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PHYSIOLOGICAL RESPONSES OF *Porphyra haitanesis* TO DIFFERENT COPPER AND ZINC CONCENTRATIONS

Ying Xia Li[1], Suo Zhou[1], Feng Juan Zhao[2], Yan Liu[3], Pan Pan Fan[1], Guang Ce Wang[4]*

[1]School of Life Science and Technology, Nanyang Normal University (Nanyang, China, 473061)

[2]Department of Biological Sciences, BingZhou University (Bingzhou, China, 256600)

[3]School of Life science, Wuhan University of Science and Technology Zhongnan Brach (Wuhan, China, 430223)

[4]Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences (Qingdao, China, 266071)

*Correspoding author: liyx108@163.com

ABSTRACT

In the present study, several physiological responses of the red marine alga *Porphyra haitanesis* to elevated concentrations of copper (up to 50 μM) and zinc (up to 100 μM) were investigated. Our results showed that the effects of Cu^{2+} and Zn^{2+} on growth, photosynthetic pigments (chlorophylls and carotenoids), phycobiliprotein and metabolism (the fluorescence emission spectra and the activities of photosystem Ⅱ) did not follow the same pattern. The relative growth rate was inhibited by different concentrations of Cu^{2+}, and was slightly increased at lower concentrations (up to 10 μM) and inhibited at higher Zn^{2+}concentrations. On the other hand, the phycoerythrin contents were slightly increased at relatively low concentrations (up to 1 μM Cu^{2+} or 20 μM Zn^{2+}) and inhibited by high Cu^{2+} and Zn^{2+} concentrations. Moreover, photosynthesis and respiration showed an increase in the amount of oxygen exchange in response to relatively low Cu^{2+} (up to 1 μM) and Zn^{2+} concentrations (up to 10 μM), and a reduction to relatively high Cu^{2+} and Zn^{2+} concentrations. Oxygen evolution was more sensitive than oxygen uptake to Cu^{2+} and Zn^{2+}. In addition, the photoreductive activities and fluorescence emission of photosystem II (PS II) were enhanced by lower concentrations of Cu^{2+} (up to 0.1 μM) and Zn^{2+} (up to 10 μM) and inhibited by higher concentrations. Furthermore, the intensity of chlorophyll a fluorescence and the active PSII reaction centers followed a similar pattern in response to elevated concentrations of Cu^{2+} and Zn^{2+}. These results suggest that lower concentrations of Cu^{2+} and Zn^{2+} affected the metabolism of *P. haitanesis*, which was inhibited by higher concentrations of these metals.

RESUMO

No presente estudo foram investigadas as respostas fisiológicas da alga vermelha *Porphyra haitanesis* às elevadas concentrações de cobre (acima de 50 μM) e de zinco (acima de 100 μM). Os resultados mostram que os efeitos de Cu^{2+} e Zn^{2+} sobre o crescimento, pigmentos fotossintéticos (clorofilas e carotenóides), ficobiliproteína e metabolismo (o espectro de emissão de fluorescência e as atividades do fotossistema) não seguem o mesmo padrão. A taxa de crescimento relativo foi inibida por diferentes concentrações de Cu^{2+} e, em presença de Zn^{2+}, aumentou ligeiramente em baixas concentrações (abaixo de 10 μM) e foi inibida em altas concentrações. Por outro lado, os teores de ficoeritrina apresentaram leve aumento em concentrações relativamente baixas de Cu^{2+} e Zn^{2+} (até 1 μM Cu^{2+} e até 20 μM Zn^{2+}, respectivamente) e foram inibidas por altas concentrações. Além disso, tanto a fotossíntese quanto a respiração mostraram aumento nas trocas de oxigênio em resposta às concentrações relativamente baixas de Cu^{2+} (até 1 μM) e de Zn^{2+} (até 10 μM), além da redução em concentrações relativamente altas desses metais. Adicionalmente, as atividades fotoredutoras e as emissões de fluorescência do fotossistema II (PSII) foram incrementadas em baixas concentrações de Cu^{2+} (até 0,1 μM) e de Zn^{2+} (até 10 μM) e inibidas por altas concentrações. Desta forma, a intensidade da fluorescência da clorofila-a e dos centros de reação ativa PSII seguiram um padrão semelhante em resposta às elevadas concentrações de Cu^{2+} e Zn^{2+}. Esses resultados sugerem que baixas concentrações de Cu^{2+} e Zn^{2+} afetam o metabolismo de *P. haitanesis*, que se torna inibido por altas concentrações desses metais.

Descriptors**:** *Porphyra haitanesis*, Copper, Zinc, Photosystem II.
Descritores: *Porphyra haitanesis*, Cobre, Zinco, Fotossistema II.

INTRODUCTION

Among the modern pollutants interfering with photosynthetic organism metabolism, heavy metals are one of the most common nonbiodegradable pollutants reported at elevated concentration in many parts of the world (MALLICK; RAI, 2001). Mining of metals, geo- chemical structure, industrial effluents and wastes, create a potential source of heavy metal pollution in the aquatic environment (GUMGUM et al., 1994). The toxic metals can be divided into two groups: essential and non-essential (REDDY; PRASAD, 1990). The first group includes Pb, Hg, Ur, Ag and Be, all of them are highly poisonous without any nutritional value (INTHORN, 2001). The second group consists of metals such as that are essential as nutritional requirements at trace amount for many organisms but are toxic at high level. This group consists of Fe, Mn, Cu, Mo, Zn and Co (SOLISIO et al., 2008).

Copper is the most commonly used toxic heavy metal for industrial purposes and its presence in aquatic system sarises from both naturally occurring and man-made origin (PERALES-VELA et al., 2007). Copper is ubiquitous in the environment. Various sources of copper (Cu), including industrial and domestic wastes, agricultural practices, copper marine drainage, copper-based pesticides, and antifouling paints, have leaded to a clear increase in Cu concentrations in aquatic environments (CALLOW; CALLOW, 2002). Cu is essential for macroalgae, which participates in important biological reactions as an enzymatic cofactor and electron carrier in the photosynthetic and respiratory processes at low concentrations (ANDRADE et al., 2004). It can interfere with numerous physiological processes and is considered to be potentially cytotoxic when applied in amounts higher than its particularly level, and its sensitivity varies among different macroalgae (FERNANDES; HENRIQUES, 1991; CHANG; SIBLEY, 1993). The toxicity of copper is mainly related to free ions and is a potent inhibitor of photosynthesis in macroalgae (KÜPPER et al., 2002).

Zinc (Zn) is a well- known essential micronutrient for normal growth of algae, which is widely required in many biological processes and is present in nearly 300 enzymes that perform many different metabolic functions (VALLEE; AULD, 1990). It has the adverse effects of this non- redox active metal as oxidative stress factor when in excess (CHAOUI et al., 1997).

Porphyra is one of the most important marine macroalgae with respect to its global distribution and economical importance, which is also important for aquatic ecosystems and as a food, biochemicals, and pharmaceuticals. *Porphyra haitanensis* Chang et Zheng, an intertidal red alga with high economic value, only habits and widely cultured in south of China (GAO et al., 2004a). Many studies have been devoted to the interference of copper and zinc with a number of physiological processes, while there is a general lack of information to follow and correlate both these metal induced responses in macroalgae. The aim of the present study was to investigate the effects of copper and zinc on growth, photosynthesis, pigments, proteins, fluorescence intensities and PSII activities of *P. haitanesis* in response to elevated concentrations of copper and zinc.

MATERIAL AND METHOD

Alga Harvest

The gametophytic blade of *Porphyra haitanesis* Chang et Zheng was collected from the seashore of Xiamen, China. Discs of approximately 1.2 cm in diameter were cut from the gametophytic blade of *P. haitanesis* and incubated in nutritional seawater in which 0.1 M $NaNO_3$ and 0.1 M $NH_4H_2PO_4$ was added. Plants were grown at 18ºC in 16: 8 light and dark cycles with 50 µmol photons m^{-2} s^{-1} provided by cool- white fluorescent bulbs. Experiments were conducted in 500 ml flasks that had been autoclaved at 121^{o} C for 20 min. The copper and zinc stock solutions were prepared from their analytical grade metallic salts (i.e. $CuSO_4.5H_2O$ and $ZnSO_4$. $5H_2O$, respectively) dissolved in deionized water. Cu^{2+} and Zn^{2+} solutions in the range 0-50 µM and 0- 100 µM, respectively were prepared by the dilution of a concentrated stock solution. Algal samples were taken after seven days of incubation.

Growth Rate

The relative growth rate (R), expressed as % day^{-1}, was computed from the following expression (KAIN, 1987):

$$R = (\text{In } a_t - \text{In} a_o) / t$$

where a_t is the area measured at time (t) in days and a_o is the area at the initial time. Three replicates were taken for each treatment. Disc area was determined using an image analysis software.

Oxygen Exchange

The oxygen exchange was measured with a commercial Clark-type oxygen electrode (Hansatech Instruments Ltd., England), at 18℃. *P. haitanesis* fronds were placed in an electrode chamber containing bicarbonate buffer, pH 7.6, to provide constant CO_2 concentration in the medium. The changes in oxygen concentration in the darkness and in the light (50 µmol $m^{-2}s^{-1}$ illumination) were recorded under constant stirring of the sample.

Photosynthetic Pigments

Two discs of approximately 10- 20 mg fresh weight (FW) per sample were extracted in 80% acetone at 4ºC in darkness. The resulting suspension was centrifuged at 10,000 g for 5 min. The content of Chl *a* and carotenoids were determined as described by Kursar and Alberte (1983).

Phycobiliprotein Content

One tenth of *P. haitanesis* gametophytic blade was extracted in 2 ml of 0.1 M Na-phosphate buffer (pH 7.0) at 4℃ in darkness.. The resulting suspension was centrifuged at 10,000 g for 5 min. The supernatant was collected for in vivo absorption spectra measurement at room temperature. The contents of phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC) in the cell extracts of *P. haitanesis* were made using the extinction coefficients, as described by Kursar et al. (1983).

Isolation of Photosystem Ⅱ

The photosystem Ⅱ (PSⅡ) was isolated according to the method of Gao et al. (2004b). The fragmented alga was centrifuged at 5, 000 g for 5 min to remove large debris. The supernatant was collected and centrifuged at 140,000 g (Beckman L8-80, Ti- 45 rotor) for 1h at 4℃. The resulting pellet was suspended and centrifuged at 140,000 g on the sucrose density gradient consisting of 60%, 50%, 40%, 30% and 20% (w/v) sucrose in proportions of 1:1:1:1:1 (Beckman L8-80, Sw-40 rotor) for 3.5 h at 4℃. The thylakoid membrane was isolated in 50-60% sucrose layer, and treated with SDS, then loaded onto the sucrose density gradient consisting of 60%, 50%, 40%, 30%, 20%, 15%, 10% (w/v) sucrose in proportions of 1:1:1:1:1:1:1 containing 0.2 % SDS, and ultracentrifuged at 140,000 g for 15 h at 4℃. The band in 40% sucrose layer was PSII.

The Activities of Photosystem Ⅱ

The DCIP (2, 6- dichloroindophenol) photoreduction rates of potosystemⅡ (PSⅡ) obtained from the sucrose density gradient ultracentrifugation, either with or without added artificial electron donor DPC (1,5-diphenylcarbazide), were measured spectrophotometrically at 580 nm (12.9 $mM^{-1}. cm^{-1}$), in a medium containing 40 μM DCIP and 30 mM MES-NaOH (pH 6.8). The concentration of samples was equivalent to 10 μg Chl *a*. mL^{-1}.

The fluorescence emission spectra of PSII were recorded at room temperature by a Hitachi 850 fluorescence spectrophotometer. The concentrations of samples were equivalent to 10 μg Chl *a*. ml^{-1}.

Statistical Analysis

All data were presented as the mean ± SD (n= 3). The statistical analyses were performed using SAS software. The data were analyzed using Duncan's multiple range test at the 5% level.

RESULT

Effect of Cu^{2+} and Zn^{2+} on Growth

The relative growth rates of *P. haitanesis* decreased as Cu^{2+} concentration increased in the culture medium. Inhibition of relative growth rates was not significant at 0.1 μM Cu^{2+}, whereas at 1 μM, a reduction in relative growth was apparent. At the end of the experiment, the relative growth rate in the control was 1.2% day^{-1} and was 0.05% day^{-1} at 50 μM Cu^{2+} (Fig. 1a). On the other hand, lower concentrations (0.1 and 1 μM) of Zn^{2+} led to an increase in the relative growth of *P. haitanesis*. Thus, a growth stimulation of 7.7% and 1.7% was observed in the cultures treated with 0.1 and 1 μM Zn^{2+}, respectively. Higher concentrations (20, 50 and 100 μM) exerted a progressive inhibitory effect on algal growth (Fig. 1b).

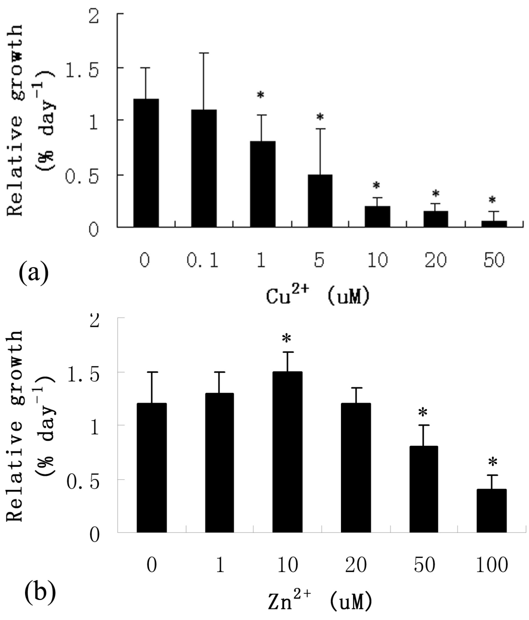

Fig. 1. Effect of Cu^{2+} (a) and Zn^{2+} (b) on relative growth rates (% day^{-1}) in *Porphyra haitanesis* following metal exposure for 168 h. Significant levels between control and treatments are indicated by asterisks (P < 0.05).

Effect of Cu^{2+} and Zn^{2+} on Photosynthesis and Respiration

Metabolic rates were stimulated at lower Cu^{2+} and Zn^{2+} concentrations and inhibited at higher Cu^{2+} and Zn^{2+} concentrations (Fig. 2). Photosynthetic oxygen evolution reached a maximum at 1 µM Cu^{2+} and was 68.5% higher than the control. At 5 µM Cu^{2+}, the photosynthetic rates decreased greatly and at 50µM, which was the highest concentration tested, the photosynthetic process was inhibited by 67.6% compared to the control. Respiratory rates increased to a maximum at 5 µM Cu^{2+} and were 108% higher than that of the control, then decreased with increasing Cu^{2+} concentration (a). On the other hand, lower concentrations of Zn^{2+} (1 and 10 µM) gradually increased oxygen evolution and oxygen uptake (b). A considerable decrease in oxygen evolution was observed at higher concentrations. Oxygen evolution was reduced by 27.8, 50 and 76.9% compared to the control when treated with 20, 50 and 100 µM Zn^{2+}, respectively. At the same time, oxygen uptake was reduced by 21.2, 34.6 and 19.2% compared with the control when treated with 20, 50 and 100 µM Zn^{2+}, respectively.

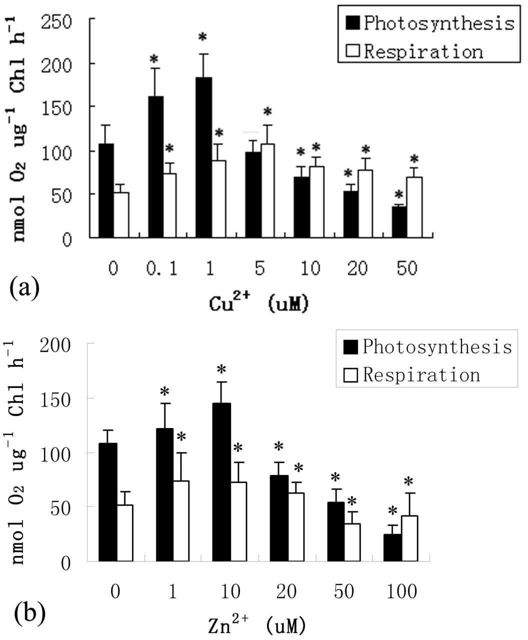

Fig. 2. Effect of Cu^{2+} (a) and Zn^{2+} (b) on the O_2 evolution (photosynthesis) and O_2 consumption (respiration) in *Porphyra haitanesis* after 168 h of metal treatment. Significant levels between control and treatments are indicated by asterisks ($P < 0.05$).

Effect of Cu^{2+} and Zn^{2+} on Photosynthetic Pigment

As shown in Figure 3a, the application of 0.1 and 1 µM Cu^{2+} increased chlorophyll a (Chl *a*) content by 7.3 and 39.1% above the control level, respectively. However, Chl *a* content decreased significantly with increased Cu^{2+} concentrations. Thus, 5, 10, 20, 50 µM Cu^{2+} led to a reduction of 7.3, 21.8, 36.4 and 58.2% compared with the control level, respectively. Lower Cu^{2+} concentrations (0.1 and 1 µM) stimulated the biosynthesis of carotenoids. Whereas, higher Cu^{2+} concentrations resulted in lower reductions in carotenoids when compared with Chl *a*. The magnitude of this reduction was 56.5% for cultures treated with 50 µM Cu^{2+} (Fig. 3a). On the other hand, application of 1, 10 and 20 µM Zn^{2+} stimulated an increase in Chl *a* content, and a pronounced increase in carotenoids was only achieved in 10 µM Zn^{2+}. The application of 50 and 100 µM Zn^{2+} resulted in an apparent decrease in Chl *a* and carotenoids (Fig. 3b).

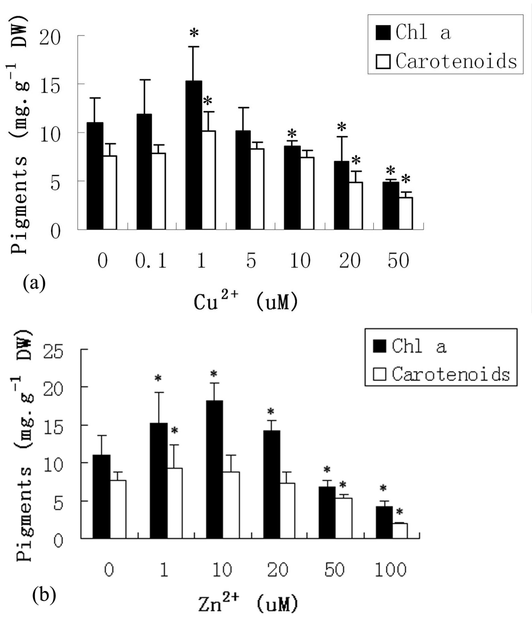

Fig. 3. Effect of Cu^{2+} (a) and Zn^{2+} (b) on the content of Chl *a* and carotenoids in *Porphyra haitanesis* after 168 h of metal treatment. Significant levels between control and treatments are indicated by asterisks ($P < 0.05$).

Effect of Cu^{2+} and Zn^{2+} on Phycobiliprotein

As shown in Figure 4**,** lower concentrations of Cu^{2+} and Zn^{2+} stimulated the biosynthesis of PE, PC and APC, and higher concentration of Cu^{2+} and Zn^{2+} inhibited the biosynthesis of PE, PC and APC. The contents of PE and APC were maximal at 1 µM Cu^{2+}, and PC was maximal at 0.1 µM Cu^{2+}. Maximum reductions in PE (54.4%), PC (41.6%) and APC (44.8%) were recorded at 50 µM Cu^{2+} (a). Increases in PE, PC and APC were 34.2, 38.2 and 8.6 % at 20 µM Zn^{2+}, respectively. Maximum reductions in PE (46.7%), PC (43.8%) and APC (39.7%) were recorded at 100 µM Zn^{2+} (b).

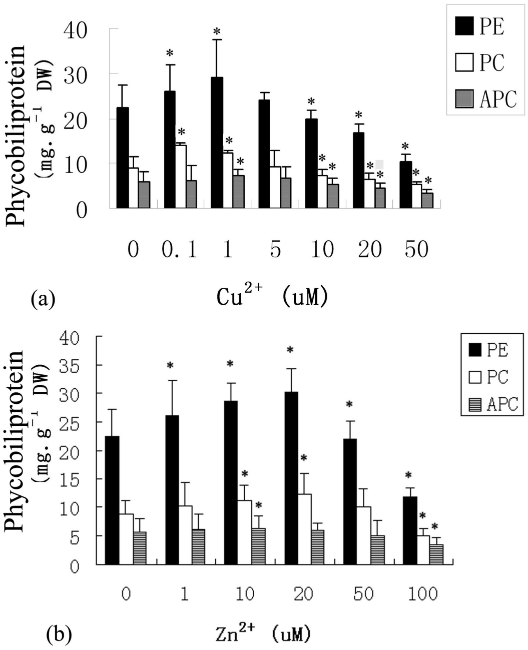

Fig. 4. Effect of Cu^{2+} (a) and Zn^{2+} (b) on phycobiliprotein in *Porphyra haitanesis* after 168 h of metal treatment. Significant levels between control and treatments are indicated by asterisks ($P < 0.05$).

Effect of Cu $^{2+}$ and Zn^{2+} on PSII activities

Figure 5 shows that the application of 0.1 and 1 µM Cu^{2+} increased the photoreduction activities of PSII, with values of 38.5 and 21.8% above the control level, respectively. Higher concentrations of Cu^{2+} resulted in a pronounced reduction in the photoreduction activities of PSII. Maximum reduction was recorded in the culture treated with 50 µM Cu^{2+}, with a value of 87.1% below the control level (a). On the other hand, the application of 1, 10 and 20 µM Zn^{2+} led to a 45.4, 84.6 and 50.8% increase above the control value, and the application of 50 and 100 µM Zn^{2+} resulted in a pronounced reduction in the photoreduction activities of PSII(b).

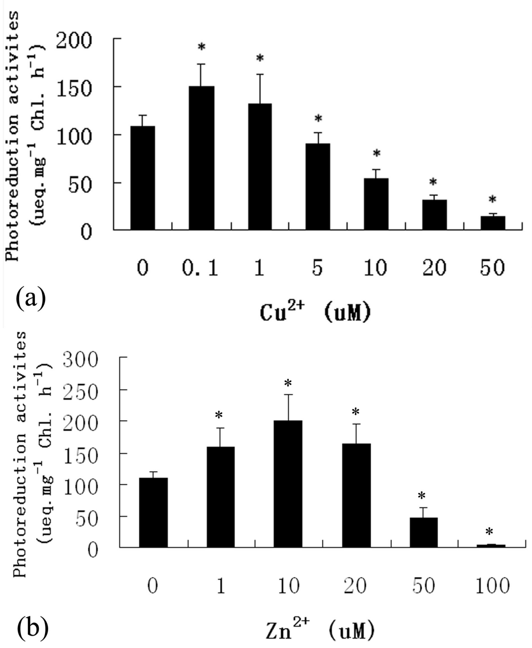

Fig. 5. Effect of Cu^{2+} (a) and Zn^{2+} (b) on the PSII activities in *Porphyra haitanesis* after 168 h of metal treatment. Significant levels between control and treatments are indicated by asterisks ($P < 0.05$).

As shown in Figure 6, lower concentrations of Cu^{2+} (0.1 µM) and Zn^{2+} (1 and 10 µM) enhanced the fluorescence emission of PSII, and higher concentrations of Cu^{2+} (1, 5, 10, 20 and 50 µM) and Zn^{2+} (20, 50 and 100 µM) inhibited the fluorescence emission of PSII.

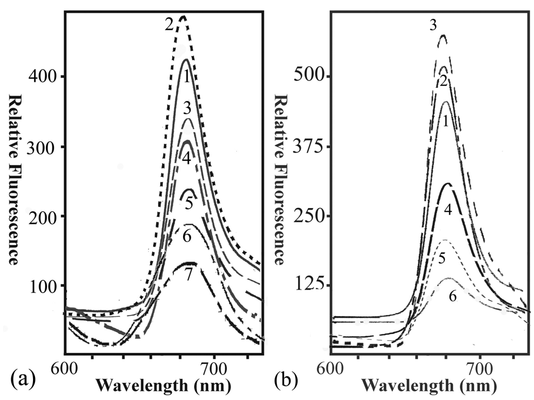

Fig. 6. Effect of Cu^{2+} (a) and Zn^{2+} (b) on the fluorescence emission spectra of Chl *a* in *Porphyra haitanesis* after 168 h of metal treatment. a: (1) control, (2) 0.1 µM Cu^{2+}, (2) 1 µM Cu^{2+}, (2) 5 µM Cu^{2+}, (2) 10 µM Cu^{2+}, (2) 20 µM Cu^{2+}, (2) 50 µM Cu^{2+}; b: (1) control, (2) 1 µM Zn^{2+}, (3) 10 µM Zn^{2+}, (4) 20 µM Zn^{2+}, (5) 50 µM Zn^{2+}, (6) 100 µM Zn^{2+}.

DISCUSSION

All the studied parameters with the exception of relative growth rate, namely, pigment content, oxygen evolution, PSⅡ activities and fluorescence intensities of *P. haitanesis*, were promoted in lower concentrations (up to 0.1 μM Cu^{2+} or 10 μM Zn^{2+}) and inhibited in higher concentrations of Cu^{2+} (greater than 5 μM) and Zn^{2+} (greater than 50 μM), indicating that Cu^{2+} and Zn^{2+} are essential nutritional requirements, while excess copper and zinc might interfere with several aspects of plant biochemistry including photosynthesis, pigment synthesis, PSⅡ activities and photosynthetic electron transport.

At 1 μM Cu^{2+} or 20 μM Zn^{2+}, growth, photosynthetic pigment, photosynthesis, PSⅡ activities and electron transport showed different sensitivities. The reason for this may be due to the inhibition of different enzymes involved in a given process or induction of enzymes which can be beneficial or detrimental to a process or pathway in the cell.

Growth decreased as Cu^{2+} concentration increased in the culture, a similar phenomenon was observed in *Schenedemus incrassatus* (PERALES-VELA et al., 2007). The effect of Cu^{2+} on algal growth has been attributed to a massive failure of many cellular processes (FERNANDES; HENRIQUES, 1991). It is well known that Cu^{2+} has toxic effects on chromosomal morphology and the mitosis cycle (JIANG et al., 2001). In this study, algal growth was more sensitive to Cu^{2+} than metabolism. The reason for this may be that growth is the conclusion of photosynthetic processes including correct electromagnetic energy absorption which is then changed into chemical energy and the efficient utilization of this chemical energy for CO_2 fixation (PERALES-VELA et al., 2007). These cellular processes have different sensitivities to different heavy metals, thus growth of *P. haitanesis* showed different sensitivities to Cu^{2+} and Zn^{2+}.

Three reasons ma bye responsible for the inhibitory effect on Chl *a* and carotenoids seen in excess Cu^{2+} and Zn^{2+}: Firstly, Cu^{2+} or Zn^{2+} probably induce production of reactive oxygen species and inhibit the reductive steps in the biosynthesis pathway of these pigments (CLIJSTERS et al., 1999). Secondly, they can directly destroy the structure and function of chloroplast by binding with SH group of enzyme and overall chlorophyll biosynthesis (SINGH, 1995). Lastly. They may activate pigment enzyme and accelerate the decomposition of pigment (HOU et al., 2007). Moreover, carotenoids appeared to be more resistant to Cu^{2+} and Zn^{2+} phytotoxicity than Chl *a* because the change in Chl *a* was apparent compared to that of carotenoids.

The photosynthesis and respiration results showed that the photosynthetic process was still active in samples following treatment with 1 μM Cu^{2+} and 50 μM Zn^{2+}. However, negative results for the 5 μM Cu^{2+} and 100 μM Zn^{2+} treatments indicated that consumption of oxygen during respiration was higher than that produced by photosynthesis, confirming the damage to metabolism caused by Cu^{2+} and Zn^{2+}. The significantly reduced oxygen evolution parameters in *P. haitanesis* were correlated with the relative decrease in Chl *a* concentrations at 10 μM Cu^{2+}. This was in agreement with the results of Andrade et al. (2004). Moreover, a higher concentration of Zn^{2+} (20 μM) also decreased photosynthesis and Chl *a* content. Above results indicated that Cu^{2+} and Zn^{2+} also exerts their toxicity on photosynthesis mainly due to the loss of Chl *a*. Moreover, increased generation of reactive oxygen species induced by these metals can induce membrane lipid peroxidation and increase unstaching of thylakoids (CLIJSTERS et al., 1999).

The changes of Chl *a* fluorescence and PSII activites showed the same pattern indicating that changes in room temperature Chl *a* fluorescence intensity are intimately association with PS□ activity and reflect the primary acceptor of PSII (RENGER; SCHREIBER, 1986). In this study, marked decreases in chlorophyll fluorescence and PSII activities were observed in response to exposure to higher concentrations of Cu^{2+} and Zn^{2+} due to the substitution of Mg^{2+} in Chl *a* molecules bound to the PS II reaction center (KÜPPER et al., 1996, 1998, 2002).

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