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Effect of temperature and irradiance on the growth and reproduction of *Enteromorpha prolifera* J. Ag. (Chlorophycophyta, Chlorophyceae)*

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Abstract Effect of temperature and irradiance on growth and reproduction of *Enteromorpha prolifera* that bloomed offshore along the Qingdao coast in summer 2008, was studied. It was showed that *E. prolifera* propagated mainly asexually with specific growth rate (SGR) of 10.47 at 25°C/40 μmol $m^{-2}s^{-1}$. Under this condition, gametes with two flagellate formed and released in 5 days. At the beginning of the development, the unicell gamete divided into two cells with heteropolarity, and then the apical cell developed into thalli primordial cells, whereas the basal cell developed into rhizoid primordial cells. In 8-day culture, the monoplast gamete developed into juvenile germling of 240 μm in length. Unreleased gametes can develop directly within the alga body. *E. prolifera* could either reproduce through lateral branching or fragmenting except apomixis revealed by Microscopic observation. On aged tissue of *E. prolifera*, although the degraded pigments partially remained in faded algal filaments, numerous vegetative cells could still divide actively in the algal tissues.

Keyword: *Enteromorpha prolifera*; growth; reproduction; gamete development

1 INTRODUCTION

Started on May 31st, 2008, vast accumulations of *Enteromorpha prolifera* had been appearing in the near shore area off Qingdao. Early in June of 2008, the unprecedented scale of the occurrence in huge mass of the green algae coursed a serious concern in local city administration as the Sailing Competition of the 29th Olympic Games had been planned in early August 2008. To ensure the clean and smooth opening of the Sailing Competition, local citizens, volunteers, and army forces were motivated to remove lingered algae on beaches from polluting coastal zones. It happened last year in a considerable scale but much smaller than in 2008, which reflects the ecological and environmental change, and affects the coastal ecosystems and biodiversities in the region.

Taxonomic analysis indicated that the over-growing seaweed belongs to Chlorophycophyta, Chlorophyceae, Ulvales, *Enteromorpha* (Link) Ag. *prolifera*, and was one of common species along the Qingdao coast. Previously, various species of green benthic algae, such as *Enteromorpha*, *Ulva*, *Chaetomorpha* and *Cladophora*, were reported as "green tides" (Fletcher, 1996), such as the events over a large area on the west coasts of Finland every summer between 1992 and 2000 (Blomster et al., 2002), and green tides caused by *Ulva* spp. in many costal sites in Japan since 1970s (Hiraoka et al., 2004). At present, many ecological studies have been addressed on the green tides (Fletcher, 1996; Valiela et al., 1997).

Morphologically and ecologically, thallus of the alga is tubular with abundant proliferous branches, in about 2 m in height, and grows on upper to middle intertidal stones

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or rocky substrates; sometimes it floats in stagnant sea-waters in sheltered sites (Tseng, 1983). Isomorphic alternation of generations consists of haploid and diploid phases. The reproduction analysis has recognized seven forms (Lin et al., 2008), of which vegetative reproduction, sexual reproduction, apomixis are common ones. Wang et al. (2007) reported that *E. prolifera* can grow normally under 10–30°C and with the irradiance over 18 μmol $m^{-2}s^{-1}$, with maximal growth rate at 72 μmol $m^{-2}s^{-1}$. In addition, the salinity and pH conditions are 20.2–26.9 and 7–9, respectively (Wu and Xu, 2000).

In order to understand the mechanism of *E. prolifera* blooms, and the effects of temperature and irradiance on its growth and development, we conducted the study to verify the physiological and ecological parameters of the blooming.

2 MATERIALS AND METHODS

2.1 Sampling and pre-culture

E. prolifera were locally collected in No.1 Bathing Beach and nearby costal sites in Qingdao from June 3rd to July 6th 2008. After the collection, the algal materials were washed several times with natural seawater, then with sterilized seawater to remove epiphytes and slime, and cultured in flasks at 25±1°C, 5 μmol $m^{-2}s^{-1}$ in 12:12 h (L:D) for 92 h, the culture medium (sterilized seawater) was changed every 24 h.

2.2 Temperature and irradiance effects on growth of *E. prolifera*

Fresh *E. prolifera* thalli of 0.2 g for each was cultured in 100 ml flasks with 75 ml culture medium, which consisted of filtered seawater and 1 mol/L 错误！未定义书签。 NO_3^--N ($NaNO_3$) and 0.1 mol/L PO_4^{3-}-P (KH_2PO_4). The pH value and salinity detected were 7.9±0.1 and 30±1, respectively.

Five temperatures (5, 10, 15, 20, 25°C) in each incubators (Jiangnan Instruments Ltd. Co. Ningbo), and three irradiance levels (10, 20, 40 μmol $m^{-2}s^{-1}$) in photoperiod of 12:12 h (L:D) were designed. For each treatment, three replicates were conducted. The flasks were shaken for 2-3 times every day.

2.3 Reproduction observations

During the culture, morphological changes of *E. prolifera* were observed and recorded with optical microscope (Olympus BX51, Japan) and digital camera (Cool SNAP 5.0, Canada). To study fragment regeneration, fresh and mature thalli were chopped into 0.1 cm in length, then transferred into sterilized Petri dishes containing 15 ml culture medium, and cultured at 25°C/40 μmol $m^{-2}s^{-1}$ in the same salinity, pH value, and photoperiod described above.

2.4 Culture of gametes

After the gametes release, the algal materials were removed and the Petri dishes containing the gametes were cultured continually at 25°C, 40 μmol $m^{-2}s^{-1}$. The early development of gametes was observed and described under microscope. The culture medium was refreshed every 3 d.

2.5 Data analysis

In 7 d culture, fresh materials of *E. prolifera* were weighed and the specific growth rate (SGR) was calculated according to the formula below:

SGR（$\%d^{-1}$）= $[\mathrm{Ln}(W_t)-\mathrm{Ln}(W_0)]\times100\% / t$, where W_t means the fresh weight of *E. prolifera* after t d cultivation and W_0 means the initial fresh weight at the beginning of the experiment, t means the number of days of cultivation.

One-way ANOVA was performed to test differences of SGR between treatments. Turkey test was done at α=0.05 significance level.

3 RESULTS

3.1 Morphological structure and feature

Thallus of *E. prolifera* was dark green, with soft and abundant proliferous and slender branches (Fig.1-1). Optical microscopic observation indicated that the thallus was tubular and hollow, consisting of one sheet of cells polygonal to squares in shape (Fig.1-2). Cells arranged in rows, each with a single chloroplast usually filling the cell and pyrenoid (Fig.1-3).

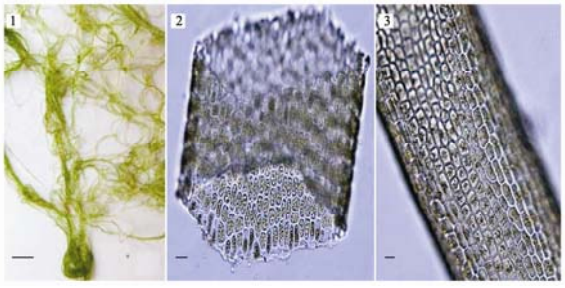

Fig.1 Morphology of *Enteromorpha prolifera*

1. Thalli of mature *E. prolifera*. Bar=1 cm; 2.Sectional observation. Bar=20 μm; 3. Surface view. Bar=20 μm

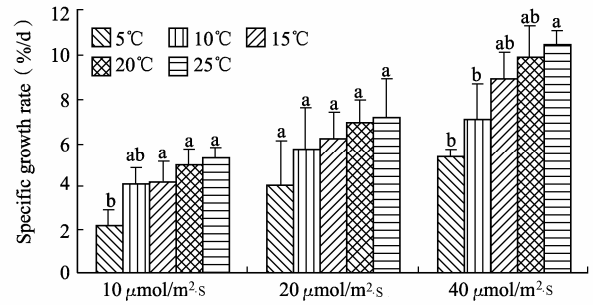

Fig.2 Effect of temperature on the growth of *E. prolifera*

Values are average + standard deviation, n=3; different letters (a, b) represent significant differences between temperature treatments (one-way ANOVA followed by the Turkey test, P<0.05)

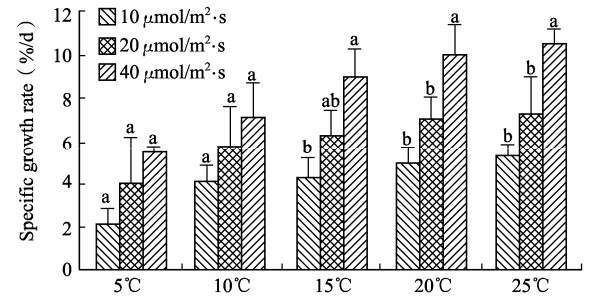

Fig.3 Effect of irradiance on the growth of *E. prolifera*

Values are average + standard deviation, n=3; Different letters (a, b) represent significant differences between irradiance treatments (one-way ANOVA followed by Turkey test, P<0.05)

3.2 Effect of temperature and irradiance on the growth

Temperature affects significantly the growth of *E. prolifera* (Fig.2). Between 5–25°C, the SGR increased with temperature, and the maximum SGR occurred at 25°C in three irradiance levels were 5.30 (10 μmol $m^{-2}s^{-1}$), 7.23 (20 μmol $m^{-2}s^{-1}$) and 10.47 (40 μmol $m^{-2}s^{-1}$).

Statistical significance existed between 5 and 10, 15, 20, and 25°C under 10 μmol $m^{-2}s^{-1}$, indicating that the proliferous ability cultured at 5°C/10 μmol $m^{-2}s^{-1}$ was inferior to those under other conditions. No significant influence was observed under 20 μmol $m^{-2}s^{-1}$, and the SGR was obviously affected by temperature, reaching the maximum SGR (10.47) at 25°C in the irradiance of 40 μmol $m^{-2}s^{-1}$.

The SGR increased significantly with irradiance from 10 to 40 μmol $m^{-2}s^{-1}$, but at low temperature range (5 and 10°C) there was no statistical significance. Under the irradiance at 40 μmol $m^{-2}s^{-1}$, the SGR increased dramatically (P<0.05) at 20 or 25°C (Fig.3).

3.3 Reproduction forms

Under laboratory conditions, three reproduction ways of *E. prolifera* were found under optical microscopic observation: branching from rhizoids, fragmenting, and apomixis.

3.3.1 Branching

In 2–3 d after culture, a great number of slender lateral branches appeared at 15, 20 or 25°C (Fig.4-1), respectively. The basal part of new branches was transparent, and formed rhizoid connecting original thalli (Fig.4-2). The rhizoid was fragile (Fig.4-3), and not all lateral branches could form rhizoid structures (Fig.4-4).

3.3.2 Fragmenting

In 9 days of nursing in 25°C/40 μmol $m^{-2}s^{-1}$ or 25°C/20 μmol $m^{-2}s^{-1}$, *E. prolifera* fragments formed transparent and massive callus at both ends of the breakage. Callus from the upper ends developed into new germling (Fig.5a), whereas the opposite developed into rhizoids (Fig.5b).

3.3.3 Gametes release and apomixis

At prophase of matured gametangia, protoplast assembled to the center, about 10–20 still "pre-gametes" formed in every cell via mitosis. AS gametangia matured, "pre-gametes" turned active and kept moving. In 5 d at 25°C/40 μmol $m^{-2}s^{-1}$, the matured gametangia released numerous gametes (Fig.6). The released gametes are sphere or ellipse in shape and 5 μm in diameter, with pigments assembled in the back hemisphere and two flagellate appeared in front of the other hemisphere (Fig.7-1). Only the cell walls remained after gametes release.

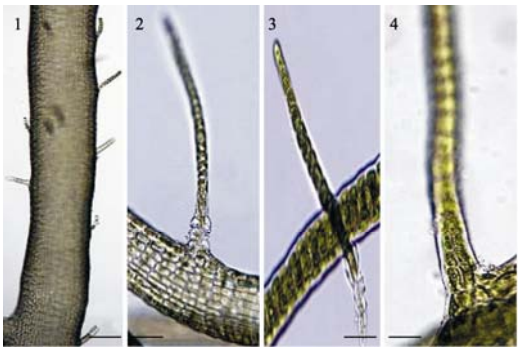

Fig.4 Reproduction through branches with rhizoid of *E. prolifera*

1. Lateral branches sprouted out. Bar=20 μm; 2. Branch with rhizoid structure, black arrow indicates rhizoid. Bar=10 μm; 3. Branch separated from original thalli. Bar=10 μm; 4. Branch without rhizoid. Bar=5 μm

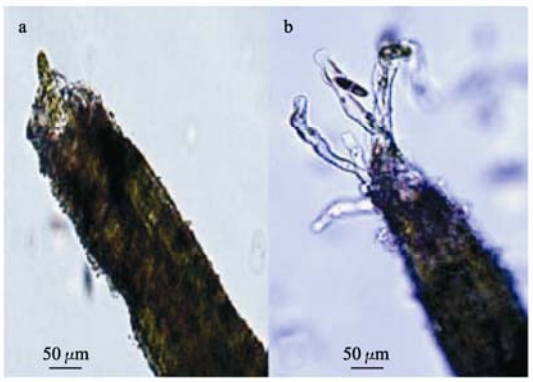

Fig.5 Reproduction through fragment of *E. prolifera*

a. Germling at the upper end of the fragment; b. Rhizoids at the lower end

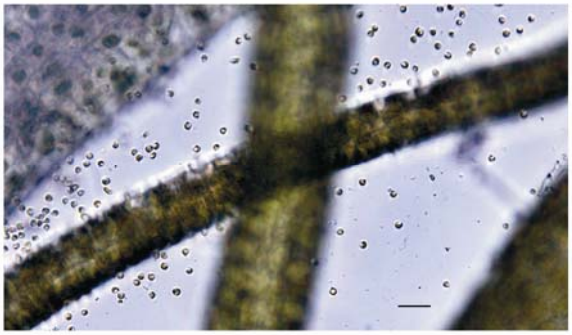

Fig. 6 Release of biflagellate gametes (bar=20μm)

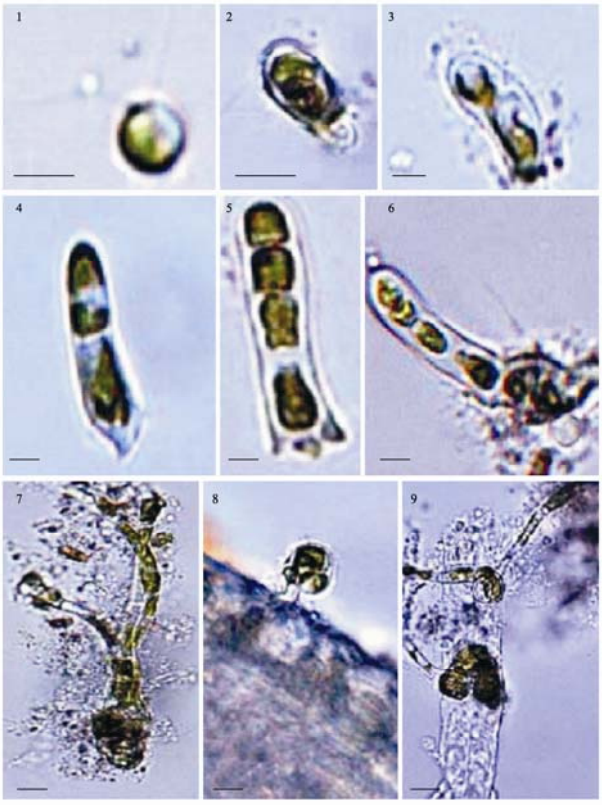

Fig.7 Germination and development of gametes

1. Gametes with two flagellate, arrows indicate flagella. Bar=5 μm; 2. Extend to ellipse in shape. Bar=20 μm; 3. Protoplast divided into two parts. Bar=10 μm; 4. The apical cell developed into thalli primordium. Bar=10 μm; 5. The basal cell developed into rhizoid primordium. Bar=10 μm; 6. Rhizoid attached to substratum. Bar=10 μm; 7. New thalli developed from gamete. Bar=20 μm; 8. Gametes germinated attaching to protoalga. Bar=10 μm. 9. Juvenile germling developed within protoalga. Bar=20 μm

The fresh gametes moved rapidly with flagellate. However, soon after being released, they settled down on immersed cove slips (10 mm×10 mm) in the Petri dishes. The flagellate disappeared and cells extended when gametes started germinating (Fig.7-2), followed by protoplast divinizing with two parts which moved to the both ends separately. Cell plate occurred and two cells with polarity formed longitudinally (Fig.7-3). Later, the apical cell kept dividing and developed into thalli primordial cells (Fig.7-4), whereas the basal cell developed into rhizoid primordial cells (Fig.7-5) making the algal attached to substratum firmly (Fig.7-6). In 8 days at 25°C/40 μmol $m^{-2}s^{-1}$, the gametes developed into new thalli 0.24 mm in length (Fig.7-7). The rhizoid primordial formation varied among different individuals. Some rhizoid formed rhizoid primordium when cell number reached 2–4, while some others did not appear even the cell number reached up to 8.

It is interesting that some gametes remained still in gametangium, and could germinate into new juvenile germling (Fig.7-9) directly, or use the protoalga as substratum for germination (Fig.7-8).

3.3.4 Vegetative cells division and development

Usually, aged or dead thalli of *E. prolifera* are grayish-white and flocculent because of pigments degradation and fading. However, many vegetative cells were still able to divide. Under the conditions of 25°C/40 μmol $m^{-2}s^{-1}$, protoplast of such type of cells could extend and divide into two parts (Fig.8), and could keep dividing and developed into new thalli with 5–6 cells in 8–9 days.

4 DICUSSION

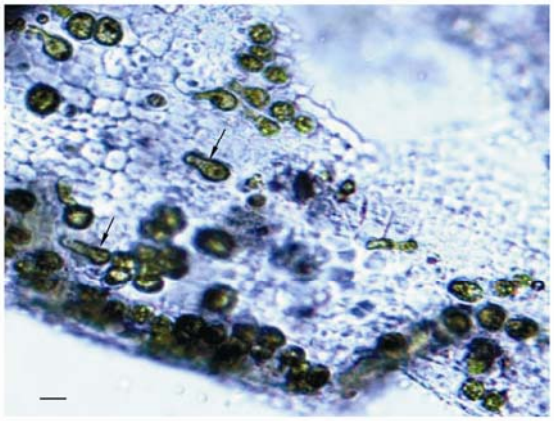

Fig. 8 Division of vegetative cells survived in grayish-white algae
Arrows indicate cell extending, bar=40μm

Many studies have indicated that costal eutrophication and tropic changes interact strongly with the biotic communities, and with associated macrophytes in particular, which can result in abnormal growth of nuisance seaweeds and decline in species diversity (Orth and Moore, 1983; Twilley et al., 1985; Taylor et al., 1995; Sfriso and Macromini, 1996). Similar phenomenon was reported in other places of the world (Dion and Bozec, 1996; Flindt et al., 1997; Raffaelli et al., 1998; Martins et al., 2001; Naldi and Viaroli, 2002; Huang et al., 2003;

Kammer et al., 2004). On the occurrence of "green tide" in Qingdao, it was possibly involved in costal eutrophication and ecosystem change.

Normally, the water temperature along the local coast in Qingdao is 27±1°C in summer, which was one of the main reasons for the algal blooming. Previously, Wang et al. (2007) suggested that *E. prolifera* is strong against high irradiance, meaning that the green algae could fully take the usage of light to elevate the efficiency of photosynthesis. Moreover, the concentrations dissolved inorganic nitrogen (DIN = NO_3^-+ NO_2^-+ NH_4^-) and the dissolved inorganic phosphate (DIP) are usually high in the study area from June to August (Liu et al., 2007), providing sufficient nutrition for the alga growth. Obviously, reproduction in various forms enables the algal to bloom within a short period of time. It was reported that almost every vegetative cell of gametophytes can transform into gametangia (Dan et al., 2002), which has been confirmed in our observation. In addition, the definition of the thalli stage can not be simply determined by the appearance, because vegetative cells survived in aged thalli can develop again once the conditions favor. Therefore, microscopic examination is necessary to determine the algal growth state. Ding et al. (2006) reported earlier that gametes of *E. linza* could germinate from protoalga, for *E. prolifera*; however, it was reported for the first time.

Interestingly, *E. intestinalis* and *E. linza* are dominant species in the study area but *E. prolifera*. Satellite remo-sensing data show that *E. prolifera* drifted massively from southern Yellow Sea in late May, during which it competed with other two dominant species. Blooming of the non-dominant species within such a short time became a serious threat to the integrity of marine ecosystems and local flora inhabitants, and brought up a very negative impact on local marine floral communities, as well as coastal pollution that damaged the tourism resources. Therefore, analyzing the molecular mechanism of the fast blooming is a must.

For this purpose, sampling has been conducted at different blooming sites for several times during the bloom event. Not only floating green algae were observed and sampled, but many algae were found sunk to the bottom of the sea, for which two questions were highlighted: Why did the massive floating algae settle down to sea-bottom? What's the destiny of the algae sediment? In general, thinly distributed algae and abundant oxygenic bubbles are the reasons causing floatation of *E. prolifera*. On the other hand, massive high density algae and aged algae with low efficiency in photosynthesis would sink. Three possibilities for *E. prolifera* sedimentation are presumed: 1. Natural decease due to low solar lighting, which inhibited the algal growth, and few *E. prolifera* could grow normally at sea-bottom (data unpublished); 2. Zooplankton consumption as observed in the samples collected at sea-bottom under optical microscope many zooplanktons were found who might eat the gametes and cells (Fig. 6). 3. Floating to sea surface again by up-welling current.

In parallel indoor tank tests, about 2 kg fresh *E. prolifera* were cultured in a tank (6 m^3) at 23±1°C and 8 μmol $m^{-2}s^{-1}$. In 24 h, almost all floating algae sank, and the alga could not float up when the irradiance reached 40–60 μmol $m^{-2}s^{-1}$. The artificial upward currents were exerted, which made the algae float up to the water surface again. The algae could grow under high irradiance for several days, which has been observed in this investigation on the massive green algae bloom from June to July.

5 CONCLUSIONS

In conclusion, the massive bloom of *E. prolifera* off and along the Qingdao coast was resulted from changes in climate, ecology, and environmental condition in coastal zone. Outbreak of usually non-dominant blooming green algal species was the results of combination of local ecosystems and marine environment change, which impacted the local marine flora and fauna very negatively in the summer 2008, causing considerable costs to local economic and cultural sectors.

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