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Cobalt and manganese stress in the microalga *Pavlova viridis* (Prymnesiophyceae): Effects on lipid peroxidation and antioxidant enzymes

LI Mei[1], ZHU Qin[2], HU Chang-wei[2], CHEN Li[2], LIU Zhi-li[2], KONG Zhi-ming[1,*]

1. National Key Laboratory of Pollution Control and Resource Reuse of China, School of the Environment, Nanjing University, Nanjing 210093, China. E-mail: meili@nju.edu.cn
2. School of Life Science, Nanjing University, Nanjing 210093, China

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Abstract

Pollution of marine environment has become an issue of major concern in recent years. Serious environmental pollution by heavy metals results from their increasing utilization in industrial processes and because most heavy metals are transported into the marine environment and accumulated without decomposition. The aim of the present study is to investigate the effects on growth, pigments, lipid peroxidation, and some antioxidant enzyme activities of marine microalga *Pavlova viridis*, in response to elevated concentrations of cobalt (Co) and manganese (Mn), especially with regard to the involvement of antioxidative defences against heavy metal-induced oxidative stress. In response to Co^{2+}, lipid peroxidation was enhanced compared to the control, as an indication of the oxidative damage caused by metal concentration assayed in the microalgal cells but not Mn^{2+}. Exposure of *Pavlova viridis* to the two metals caused changes in enzyme activities in a different manner, depending on the metal assayed: after Co^{2+} treatments, total superoxide dismutase (SOD) activity was irregular, although it was not significantly affected by Mn^{2+} exposure. Co^{2+} and Mn^{2+} stimulated the activities of catalase (CAT) and glutathione (GSH), whereas, glutathione peroxidase (GPX) showed a remarkable increase in activity in response to Co^{2+} treatments and decreased gradually with Mn^{2+} concentration, up to 50 μmol/L, and then rose very rapidly, reaching to about 38.98% at 200 μmol/L Mn^{2+}. These results suggest that an activation of some antioxidant enzymes was enhanced, to counteract the oxidative stress induced by the two metals at higher concentration.

Key words: antioxidant enzymes; cobalt; manganese; malondialdehyde (MDA); *Pavlova viridis*

Introduction

Pollution by heavy metals results from their increased utilization in industrial processes, as in the case of cobalt and manganese contamination that has been reported to have come from industrial areas, metal refineries, and the vicinity of tailings dumps. Mining, in particular, metal ore extraction is the second source of heavy metal contamination in soil after sewage sludge. The manganese (Mn) concentration is more than 10000 mg/kg and the levels of cobalt in soils are found to be as high as 12700 mg/kg, which is 2000 times that of the average (Abraham and Hunt, 1995). In recent years, most heavy metals are transported into the marine environment and accumulate without decomposition, marine pollution has become an issue of major concern. Cobalt (Co), one of the essential micronutrient elements, is needed by the phytoplankton cell for the synthesis of cyanocobalamin (vitamin B_{12}) and is therefore an important trace metal (Sunda, 1989). It has been seen to affect the growth and metabolism of plants to varying degrees, depending on the concentration and status of cobalt in the rhizosphere (Palit *et al*., 1994). Cobalt also induces toxicity in many organisms at higher concentrations, the critical level varying with the species (Dies, 1999). The mechanisms by which cobalt affects plants are not yet clearly known, although it has been proposed that cobalt interacts with the uptake of other macro- and microelements (Palit and Talukder, 1994). Manganese is also an essential nutrient, and numerous enzymes utilize the redox properties of this element (Larson and Pecorato, 1992). Hence, the growth of phytoplankton might be limited at high manganese concentrations (Sunda, 1989).

Marine phytoplankton forms the basis of the marine food chain and is essential for the normal functioning of ecosystems. Any disturbance to this component, because of the release and accumulation of toxic compounds is likely to have an impact on higher trophic levels. An understanding of the regulatory mechanisms of metal tolerance and the components involved in them will help in metal removal from aquatic ecosystems. Tolerant and protective mechanisms have evolved to scavenge free radicals and peroxides generated during various metabolic reactions.

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E-mail: zmkong@nju.edu.cn.

As oxidative stress can be produced in plants exposed to high metal concentration, the induction of some antioxidant enzymes is of great importance (Robinson *et al*., 1993). Cells have evolved efficient strategies to counteract oxidative stress. Enzymatic and nonenzymatic reactions of free radical scavengers minimize cellular oxidations: superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and antioxidant compounds, such as, glutathione (GSH) and carotenoids (CAR), can play a key role in controlling cellular levels of metal-induced reactive oxygen species (ROS) (Weckx and Clijsters, 1996). All aerobic organisms possess the means to protect themselves from the toxic effects of reduced oxygen species. Thus it is of great importance to study metal-induced oxidative stress to understand the mechanisms of metal tolerance evolved by plants.

There are many reports concerning the response of the antioxidant systems in plants to metal stress (Chaoui *et al*., 1997; Mazhoudi *et al*., 1997). In recent years, there is more in the literature about oxidative stress stimulated by heavy metals in marine algae studies on microalgae (Okamoto *et al*., 1996, 1998; Lee and Shin, 2003; Morelli and Scarano, 2004; Elisabetta and Gioacchino, 2004; Tripathi *et al*., 2006; Li *et al*., 2006). However, there are few studies on antioxidant systems of microalgae exposed to Co^{2+} and Mg^{2+} stress. The aim of the present study was to investigate the effects on growth, lipid peroxidation and activities of some antioxidant enzymes of marine microalga *Pavlova viridis* in response to elevated concentrations of cobalt and manganese, especially with regard to the involvement of antioxidative defences against heavy metal-induced oxidative stress.

1 Materials and methods

1.1 Microalgal culture

The marine microalga *Pavlova viridis* (3012) was provided by the Institute of Oceanology, Chinese Academy of Sciences, Qingdao. It was maintained in sterilized artificial seawater consisting of the following composition in g/L: NaCl, 25; $MgSO_4{\cdot}7H_2O$, 3.5; $MgCl_2{\cdot}6H_2O$, 2.5; $CaCl_2$, 1.2; KCl, 0.8; $NaHCO_3$, 0.2; KNO_3, 0.25, and KH_2PO_4, 0.015 (Yang *et al*., 2000). The artificial seawater was enriched with f/2 medium (Guillard and Ryther, 1962) in which the vitamins were doubled (B_1 200 μg/L, B_2 1 μg/L and B_{12} 1 μg/L). Cultures were grown and maintained at 23 ± 1°C under 12-h light/12-h dark cycle provided by cool white fluorescence lights at 85–90 μmol photon/($m^2{\cdot}s$) irradiance.

Experiments were conducted in 500 ml flasks that had been autoclaved at 121°C for 20 min. The Co^{2+} and Mn^{2+} stock solutions were prepared from their analytical grade metallic salts (i.e., $CoCl_2{\cdot}6H_2O$ and $MnCl_2{\cdot}4H_2O$, respectively) dissolved in deionized distilled water with other trace elements. Co^{2+} and Mn^{2+} solutions in the range 0 to 200 μmol/L were prepared by the dilution of a concentrated stock solution. The initial culture was harvested by centrifugation at 4000×*g* for 5 min before the day of experiment, washed twice with artificial seawater without any nutrition and then the microalgal pellet was homogenized in 200 ml artificial seawater as the inoculums. The inoculums were mixed in media of different metal ion concentrations during the experiments. The initial cellular concentration was about 3.5×10^6 cell/ml for cobalt treatment and 2.5×10^6 cell/ml for manganese treatment. The experiments were carried out under the conditions described earlier. Three replicates were considered in all the experiments.

1.2 Growth bioassay

The algal growth as cell density was measured spectrophotometrically (VIS-7220 spectrophotometer, Rayleigh Analytical Instrument Corp., Beijing) at a wavelength of 645 nm in a cuvette with a 1-cm light path. OD_{645nm} values were converted to cell counts using the liner regressions between optical density (OD) and cell counts, which had been determined in the preliminary experiments ($y = 167.59OD_{645nm} - 1.32$, $r = 0.999$, cell counts $= y \times 10^5$). Pigments were extracted with 90% acetone and determined in accordance with Jeffrey and Humphrey (1975), for chlorophyll-*a* contents and Strickland and Parsons (1972), for carotenoid contents. The levels were expressed in μg/10^6 cells.

1.3 Antioxidative enzymes assay

For enzyme assays, *P. viridis* samples cultured for 13–15 d were centrifuged at 6000×*g* for 10 min and resuspended in 0.067 mmol/L of precooled sodium phosphate buffer (pH 7.0). After sonication for 5 min in an ice bath, the cell debris was removed by centrifugation at 12000×*g* for 20 min at 4°C. The content of lipid peroxidation products, malondialdehyde (MDA), was determined according to the methods provided by Heath and Parker (1968). The results were expressed as nmol 10^6/cells. Total superoxide dismutase activity (SOD, EC 1.15.1.1) was determined by the ferric cytochrome *c* method using xanthine/xanthine oxidase as the source of superoxide radicals, and a unit of activity was defined as that described in McCord and Fridovich (1969), which was expressed as U 10^6/cells. Catalase activity (CAT, EC.1.11.1.6) was assayed spectrophotometrically with a Hitachi U-3000, by measuring the decrease of absorbance at 240 nm because of H_2O_2 decomposition (Rao *et al*., 1996). Glutathione peroxidase (GPX, EC.1.11.1.9) activity was tested in the presence of added glutathione (Thomas *et al*., 1990). The concentration of reduced glutathione (GSH) was determined spectrophotometrically with dithionitrobenzoic acid (DTNB) at 412 nm (Rijstenbil *et al*., 1994). Protein content of homogenates was determined by the reaction with Coomassie Blue dye, using bovine serum albumin as the standard, in a VIS-7220 spectrophotometer (Bradford, 1976).

1.4 Statistical analysis

The differences between the control and treated samples were analyzed by one-way ANOVA using Origin 7.0 statistical software, taking $P < 0.05$ as significant according to Tukey's multiple range tests. Each experiment had three

replicates. The mean values ± SD are reported in figures.

2 Results and discussion

2.1 Metal effects on the algal growth

The growth of microalga *P. viridis* was more sensitive to Co^{2+} than Mn^{2+}. Fig.1a shows that *P. viridis* could grow under all experimental conditions when cultured in manganese media, but when in cobalt media the microalgal cells grew poorly except for the control (Fig.1b). The inhibition of growth was not significant at other Mn^{2+} concentrations, whereas, in Co^{2+} treatments higher than 10 μmol/L, a significant reduction in the number of microalgal cells was apparent. Low concentrations of Mn^{2+} (10–20 μmol/L) could promote the growth whereas, high concentrations inhibited it. At the end of the experiment, the number of cells in the control was in the range of 12.32×10^6 cell/ml, whereas, at 200 μmol/L it was 3.09×10^6 cell/ml. Growth was inhibited by 74.95% and 4.51%, respectively, when compared to the control at treatment of 200 μmol/L Co^{2+} and Mn^{2+}.

2.2 Metal effects on content of chlorophyll-*a* and carotenoids

Figures 2a and 2b show the chlorophyll-*a* and carotenoids content of *P. viridis*, calculated from the spectrophotometric data. There is a sharp increase in the chlorophyll-*a* and carotenoids content, which is expressed by μg/10^6 cells after 13 d of treatment, induced by Co^{2+}, and higher concentrations have resulted in a decrease, but they are still higher than the control (Fig.2a). As for the Mn^{2+} treatment the group of 10 μmol/L Mn^{2+} has the highest chlorophyll-*a* and carotenoids content (Fig.2b). Results indicate that chlorophyll-*a* and carotenoids content ranged from 0.10×10^6 to 0.20×10^6 μg/cells and 0.05×10^6 to 0.11×10^6 μg/cells at Mn^{2+} treatments, respectively. An increase of carotenoids and carotenoids to chlorophylls ratio (data not shown) in microalgae *P. viridis* treatment with Co^{2+} and Mn^{2+} might have a protective effect as carotenoids are known to be potent quenchers of ROS, particularly singlet oxygen (Larson, 1988). It is possible that the higher concentration of heavy metal contributes to the accumulation of carotenoids in relation to per 10^6 cells.

2.3 Metal effects on content of soluble protein and lipid peroxidation products

Protein content of Co^{2+} showed a significant decrease at 10–500 μmol/L concentrations (Fig.3). It decreased to 38.20%, 45.09%, 37.93%, 58.36%, and 56.76% in control at 10, 20, 50, 100, and 200 μmol/L Co^{2+} respectively. However, the effect was not found with Mn^{2+} supply where it was significantly higher than that of control, which increased by 14.48%, 15.17%, and 10% at 10, 20, and 50 μmol/L Mn^{2+} respectively. Co^{2+} markedly enhanced

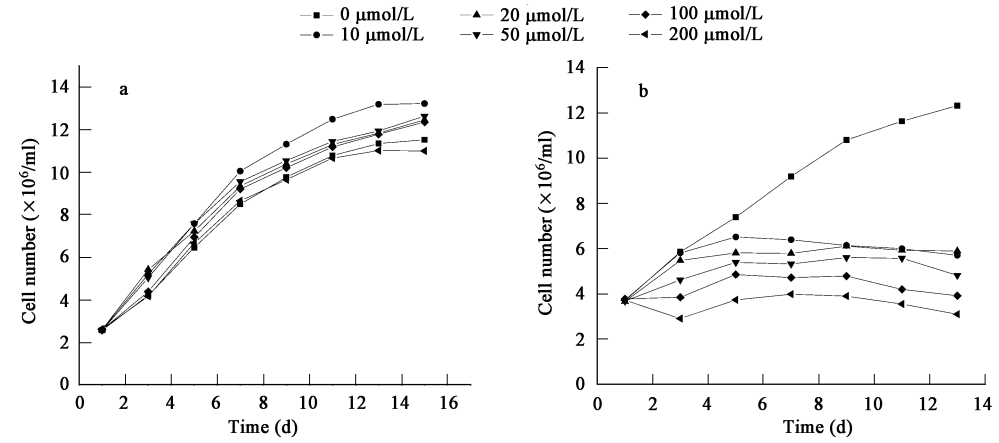

Fig. 1 Growth curves of *P. viridis* supplemented with 0–200 μmol/L Mn^{2+} (a) and Co^{2+} (b). Algae were cultured at 23 ± 1°C, 85–90 μmol photon/(m^2·s) irradiance and 12:12 h light/dark cycle. The results are the mean of three replicates for each treatment (error bars not shown).

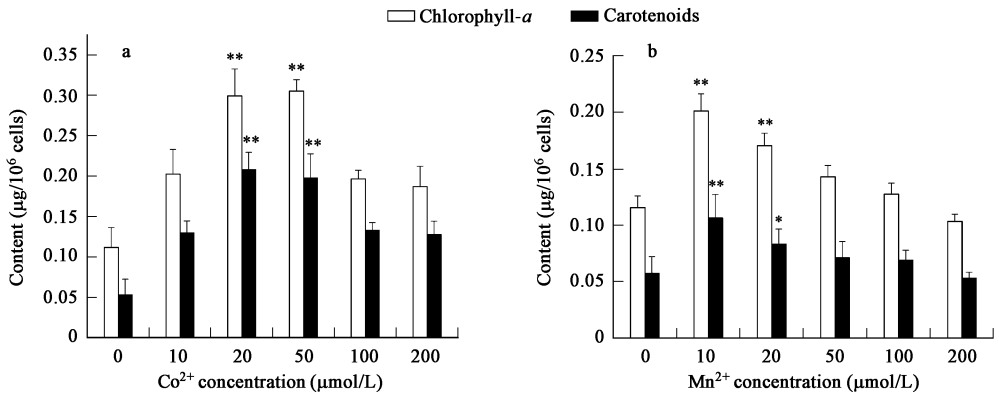

Fig. 2 Chlorophyll-*a* and carotenoids contents of *P. viridis* supplemented with different concentrations of Co^{2+} (a) and Mn^{2+} (b). Data are the means ± SD of three replicates. **P*<0.05, ***P*<0.01.

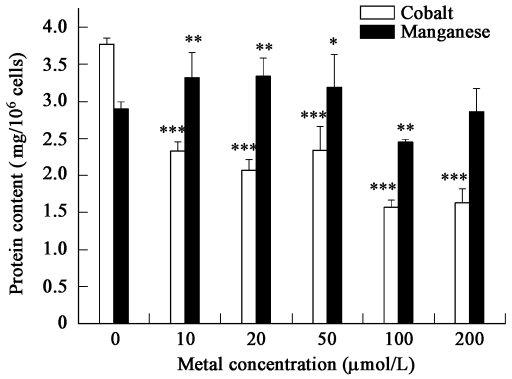

Fig. 3 Soluble protein contents of *P. viridis* supplemented with different concentrations of Co^{2+} and Mn^{2+}. Protein content of homogenates was determined by reaction with Coomassie Blue dye using bovine serum albumin as the standard. Data are the means ± SD of three replicates. **P*<0.05, ***P*<0.01, ****P*<0.001.

lipid peroxidation over control values, measured as total MDA content, in *P. viridis* whereas at 10–200 Mn^{2+} concentrations there were no significant changes when compared to the control (Fig.4). Membrane destabilization is generally attributed to lipid peroxidation resulting from an increased production of ROS (Mead *et al*., 1982), and lipid peroxidation can be initiated by redox activating metal ions themselves (Chaoui *et al*., 1997). The increase in lipid peroxidation observed in this study is considered as an indicator of increased oxidative damage caused by Co^{2+}, but not Mn^{2+}, in cells. Cobalt is defined as an oxidative stress-inducing factor as Co^{2+} has been shown to react with hydrogen peroxide by a Fenton-type reaction to produce hydroxyl radicals (Moorhouse *et al*., 1985). The activation of free radical formation is usually judged by the dynamics of lipid peroxidation. High concentrations of Co^{2+} have a toxic effect on cell division and induce chromosomal aberrations in the root tips of *Allium cepa* (Liu *et al*., 1994).

2.4 Metal effects on the activities of antioxidant enzymes and glutathione (GSH) content

It is well known that ROS are produced in cells when exposed to environmental stresses, for example exposure

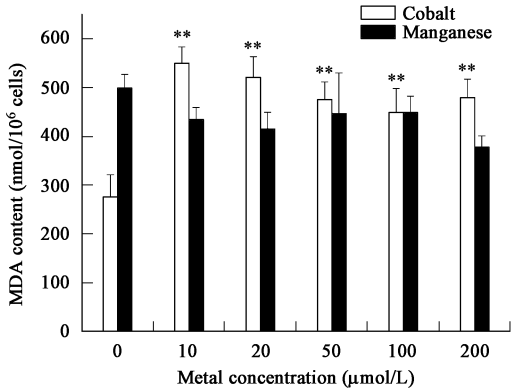

Fig. 4 Level of lipid peroxidation products (MDA) measured as thiobarbituric acid reactive substances in *P. viridis* exposed to different concentrations of Co^{2+} and Mn^{2+}. Data are the means ± SD of three replicates. ***P*<0.01.

to high light intensities, UV radiation, metals. Increasing the levels of ROS can lead to severe cellular injury or death. Therefore, induction of antioxidant enzymes is an important protective mechanism to minimize cell oxidative damage in polluted environments. Several antioxidant enzymes and antioxidant substances are involved in protective mechanisms adapted by plants to scavenge free radicals and peroxides (Allen, 1995). The antioxidant enzymes are important components in preventing oxidative stress in plants, because the activity of one or more of these enzymes is generally increased when exposed to stressful conditions and these elevated activities correlate to increased stress tolerance (Allen, 1995; Mazhoudi *et al*., 1997).

The mechanism underlying the toxicity of heavy metals is not always clear. In some cases, such as, the inhibition of photosynthesis in the marine diatom *Phaeodactylum tricornutum* by Cd, inhibition of diatoxanthin epoxidation to diadinoxanthin in the xanthophyll cycle is suggested to be responsible (Bertrand *et al*., 2001). More generally, however, heavy metal toxicity is related, at least in part, to the oxidative stress induced in living systems (Quinlan *et al*., 1988; Okamoto and Colepicolo, 1998; Livingstone, 2001). In contrast to higher plants, the antioxidant response to oxidative and environmental stress has not been extensively investigated in algae (Reed and Gada, 1990; Okamoto *et al*., 1996).

The results of these experiments showed that activities of these enzymes were significantly correlated with the concentrations of the metal. SOD activity rose gradually from 221.73%, in control, in *P. viridis* exposed to 10 μmol/L Co^{2+}, to 272.35% in those treated with 20 μmol/L Co^{2+}. When Co^{2+} concentration increased to 50 μmol/L, SOD activity decreased and reached about 17.62% of control, whereas, in the Mn^{2+} treatment SOD activity was not significantly affected (Fig.5a). With respect to algae, SOD activity could be induced by both copper exposure in the diatom *Ditylum brightwellii* and Cd exposure in *Tetraselmis gracilis* (Rijstenbil *et al*., 1994; Okamoto *et al*., 1996), but it was found that in these microalgal cells, after being treated by Co^{2+} and Mn^{2+}, the level of SOD activity was not induced.

The increase of CAT when treated with Co^{2+} or Mn^{2+} was particularly high when the concentration in the growth media was 100–200 μmol/L (Fig.5b). It was 754.12% at 100 μmol/L and 1418.98% at 200 μmol/L of control respectively in Co^{2+} treatment, as well as, 358.78% at 100 μmol/L and 216.86% at 200 μmol/L in Mn^{2+}. A very great increase of GPX activity was observed under Co^{2+} treatment, from 191.67% at 20 μmol/L of control to 414.79% at 50 μmol/L. In *P. viridis* grown under excess Mn^{2+} the GPX contents decreased gradually with Mn^{2+} concentration in the growth medium, up to 50 μmol/L, then they rose very rapidly, reaching about 38.98% at 200 μmol/L Mn^{2+} (Fig.6a). It is well known that CAT plays an important role in reducing oxidative stress by catalyzing the oxidation of H_2O_2 (Weckx and Clijesters, 1996). Thus, the increase in the activity of this enzyme by cobalt suggests the production of H_2O_2 increased, and the

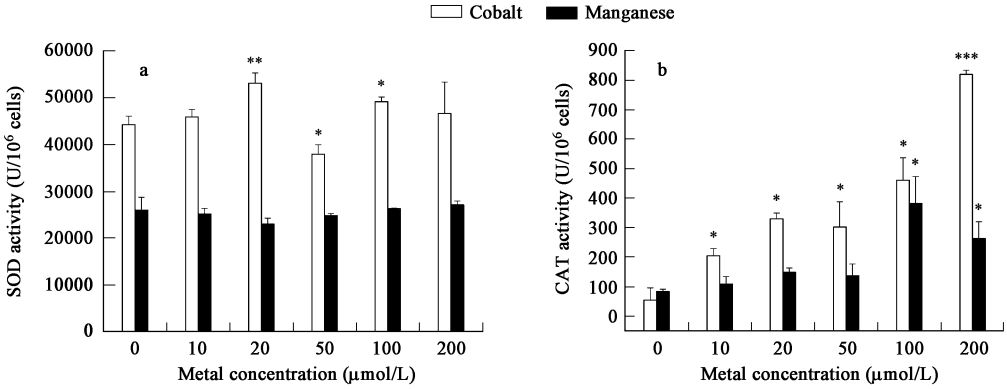

Fig. 5 Total SOD activity (a) and CAT activity (b) of *P. viridis* supplemented with different concentrations of Co^{2+} and Mn^{2+}. Data are the means ± SD of three replicates. **P*<0.05, ***P*<0.01, ****P*<0.001.

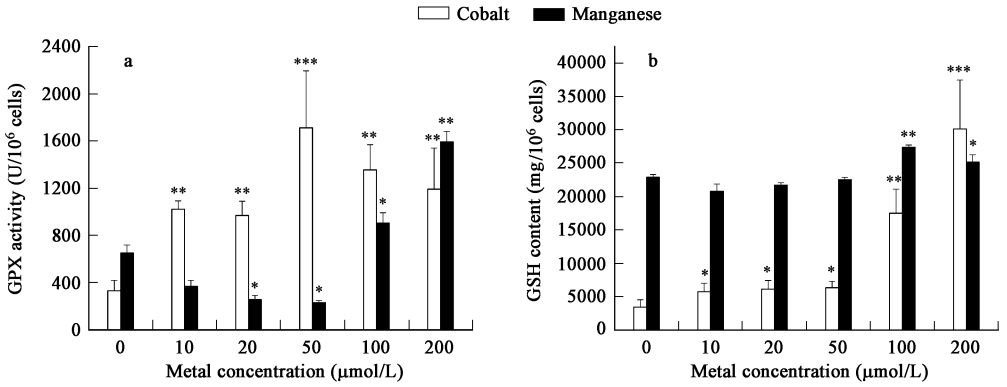

Fig. 6 GPX activity (a) and GSH content (b) of *P. viridis* supplemented with different concentrations of Co^{2+} and Mn^{2+}. Data are the means ± SD of three replicates. **P*<0.05, ***P*<0.01, ****P*<0.001.

markedly elevated levels of CAT and GPX following Co^{2+} treatment at higher concentration indicated the protective role of these enzymes against cobalt-induced oxidative stress. Lee *et al*. (2003) also found CAT activity increased slightly when microalgae *Nannochloropsis oculata* were exposed to cadmium stress. Such increase in the activity of these enzymes had been reported with a variety of heavy metals applied for treatments (Van assche and Clijsters, 1990), and the extent of increase varied with metal, metal concentration, the enzyme tested, and plant species. However, detailed information concerning activation of the antioxidant system for the detoxification of metal ions in marine algae is scarce, although algae are recommended as biological monitors for heavy metal pollution (Whitton *et al*., 1989).

Apart from these enzymes, some antioxidants like GSH may play a role in inducing resistance to metals by protecting labile macromolecules against the attack by free radicals that are formed during various metabolic reactions and lead to oxidative stress (Alscher, 1988). In the present study, the level of antioxidant (GSH) exhibited varied responses to the cobalt and manganese treatments, which was dependent on metal concentration. Co^{2+} concentrations higher than 100 μmol/L, significantly increased (407.14% and 773.26%, respectively) the GSH content compared to the control. The content of GSH at lower manganese concentrations did not differ from that of the control, whereas, at higher concentrations (100 and 200 μmol/L) it increased by 19.63% and 9.93% respectively (Fig.6b). Earlier Tukendorf and Rauser (1990), reported an increase in GSH content with the treatment of Cd because of activation of GSH synthesizing enzymes. Such an activity of enzymes involved in GSH biosynthesis is known to be as a result of metal stress (Scheller *et al*., 1987), but the depletion of GSH content with increasing concentration of copper was also observed in some algae (Nagalakshmi and Prasad, 2001). Thus, the observed relation between cobalt and manganese induced oxidative stress (lipid peroxidation) and antioxidative capacity (antioxidant enzymes, CAR and GSH) in *P. viridis* cells, which suggests that the tolerance capacity of the plants to the metal depends on the balance of the factors favoring oxidative stress and the factors reducing oxidative stress.

Taken together the results indicate that to cope with Co and Mn toxicity, especially Co^{2+}, the marine microlage *P. viridis* is able to carry a cellular strategy involving the activation of antioxidant enzymes and nonenzyme antioxidative substances. Prolonged exposure inhibits microalgae growth, enhances the activity of antioxidant enzymes, showing that an oxidative stress has occurred, and induces lipid peroxidation indicative of membrane damages in Co^{2+} treatment.

3 Conclusions

In conclusion, it is evident that Co^{2+} concentration in the

nutrient medium has a direct effect on the production or scavenging of the ROS. The presented results show that higher Co^{2+} concentrations may promote oxidative damage to the membrane. The present study also suggests that the antioxidant enzymes (CAT and GPX) and antioxidant compounds (GSH and CAR) have a combined effect on the protection of biomembranes against the damaging effects of the ROS.

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