider range of 11.8–37.3°C with the maximum at 24.5°C; the maximal growth rate in summer plants was about twofold that of winter plants (Figure 4). Temperature also affected significantly the recovery specific growth rate (ANCOVA, $p=0.0428$). Winter plants showed a positive recovery specific growth rate in a range of 13.4–27.6°C with the maximum at 20.8°C and a range of 14.7–34.9°C for summer plants with the maximum at 25°C. Plants at 40°C (open circle) were not included in the regression analysis due to death (Figure 5).

Net Photosynthesis and Pigment Contents

Net photosynthesis was not significantly affected by year (three-way ANOVA, $p=0.5175$), pond ($p=0.7385$) or season ($p=0.7964$), but it was significantly affected by temperature (ANCOVA, $p=0.0021$). Net photosynthesis in winter plants was positive between 10.1–34.6°C with the maximum at 24.2°C while that in summer plants was positive between 12.4–40.1°C with the maximum at 25.8°C. The maximal rate in summer plants was about 60% higher than in winter plants (Figure 6).

The levels of chlorophyll a were not significantly affected by year (three-way ANOVA, $p=0.451$), pond ($p=0.4783$), or season ($p=0.2386$), but they were significantly affected by temperature (ANOVA, $p=0.0068$). The levels of chlorophyll a rose as temperature increased from 10 to around 24.5°C and then decreased (Figure 7).

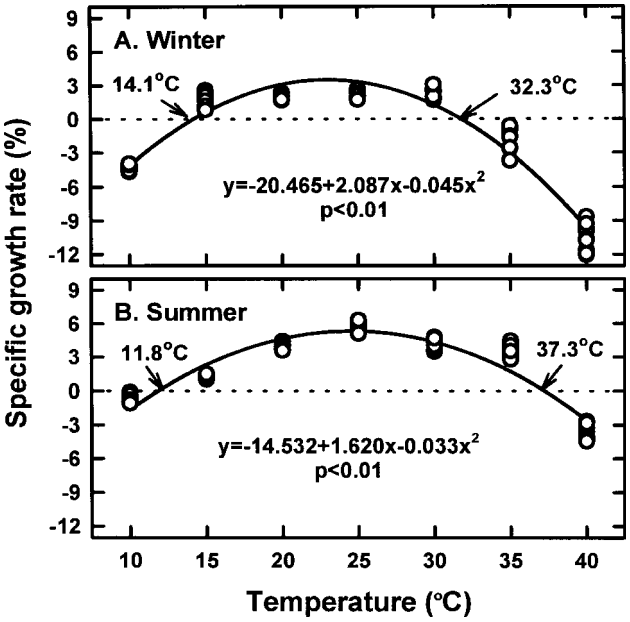

Figure 4. Effects of temperature on specific growth rate (% d^{-1}, n=3×3) of winter (A) and summer (B) *Gracilaria tenuistipitata*.

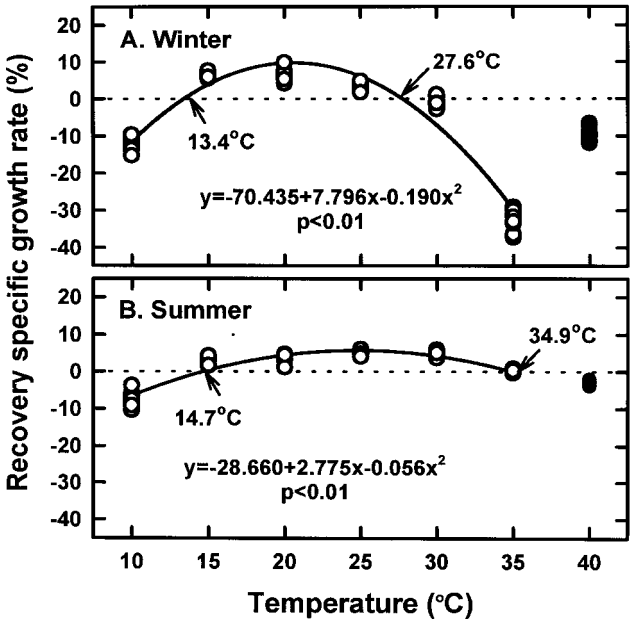

Figure 5. Effects of temperature on recovery specific growth rate (% d^{-1}, n=3×3) of winter (A) and summer (B) *Gracilaria tenuistipitata*.

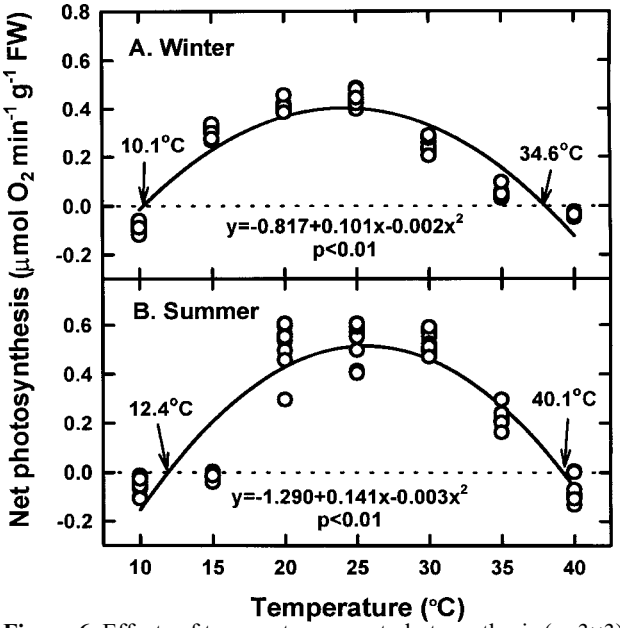

Figure 6. Effects of temperature on net photosynthesis (n=3×3) of winter (A) and summer (B) *Gracilaria tenuistipitata*.

The levels of allophycocyanin, phycocyanin, and phycoerythrin were not affected by year (three-way ANCOVA, p=0.2135 for allophycocyanin; p=0.5425 for phycocyanin; p=0.4931 for phycoerythrin), pond (p=0.5310 for allophycocyanin; p=0.5066 for phycocyanin; p=0.5629 for phycoerythrin), or season (p=0.5617 for allophycocyanin; p=0.6183 for phycocyanin; p=0.5862 for phycoerythrin). However, temperature significantly affected the levels of allophycocyanin (ANCOVA, p=0.0062), phycocyanin (p=0.0097), and phycoerythrin (p=0.0015). Levels in winter plants increased with temperature and reached the maximum at 22.4, 21.9 and 22.8°C, respectively, while those in summer plants reached the maximum at 25.3, 24.6 and 25.2°C, respectively (Figure 7).

Levels of Soluble Protein, Free Amino Acid and Free Proline

Temperature (ANCOVA, p=0.0031 for soluble protein; p=0.0074 for free amino acid), but not year (three-way ANOVA, p=0.4795 for soluble protein; p=0.5083 for free amino acid), pond (p=0.5365 for soluble protein; p=0.6207 for free amino acid), or season (p=0.3932 for soluble protein; p=0.7665 for free amino acid) affected the levels of both soluble protein and free amino acid. The levels of soluble protein in both winter and summer plants increased as temperature increased, reaching a maximum at 21.8 and 24.3°C, respectively, and then declining (Figure 8). Changes in the levels of free amino acid to temperatures showed a similar trend with the maximum at 21.6 and 22.1°C, respectively (Figure 8).

Neither year (three-way ANOVA, p=0.0681) nor pond (p=0.2975) had any significant affect on the levels of free proline. Both season (three-way ANOVA, p=0.0001) and

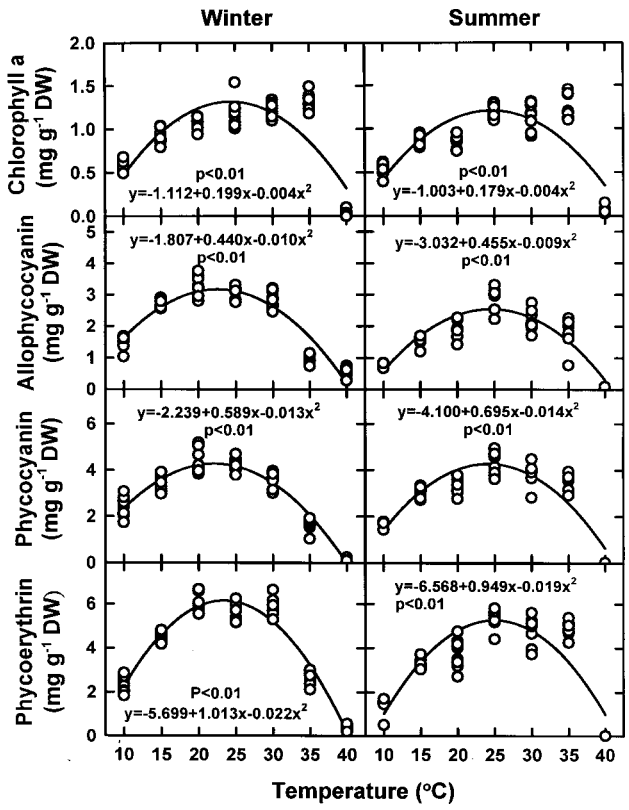

Figure 7. Effects of temperature on levels (n=3×3) of chlorophyll a and phycobiliproteins in winter (A) and summer (B) *Gracilaria tenuistipitata*.

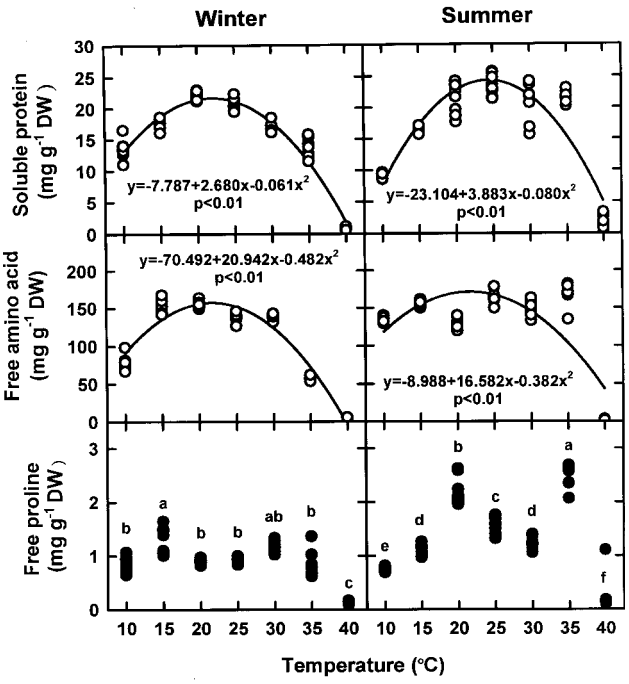

Figure 8. Effects of temperature on levels (n=3×3) of soluble protein, free amino acid, and free proline in winter and summer *Gracilaria tenuistipitata*. Different symbols for free proline levels indicate significant difference at p<0.05.

temperature (ANCOVA, p=0.0001) significantly influenced the levels of free proline. These levels in winter plants were similar over all temperatures, but those in summer plants increased at 20 or 35°C, the temperatures near the extreme (Figure 8, Duncan's multiple range test, $p<0.05$). In summer plants, the levels of accumulated free proline in 35°C-treated plants was about twofold that of the 25°C controls (Figure 8). This extreme temperature-induced free proline accumulation was also found in summer plants collected in August, 1996 and 1998 (data not shown).

Discussion

This study shows that quantitative changes in the physiological parameters in *G. tenusitipitata* are not affected by year or pond. Only the factor of season affected the specific growth rate, the recovery specific growth rate, and the levels of free proline. It is apparent that growth and free proline levels are associated with seasonal acclimation.

Temperature significantly affected all the physiological parameters in this study. The temperature range for the growth of winter plants was relatively narrow compared with summer plants although the optimum temperature was around 24°C for both. Based on repairing ability, judged by recovery specific growth rate, the upper temperature limit in summer plants was higher than that in winter plants. The temperature range for net photosynthesis was in agreement with that for growth in summer *G. tenuistipitata* but not for winter. Possibly, factors other than photosynthesis are involved in growth regulation. Nitrogen metabolism is known to have an impact on algal growth (Friedlander and Dawes, 1985; Lapointe, 1981; Rosenberg and Ramus, 1982). Compared with summer plants, low availability in nitrogen (soluble protein and free amino acid) at high temperatures may explain a growth cessation of winter plants when photosynthesis was still active.

The amount of both phycoerythrin and chlorophyll a is known to parallel photosynthetic activity in *G. verrucosa* (Dawes et al., 1984); that is, the increase in photosynthetic pigments, especially phycobiliproteins, leads to a rise of photosynthetic capacity and the fixed carbon capital available for growth. The present study also shows a similar trend in both winter and summer *G. tenuistipitata*. Changes in the amount of phycobiliproteins rather than chlorophyll a coincided with changes in net photosynthesis in response to various temperatures.

Since the low limit for repairing is approximately 14°C, chilling stress may be a factor limiting the growth of *G. tenuistipitata* in winter when water temperature falls below 15°C (Figure 1). Ryther et al. (1984) reported a similar result for *Gracilaria*, which grew half as fast in Florida during periods of low temperature.

In conclusion, a seasonal change triggers a shift in both growth activity and free proline levels in *G. tenuistipitata*. The low growth activity in winter plants may mean (1) a weaker condition for *G. tenuistipitata*, which enables pests to a larger extent, and (2) a reduced resistance to environments. Now, the relationships between proline metabolism and tolerance to extreme high temperature in summer *G. tenuistipitata* are being undertaken.

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