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Responses of the macroalga *Gracilaria tenuistipitata* var. *liui* (Rhodophyta) to iron stress

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Abstract

Chlorophyll (Chl), phycoerythrin (PE), total nitrogen (TN% dw) and Fe in tissues were measured in Fe-deficient cultures of *Gracilaria tenuistipitata* var. *liui* over a period of 60 days. ^{55}Fe uptake and photosynthetic carbon fixation ($NaH^{14}CO_3$) were compared in Fe-rich and Fe-deficient cultures and analyzed the effects of Fe-deficiency on the ultrastructure. The maximum carbon fixation decreased significantly ($p < 0.01$) under Fe-deficiency. The chlorophyll and phycoerythrin contents also declined with decreasing tissue iron content, falling, respectively, to 7.9 and 33.8% of their original level. Photosynthesis in Fe-deficient cells became light-saturated at lower irradiance than the control. Total N in tissue decreased from 3.65 to 2.49%. ^{55}Fe uptake rate for cultures grown on NO_3^- was measured following resuspension in either NH_4^+ or NO_3^- as N source. Enhanced Fe uptake developed under Fe stress, especially with cells resuspended in NH_4^+-N medium. The *Vmax* for Fe uptake was higher with NH_4^+ than NO_3^- (62.8 versus 12.1 pmol mg dw^{-1} h^{-1}). The requirement for N accelerates further Fe uptake. Ultrastructural observations of Fe-deficient cells showed reductions in chloroplast number, degeneration of lamellar organization, decrease in mitochondrial matrix density and variation in accumulation body number and morphology. During Fe-deficiency, the growth rate continued to decline and after 40 days of iron deficiency, no further growth was detectable, and eventually iron deficiency resulted in chlorosis. The results suggest that the lower growth rate of *Gracilaria tenuistipitata* var. *liui* under Fe-deficiency may result largely from inhibition of photosynthesis and nitrogen utilization.

Introduction

Iron is an important nutritional element for phototrophs. Photosynthesis is especially dependent on iron, because of its requirement during chlorophyll synthesis. Iron is also essential for cytochromes, ferredoxin, and Fe-S protein. Other biochemical roles are those linked closely to certain aspects of nitrogen metabolism. Many of the major enzymes of nitrogen assimilation contain Fe (NR, NiR, GS) and the catalytic activity of some or all forms of various enzymes, including ribonucleotide reductase (β_2 dimer), RNA polymerase III, superoxide dismutase, catalase, peroxidase, aconitase and hydrogenase also require iron (Weinberg, 1989). Iron deficiency has been hypothesised to affect nitrogen utilization in marine phytoplankton (Raven et al., 1988; Milligan & Harrison, 2000). The overall effect of iron on cell growth depends on its importance in pathways for nitrogen and carbon assimilation.

In aquatic ecosystems, iron concentration is an important regulatory factor. The stable oxidation state of iron in oxide seawater is Fe (III) with a low solubility. There is accumulating evidence that phytoplankton grown in marine water may be potentially rate limited by the availability of iron, such as in the North Pacific gyre (Martin & Fitzwater, 1988; Coale et al., 1996; Boyd et al., 1996), the equatorial Pacific (Price

et al., 1991) and the Southern Ocean (Timmermans et al., 1998). These areas have been termed high nutrient (nitrogen or phosphate), low chlorophyll (HNLC) areas. In addition, studies of coastal ponds (Howarth, 1988), estuaries and saline lake systems (Howarth et al., 1988; Evans & Prepas, 1997) indicate that N availability becomes increasingly important in limiting phytoplankton biomass. Reducing the alkalinity of the bioassay water stimulated phytoplankton growth in the same manner as the Fe additions, suggesting that the bioavailability of the Fe was severely restricted by lakewater alkalinity (Evans & Prepas, 1997). These systems appear to have been limited by iron because of high alkalinity and salinity, and thus affect N_2-fixers. It is possible that iron availability has played a crucial role in global productivity and consequently in atmospheric CO_2 concentration during the earth's history (Martin, 1990).

In spite of its biological importance, the iron requirement of marine plants is not yet fully understood. The physiological responses of iron limitation by phytoplankton and cyanobacteria have been studied extensively (Martin & Fitzwater, 1988; Doucette & Harrison, 1991; Hudson & Morel, 1990; Sunda & Huntsman, 1997; Kudo & Harrison, 1997; Marquardt et al., 1991). These results indicated that iron deficiency showed lower phytoplankton growth, utilization of nitrogen and some changes of biochemical composition and ultrastructure of the photosynthetic apparatus. However, there have been fewer studies of iron uptake kinetic by macroalgae (Manley, 1981; Suzuki et al., 1995) and little is known about the effects of iron deficiency on *Gracilaria*. In this study *Gracilaria tenuistipitata* var. *liui* was chosen to examine the effect of iron deficiency on amacroalga.

Materials and methods

Culture medium

All experiments were conducted in artificial seawater, Aquil (Morel & Rueter, 1979) with several adjustment to the original medium (containing 250 μmol L^{-1} $NaNO_3$ as sole N resource, 15 μmol $^{L-1}$ $NaH_2PO_4{\cdot}H_2O$, 5×10^{-6} mol^{L-1} $FeCl_3{\cdot}6H_2O$, silicate and $NH_4{}^+$ were omitted). Trace metals and EDTA were added after the bulk medium was passed through a chelex-100 ion exchange resin (Morel & Rueter, 1979) to reduce the trace metal background and contamination (the background concentration of Fe $< 5 \times 10^{-9}$ mol L^{-1}). Each stock solution was kept in dark with acid-washed container.

General culture

Experimental studies were conducted with cultures of *G. tenuistipitata* var. *liui* Zhang et Xia collected from Shantou, the south of China. The epiphytic algae on the macroalga were removed under microscope, and the macroalga were rinsed with filtered Aquil, then cultured in 3 L Aquil under conditions of 25 °C, 21‰, pH 7.8 and continuous aeration. Light was provided at 150 μmol m^{-2} s^{-1} 12L:12D from fluorescent cool-white bulbs. These lighting, temperature, salinity and pH conditions were maintained during the experiments. Fronds were preconditioned in Aquil for 10 days prior to the experiments.

Fe-deficiency culture

Alga cultured for Fe-deficiency was first transferred to an acid-washed glass beaker containing 0.02 mol^{L-1} Ti (III)-citrate EDTA solution (Hudson & Morel, 1989) (0.02 mol^{L-1} $TiCl_3$, $Na_2EDTA{\cdot}2H_2O$ and $Na_3citrate{\cdot}2H_2O$ in filtered Aquil medium), followed by adjusting pH to 7.8 to remove extracellularly adsorbed iron on tissue surface. The fronds were transferred to 3-L (polycarbonate flasks) iron-deficiency medium (Fe-EDTA was replaced with Na-EDTA) and lasted for 60 days. The medium was changed once a week. Duplicate cultures were maintained.

Growth rate, tissue iron, pigment and carbon fixation

Growth rate, Total nitrogen, tissue Fe, Chlorophyll (Chl), phycoerythrin (PE) (triplicate samples) were monitored initially and then every 10 days during the course of 60 days Fe-deficiency. Chl was determined by the N, N'-dimethylformamide extraction technique (Moran, 1982), and PE was extracted with phosphate buffer (Naldi & Wheeler, 1999). The alga was harvested, parts of issue samples were removed and dried to a constant weight (24 to 48 h at 60 °C) in order to normalize the Chl, PE, TN, ^{14}C fixation rate and the following iron uptake rate to dry weight of the fronds. The remainder was rinsed quickly with DDW, frozen, lyophilized and ground in a Wiley mill to pass a 20-mesh sieve. 0.2 g dried material was wet-ashed in concentrated reagent-grade nitric acid for flameless atomic absorption determination of iron. TN content was analyzed with a Carlo-Erba CHN analyzer.

Carbon fixation rate was estimated by the uptake of ^{14}C at 0 and 30 days after Fe-deficiency. The stock $NaH^{14}CO_3$ solution was purified by passing through Chelex-100 to prevent trace metal contamination. The alga was dark-adapted for a period of 30 min prior to the introduction of approximately 10 μCi mL^{-1} radiotracer. Photosynthetic carbon uptake was monitored under light density of 50–250 μmol m^{-2} s^{-1}. A parallel set of samples were maintained in the dark in order to correct for a dark fixation of carbon into the cell. After incubation, the fronds were washed for several minutes in filtered unlabeled Aquil for radioactivity extraction. Subsampled tissues were exhaustively extracted with 80% ethanol acidified with 2 mol L^{-1} HCl to remove any residual cell wall bound $H^{14}CO_3^-$. Ethanol insoluble material was solubilized using the procedure of Gagne et al. (1979) modified by Manley (1981). Both ethanol soluble and insoluble concentrates were corrected for quench (ESCR) and background.

Determination of iron uptake rate under Fe-deficiency condition

Uptake experiments utilized ^{55}Fe as $FeCl_3$ in 0.05 molL^{-1} HCl (U.S.A. Nuclear, 10 mCi mL^{-1}) were conduced at initial and 30 days after Fe-deficiency. All containers and labware for iron uptake underwent an initial acid soak (50% HCl, 50%HNO_3), and cleaning in DDDW. Whole fronds (0.1–0.5 g fresh weight) were used for iron uptake experiments. Triplet samples were transferred to 50ml Aquil (polycarbonate containers) of known total Fe concentration containing ^{55}Fe (total iron final concentration was ranged 0.05–20 μ mol^{L-1}).

Iron uptake rates included determinations made in the presence of either added NO_3^- (Fe/NO_3^-) or NH_4^+ (Fe/NH_4^+) medium (60 μ mol^{L-1} nitrate or ammonium and 3.5 μ mol^{L-1} phosphate). Light, temperature, salinity and pH kept the same as preincubation. After 12-h uptake experiments, the tissue were dipped in 50 mL of 0.02 mol^{L-1} Ti (III)-citrate EDTA solution to rapidly remove extracellularly adsorbed iron on frond tissue surface, and then were treated to be totally dissolved by the procedure of Manley (1981). The samples were placed in scintillation vials, and then treated with hydrogenperoxide to degrade Chlorophyll. All samples were dark adapted at 35–40 °C for 24 h prior to counting to eliminate chemiluminescence and phosphorescence. Quench correction for tissue samples was determined by production of quenched standards (ESCR). Average counting efficiency was 38% ± 1.2%. The variation in counting efficiencies of experimental material did not exceed 5%.

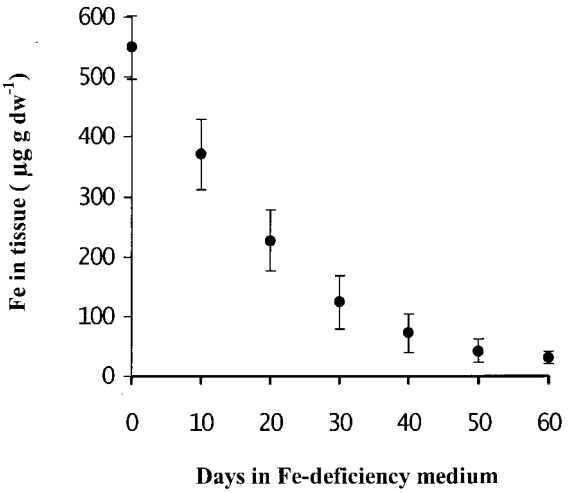

Figure 1. Exponential fits of the decline in tissue Fe in *G. tenuistipitata* var. *liui* during 60 days of Fe-deficiency. (mean ± SD, n = 3).

Ultrastructure

The alga was sampled for transmission electron microscopy on the 0, 30,60 days after Fe-deficiency. At the same time, the algal N- and N, P-double deficiency were also prepared for comparison. Fronds were cut into 1mm with the middle part, then washed several times in 0.1mol^{L-1} sodium cacodylate buffer, and fixed in 4% (v/v) glutaraldehyde in cacodylate buffer. After washed with the buffer, the samples were fixed in 2% OsO_4 in the buffer, dehydrated using increasing concentrations of ethanol, embedded with Epon 812 and stained with saturated uranyl acetate (in 50% methanol) and lead citrate. Electron microscopy was performed with a H-7000 transmission electron microscope.

Results

Tissue iron, pigment, growth rate and carbon fixation

Iron content in tissue declined exponentially with increasing Fe-deficiency, dropping from 549 to 30.4 μg g dw^{-1} (Figure 1, p <0.01), which was largely due to the dilution of existing Fe pool by the addition of new biomass from macroalgal growth.

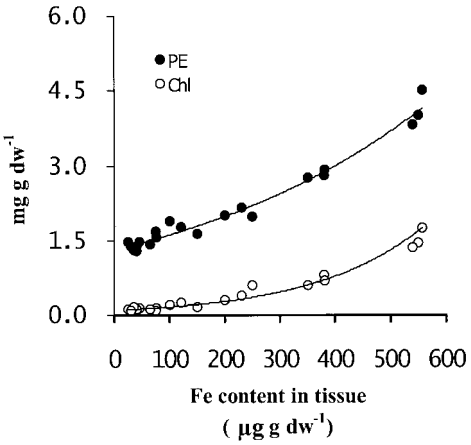

Figure 2. Relationship between Chl, PE and tissue iron in *G. tenuistipitata* var. *liui* (n = 3) PE = $1.3116e^{0.0021x}$ (r^2= 0.9691), Chl = $0.0883e^{0.0054x}$ (r^2 = 0.9411).

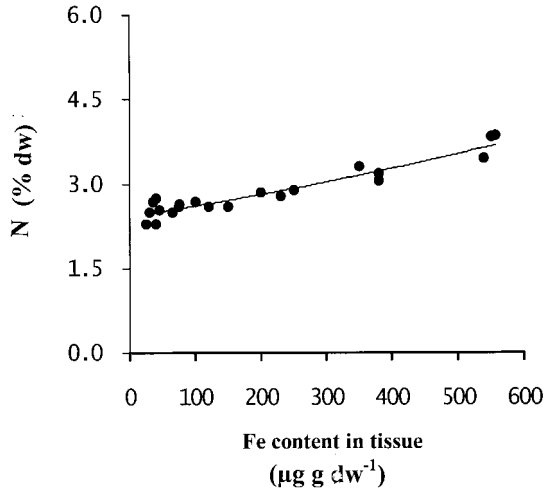

Figure 3. Relationship between N% and tissue iron in *G. tenuistipitata* var. *liui* (n = 3) y = $2.4255e^{0.0008x}$ (r^2 = 0.8971).

During the period of Fe-deficiency, the color of the fronds became pale strawed-yellow and resulted in chlorosis, which indicated a change in the pigment of the cells.

Chl and PE content decreased significantly with decreasing iron in tissue during Fe-deficiency, especially in the first 10 days (Figure 2, $p < 0.01$). Subsequently, the reduction rate decreased continuously, and after approximately 5 wk the pigment content remained constant at a low level. TN in tissue also decreased from 3.65 to 2.49% dw (Figure 3, $p < 0.05$).

During the Fe-deficiency, the specific growth rate of the macroalga decreased rapidly and positively related to Fe content in tissue (Figure 4). After 40

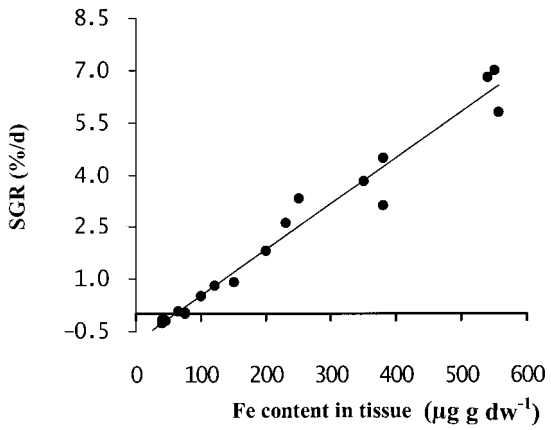

Figure 4. Relationship between specific growth rate and tissue iron in *G. tenuistipitata* var. *liui* (n = 3) y = 0.0132x – 0.8086 (r^2 = 0.9683).

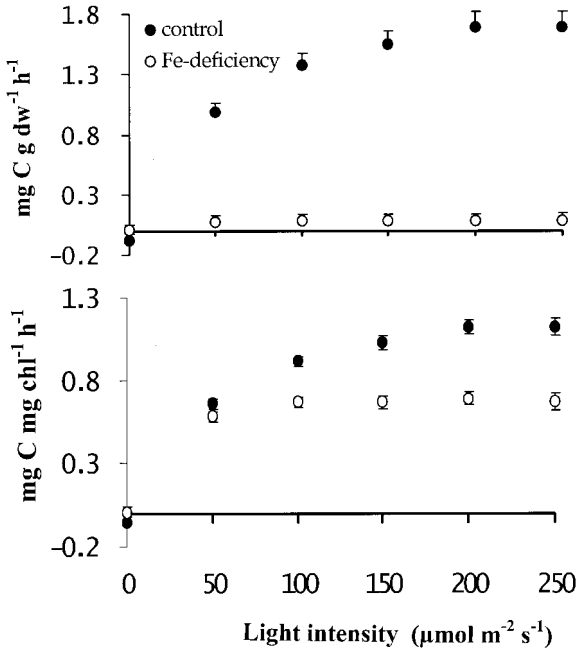

Figure 5. Photosynthetic carbon fixation rate vs. Irradiance (Weight-specific and Chl-specific) for Fe-deficient and control *G. tenuistipitata* var. *liui*. (mean ± SD, n = 3).

days of iron deficiency, no further growth was detectable. Correlation analysis of Fe content in tissue with Chl, PE, TN and growth rates revealed significant relationships (Table 1).

Compared with controls, the maximum carbon fixation rate of the macroalga decreased by 18.9 fold (expressed as mg C g dw^{-1} h^{-1}). The light-saturated

Table 1. Characteristics of *Gracilaria tenuistipitata* var. *liui* under iron deficiency (mean ± SD, n = 3) (at initial T_i and final T_f) * $p < 0.05$; ** $p < 0.01$

Parameter	$T_{initial}$	T_{final}
Specific growth rate (% d^{-1})	6.54 ± 1.3	–0.05 ± 0.66**
Tissue Fe content (μg gdw^{-1})	549 ± 52.6	30.4 ± 10.2**
Chl (mg g dw^{-1})	1.51 ± 0.18	0.12 ± 0.03**
PE (mg g dw^{-1})	4.11 ± 0.35	1.39 ± 0.18**
TN (% dw)	3.65 ± 0.41	2.49 ± 0.32*
^{14}C fixation rate (30 d after Fe-deficiency)		
(mg C g dw^{-1} h^{-1})	1.69 ± 0.13	0.475 ± 0.07**
PI (mg C mg $Chl^{-1}h^{-1}$)	1.13 ± 0.01	0.896 ± 0.05*

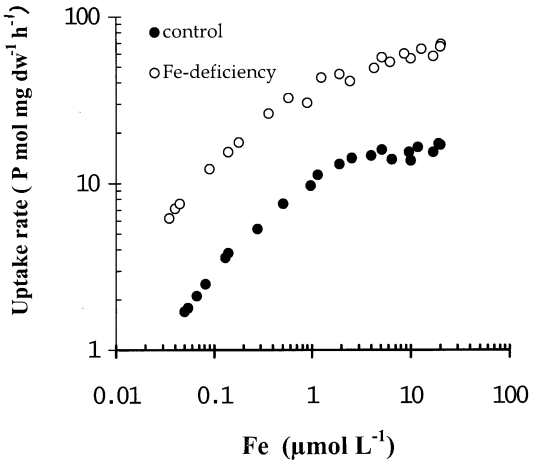

Figure 6. Iron uptake rate versus iron concentration in Aquil with Fe-deficient and control *G.tenuistipitata.* var. *liui* resuspended in NH_4^+-N medium (n = 3).

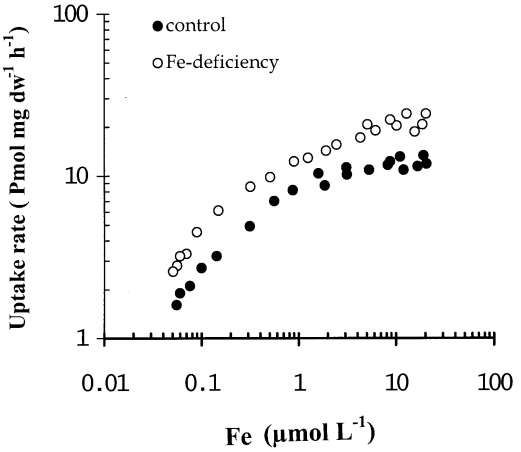

Figure 7. Iron uptake rate versus iron concentration in Aquil with Fe-deficient and control *G.tenuistipitata.* var. *liui* resuspended in NO_3^--N medium (n = 3).

for photosynthetic carbon fixation in Fe-deficient cells was lower than that of controls (Figure 5).

Iron uptake

The saturated iron uptake rates presented both in + Fe, - Fe/NH_4^+ and + Fe, - Fe/NO_3^- (Figures 6, 7). The maximum uptake rate of - Fe/NH_4^+ and - Fe/NO_3^- exceeded that measured for + Fe/NH_4^+ and + Fe/NO_3^- by 4.03 and 1.79 fold, respectively. The highest Fe uptake rate occurred by - Fe/NH_4^+ and the maximum Fe uptake rates arranged as - Fe/NH_4^+>- Fe/NO_3^-> + Fe/NH_4^+>+ Fe/NO_3^- (Table 2).

Ultrastructure

A control cell is shown in Figure 8A, 8B. Large lobes of the plastid occupy most of the volume along the cell periphery, and the remainder is filled by nucleus, mitochondria, vacuoles, starch grains and a few small osmiophilic droplets. Between the cell wall and plasma membrane there are abundant and well-distributed algal gum layers. The plastid is packed with thylakoids, most of them oriented in parallel. The thylakoids are densely covered with phycobilisomes.

Figure 8C, 8D shows that the chloroplasts after 30 days of iron deficiency were considerably different from those of control. There is a significant accumulation of starch grains and there are many big osmiophilic granules. The area of plastid lobes per cell section is reduced, and there appear wide and narrow separations between thylakoid lamellae. Algal gum layer also changed with loose middle and dense bothside.

After 60 days of iron-deficiency (Figure 8E, 8F, 8G), only a few small plastid lobe sections con-

Table 2. Effect of Fe-deficiency on Fe uptake rates on resuspension in NH_4-N or NO_3-N medium by *Gracilaria tenuistipitata* var. *liui*. (mean ± SD, n = 3)

Kinetic parameters	– Fe/NH_4	– Fe/NO_3	+ Fe/NH_4	+ Fe/NO_3
Vmax (pmol mg $dw^{-1}h^{-1}$)	62.8 ± 6.75	21.6 ± 2.69	15.6 ± 1.92	12.1 ± 0.60
Ks (pμmol)	0.89 ± 0.21	0.49 ± 0.16	0.52 ± 0.11	0.37 ± 0.13

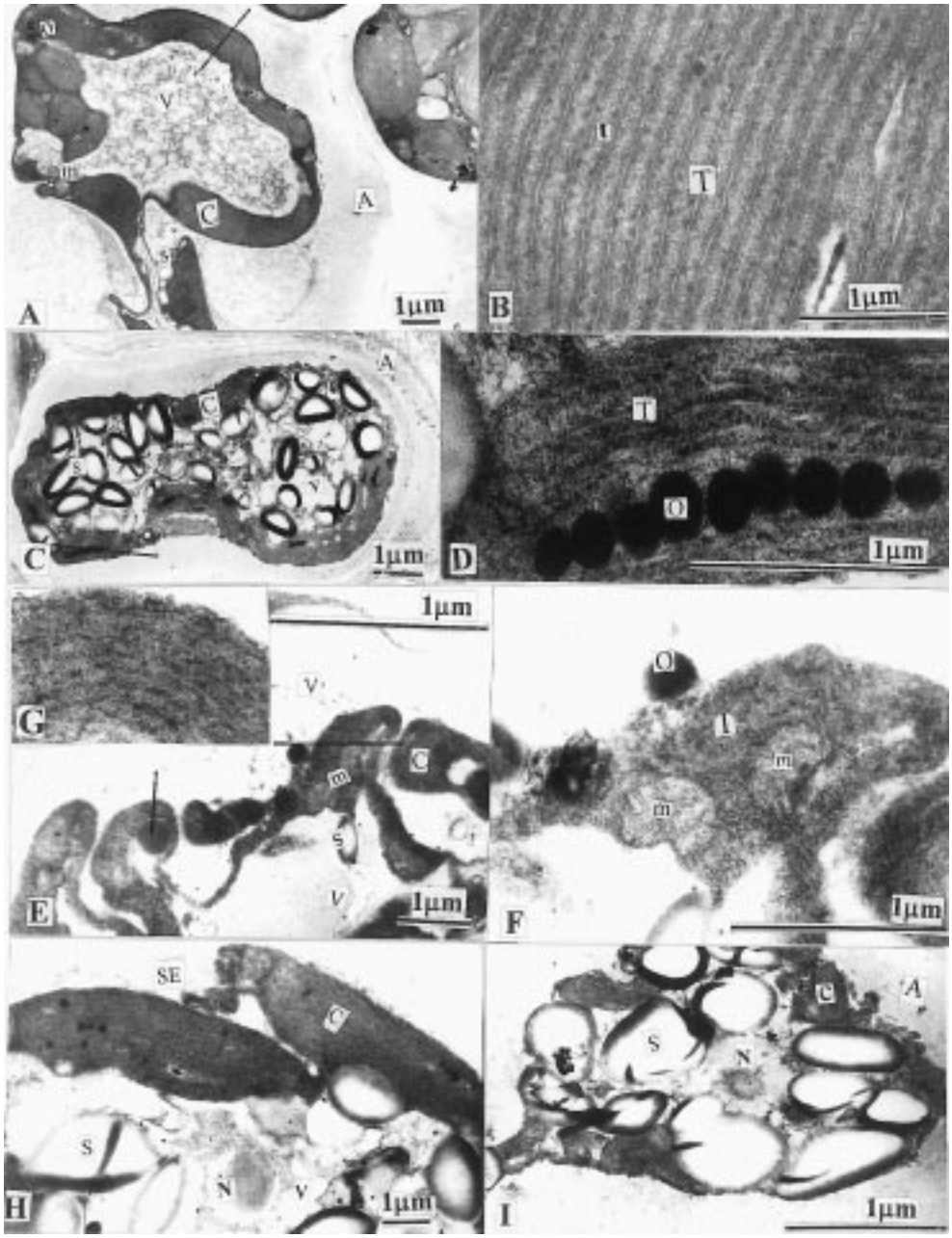

Figure 8. Ultrastructure of *G. tenuistipitata* var. *liui* A, B) Control cells; C, D) Fe-deficiency for 30 d; E, F, G) Fe-deficiency for 60 d; H) N-deficiency for 60 d; I) N, P-double deficiency for 60 d. Arrowheads indicates phycobilisome rows in thylakoid; C, chloroplast; T, thylakoid; S, starch grain; N, nucleus; M, mitochondrion; V, vacuole; SE, secretion; O, osmiophikic granules and A, algal gum layer.

tain thylakoids. Many severely degenerative structural changes are evident. Some chloroplast lamellae and their constituent thylakoids do still retain a normal appearance and there are some visible phycobilisomes on them (Figure 8G). The size of the cells is the same as the control cells, but the number of starch grains is reduced significantly compared with that of cells after 30 days. The severely Fe-deficient chloroplast appears to be sausage-like in shape with dissolved lamellae and big osmiophilic granules. Figure 8H, 8I shows the chloroplasts structure after 60 days of N- and N, P-double deficiency respectively. There are abundant starch grains in the cells. The number of chloroplast are reduced and some chloroplast thylakoids have dissolved. There appear some secretions outside the membrane.

Discussion

Iron-deficient cells consistently showed higher rates of iron uptake than the control and followed classical Michaelis-Menten kinetics with the highest V_{max} for - Fe/NH_4^+, and lowest V_{max} for + Fe/NO_3^- (Table 2, Figures 6, 7). The results demonstrate an enhancement of iron uptake for a Fe-stressed macroalga similar to phytoplankton (Doucette & Harrison, 1990). There have been reports of siderophore production by phytoplankton (Trick et al., 1983; Gledhill et al., 1998), and many organic compounds (such as in cyanobacterial) could enhance Fe (III) transport by either reducing or complexing Fe under iron stress (Boyer et al., 1987). These results indicate that algae have developed the same type of homoeostatic physiological mechanisms to regulate cellular trace element concentrations as those for regulation of major nutrients. Iron maximum uptake rates resuspensed in NH_4^+-N were higher than that of in NO_3^--N either for Fe-deficiency or control (Figures 6, 7). In our experiments, alga cultured for iron uptake was preincubated with NO_3^--N as sole N source. The cells grown on NO_3^- require much more iron to sustain the growth rate, which is attributable to the iron content of nitrate and nitrite reductase, and the additional electron transport components needed to provide the necessary reductant. Fe-deficiency resulted in lower enzyme active and content (NR, NiR) (Milligan & Harrison, 2000), which in turn deprived the utilization of NO_3^-. Under this condition, iron deficiency resulted in both iron and nitrogen stress and the later may be even more severe. In addition, some researches have reported that NH_4^+ uptake kinetics of many macroalgal species showed an enhanced uptake rates during the first hours of N re-supply after N-limitation (McGlathery, 1996). When the macroalga under Fe-, N- double deficiency was changed in N source from NO_3^- to NH_4^+, the fast utilization for NH_4^+ accelerate further Fe uptake in order to maintain the normal ratio of N/Fe in the tissue. These results in *Gracilaria* are consistent with in phytoplankton reported by Doucette et al. (1990) in *Gymnodinium sanguinium* and by Harrison et al. (1986) in *Thalassiosira weissflogii*.

The cellular content of Fe and TN diminished under iron stress (Table 1), which resulted in a decrease in photosynthetic pigment content (especially Chl). Because Chl is derived from the tetropyrrole biosynthetic pathway in which iron participates as a cofactor of certain enzymes and nitrogen also is an important constituent of the pigment. Chl may function as a sensitive indicator of Fe-deficiency. TN in tissue decreased, but not much under Fe-deficiency (Table 1). A similar result has been reported for the planktonic *Phaeodactylum tricornutum* (Greene et al., 1991). This may be a physiological reflection of photosynthetic carbon fixation to iron stress. TN levels and photosynthetic carbon fixation are strongly coupled in all plants because the energy and C skeletons required for N uptake and assimilation are provided by photosynthesis, and over 50% of tissue N is allocated to the chloroplast (McGlathery & Perdersen, 1999). The degree of iron deficiency is reflected in the proportion of total photosynthate that goes into protein.

G. tenuistipitata cells grown under Fe-deficiency conditions share many features with Fe-depleted phytoplankton *Rhodella violacea* (Marquardt et al., 1999), but the extent of chloroplast damage of the former was more severe. As shown by electron microscopy, there is extensive reduction of photosynthetic membranes and an accumulation of polysaccharide granules. Most thylakoids are dissolved completely under severe Fe-deficiency. A similar altered thylakoid structure occurred in N-deficient cells (Figure 8H, 8I). On the other hand, severe N, P deficiency resulted in breakdown of the nuclear membrane and a radio-shaped nucleus.

Photosynthetic pigment content decreased and the chloroplast ultrastructure changed with progressive Fe stress (Figures 2, 8), which was reflected in the reduction in carbon fixation rate (Table 1). On the other hand, many Fe-containing proteins are involved in mediation photosynthetic electron transport and Chl biosynthesis, so Fe deficiency may be expected to affect directly inorganic carbon fixation. The reduction of

carbon fixation in Fe-deficient cells can be attributed to a reduction in both cell Chl content and Chl-specific light-saturated photosynthesis. Thus Fe-deficient cells become light saturated at lower irradiance than the control (Figure 5). It was the combination of decline in nitrogen utilization, degenerated chloroplast structure, reduced pigment content and photosynthetic capacity in the macroalga under iron stress that resulted in its lower growth rate, even chlorosis and death.

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