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Full Length Research Paper

Effect of copper on growth, bioactive metabolites, antioxidant enzymes and photosynthesis-related gene transcription in *Chlorella vulgaris*

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A significant increase in carotenoids, intracellular proline contents, and activity of antioxidative enzymes; catalase, peroxidise, polyphenol oxidase, and superoxide dismutase, was observed in the green microalga *Chlorella vulgaris* following copper exposure. In contrast, a reduction in Chl a, Chl b, free amino acids and total protein contents was also observed. Real-time PCR shows that Cu treatment reduced significantly the transcript abundance of *psb*C and *rbc*L at moderate and high Cu concentrations, whereas the transcript abundance of *psa*B synergistically increased at lower Cu concentrations and thereafter decline with increasing Cu doses. These results demonstrate that Cu inhibits PSII activity and CO_2 assimilation, but increase the activity of the antioxidative enzymes. Consequently, *Chlorella vulgaris* showed diverse response to Cu stress on physiological, biochemical and molecular levels.

Key words: *Chlorella vulgaris*, copper, antioxidant enzymes, bioactive metabolites, photosynthetic genes transcripts.

INTRODUCTION

Metal compounds are one of the most persistent pollutants in soil and aquatic environments. Large quantities of metal compounds are discharged into freshwater ecosystems due to their widespread industrial use and their levels have increased substantially world-wide over the last century (Penuelas and Filella, 2002). A general increase in the level of heavy metals poses a pervasive threat to the natural ecosystem. Although many heavy metals; including copper; when in trace amounts are essential for various metabolic processes in organisms, they create physiological stress leading to generation of free radicals especially at high concentration. Unlike complex organic pollutants, metal compounds cannot be degraded by microorganisms or chemical degradation; instead, they can be accumulated by organisms and also take part in the process of bioaccumulation throughout the food chain, thus threatening human health (Kong et al., 1995). In aquatic ecosystems, algae provide oxygen and organic substances to other life forms. Microalgae are sensitive indicators of environmental changes and, as the basis of most freshwater and marine ecosystems, they have been widely used to evaluate the impacts of metal, herbicide and other persistent xenobiotic contamination and bioavailability in aquatic systems (Stauber and Davies, 2000; Qian et al., 2008). They are also used in the assessment of risk and development of environmental regulations for metals (Levy et al., 2007). Therefore,

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evaluating the impact of heavy metal stress on marine microalgae (e.g. *Chlorella*) at the biochemical and molecular levels will provide new biological markers for heavy metals' toxicity in contaminated wastewater and sediments. This will also help to provide new insights for developing new strategies in producing genetically modified microalgae with enhanced heavy metal specificity and binding capacity.

Copper is an essential micronutrient for numerous physiological processes at low concentrations but it has a toxic effect at higher concentrations (Gaetke and Chow, 2003). It is required in the synthesis of chlorophyll and is important for maintaining optimum plant metabolism. Copper deficiency results in reduced synthesis of the copper-containing electron carriers; plastocyanin and cytochrome oxidase. The reduction of these electron carriers reduces photosynthesis and respiration (Baro'n et al., 1995). However, copper becomes toxic to aquatic biota when biological requirements are exceeded (Andrade et al., 2004). Therefore, Cu was selected for this study because it plays a relevant role in aquatic pollution and is potentially toxic to freshwater organisms. Various species of *Chlorella* have often been used in toxicity bioassays (Shubert, 1984) and has a remarkable ability to accumulate high concentrations of various heavy metals. High concentrations of all metals exert toxic effects on the metabolic machinery of algae through the enhanced production of reactive oxygen species (ROS). Under normal circumstances, concentration of oxygen radicals remains low because of the activity of protective enzymes, including superoxide dismutase, catalase, ascorbate peroxidase , and polyphenol oxidase (Asada, 1984). Such antioxidant enzymes provide a defence mechanism for the survival of aerobic organisms (Beyer et al., 1991). Although there is an extensive literature on the biochemical and physiological influence of copper on algae (Baro'n et al., 1995; Gaetke and Chow, 2003; Levy et al., 2007), to our knowledge, the effects of copper on phytoplankton have rarely been assessed at the gene transcription level. Therefore, the aim of the present study was to assess the effects of copper on the activity of antioxidant enzymes, levels of bioactive metabolites, and photosynthesis related gene expressions in the unicellular green alga *Chlorella vulgaris*. Transcript accumulations of three photosynthesis-related genes were measured under copper stress: *rbc*L, *psa*B, and *psb*C. *rbc*L encodes the large subunit of Rubisco, the key enzyme of the Calvin cycle that catalyzes the primary step in CO_2 assimilated into organic carbon. *psa*B is part of the *psa*A/B operon of the chloroplast genome and encodes the photosystem I (PSI) reaction center protein. *psb*C encodes an integral membrane protein component of photosystem II (PSII) that plays a major role in transduction of excitation energy from the light harvesting proteins to the photochemical reaction center. To our knowledge, this is the first approach considering the link between bioactive metabolites production, antioxidant enzymes activity, and photosynthetic related gene transcription under relatively high Cu concentrations in *C. vulgaris*.

MATERIALS AND METHODS

Algal growth, copper treatment, and biochemical analyses

All toxicity tests were conducted using the freshwater unicellular green alga, *C. vulgaris*, obtained from the Egyptian National Research Center (NRC), Cairo, Egypt. The alga was cultured in 250 ml flasks containing 100 ml sterilized Bold Basal Medium (Bischoff and Bold, 1963) supplemented with sterile compressed air and kept under fluorescent light (50 µmol $m^{-2}s^{-1}$) with 16 h light period at 25 ±2°C temperature. Exponential phase of growth of *C. vulgaris* was determined in cultures, where it was grown for 14 days in media containing additional $CuSO_4.5H_2O$ concentrations of 0, 0.5, 1.5, 3.0, and 4.5 µM. The starting cultures were adjusted to contain 0.125 cell × 10^6 ml^{-1} medium. *Chlorella* was harvested (by centrifugation) after seven days from cultures, which were treated with the above mentioned concentrations of copper. Reference controls contained untreated culture media. All treatments were replicates of at least three independent cultures. The algal growth was determined by measuring the optical density at 678 nm as described by Robert (1979). The growth curve of the alga was estimated every 2x day intervals. Chlorophyll pigments, and β-carotene were determined according to Metzner et al. (1965). Soluble and insoluble proteins were determined as described by Badour (1959). Total carbohydrates were determined as described by Dubois et al. (1956). Proteins were measured according to the method of Lowry et al. (1951). Free amino acids were extracted by grinding known algal dry weight in 70% ethanol. Alcoholic algal extract was obtained by centrifugation at 5000 rpm for 10 min and used to determine total free amino acid according to Lee and Takahashi (1966). Glycine was used as a standard. Amino acid content was calculated as mg/g dry weight. Proline content was determined following the protocol described by Bates et al. (1973). Algal cells were homogenized in 10 ml of 3% sulphosalicylic acid. Supernatant was obtained by centrifugation at 5000 rpm for 10 min. Proline content was calculated as µg/g dry weight.

Enzyme extraction and assays

The fresh algal weight was homogenized in cold phosphate buffer (0.05 M at pH 7.5). The homogenate was centrifuged at 12000 rpm for 20 min at 4°C (Kar and Mishra, 1976). The supernatant was used for analyzing Catalase (CAT), Peroxidise (POD), Polyphenol oxidase (PPO), and Superoxide dismutase (SOD) activity assays.

Catalase assay

Catalase (CAT, EC 1.11.1.6) activity was determined spectrophotometrically (UV/VIS, Model T80+, PG Instrument, UK) at 25°C according to the methods described by Xu et al. (2008). The reaction mixture containing 1.5 ml of 0.05 M sodium phosphate buffer (pH 7.8), 1 ml de-ionized water, and 0.3 ml of 0.1 M H_2O_2 prepared immediately before use, and then 0.2 ml enzyme extract was added. CAT activity was measured by monitoring the decrease in absorbance at 240 nm as a consequence of H_2O_2 consumption. Catalase activity was expressed as µmol H_2O_2 destroyed/mg protein/minute.

Peroxidase assay

The reaction mixtures for peroxidase (POD, EC 1.11.1.7) assay, containing 0.1 M potassium phosphate buffer (pH 6.8), 50 µM H_2O_2, and 50 µM catechol prepared fresh before use. 0.5 ml of enzyme extract was then added to 2.5 ml reaction mixtures. Increase in absorbance was measured at 430 nm at 0.5 min intervals up to 3 min using a UV/VIS spectrophotometer (PG Instrument, UK). The enzyme activity was expressed as the change in the optical density/mg protein/minute according to Racusen and Foote (1965).

Polyphenol oxidase assay

Polyphenol oxidase (PPO, EC 1.14.18.1) assay was performed according to the method described by Kar and Mishra (1976). 5 ml of the assay mixture contained 125 µM of phosphate buffer (pH 6.8), 100 µM of pyrogallol and 1 ml of crude enzyme extract. After incubation at 25°C for 5 min, the reaction was stopped with the addition of 1 ml of 10% H_2SO_4. The colour intensity was read at 430 nm and the enzyme activity was expressed as the change in the optical density/mg protein/h.

Superoxide dismutase assay

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined using the modified method proposed by Zhang and Zhai (2003). The reaction mixture containing 14.5 mM methionine, 2.25 mM nitroblue tetrazolium, 3 µM EDTA, 60 µM riboflavin, and these reagents were prepared with 0.05 M phosphate buffer (pH 7.8) except riboflavin which was prepared with deionized water (Zhang and Zhai, 2003). 0.2 ml enzyme extract was then added to 3 ml reaction mixtures in tubes. The reaction started by placing the tubes below two 40-W fluorescent lamps for 10 min. The reaction was finished by keeping the tubes in the dark for 10 min. The developed purple colour was then measured at 560 nm using a UV/VIS spectrophotometer (PG Instrument, UK). One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of the photochemical reduction of NBT. SOD activity was expressed as units/mg protein.

RNA extraction and real-time RT-PCR analysis

RNA was prepared from 50 ml *C. vulgaris* cell cultures following the BCP (1-bromo-3-chlorpropane) protocol (Chomczynski and Mackey, 1995). Nucleic acid concentrations were measured spectrophotometrically at 260 nm. The 260/280 nm ratios were determined and referred to as the purity of the total RNA extracted. The integrity was tested by electrophoresis on a 1% agarose gel. Preparation of first strand cDNA was performed as described by Niessen et al. (2007). Quantitative PCRs were performed on an ABI PRISM® 7300 Sequence Detection System (Applied Biosystems, USA) following the manufacturer's instructions. Gene-specific primer pairs of *psa*B, *psb*C, *rbc*L, and housekeeping gene used for PCR are listed in Table 1. The 18S rRNA transcript was used to standardize the results by eliminating variations in the quantity and quality of mRNA and cDNA. Each mRNA level was expressed as the ratio of itself to 18S rRNA. RT-PCR amplifications were performed in the presence of SYBR Green (SYBR® GreenER™ qPCR SuperMixes; Invitrogen), and oligonucleotides were purchased from Metabion, Planegg, Germany. The final primer concentration was 200 nM in the reaction mixture. Amplification conditions were 10 min of initial denaturation at 95°C, followed by 40 cycles each of 15 s denaturation at 95°C and 1 min combined annealing and extension at 60°C.

Statistical analysis

Data are presented as mean ± standard error of the mean. Significance was determined according to Student's *t*-test using Excel software (Microsoft). Two-sided tests were performed for homoscedastic matrices.

RESULTS

Effect on algal growth

The growth of *C. vulgaris* was measured in presence of different concentrations of copper as shown in Figure 1. It can be seen that *C. vulgaris* could grow under all experimental conditions. Short lag phases for the alga was observed at all concentrations of copper (that is, 0.5, 1.5, 3.0, and 4.5 µM) and control culture. This indicates that the alga has a well adaptability to these growth conditions. The growth patterns of *Chlorella*, at all Cu concentrations, have log phases extended for 15 days then passes to the stationary phase. The exponential phase started with the inoculums and the overall growth was copper dependent where the alga before and after primary settling had highly overlapped growth curves along the experiment time.

Table 1. Sequence of primer pairs used in real time RT-PCR.

Gene name	Primer	GenBank accession no.
18S rRNA	Forward 5`-TTCTATGGGTGGTGGTGCAT-3` Reverse 5`-GCGAACCAACCGTGACTATT-3`	X13688
*psa*B	Forward 5`-TGCCACTGGGTTTATGTTCC-3` Reverse 5`-GCCATCGTACGAGATTTGCT-3`	GeneID:809130
psbC	Forward 5`-GAACGTCGTGCTGCTGAATA-3` Reverse 5`-CCAACTACGCGGAGAAACAT-3`	GeneID:809108
*rbc*L	Forward 5`-CGGTGGTGGTACTTTAGTC-3` Reverse 5`-TCACGAGCAAGATCACGACC-3`	AF499684

*psa*B: Photosystem I reaction center protein subunit B, *psbC*: Photosystem II reaction center protein subunit C, and *rbc*L: Large subunit of Rubisco.

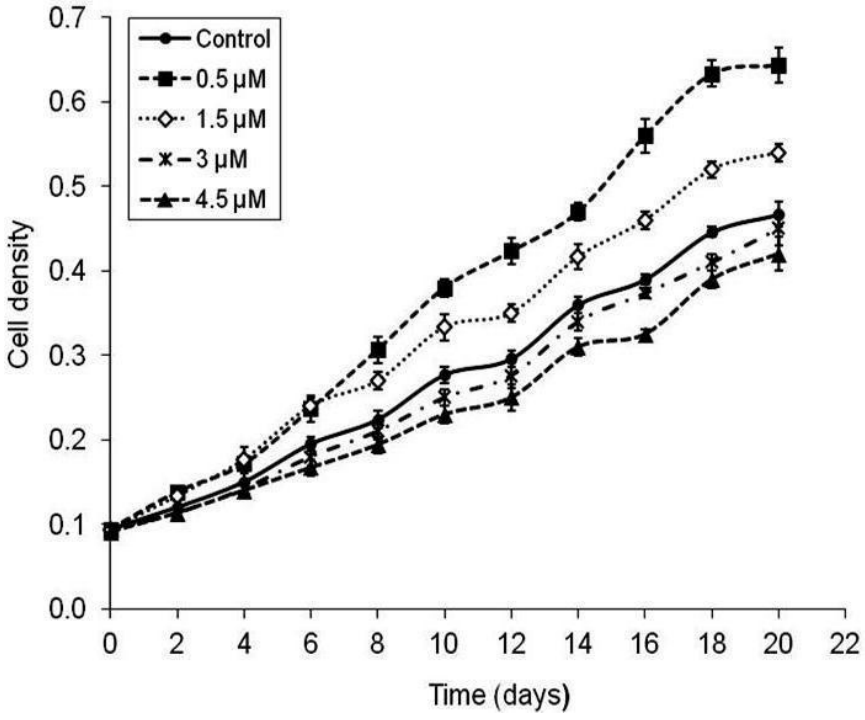

Figure 1. Growth response curves of *Chlorella vulgaris* upon exposeure to different copper concentrations.

Effect of Cu treatment on growth response curves of *Chlorella vulgaris*. The alga was cultured in 250 ml flasks containing 100 ml sterilized Bold Basal Medium and kept under fluorescent light (50 µmol $m^{-2}s^{-1}$) with 16 h light period at 25 ±2 ºC. The cell density was determined by measuring the optical density at 678 nm according to Robert (1979), 2 days intervals. Vertical bars represent standard error of at least three independent measurements.

However, it was obvious that higher concentrations of copper (that is, 3 and 4.5 µM) showed a relative reduction in algal growth rate and cell density starting from day four of copper exposure when compared to their corresponding

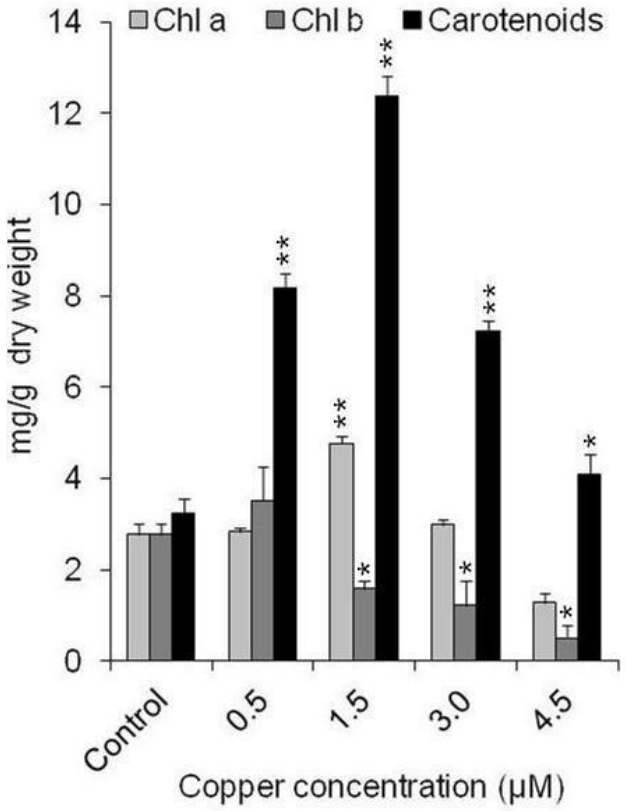

Figure 2. Effect of copper treatment on Chl a, Chl b, and carotenoid contents in *Chlorella vulgaris*. Chlorophyll a (Chl a), Chlorophyll b (Chl b), and carotenoid contents measured from *Chlorella vulgaris* treated with different concentrations of Cu; 0, 0.5, 1.5, 3.0, and 4.5 µM; for 10 days. Data are means of three independent measurements. Vertical bars represent standard error. (*) and (**) represent statistically significant differences when compared with the control without copper treatment at $p < 0.05$ and at $p < 0.01$ levels, respectively.

controls. The relative reduction in *Chlorella* growth rate at higher copper concentrations may be considered a reasonable determinant of its toxic effect.

Effect on chlorophyll content

The bioassay results, as illustrated in Figure 2, show clear differences in pigment contents (that is, chlorophyll a, b, and carotenoids) of algal cells after exposure to different concentrations of copper. There is a gradual increase in Chl a in the algal culture supplemented with 1.5 and 3 µM of copper, whereas higher concentrations of copper (that is, 4.5 µM) cause a reduction in chl a content in *C. vulgaris* as shown in Figure 2. Moreover, there is a clear reduction in Chl b contents measured at moderate and higher concentrations of copper (1.5, 3, 4.5 µM). On the contrary, the relatively higher concentrations of copper support the biosynthesis of carotenoids rather than Chl a and b. The values recorded for carotenoids are 2.5, 3.7, 2.1, and 1.4 fold at 0.5, 1.5, 3, and 4.5 µM Cu; respectively compared to control samples (Figure 2). This indicates that the algal cells tend to stimulate carotenoids biosynthesis rather than chl a and b under copper stress.

Effect on total free amino acids and proline content

The data indicated in Figure 3A show approximately 50% reduction of the total free amino acids measured from *C. vulgaris* upon exposure to Cu compared to control samples. Figure 3B shows also a gradual rise in proline content reaching a maximum values at copper concentrations of 3 and 4.5 µM accounting for 71.4 and 55.2% increase in proline content, respectively compared to their corresponding controls. This copper dependent increase in intracellular proline pool in *C. vulgaris* can be considered as an adaptive mechanism for reducing the level of accumulated NADH and H^+ upon exposure to copper. Thus, the results of this study indicate that proline accumulation plays an important role in alleviating the toxic effects of excess heavy metal concentrations rather than other free amino acids.

Effect on total soluble, insoluble protein, and carbohydrate contents

Gradual decrease in the level of soluble and insoluble protein contents was observed in the green alga *C. vulgaris* after 10 days of exposure to variable concentrations of copper (Figure 4A). The maximum decrease was recorded at copper concentrations of 3, and 4.5 µM. Total insoluble protein show a decrease of 22.2 and 38.8%; respectively, whereas total soluble protein show a decrease of 20 and 27%; respectively, compared to their corresponding values recorded for the control samples. Total carbohydrate contents measured from *Chlorella* cultures grown under various concentrations of copper are illustrated in Figure 4B. Results revealed that copper stimulates the production of higher levels of carbohydrates at low and moderate concentrations of copper (that is, 0.5 - 3 µM). The maximum values recorded for carbohydrate accumulation in *C. vulgaris* are 2.2, 2.4, and 1.8 fold at 0.5, 1.5, and 3 µM Cu; respectively, compared to the corresponding control cultures. Thus, our measurements show a significant increase in carbohydrate levels that correlates with a clear reduction in protein levels upon exposure to low (0.5 µM), moderate (1.5 µM), and relatively high (3 µM) concentrations of copper.

Effect on antioxidant enzymes

Figure 5 represents the response of various antioxidant

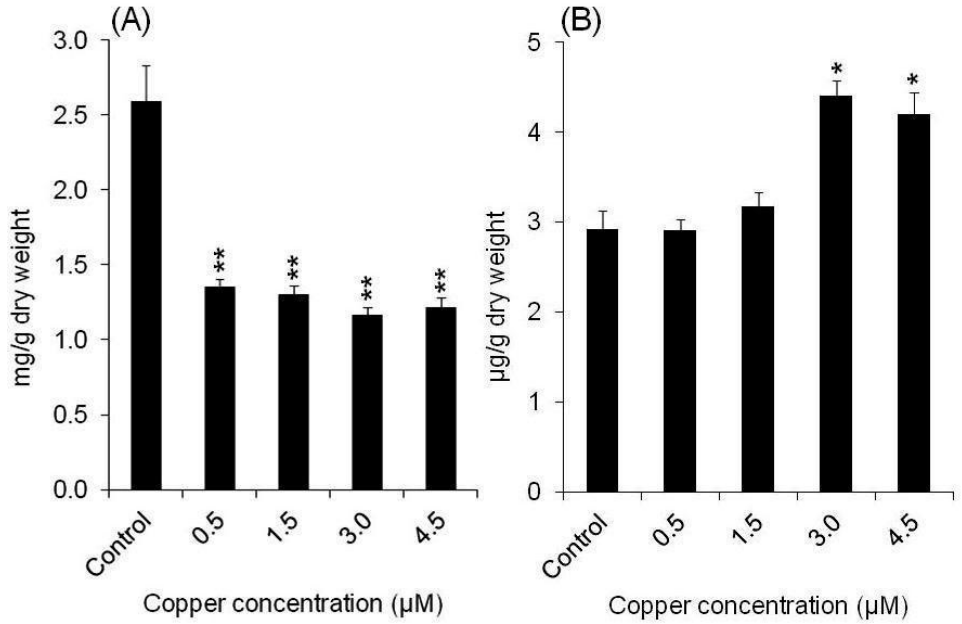

Figure 3. Effect of copper treatment on total free amino acids and proline contents in *Chlorella vulgaris*. Total free amino acids (A) and proline content (B) measured from *chlorella vulgaris* exposed to different Cu concentrations; 0, 0.5, 1.5, 3.0, and 4.5 µM; for 10 days. Data are means of three independent measurements. Vertical bars represent standard error. (*) and (**) represent statistically significant differences when compared with the control without copper treatment at $p < 0.05$ and at $p < 0.01$ levels, respectively.

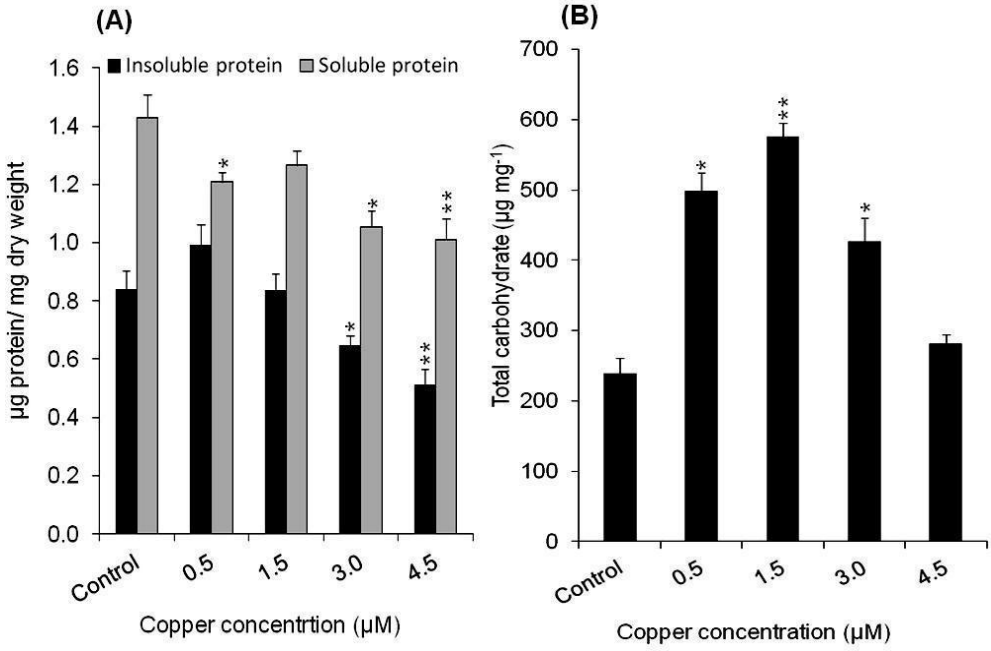

Figure 4. Effect of copper on soluble, insoluble protein, and total carbohydrate contents in *Chlorella vulgaris*. Total soluble and insoluble protein (A), and total carbohydrate contents (B) measured from *Chlorella vulgaris* after exposure to different concentrations of Cu; 0, 0.5, 1.5, 3.0, and 4.5 µM; for 10 days. Data are means of three independent measurements. Vertical bars represent standard error. (*) and (**) represent statistically significant differences when compared with the control without copper treatment at $p < 0.05$ and at $p < 0.01$ levels; respectively.

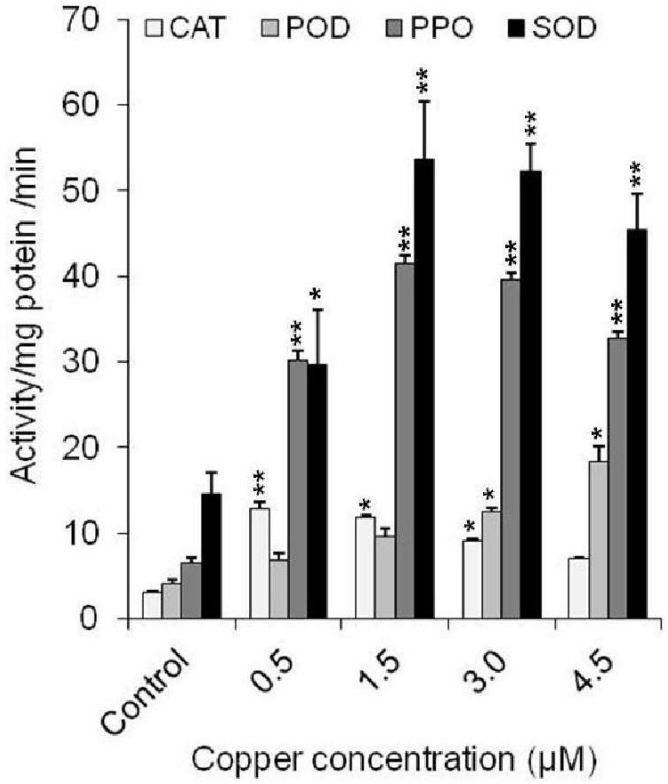

Figure 5. Effect of copper treatment on the activity of Catalase, Peroxidise, Polyphenol oxidase, and Superoxide dismutase in *Chlorella vulgaris*. Catalyse (CAT), Peroxidase (POD), Polyphenol oxidase (PPO), and Superoxide dismutase (SOD) enzymatic activities measured from *Chlorella vulgaris* cultures treated with varying concentrations of copper; 0, 0.5, 1.5, 3.0, and 4.5 µM; for 10 days. Vertical bars represent standard error. (*) and (**) represent statistically significant differences when compared with the control without copper treatment at $p < 0.05$ and at $p < 0.01$ levels, respectively.

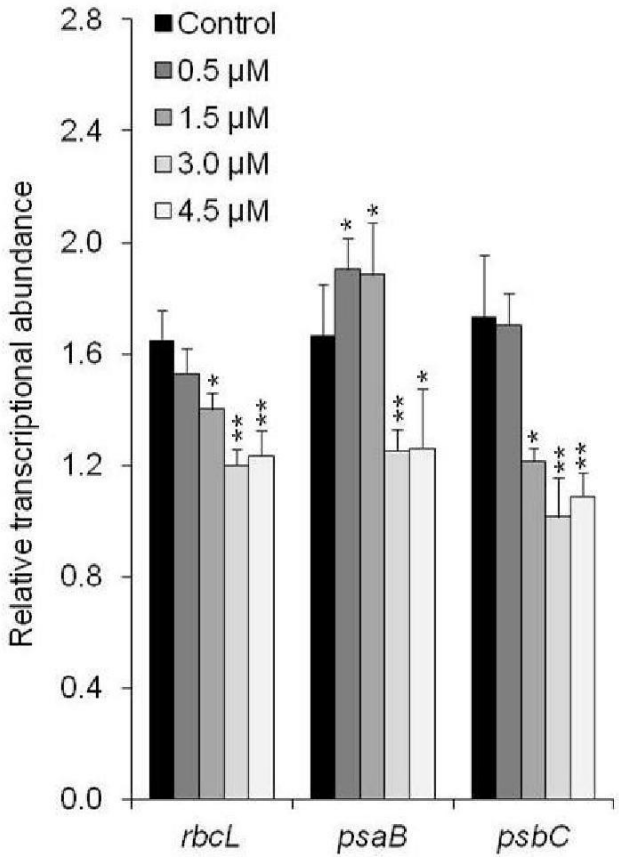

Figure 6. Real time RT-PCR analysis of mRNA transcripts of *rbc*L, *psa*B, and *psb*C in *Chlorella vulgaris* after exposure to varying copper concentrations. Transcripts of large subunit of Rubisco (*rbc*L), photosystem I reaction center protein subunits B (*psa*B), and an integral membrane protein component of photosystem II (*psb*C) in *Chlorella vulgaris* exposed to varying concentrations of copper; 0, 0.5, 1.5, 3.0, and 4.5 µM.. Copper treatment was performed at the logarithmic growth phase of *Chlorella* and the measurements were performed 48h after copper application. Values were normalized against 18S rRNA. Each data point is based on at least three independent RNA preparations. Vertical bars show standard error. (*) and (**) represent statistically significant differences when compared with the control without $CuSO_4.5H_2O$ treatment at $p < 0.05$ and at $p < 0.01$ levels, respectively.

enzymes (CAT, POD, PPO and SOD) in *C. vulgaris* cultures treated with different concentrations of Cu. Control cultures of *C. vulgaris* possessed the minimum value of antioxidant enzymes. A relatively concentration-dependent increase in antioxidant enzymes activities is evident. Regarding to PPO and SOD, it is obvious that copper stress increases their activities and reach to their maximum value at 1.5 µM with maximum ratios of 5.6 and 3.4 fold; respectively compared to corresponding controls. Moreover, a clear stimulation in the activity of CAT was also observed at all Cu concentrations. The maximum rate of stimulation (4 fold) was observed at 0.5 µM compared to its corresponding controls. Stimulation in the activity of POD was also observed under copper stress. The maximum rate of stimulation recorded at copper concentration of 4.5 µM was 4.5 fold more than its corresponding controls. Thus, copper treatment provoked the activity of CAT, POD, PPO, and SOD in *C. vulgaris*.

Effect on photosynthesis-related gene transcription

Figure 6 shows the effects of different Cu concentrations after two days of exposure on the relative transcriptional abundance of *rbc*L, *psa*B, and *psb*C genes. Rubisco is the carboxylase that controls the rate limiting step of carbon assimilation as it enables the continuation of growth and development of plants. Real-time PCR was performed to evaluate the abundance of *rbc*L, which codes for the large subunit of Rubisco. Abundance of *rbc*L transcript was significantly affected by Cu treatments at concentrations of

1.5, 3, and 4.5 µM (Figure 6). After two days of exposure, the abundance of *rbc*L transcripts was reduced by ~25% compared to the corresponding values measured from the control samples. *psa*B is part of the psaA/B operon of the chloroplast genome and encodes P700 chlorophyllA2 apoproteins. Exposure to low (0.5 µM) and moderate (1.5 µM) Cu concentrations result in a higher transcript abundance of *psa*B gene. *psa*B transcription was increased by 18% compared to their corresponding control samples (Figure 6). However, this level was significant which thereafter decline by 41% upon exposure to higher Cu concentrations (that is, 3 and 4.5 µM). It is also obvious that Cu treatments inhibit the transcriptional abundance of *psb*C gene in *C. vulgaris* especially at 1.5, 3, and 4.5 µM. *psb*C is a gene that codes for an integral membrane protein of photosystem II (PSII). 47% inhibition in *psb*C transcription was observed at high Cu concentrations (that is, 3, and 4.5 µM). However, low Cu treatment has no clear effect on *psb*C transcription. Thus, moderate and high concentrations of Cu cause a clear reduction in the transcriptional abundance of *rbcL* and *psbC* that in turns affect not only the efficiency of PS II and electron transfer rates, but also the CO_2 assimilation rates in *Chlorella vulgaris*.

DISCUSSION

This study was performed to evaluate the Cu stress response on physiological, biochemical, and gene transcriptional levels in *C. vulgaris*. The results of this study showed a relative reduction in algal growth rate and cell density at higher concentrations of copper (Figure 1). Such growth retardation effect is similar to that observed for *Spirulina platensis*-S5 (Choudhary et al., 2007), *Synechocystis aquatilis* (Shavyrina et al., 2001) and *Asparagopsis armata* (Segot et al., 1983); since, the reduction in growth is due to inhibition of normal cell division induced by excess Cu concentration. Although excess Cu concentration is harmful to cell growth, the algal cells, in order to survive metal stress conditions, tend to stimulate carotenoids biosynthesis rather than Chl a and b (Figure 2). The significant rise in carotenoids content as noticed under copper stress (Figure 2) might offer protection to chlorophyll and photosynthetic membrane from photo-oxidative damage (Siefermann-Harms, 1987; Shanab et al., 2012). Therefore, carotenoids appeared to be more resistant to Cu^{2+} phytotoxicity than chlorophylls because the change in chlorophyll was apparent compared to that of carotenoids. Our results also showed a gradual increased in proline contents at higher Cu concentrations (Figure 3B). This copper dependent increase in intracellular proline pool in *C. vulgaris* is in accordance with the reports on higher plants and algae (Alia and Saradhi, 1991; Bassi and Sharma, 1993). It is also proposed that proline acts as a source of carbon and nitrogen for rapid recovery from the stress, and also serves as stabilizer of plasma membrane and some macro-molecules and acts as free radical scavenger (Jain et al., 2001). Therefore, higher proline levels observed in the current study for *C. vulgaris* can be considered as a protective mechanism against Cu stress. Moreover, suppression of total protein accumulation in *C. vulgaris* upon exposure to Cu stress (Figure 4A) may be attributed to shortage of carbon skeleton results from low photosynthetic rate. These results are consistent with those observed by Fathi et al. (2000) and Li et al. (2006). Torres et al. (1998) demonstrated that the alga,

Cylindrothica fusiformis, produce carbohydrate as a defense mechanism against copper toxicity in stationary phase when cells are exposed to 0.5 mg Cu l^{-1}. The decrease in chlorophyll synthesis (Figure 2) coupled with severe drop in protein levels (Figure 4A) enhance consequently the rate of carbohydrates biosynthesis. Therefore, the increased level of total carbohydrate accumulation under moderate and high Cu concentrations (Figure 4B) can be considered as a defense mechanism against Cu toxicity. In higher plants, heavy metals induce generation of superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-), and singlet oxygen ($^1O_2$), collectively termed ROS that exert a variety of damaging effects (Devi and Prasad, 1998). When the levels of ROS formed exceed the ability of the antioxidant system to cope with them, damage to cellular components occurs. The dismutation of O_2^- radicals by SOD results in H_2O_2, which can be scavenged by both CAT and peroxidises (Elisabetta and Gioacchino, 2004). The induction of antioxidant enzymes is therefore an important protective mechanism to minimize cell oxidative damage in polluted environments (Sabatini et al., 2009; Sheih et al., 2009). Thus, the markedly elevated levels of CAT, POD, PPO, and SOD observed for *C. vulgaris* in the current study (Figure 5) indicates the protective role of these enzymes against copper-induced oxidative stress. Real- time RT-PCR analysis of the photosynthetic related gene transcription reveals a clear reduction in *psb*C, *psa*B, and *rbc*L transcription levels at moderate and high Cu concentrations (Figure 6). The inhibition of *psb*C mRNA transcripts may decrease the activity of photosystem II (PSII) and electron transfer rates in *C. vulgaris*, which was observed by a decrease in chlorophyll content (Figure 2). The increase in *psa*B transcript abundance at relatively low Cu concentrations should improve the activity of PSI. However, this increase in PSI activity increases ATP synthesis but decreases the generation of NADPH (Zhou et al., 2006). Electron transport occurs at PSII first, and then is relayed to PSI. When electron transport is blocked at PSII, algal cells tend to increase related protein at PSI to enhance electron receptivity from PSII. This behaviour may enable photosynthesis to proceed normally, especially under stress conditions (Pfannschmidt, 2003).

When the metal stress pressures exceed the organism's ability to tolerate the stress, normal metabolism is

inevitably disrupted, resulting in subsequent decrease in the transcript abundance of these photosynthetic related genes. By decreasing the abundance of *rbc*L (Figure 6) and blocking carbon assimilation and photorespiration, Cu may cause the accumulation of a mass of reducing equivalents which could be scavenged by the accumulated proline (Figure 3B) under Cu stress. Excess electrons can lead to decreased transcription of PSI and PSII genes. This, in turn, reduces electron flow through PSI and PSII (Qian et al., 2008). Thus, Cu treatment decreases the assimilation of CO_2 not only by influencing the activities of enzymes, but also by inhibiting the mRNA expression of genes coding for related enzymes. However, the effects of Cu on *C. vulgaris* growth, bioactive metabolites, antioxidative enzymes activity, and the transcription of photosynthesis related genes have been assessed in this study. The results show that Cu induces the activity of antioxidant enzymes, increases the accumulation of carotenoids, proline, and carbohydrates. Furthermore, Cu inhibits *rbc*L, *psb*C, and *psa*B gene expression which negatively affect PS II efficiency and decreases chlorophyll content synergistically.

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