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Comparison in copper accumulation and physiological responses of *Gracilaria lemaneiformis* and *G. lichenoides* (Rhodophyceae)*

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Abstract Heavy metal pollution has become a worldwide problem in aquaculture. We studied copper (Cu^{2+}) accumulation and physiological responses of two red algae *Gracilaria lemaneiformis* and *Gracilaria lichenoides* from China under Cu^{2+} exposure of 0–500 μg/L in concentration. Compared with *G. lemaneiformis*, *G. lichenoides* was more capable in accumulating Cu^{2+}, specifically, more Cu^{2+} on extracellular side (cell wall) than on intracellular side (cytoplasm) and in cell organelles (especially chloroplast, cell nucleus, mitochondria, and ribosome). In addition, *G. lichenoides* contained more insoluble polysaccharide in cell wall, which might promote the extracellular Cu^{2+}-binding as an efficient barrier against metal toxicity. Conversely, *G. lemaneiformis* was more vulnerable than *G. lichenoides* to Cu^{2+} toxin for decreases in growth, pigment (chlorophyll *a*, chlorophyll *b*, phycobiliprotein, and β-carotene) content, and photosynthetic activity. Moreover, more serious oxidative damages in *G. lemaneiformis* than in *G. lichenoides*, in accumulation of reactive oxidative species and malondialdehyde, and in electrolyte leakage, because of lower antioxidant enzyme (superoxide dismutase and glutathione reductase) activities. Therefore, *G. lichenoides* was less susceptible to Cu^{2+} stress than *G. lemaneiformis*.

Keyword: copper (II) pollution; *Gracilaria*; physiological response; reactive oxidative species; chlorophyll fluorescence parameters

1 INTRODUCTION

In recent years, Cu^{2+} has become one of the most significant aquatic environment pollutants in the world. In offshore seawater, total Cu^{2+} concentration varies from 10–100 nmol/L (0.64–6.4 μg/L), and the concentration may even higher in coastal aquatic circumstance with industrial and agricultural activities (Martins et al., 2011). Furthermore, Cu^{2+} concentration is often set in the range of 500–2 000 μg/L in an aquaculture system to depress aquatic macrophyte and algal proliferation (Boyd and Massaut, 1999). However, long-lasting contamination at even low Cu^{2+} concentrations could cause coastal biotic accumulation, posing serious health hazards to animals and humans, which is very adverse to fisheries development (Cotou et al., 2012).

Red macroalga *Gracilaria* (Gracilariae, Rhodophyta) has been used as an effective biofilter to uptake and utilize dissolved nutrients released by animals in the integrated multi-trophic aquaculture system (Zhou et al., 2006; Huo et al., 2012), and as a bio-device for eutrophication control in Chinese coastal waters (Tang et al., 2003; Yang et al., 2006; Huo et al., 2011). Compared with other *Gracilaria* species, *G. lemaneiformis* and *G. lichenoides* are two important aquaculture species in good potential for bioremediation for having high growth rate and nutrient uptake ability, and strong thermo tolerance as

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well (Yang et al., 2006; Xu et al., 2008). However, heavy metal accumulation in their tissues and their responses to metal pollution remained unclear.

Therefore, we conducted a study on Cu^{2+} accumulation in the two *Gracilaria* species and compared their physiological responses, to seek for information of their cultivation and their potential roles as bioindicators or as bioremediation species of metal pollution. To assess relative strength of tolerance to copper pollution and the mechanisms of the two species, several physiological end-points affected by Cu^{2+} were selected according to the results of previous experiments (Prasad et al., 2001; Mal et al., 2002; Tanyolac et al., 2007), including growth, photosynthetic pigments and activity, antioxidative enzymes activities, oxidative damage to lipids and membrane integrity, as well as accumulation of Cu^{2+} in extra- or intra-cellular spaces and cell organelles.

2 MATERIAL AND METHOD

2.1 Algal material and cultivation conditions

Two algae species, *G. lemaneiformis* and *G. lichenoides* (Rhodophyta), were collected from a culture pond in Nantong, Jiangsu, East China (30°2′26″N, 130°2′26″E), where no metal contamination was observed. In laboratory, samples were washed to remove microorganisms and transferred to glass aquariums filled with sterilized and filtered seawater. Cultures were enriched with nutrients according to Haglund et al. (1996) every three days for 20 days until one day before experiment. Initial concentrations of Cu^{2+} treatments were set at 0 (control), 50, 100, 250, and 500 μg/L Cu^{2+}, by adding $Cu_2SO_4{\cdot}H_2O$ (Sigma). Each treatment was carried out six replicates. No chelators or nutrients were added during experiment. An amount of 3.0±0.05 g in fresh weight (FW in gram) was cultured in flasks containing 1-L medium of different Cu^{2+} concentrations under the optimal conditions obtained from this and previous experiments (Collén et al., 2003): temperature 22±1°C, photon flux density 80 photons μmol/(m^2·s) on a 12-h photoperiod, pH 7.8, and salinity 25. The experiment was conducted in 6 days.

2.2 Determination of growth parameters

Algae samples were harvested, blotted with paper towels, and FW was measured to compare growth in different Cu^{2+} concentrations in 6 days. Specific growth rates (SGR, %/d) were calculated by SGR= $[(W_t/W_0)^{1/t}-1]\times100\%$, where W_0 is the initial FW, W_t the FW at time t, and t is the number of days (Haglund et al., 1996).

2.3 Determination of cell wall polysaccharides, total and extra- or intra-cellular and cell organelle copper quantification

Vegetative thalli of the two *Gracilaria* species were cleaned, freeze-dried, and pulverized. Cell wall polysaccharides were extracted from dried algae powder by digestion and partially purified by cetylpyridinium chloride as described by Farias et al. (2000). Soluble fractions were segregated from insoluble sulfated polysaccharides as per Virginia et al. (2007). Fractions obtained were lyophilized and weighed.

Algae samples were rinsed and divided into three parts. One part was used to measure intracellular Cu^{2+} by being washed with 5 mmol/L EDTA at pH 8 for 10 min to remove any extracellular Cu^{2+} adsorbed according to Virginia et al. (2007). The second part was used to measure Cu^{2+} distribution in different cell organelles of algae samples by differential centrifugation described by Brooks et al. (1981). The third part was used to determine the total intra (-cellular and extra-cellular) Cu^{2+} amount in untreated samples by EDTA. All parts were freeze-dried for calculation of dry weight. Cu^{2+} content in the sample was determined with coupled plasma mass spectrometry (ICP-MS) according to Fang (1991).

2.4 Chlorophyll fluorescence measurements

Kinetic fluorescent parameters were measured on Modulated Chlorophyll Fluorometer (Dual-PAM-100, Walz, Germany). Algal pieces were placed in darkness for 15 min at 20°C prior to each measurement. The maximum fluorescence yield (F_m), initial fluorescence yield (F_0), the ratio of variable to maximal fluorescence (F_v/F_m), and relative electron transport rates (ETR) were calculated from ETRmax tanh (αI/ETRmax) according to Han et al. (2008). Non-photochemical quenching (NPQ) was calculated by $(F_m-F'_m)/F'_m$ (Maxwell and Johnson, 2000). Parameters of photochemical quenching (qP)=$(F'_m-F_s)/(F'_m-F'_0)$ were calculated as per Schreiber et al. (1986).

2.5 Pigment analysis

Pigments were extracted in acetone (90%) by grinding 300 mg FW in a mortar. Extracts were then centrifuged (8 000×*g*, 10 min, 4°C) and the absorbance

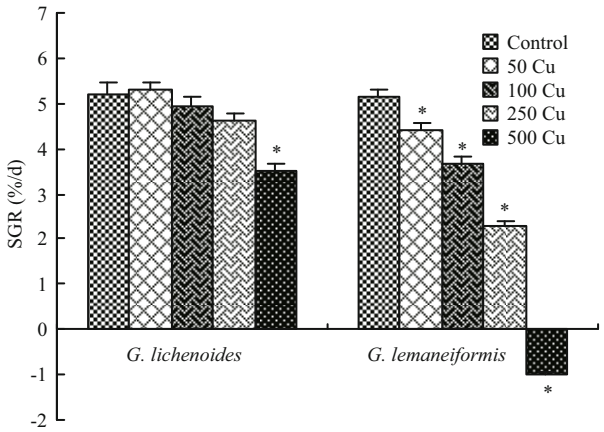

Fig.1 Effects of copper concentrations on specific growth rate (SGR) of *Gracilaria* species following exposure for 6 d

Values are means±S.D. (n=6). Asterisk indicates significantly differences from controls (P<0.05).

of the supernatants was measured at 470, 652.4, and 665.2 nm for determining chlorophyll *a*, *b*, and β-carotene contents as per Wellburn (1994). Phycobilins were extracted in 50 mmol/L phosphate buffer (pH=5.7, 4°C). The extracts were then centrifuged for 10 min at 10 000×*g*. Phycoerythrin (R-PE), phycocyanin (R-PC), and allophycocyanin (APC) contents were determined spectrophotometrically at 498, 551, 614, and 651 nm, respectively, using the equations described by Kursar et a1. (1983).

2.6 Determination of ROS content, MDA content, and electrolyte leakage

Reactive oxidative species (ROS) contents were measured using the kits of Jiancheng Bioengineering Institute, Nanjing, China. Estimation of malondialdehyde (MDA) content followed methods of Lei et al. (2007). Electrolyte leakage was assayed in method of Axelsson and Axelsson (1987).

2.7 Estimation of antioxidative enzymes

SOD (EC 1.15.1.1) activity was measured as per Giannopolitis and Ries (1977). One unit of SOD activity was defined as the quantity of enzyme demanded to result in 50% the reduction of nitro blue tetrazolium. APX (EC 1.11.1.11) activity was determined as per Nakano and Asada (1981) by measuring the decrease of ascorbate in a buffer containing H_2O_2. Activity of GR (EC 1.6.4.2) was determined following the method of Sgherri et al. (1994) by measuring the decrement of NADPH in reaction system.

2.8 Statistical analysis

The experimental measurements obtained in a randomized order were used to test species and Cu^{2+} treatment effects. Independent data from six replicates were analyzed by one-way ANOVA (Sigma Plot 10.0 and SPSS 13.0) followed by Tukey's multiple comparison test or *t*-test to detect least significant differences at 5% level among treatments.

3 RESULT

3.1 Effects of copper on growth

The effect of copper in deferent concentrations on SGR varied between the two *Gracilaria* species (Fig.1). A big reduction in SGR for 14.54% at 50 μg/L Cu^{2+}concentration from that of the control and even a negative mean SGR (-1.02%/d) at 500 μg/L Cu^{2+} in *G. lemaneiformis* was observed. For *G. lichenoides*, however, no notable decrease in SGR at levels of 50–250 μg/L Cu^{2+} was noticed, only at high level of 500 μg/L Cu^{2+} the mean SGR was significantly reduced by 32.11% compared to those of control (Fig.1).

3.2 Cell wall polysaccharides and accumulation of copper extra- and intra-cellularly and in cell organelles

No distinct variance in soluble or insoluble polysaccharide contents in 0–6 d Cu^{2+} exposure was shown in both *Gracilaria* species (Table 1). In *G. lichenoides*, 57.97%–58.47% of total polysaccharides (27.25%–27.39% of algae in dry weight) was insoluble (157.94–160.15 mg/g DW) and 41.53%–42.03% was soluble. In *G. lemaneiformis*, however, insoluble polysaccharide content (96.54–97.93 mg/g DW) was only 30.86%–31.95% of the total polysaccharide content (30.65%–31.28% of algae in dry weight) and it decreased greatly for 60.28%–62.00% compared with that of *G. lichenoide*, although soluble polysaccharide content was remarkably increased for 182.16%–190.13% compared with that of *G. lichenoide* (Table 1).

The intra-cellular and extra-cellular Cu^{2+} content increased significantly with increasing Cu^{2+} exposure (P<0.001), most of the metal accumulated extracellularly in both species (Fig.2a, b). Conversely, the intracellular Cu^{2+} content of *G. lichenoides* was lower than that of *G. lemaneiformis* while the extracellular Cu^{2+} content was higher than that of *G. lemaneiformis* in all treatments.

Table 1 Soluble and insoluble polysaccharide content in the cell wall of two *Gracilaria* species

Species	Cu^{2+}concentration (μg/L)	Total polysaccharide (mg/g DW)	Soluble polysaccharide (mg/g DW (% of total))	Insoluble polysaccharide (mg/g DW (% of total))
G. lichenoides	0	272.46±12.51[a]	114.52±8.57 (42.03)[b]	157.94±8.76 (57.97)[a]
	500	273.89±13.02[a]	113.74±9.65 (41.53)[b]	160.15±10.71 (58.47)[a]
G. tenuistipitata	0	306.54±15.33[a]	208.61±10.24 (68.05)[a]	97.93±6.53 (31.95)[b]
	500	312.79±16.27[a]	216.25±10.81 (69.14)[a]	96.54±6.36 (30.86)[b]

Values are means±S.D. (n=6). The different letters in the same column indicate significantly differences (P<0.05).

Table 2 Total intracellular and cell organelle copper accumulation in two *Gracilaria* species under 500 μg/L Cu^{2+} after 6 d

Species	Total Cu^{2+}	Extracellular	Intracellular	Chloroplast	Nucleus	Mitochondria	Ribosome
G. lichenoides	349.20±10.64[b]	303.96±8.69[b]	45.24±1.85[a]	16.38±0.57[a]	4.34±0.17[a]	5.14±0.23[a]	12.13±0.51[a]
G. lemaneiformis	303.25±10.16[a]	224.52±8.26[a]	78.73±2.32[b]	21.92±0.93[a]	7.09±0.20[b]	8.16±0.18[b]	16.67±0.43[b]

Units: μg/g DW. Values are means±S.D. (n=6). The different letters in the same column indicate significantly differences (P<0.05)

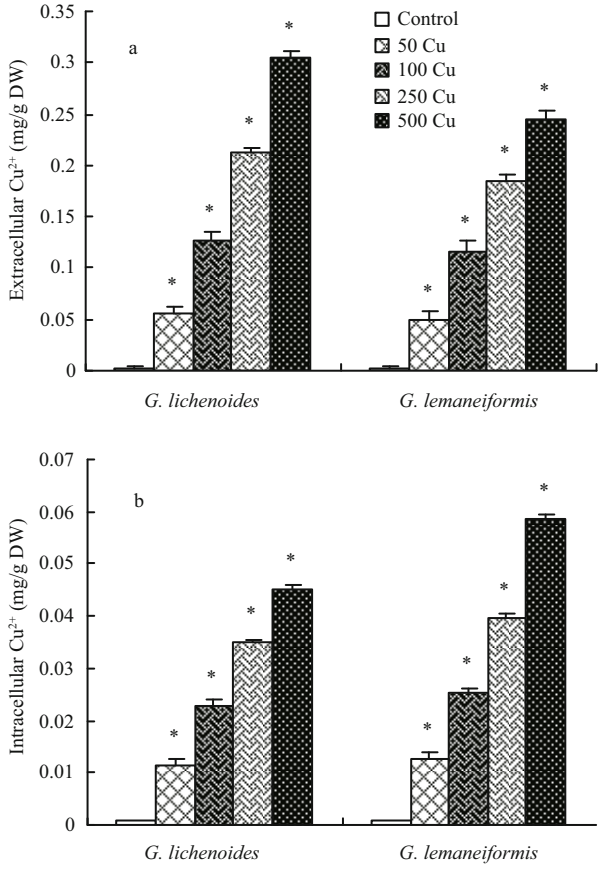

Fig.2 Effect of copper concentration on intra-cellular (a) and extra-cellular Cu^{2+} content (b) of two *Gracilaria* species

Values are means±S.D. (n=6). Asterisk indicates significantly differences from controls (P<0.05).

Under 500-μg/L Cu^{2+} for 6 days, the total and extra-cellular Cu^{2+} content of *G. lichenoides* was 15.15% and 35.38% higher than those of *G. lemaneiformis*, respectively (Table 2). In contrast, under the same treatment, Cu^{2+} accumulation of *G. lichenoides* in intra-cellular, chloroplast, cell nucleus, mitochondria, and ribosome was 42.54%, 25.27%, 38.79%, 37.01%, and 27.23% less than that of *G. lemaneiformis* (P<0.05), respectively (Table 2).

3.3 The effect of copper on chlorophyll fluorescence parameters

Analysis of chlorophyll fluorescence parameters showed that F_0, F_v/F_m, F_m, ETR, qP, and NPQ values decreased more in *G. lemaneiformis* than those of *G. lichenoides* under higher Cu^{2+} concentrations (Fig.3). *G. lichenoides* exhibited a relatively stable F_0, F_v/F_m, ETR, and NPQ up to 250 μg/L Cu^{2+}; however, those values declined significantly at 500 μg/L Cu^{2+} (P<0.05) (Fig.3a, c, d, f). In *G. lemaneiformis*, however, F_0, F_m, F_v/F_m, and NPQ values were significantly lower at 100 μg/L Cu^{2+} (P<0.05) (Fig.3a, b, c, f), with the exception of ETR at 50 μg/L, (Fig.3d) compared to that of control. However, this effect was found significant at 250 μg/L Cu^{2+} in *G. lichenoides* (P<0.05) (Fig.3b). On the contrary, qP values reduced for 22.6% and 27.6% at 250 μg/L and 500 μg/L Cu^{2+}, respectively, in *G. lemaneiformis* (P<0.05), whereas this effect had no significant difference from the control's at any Cu^{2+} concentration in *G. lichenoides* (P>0.05) (Fig.3e).

3.4 Effects of copper on pigment content

A notable decrease was observed for chlorophyll *a*

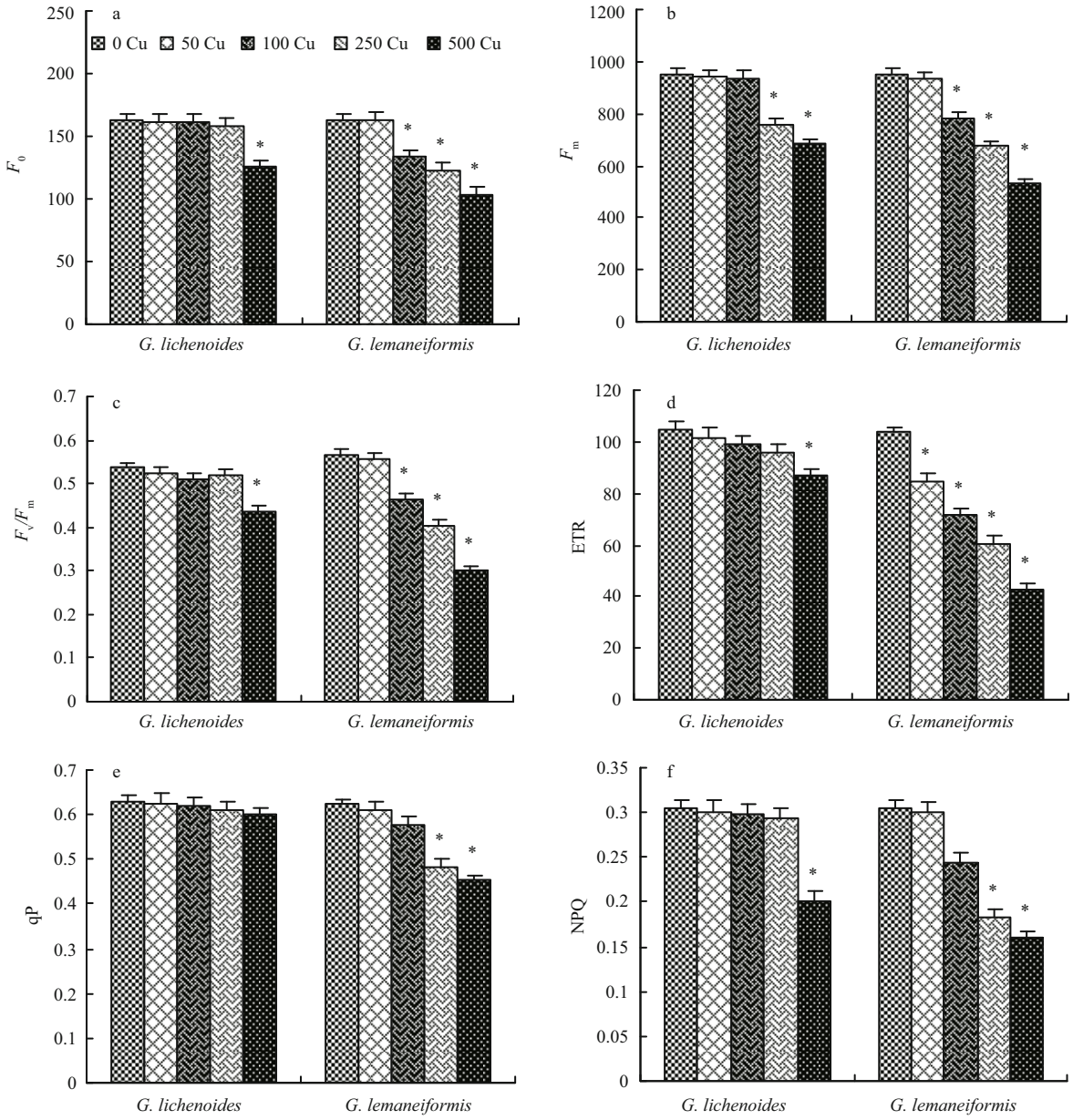

Fig.3 Effects of copper concentration on the chlorophyll fluorescence parameters of both *Gracilaria* species fronds: a. F_0; b. F_m; c. F_v/F_m; d. ETR; e. qP; f. NPQ

Values are means±S.D. (n=6). Asterisk indicates significantly differences from controls (P<0.05).

and β-carotene at 250 μg/L and 500 μg/L Cu^{2+} (P<0.05) in *G. lemaneiformis*, while no significant difference was observed for those under various Cu^{2+} concentrations tested in *G. lichenoides* (P>0.05) (Table 3). However, R-PC, R-PE, and APC were significantly decreased from the control at 500 μg/L Cu^{2+} (P<0.05) for *G. lichenoides*, while those phycobiliproteins were significantly reduced at all but the lowest Cu^{2+} concentration (50 μg/L Cu^{2+}) (P<0.05) in *G. lemaneiformis* (Table 4).

3.5 Effects of copper on ROS, antioxidative enzymes, lipid peroxidation, and electrolyte leakage

The production of ROS in *G. lichenoides* increased by 6.7%, 15.3%, 39.2% and 46.9% at 50, 100, 250, and 500 μg/L Cu^{2+}, respectively, while significant increase in ROS in *G. lemaneiformis* was 27.2%, 50.4%, 64.7% and 87.1% at 50, 100, 250, and 500 μg/L Cu^{2+} (P<0.05), respectively, in comparison against the control (Fig.4a). SOD activity increased

Table 3 Toxic effects of copper on chlorophyll *a*, *b*, and β-carotene content in two *Gracilaria* species

Concentration	*G. lichenoides*			*G. lemaneiformis*		
	Chlorophyll *a* (% of control)	Chlorophyll *b* (% of control)	β-carotene (% of control)	Chlorophyll *a* (% of control)	Chlorophyll *b* (% of control)	β-carotene (% of control)
Control	0.192±0.012 (100)	0.067±0.011 (100)	0.011±0.005 (100)	0.191±0.012 (100)	0.067±0.010 (100)	0.011±0.004 (100)
50	0.207±0.014 (107.5)	0.069±0.010 (103.0)	0.011±0.003 (99.3)	0.199±0.011 (104.0)	0.069±0.011 (103.3)	0.011±0.003 (101.0)
100	0.189±0.011 (98.6)	0.066±0.009 (98.4)	0.012±0.004 (100.4)	0.185±0.012 (96.2)	0.062±0.009 (92.4)	0.011±0.004 (98.9)
250	0.184±0.012 (96.0)	0.064±0.009 (95.1)	0.012±0.003 (102.0)	0.161±0.011* (83.9)	0.057±0.008 (85.2)	0.009±0.003* (84.1)
500	0.176±0.012 (91.6)	0.062±0.010 (92.2)	0.011±0.002 (93.5)	0.118±0.008* (61.4)	0.038±0.008* (57.0)	0.008±0.002* (74.7)

Units: Concentration: μg/L. Chlorophyll *a*, *b*, β-carotene: mg/g FW. Values are means±S.D. (n=6). Asterisk indicates significantly differences from controls (P<0.05)

Table 4 Toxic effects of copper on phycobiliprotein in two *Gracilaria* species

Concentration	*G. lichenoides*			*G. lemaneiformis*		
	R-PC (% of control)	R-PE (% of control)	APC (% of control)	R-PC (% of control)	R-PE (% of control)	APC (% of control)
Control	0.111±0.012 (100)	0.490±0.012 (100)	0.375±0.012 (100)	0.108±0.012 (100)	0.469±0.012 (100)	0.354±0.012 (100)
50	0.107±0.014 (96.3)	0.484±0.014 (98.7)	0.371±0.014 (98.7)	0.101±0.011 (93.2)	0.420±0.014 (89.4)	0.314±0.014 (88.7)
100	0.105±0.011 (94.7)	0.480±0.011 (98.1)	0.355±0.011 (94.5)	0.087±0.012* (80.7)	0.355±0.011* (75.7)	0.254±0.011* (71.6)
250	0.098±0.010 (88.2)	0.425±0.010 (86.8)	0.348±0.010 (92.7)	0.076±0.011* (70.3)	0.293±0.010* (62.4)	0.221±0.010* (62.3)
500	0.087±0.012* (78.0)	0.318±0.012* (64.8)	0.261±0.012* (69.5)	0.068±0.008* (62.7)	0.210±0.012* (44.8)	0.170±0.012* (48.0)

Units: Concentration: μg/L. R-PC, R-PE, APC: mg/g FW. Values are means±S.D. (n=6). Asterisk indicates significantly differences from controls (P<0.05).

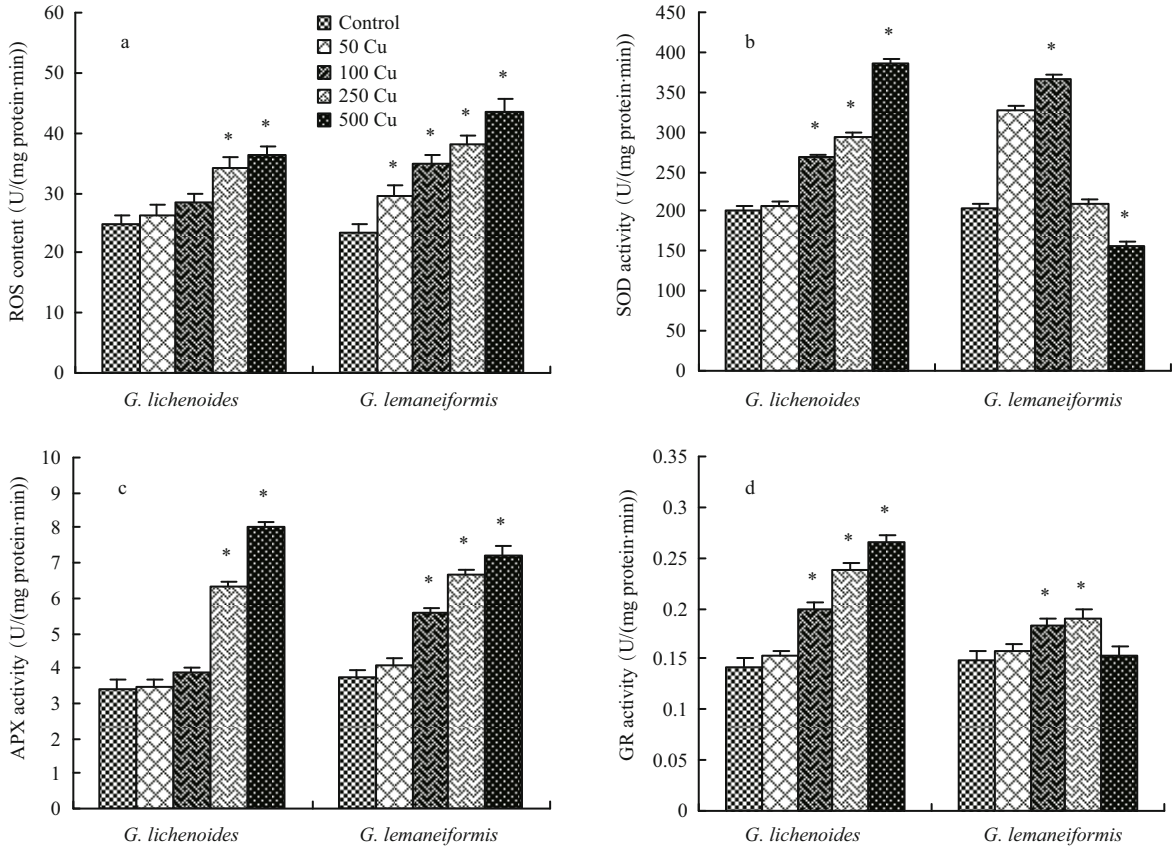

Fig.4 Effects of copper on ROS contents (a) and antioxidative enzyme activity of both *Gracilaria* species fronds (b. SOD activity; c. APX activity; d. GR activity)

Values are means±S.D. (n=6). Asterisk indicates significantly differences from controls (P<0.05).

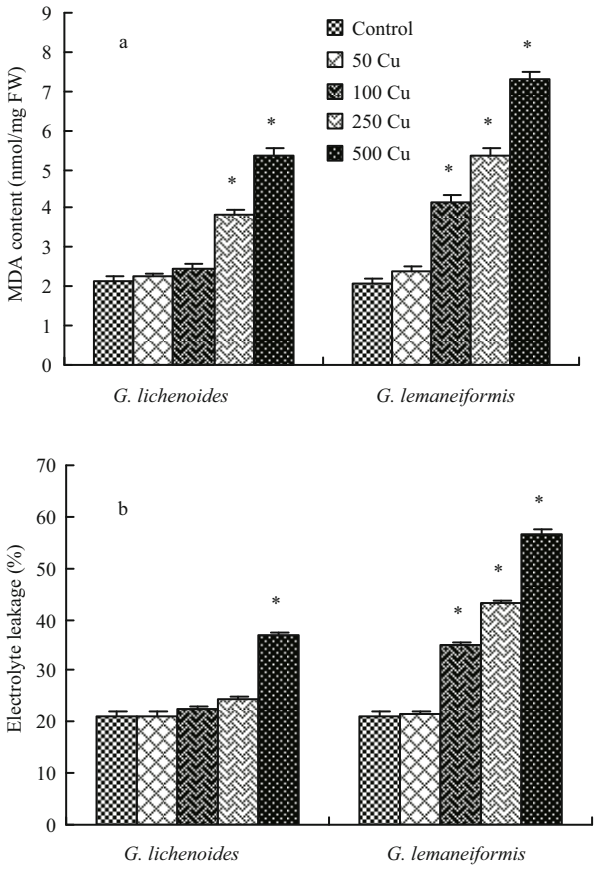

Fig.5 Effects of copper on MDA (a) and electrolyte leakage (b) of both *Gracilaria* species fronds

Values are means±S.D. (n=6). Asterisk indicates significantly differences from controls (P<0.05).

by 3.1%, 32.9%, 45.9%, and 92.0% in *G. lichenoides* at 50, 100, 250, and 500 μg/L Cu^{2+}, respectively, from that of the control (Fig.4b). However in *G. lemaneiformis*, SOD activity increased significantly by 59.4% and 79.1% at 50 and 100 μg/L Cu^{2+} (P<0.05) whereas at 250 μg/L Cu^{2+} the enzyme activity decreased down to the control level and even decreased significantly by 23.7% at 500 μg/L Cu^{2+} (P<0.05). APX activity increased significantly by 84.8% and 134.4% in *G. lichenoides* (P<0.05) and 77.8% and 93.0% in *G. lemaneiformis* (P<0.05) in comparison to the control at 250 and 500 μg/L Cu^{2+}, respectively (Fig.4c). An increase in GR activity was notable by 40.1%, 67.6%, and 87.3% at 100, 250, and 500 μg/L Cu^{2+}, respectively, for *G. lichenoides* (P<0.05), but 24.7% and 29.4% only at 100 and 250 μg/L Cu^{2+} (P<0.05), 4.3% at 500 μg/L Cu^{2+} (P>0.05), for *G. lemaneiformis*, respectively, in comparison to the control (Fig.4d).

The MDA content increased respectively by 8.9%, 78.5%, and 150.0% in *G. lichenoides*, but increased significantly by 99.0%, 154.3%, and 246.2% in *G. lemaneiformis* (P<0.05), at 100, 250 and 500 μg/L Cu^{2+}, for comparison to the control (Fig.5a). The electrolyte leakage increased by 5.6%, 14.6%, and 73.1% in *G. lichenoides*, but significantly by 64.6%, 103.8%, and 166.0% in *G. lemaneiformis*, at 100, 250, and 500 μg/L Cu^{2+}, respectively, in comparison to the control (P<0.05) (Fig.5b).

4 DISCUSSION

4.1 Effects of copper on growth

In this study, difference in SGR response of the two species to Cu^{2+} exposure was identified, the threshold concentration that caused a notable inhibition in SGR was 50 μg/L Cu^{2+} for *G. lemaneiformis* and 500 μg/L for *G. lichenoides*. Compared with other red seaweed species, the reduction in relative growth rate (RGR) for *Gracilariopsis longissima* was observed at 12.5 μg/L Cu^{2+}, with zero growth above about 300 μg/L (Brown and Newman, 2003). Collén et al. (2003) reported that the effects of short term (4 days) 60% growth inhibition was observed at 200 μg/L Cu^{2+} but no effect on was observed at 100 μg/L Cu^{2+} for *Gracilaria tenuistipitata*. Our result indicate that EC_{50} after 6 days is 218 μg/L Cu^{2+} for *G. lemaneiformis* and >500 μg/L Cu^{2+} for *G. lichenoides*. The differences in the above-mentioned findings might suggest differential tolerance response to Cu^{2+} stress between and within species. In practice, Cu^{2+} additions have been used to prevent the growth of the ship-fouling algae *Enteromorpha* spp. (Evans, 1981), epiphytic in fish farming (Lewis and Metaxas, 1991), and brown alga *Ectocarpus siliculosus* in cultivations of *Gracilaria gracilis* (van-Heerden et al., 1997). Therefore, *G. lichenoides* might be more adaptable for use in cleaning epiphytes or other macroalga by the addition of Cu^{2+} in outdoor pond cultivations than *G. lemaneiformis*.

4.2 Cell wall polysaccharides and accumulation of copper extra- and intra-cellularly and in cell organelles

In higher plants and algae, a cell wall acts as the first barrier against metal ion uptake (Macfie and Welbourn, 2000, Martins et al., 2011). The main mechanisms of binding include ionic interactions and complex formations between metal cations and ligands on cell wall surface-active groups of seaweeds (Yun et al., 2001; Ata et al., 2012). Brown algae (*Padina gymnospora*) could overproduce cell wall

polysaccharides to prevent metals adsorbed by the cytoplasm against the heavy metal toxicity (Andrade et al., 2010) and alginic acid in the cell wall of brown algae was more efficient to bind Cd^{2+} than polysaccharides found in red or green macroalgae (Hashim and Chu, 2004). However, red algae polysaccharides show a larger potential in binding and accumulating zinc ions comparing with green (Chlorophyta) algae (Stengel et al., 2004). It was also reported that *Chondrophycus poiteaui* accumulated insoluble polysaccharides intra-cellularly of nearly all Cd^{2+}, while *Gracilaria cornea* did it extra-cellularly (Virginia et al., 2007). In this study, *G. lichenoides* that contained higher content of insoluble polysaccharides in cell wall might have promoted the extra-cellular Cu^{2+} binding and formed an efficient barrier to avert metal toxicity, resulting in lower intracellular Cu^{2+} accumulation in cell organelles (including chloroplast, cell nucleus, mitochondria, and ribosome) than that of *G. lemaneiformis*. However, more studies are necessary to understand the role of insoluble polysaccharides in cell wall of red algae in Cu^{2+} accumulation.

4.3 Effect of Cu^{2+} on photochemical activity

In plants and algae, chlorophyll fluorescence analysis is a significant approach that brings useful parameters of physiological condition (Maxwell and Johnson, 2000). The results demonstrate that a notable drawdown of the end-points of F_0 was the same as phycobiliproteins but lower than chlorophylls for both species (Fig.3a, Tables 3 and 4). Cu^{2+} has a greater binding affinity to phycocyanin and phycoerythrin, thus exerting greater damage to these pigments (Han et al., 2008). F_0 is thought to relate to the amount of fluorescence emitted by the PSII reaction centers (Bolhar-Nordenkampf et al., 1989). Therefore, decrease in F_0 may be due to losses of phycobiliproteins of contributing fluorescence (Brown and Newman, 2003). F_v/F_m ratio is a measure of the functional efficiency of PSII (Maxwell and Johnson, 2000). The tendency observed in the F_v/F_m ratio of *G. lichenoides* was probably related to more slowly-degraded functional efficiency of PSII compared with *G. lemaneiformis*. In *G. lichenoides*, changes in ETR paralleled those of F_v/F_m whereas in *G. lemaneiformis*, ETR showed a significant decrease at lower Cu^{2+} concentrations than did F_v/F_m. The inequality between the responses of F_v/F_m and ETR reflects inhibition downstream of PSII for *G. lemaneiformis* exposed to Cu^{2+} (Yu et al., 2002). In this trial, the decreases in F_v/F_m, ETR, qP, and NPQ indicate that photochemical activity was more hindered in *G. lemaneiformis* than in *G. lichenoides* at high Cu^{2+} concentrations (Fig.3c, d, and e), and the mechanism needs further study.

4.4 Effect of copper on pigment content

Effect of Cu^{2+} treatments on photosynthetic pigments in two species of *Gracilaria* was somehow different: *G. lemaneiformis* was more sensitive, in which Cu^{2+} stress was more effective to induce the reduction of photosynthetic pigments than in *G. lichenoides* (Tables 3 and 4). Degradation in photosynthetic pigment content could be a result of peroxidation of pigment membranes (Table 3; Fig.5a, b) or replacing magnesium in chlorophyll molecules with Cu^{2+} (Mal et al., 2002). Our results show that the drop in rate of chlorophyll *b* was slower than that of chlorophyll *a*, β-carotenoid, and phycobiliproteins at stress of a high Cu^{2+} concentration in *G. lemaneiformis* (Tables 3 and 4). It has been suggested that this slower degradation of chlorophyll *b* involves a photooxidative mechanism in copper-induced disruption (Prasad et al., 2001). Chlorophyll *a* is an important pigment in photosynthesis; decrease of chlorophyll *a* could inhibit photosynthesis greatly (Fig.3). In this study, β-carotenoid contents were positively correlated with chlorophyll but negatively with MDA and electrolyte leakage in both *Gracilaria* species.

4.5 Effects of copper on ROS, antioxidative enzymes, lipid peroxidation, and electrolyte leakage

Cu^{2+} can catalyze harmful free radicals such as ROS via Haber-Weiss or Fenton reaction at supra-optimal concentrations in plant (Deckert, 2005), although it is a redox active metal and an essential micronutrient. Accordingly, the activity of one or more antioxidant enzymes generally increases stress tolerance in plants. SOD catalyzes superoxide anions to O_2 and H_2O_2 (Sudhakar et al., 2001). APX and GR are two important ingredients of ascorbate-glutathione cycle response to eliminate H_2O_2 in different cellular intervals (Jimenez et al., 1997). However, Cu^{2+} may inhibit GR activity by interacting with -SH binding sites and interrupting enzyme active sites (Stauber and Florence, 1987). Our study showed that at higher Cu^{2+} concentrations for *G. lemaneiformis*, SOD activity decreased remarkably but no significant change in GR activity (Fig.4b, d), whereas for *G. lichenoides*, activities of SOD, APX, and GR rose significantly (Fig.4b–d), when compared to the

control. Throughout the trial, changes in ROS, SOD, APX, and GR were synchronous to some degrees for *G. lichenoides* (Fig.4), which imply that SOD, APX, and GR play an important role in scavenging ROS in *G. lichenoides*. Meanwhile, the contents of MDA and electrolyte leakage, as represented degree of membrane disruption, which are positively correlated to ROS content (Figs.4a, 5), are lower in *G. lichenoides* than those in *G. lemaneiformis* at high Cu^{2+} concentrations. Therefore, *G. lichenoides* can readily found a balance between ROS content and antioxidative system and prevent oxidative damage, which resulted in greater tolerance to Cu^{2+}. However, *G. lemaneiformis* is more difficult to adapt to changes in Cu^{2+} concentrations of the environment, because its higher Cu^{2+} accumulation in intra-cellular and in cell organelles, moreover its lower detoxification capacity of the tissues.

The loss in membrane integrity coincided with reductions in phycobiliprotein concentrations (Table 4; Fig.5b), but did not correspond with reductions in chlorophyll *a* or *b* concentrations (Table 3; Fig.5b) were observed in our results, implying that Cu^{2+} has a greater binding affinity to phycocyanin, phycoerythrin, and allophycocyanin, thus, exerting a greater damage on those pigments than chlorophyll *a* and *b* for both *Gracilaria* species (Tables 3 and 4). Similarly, phycobiliproteins disappearance at a faster rate than chlorophyll was observed in red alga *Antithamnion plumula* (J. Ellis) Thuret (Küpper et al., 2002) and *Gracilariopsis longissima* (S. G. Gmelin) Steentoft (Brown and Newman, 2003) treated with Cu^{2+}. Our results also show that higher intra-cellular Cu^{2+} accumulation was correlated with elevated generation of ROS, MDA, and electrolyte leakage, and with decreases in SGR, pigment content, and photochemical activity in *G. lemaneiformis* than *G. lichenoides* (Figs.2 and 5), indicating that *G. lemaneiformis* is more sensitive to Cu^{2+} exposure than *G. lichenoides*.

5 CONCLUSION

The results of this study demonstrate that there are significant differences in physiology between the two species of *Gracilaria* in terms of Cu^{2+} accumulation, responses of antioxidant enzymes, and photochemical activities when exposed to Cu^{2+}. Under Cu^{2+} stress, compared to *G. lemaneiformis*, *G. lichenoides* accumulated more Cu^{2+} extra-cellularly and less intra-cellularly and in the cell organelles (especially in the chloroplast, cell nucleus, mitochondria, and ribosome), which is coincided with higher insoluble polysaccharide content in the cell wall due possibly to the establishment of an efficient barrier against intra-cellular Cu^{2+} accumulation. On the other hand, because of higher Cu^{2+} accumulation and lower SOD, APX, and GR activities, expressions of toxic Cu^{2+}effects, for example oxidative stress, in terms of ROS and MDA accumulations and electrolyte leakage, were higher in *G. lemaneiformis* than in *G. lichenoides*. In *G. lemaneiformis*, decrease in growth, pigment (chlorophyll *a*, chlorophyll *b*, phycobiliprotein, β-carotene) content and photosynthetic activity was more prominent, making it a suitable candidate of potential bioindicator to Cu^{2+} pollution.

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