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EFFECT OF COPPER ON NITROGEN COMPOUNDS, NITRATE-REDUCTASE ACTIVITY AND ASCORBATE IN CHLOROCOCCCUM HUMICOLA CELLS

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Chlorococcum humicola **(Nag) Rab. isolated from soil polluted with waste water at Sohag district was used as test organism in this investigation. The alga was grown in BG-11 medium containing different Cu^{2+} concentrations (0.0, 0.5, 50, 100, 150, 200 and 250 µM Cu SO_4) and the lethal dose was 300 µM Cu SO_4. The low concentration (0.5 µM) of Cu^{2+} caused an increase in growth {cell numbers, dry mass } and photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids). On increasing the Cu^{2+} concentrations, the cell number, dry weight and photosynthetic pigments were decreased. Moreover, total free amino acids were also inhibited with increasing Cu^{2+} concentrations. The protein content remained more or less unchanged even at the higher doses of the copper. Nitrate-reductase activity and phenolic compounds were decreased with increasing Cu^{2+} dose. On the other hand, Cu^{2+} stimulated the proline content, soluble proteins, lipoperoxides and ascorbate. The production of new polypeptide bands were appeared at the level of 150 µM Cu SO_4 (70, 28, 26, 25 KDa). The results obtained indicated that, *Chlorococcum humicola* might be tolerated the pollution with Copper in soil.**

Keyword: Copper, Ascorbate, Nitrate-reductase activity, Nitrogenous compounds, *Chlorococcum humicola*.

INTRODUCTION

Toxic effect of heavy metals on living systems is one of the main problems derived from environmental contamination. Copper is the most commonly used heavy metal for industrial purposes and its presence in the environment arises from both naturally occurring and man-made origins. Various sources of Cu^{+2}, including industrial and domestic wastes, agricultural practices, copper mine drainage, copper-based pesticides, have contributed to a progressive increase in cupper concentrations in varied environments [1, 2].

Copper can be good and evil to algae as it is an essential micronutrient for algal growth, participating important biological reactions as an enzymatic co-factor and electron carrier in the photosynthetic and respiratory processes [3]. At high concentrations, it becomes highly toxic [4]. Growth inhibition and chlorosis are common symptoms of metal phyto-toxicity in several algae, in which photosynthesis is probably the most

affected metabolic process [5]. Copper reduces growth as well as photosynthetic and respiratory activities [6, 5, 7].

The photosynthetic apparatus is particularly susceptible to copper resulting in a decrease in the activity of photo-system 11 and electron transfer rate [8, 9]. Because of its redox properties, copper induces oxidative stress by generating reactive oxygen species like superoxide and hydroxyl radicals via Haber-Weiss and Fenton reactions. Toxicity of copper may also result from the oxidation of sulphydryl groups of enzymes leading to their inhibition [10]. Oxidative stress directly damages proteins, amino acids, nucleic acids, and membrane lipids often leading to alterations in cell structure and mutagenesis [11]. Under acute conditions, however, the toxic effects of the pollutants may overwhelm the antioxidant defenses. This may result in cell death or shutting down of all cellular machinery. Toxicity may result in diverse effects, which depend on the type of algae, the nature and concentration of the metal, and the environmental conditions accompanying heavy metal stress [12].

This investigation aimed to study the effects of different concentrations of Cu SO_4 on the growth of *Chlorococcum humicola* (isolated from soil polluted with waste water at Sohag district) determining the cell numbers, dry weight and chlorophyll contents. In addition, the effects of Cu SO_4 on some antioxidant compounds and protein metabolism were also studied.

Chlorococcum humicola is unicellular green algae which can provide important information on the toxic effects of a pollutant on general metabolic processes and is often used as an indicator of pollution.

MATERIALS AND METHODS

Algal species and culturing

Chlorococcum humicola (Nag) Rab. was isolated from polluted soil with waste water at Sohag district, Egypt. The alga was grown in BG-11 medium according to [13] under the conditions of fluorescent illumination (2500 lux) and room temperature (25±2). Filtered dry air was let to bubble in the culture vessels to provide carbon dioxide and to prevent settling of algal cells.

In this experiment, different concentrations of Cu SO_4 (0.5, 50, 100, 150, 200 and 250 µM.) were added to BG-11 medium for 10 days under the same conditions mentioned above. Each treatment was made in three replicates. At the end of incubation period, the algal cells were harvested and used for growth and metabolic determinations.

Determination of growth parameters

***Cell number*:** The cell count of control and treated cultures was measured by Hemacytometer, 0.1 mm deep, having improved Naubauer ruling (A.O. Spencer "Bright fine"). The count was expressed as cells / ml algal suspension.

***Dry weight*:** Dry weight was determined according to [14] by filtering culture aliquots (50 ml) through Whatman GF/C filters. The filters were then dried and weighed.

Photosynthetic pigment extraction:

Chlorophylls *a*, *b* and caroteniods were extracted in 100% acetone at 65°C and their contents were determined spectrophotometrically (SPEKOL 11, CARL ZEISS, JENA, GERMANY) according to [15].

Biochemical determinations

Estimation of protein

Protein content was determined according to [16]. The algae in10 ml of algal suspension was extracted in distilled-water (soluble protein) and in NaOH (total protein) for 2 h at 90°C. The extract was centrifuged and the supernatants were pooled. The water-soluble protein was estimated using Folin-phenol reagents and measured spectrophotometrically (SPEKOL 11, CARL ZEISS, JENA, GERMANY.) Bovine serum albumin was used as a standard.

Estimation of proline.

Free proline content of algal suspension was determined according to [17]. Briefly, 10 ml of algal suspension was centrifuged and the pellete was extracted in 5 ml of aqueous 3% sulfosalicylic acid for 3 h. The extract was centrifuged at 4000 rpm for 10 min. Two ml of the supernatant were mixed with 2 ml of fresh acid ninhydrin solution and 2 ml glacial acetic acid in a test tube for 1 h at 100℃. The tubes were cooled, and the mixture was extracted with 4 ml toluene. The extract was vigorously stirred for 20 seconds. Therefore, the chromophore-containing toluene was aspirated from the aqueous phase, and its absorbance was measured at 520 nm. Proline was used as a standard.

Estimation of total free amino acids

Total free amino acids were determined according to [18]. The quantity of total free amino acids was calculated as μ g/mg. dry weight.

Nitrate reductase assay in vivo

For in vivo assay of nitrate reductase, the method of [19] was used. Algal cells of 10 ml algal suspension of copper-treated alga and untreated were precipitated and incubated in anaerobic dark conditions for 1 h in 5 ml of 0.1 M K-phosphate (pH=7.5) containing 50 mM KNO_3 and 1% (v/v) n-propanol at 28 ºC. The reaction was stopped by boiling in water bath for 5 min and then centrifuged. The supernatant of one ml sample mixed well with two ml1% w/v sulphonilamide in 1N HCl and two ml 0.1 % w/v N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water. The absorbance was measured by using spectrophotometer (SPEKOL 11, CARL ZEISS, JENA, GERMANY) at 540 nm. Nitrate reductase (N-R) activity was expressed as µg NO_2 /ml algal suspension h^{-1}.

Determination of phenolic compounds

Phenolic compound contents were determined according to [20]. 0.1 g algal cells were homogenized with a plastic pestle in an Eppendorf tube containing 1 ml phosphate buffer 0.1 M pH= 7.0. The homogenate was centrifuged in an Eppendorf microcentrifuge at 12800 for 10 min. Aliquots of 50 µl were added to a reaction mixture containing 3% of sodium carbonate and 0.3 M Folin reagent in a final volume of 1 ml. The reaction mixture was incubated for 2 hr. at room temperature and the absorbance was measured at 765 nm. Total phenolic compounds were expressed as nanoequivalents of gallic acid using a caliberation curve prepared with 10-50 µM of gallic acid.

Detection of lipoperoxides

Lipoperoxides were determined according to [21]. Algal tissue (1–2 g dry weight) from Cachagua and Caleta Palito was frozen in liquid nitrogen in a mortar and homogenized with a pestle. A total of 5ml of 0.1% of trichloroacetic acid was added during homogenization. The homogenate was transferred to a 30-ml glass tube and centrifuged at 7400 g for 20 min. Lipoperoxides were detected by addition of 100 ml of the clear homogenate to a reaction mixture containing 0.5% thiobarbituric acid (solubilized in 20% trichloroacetic acid) in a final volume of 1m. The reaction mixture was incubated in boiling water for 30 min, and the absorbance was measured at 512 nm. To determine the amount of lipoperoxides, the extinction coefficient of the synthesized adduct was used (ec $155mM^{-1}$ cm^{-1}).

Detection of ascorbate

The ascorbate (ASC) and dehydroascorbate (DHA) were determined according to **[22]**, using 0.5 g of dry tissue, ground in liquid nitrogen and homogenized in a mortar with a pestle. A total of 5ml of 2.5M perchloric acid was added during homogenization. The homogenate was transferred to a 30 ml

glass tube and centrifuged at 7400 g for 15 min. ASC was detected by addition 100 ml of the clear homogenate to a reaction mixture containing 2% (w/v) trichloroacetic acid, 8.8% orthophosphoric acid, 0.01% a,a α-dipyridyl, and 10 mM ferric chloride in a final volume of 1 ml. The reaction mixture was incubated for 1 h at 40 ^{0}C, and the absorbance was determined at 525 nm. The calibration curve was prepared using 10 to 300 µM of ASC in the same reaction mixture.

Determination of protein electrophoretic pattern

The algal suspension containing 10^9cells/ml. was centrifuged at 4000 rpm for 5 min. The pellet was used for the extraction of protein by the method cited by [23] using an extraction buffer as recommended by [24] and described by [25]. The method of SDS vertical polyacrylamide gel electrophoresis (SDS- PAGE) was used as described by [23], for the determination of protein electrophoretic pattern. The gels were subjected to the staining solution for 1-2 h., followed by destaining solution over night. There-after, the destained gels were photographed while wet.

RESULTS

The results of this work showed that, the growth rate of *Chlorococcum humicola* was gradually decreased by increasing concentration in culture medium. High concentrations of $CuSO_4$ (250 µM) caused reductions in the algal cell numbers and dry weights reaching 24 % and 20 % .of control respectively .(Fig.1C). The data in Fig.1 reveal that, 0.5 µM $CuSO_4$ stimulated the growth expressed as cell number (104% of control) and dry weight (103% .of control) of *Chlorococcum humicola* cells. The growth criteria decreased gradually by further increase in $CuSO_4$ concentration. The harmful effect of the Cu^{+2} was much more pronounced at the high doses (200 µM and 250 µM).

Photosynthetic pigments were also stimulated (103% of control) at 0.5 µM Cu^{+2}. A gradual reduction in photosynthetic pigments was obtained at the higher levels of copper (Table 1).

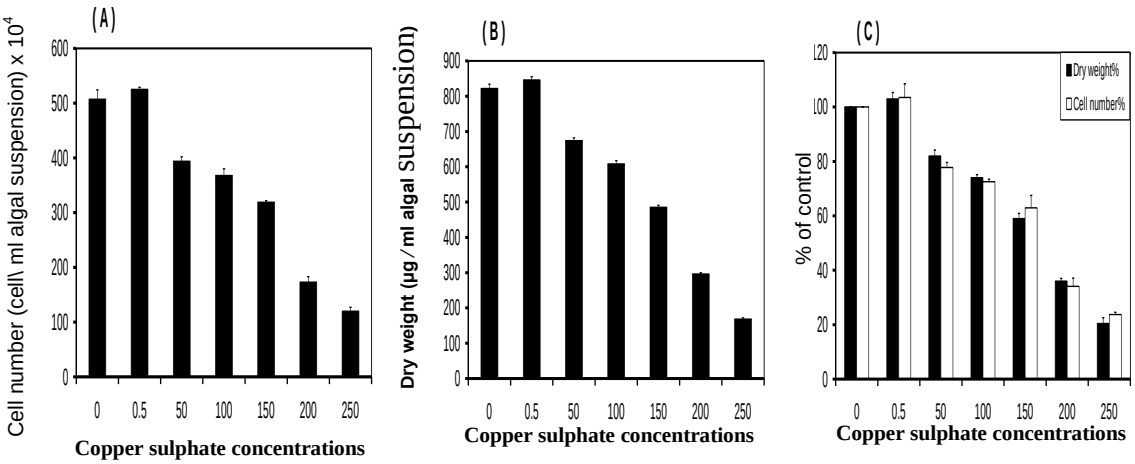

Fig. 1: Effect of different concentrations of copper sulphate (µM) on cell number (A) and dry weight (µg ml algal suspension) (B) of *Chlorococcum humicola*, and the percentage change of control of cell number and dry weight (C). The results are means of triplicate samples. The bars on the colums represent standard deviations.

Table 1: Effect of different concentrations of copper sulphate (µM) on photosynthetic pigments of *Chlorococcum humicola*, and the percentage of control . The change from the results are means of triplicate samples ± standard deviations.

Copper sulphate concentrations (µM)	µg/ml algal suspension			Total pigments	% change from control
	Chl-a	*Chl-b*	*Caroteniod*		
0.0	4.03 ± 0.30	1.69 ± 0.30	0.93 ± 0.07	6.66	100
0.5	4.12 ± 0.30	1.79 ± 0.30	0.99 ± 0.07	6.90	103.60
50	3.49 ± 0.40	1.22 ± 0.20	0.70 ± 0.16	5.41	81.23
100	3.08 ± 0.49	0.99 ± 0.34	0.55 ± 0.17	4.62	72.66
150	2.76 ± 0.16	0.88 ± 0.14	0.43 ± 0.02	4.07	61.10
200	1.49 ± 0.17	0.66 ± 0.07	0.16 ± 0.01	2.31	34.71
250	0.80 ± 0.02	0.24 ± 0.01	0.11 ± 0.02	1.15	17.23

Figure 2 shows that, protein contents were varied considerably according to the dose of the copper salt as well as the protein fraction. The insoluble protein was decreased markedly and the soluble fraction increased (166% of control) by increasing Copper sulphate concentrations. Consequently, the total protein remained more or less unchanged even at the higher doses of the applied salt and it was decreased by only 11% at the level of 250 µM copper sulphate (Fig. 2).

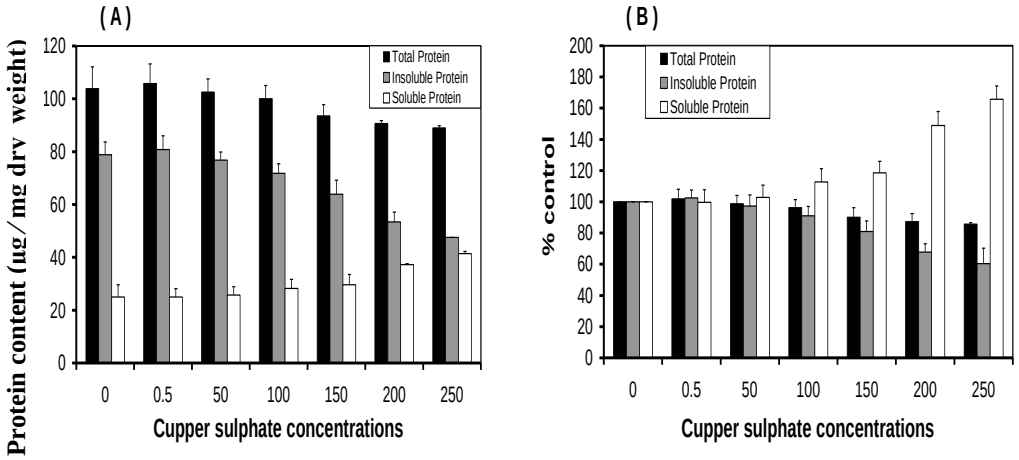

Fig. 2: Effect of different concentrations of copper sulphate (µM) on protein(total, insoluble, soluble) contents as (µg / mg) dry weight (A) of *Chlorococcum humicola* and as percentage control of protein contents from control (B). The results are means of triplicate samples. The bars on the colums represent standard deviations.

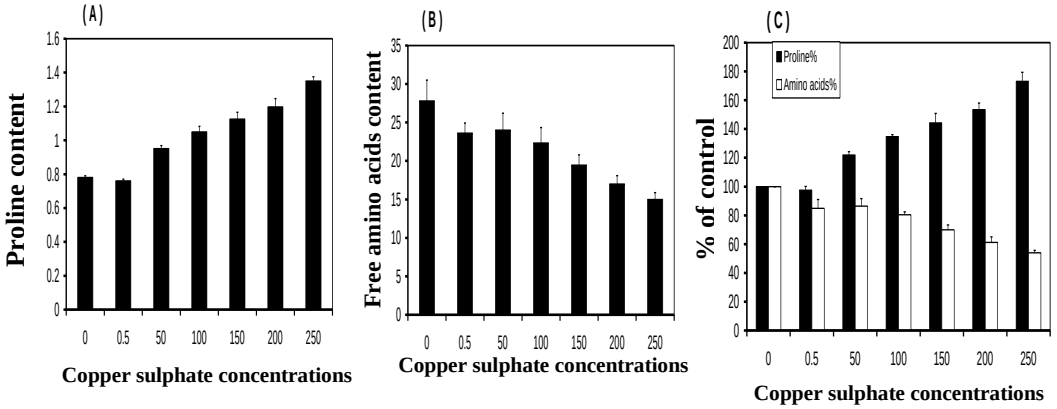

Fig. 3: Effect of different concentrations of copper sulphate (µM) on proline (µg/mg) dry weight (A) and amino acids (µg/mg) dry weight (B) of *Chlorococcum humicola*, and the percentage control of proline and amino acids (C). The results are means of triplicate samples. The bars on the colums represent standard deviations.

Total free amino acids were inhibited with increasing Cu^{+2} concentrations (54 % of control) at the level of 250 µM $CuSO_4$. Proline contents was markedly increased with increasing Copper sulphate doses reached 173% of control (Fig.3). Figure 4 shows in-*vivo* N-R activity and phenolic compounds which were decreased in response to increase of $CuSO_4$ dose. On the other hands, lipoperoxides and ascorbate (ASC) contents were increased with increasing Cu^{+2} concentrations (186 % of control and 134 % of control respectively) (Fig.5).

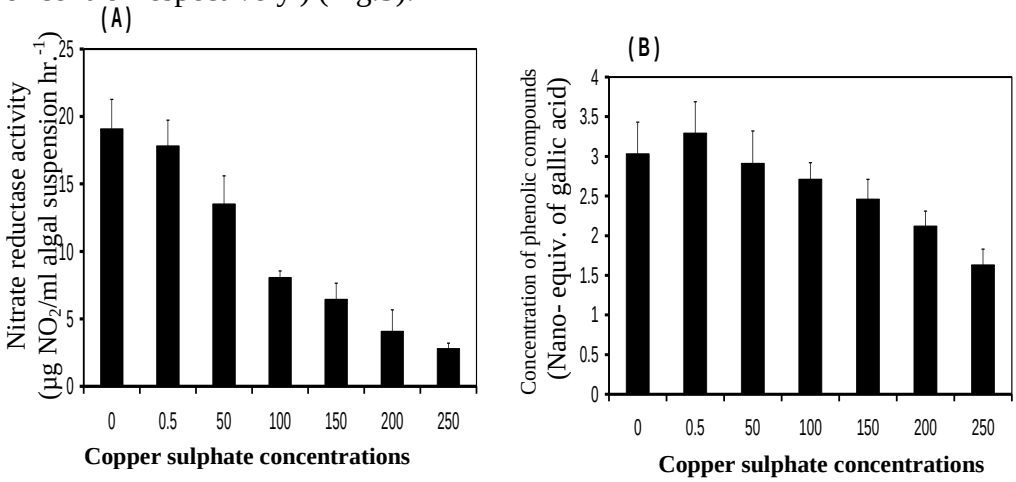

Fig.4.: Effect of different concentrations of copper sulphate (µM) on nitrate reductase activity (µgNO_2/ ml algal suspension. $hr.^{-1}$) (A) and phenolic compounds (µg /mg dry weight) (B) of *Chlorococcum humicola*. The results are means of triplicate samples. The bars on the colums represent standard deviations.

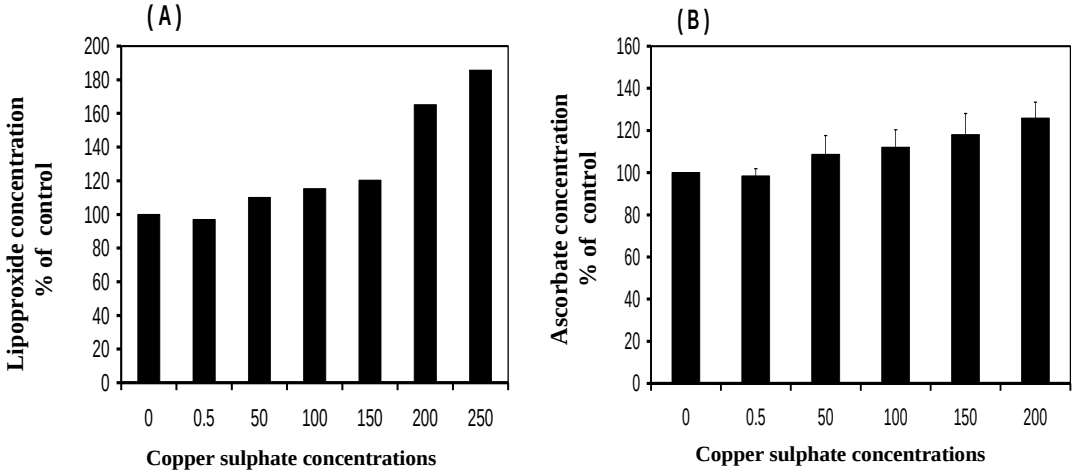

Fig.5: Effect of different concentrations of copper sulphate (µM) on lipoperoxides (A) and Ascorbate (B) of *Chlorococcum humicola* as percentages of corresponding control. The results are means of triplicate samples. The bars on the colums represent standard deviations

The protein electrophoretic patterns of *Cholococcum humicola* treated with low concentrations of $CuSO_4$ (50µM) showed reduction in the number of protein bands, compared with control. This reduction was obvious at higher doses of $CuSO_4$ (200 µM, 250 µM). Some new bands appeared as a results of cupper sulphate treatments. The higher number of these new bands were shown at 150 µM (70, 28, 26, 25 kDa) (Table 2 and Fig 6)

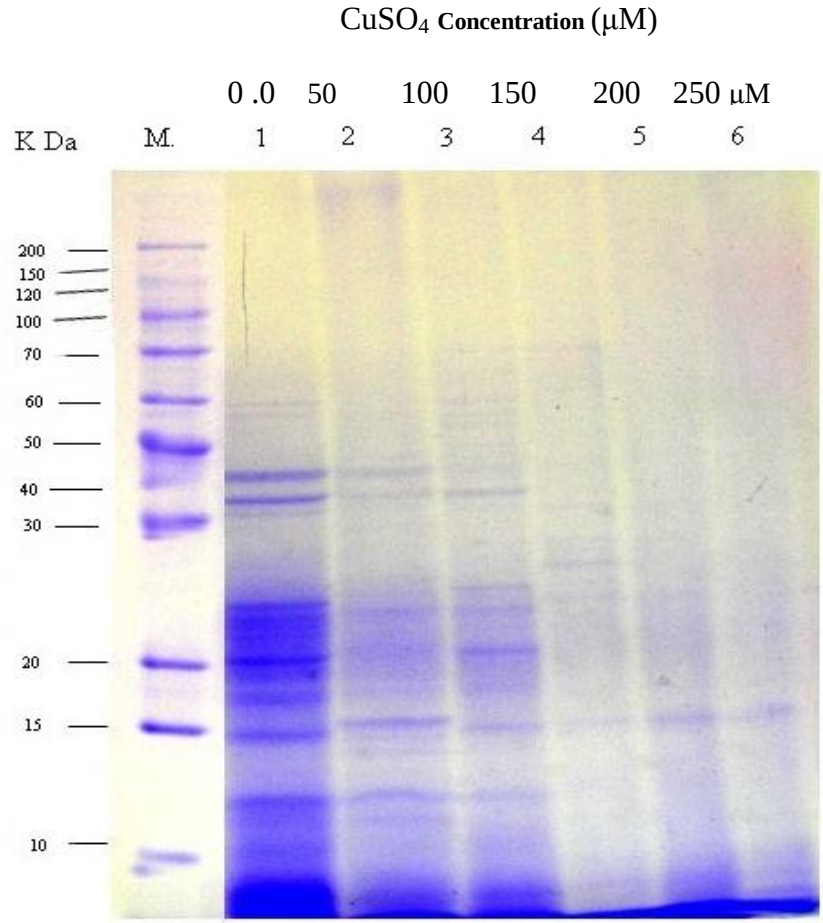

Fig.6: Effect of different concentrations of copper sulphate (µM) on the protein banding pattern of *Cholococcum humicola*. SDS PAGE was used and the gel stained with Comassie-stained. SDS-10% polyacrylamide gel containing the samples treated with different concentrations of copper sulphate (0.0 µM, lane 1; 50 µM , lane 2; 100 µM lane 3, 150 µM lane 4 and 200 µM lane 5, 250 µM lane 6). A left side are the protein molecular weight markers (kDa).

Table (2): Molecular weight (kDa). of new induced and repressed protein bands under copper stress. Data were obtained using total Lab version 1.10 electrophoresis data system program.

Copper conc. (µM)	Induced bands(kDa)	Repressed bands (kDa) .
50	13	57, 50, 36, 30, 22, 20, 18, 10, 9, 5, 3
100	60, 25	57, 50, 36, 32, 30, 22, 19, 18, 17, 10, 7, 5, 3
150	70, 28, 26, 25	57, 50, 36, 32, 23, 22, 20, 19, 18, 17, 10, 9, 7, 5, 3, 1
200	16	57, 50, 36, 32, 30, 23, 22, 20, 19, 17, 15, 10, 9, 7, 5, 3, 2

DISCUSSION

In this work the sub-lethal doses of copper sulphate reduced the dry mass and numbers of cells in the growth media of *Chlorococcum humicola*. In plant and algae the Cu^{+2} effect on growth has been attributed to a massive failure of many cellular processes. The exact inhibitory mechanism of Cu^{+2} on cell division process has been less studied. It is known that Cu^{+2} has toxic effects on chromosomal morphology and mitotic cycle [26].

The data of the present work reveal that, the effect of Cu^{+2} on growth and photosynthetic pigments (chlorophylls and carotenoids) follow nearly thesimilar trends. The concentration of chlorophylls in the cell has been used many times as a parameter to follow the growth of the algal culturs. The negative effect of Cu^{+2}on photosynthetic pigments in algae had been reported by other authers [27, 28, 29, 30]. At high concentrations, it was found that Cu^{+2} inhibits the synthesis of δ-aminolevulinic acid and the photochlorophyllide reductase [31]. Peroxidative of pigments and membrane lipids by reactive oxygen species has been also reported [32].

Inhibition of protein accumulation induced by high concentrations of heavy metals may be attributed to the toxic action of these heavy metals on the enzymatic reactions responsible for protein biosynthesis [33, 34, 35]. In the present work, high accumulation of soluble protein at the high doses of copper was accompanied with a marked and progressive decline in the free amino acids. Thus it might be assumed that, the accumulation of soluble protein could be at the expense of free amino acids. Copper stimulated the incorporation of amino acids into protein as a result of $CuSO_4$ treatments, which indicated the osmo-regulatory role of soluble protein. Proline was also found to be accumulated in this investigation under copper stress. This accumulation might be at the expense of other amino acids. This led us to conclude that, $CuSO_4$ treatments might stimulate the incorporation of free

amino acids into soluble proteins. However, copper stressed *Chlorococcum humicola* used soluble protein, free amino acids and proline for osmo-regulation. This is in agreement with the conclusion of [36]. The mechanisms of proline action are not fully understood, but it has been suggested that increased accumulation permits osmotic adjustment and provides protection for enzymes, biological membranes and polyribosomes [37, 38].

The present results also showed inhibition of N-R activity of *Chlorococcum humicola* by elevated Cu^2 levels. The inhibition of N-R activity in *Chlorococcum* is likely due to impaired NO_3^- uptake in the presence of elevated levels of the test metals. Proteins were not affected with the pronounced drop in the activity of nitrate reductase, especially at higher doses of $CuSO_4$.This might indicate that the two processes (the activity of nitrate reductase and the machinery of protein synthesis did not necessary be linked. This agreed well with the previous reports of other workers [39, 40]. The other possibility could be direct inhibition of N-R activity by the test metals [41].

A positive correlation has been recorded in the trends of phenolic compounds and growth criteria (enhanced at the lowest concentration of $Cu^{2+}$and reduced considerably by further increase), which indicated that the concentration of phenolic compounds might be use suitable criterion for the heavy metal tolerance of *Chlorococcum humicola*. Changes in the antioxidant compounds are much closer to those reported for other macro-algae exposed to various abiotic stresses [42, 43].

The accumulation of high levels of copper in the tissues of *Chlorococcum humicola* was associated with a significant increase in lipoperoxide levels. This result is in agreement with that of [44]. In algae under copper stress, the ascorbate (ASC) was accumulated with increasing $CuSO_4$ concentrations in culture medium. High levels of ASC seem to result from recycling DHA into ASC through DHA reductase activity [45].

The protein electrophoretic patterns of *Cholococcum humicola* treated with low concentrations of copper sulphate did not show any significant changes, compared with control. At relatively high concentious, the induction of low molecular weight proteins might represent a possible tolerance mechanism. This result is in accordance with [46].

CONCLUSION

Chlorococcum humicola isolated from polluted soil with waste water at Sohag district tolerated pollution with copper. The algal cells increased the contents of proline, soluble protein and many new polypeptides with low molecular weight for osmo-regulation. High copper concentrations also

increased lipoperoxidation content and ascorbate. Thus, this alga can be used as an indicator of pollution.

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تأثير النحاس على المركبات النيتروجينية، انزيم مختزل النترات و الأسكوربات فى خلايا طحلب كلوروكوكم هيوميكولا

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في هذا البحث تم عزل طحلب **كلوروكوكم هيوميكولا من تربة ملوثة** بمياه الصرف الصحي في الكولا منطقة سوهاج- مصر . ولقد تم استخدام هذا الطحلب لدراسة تأثير التركيزات المختلفة من كبريتات النحاس على نمو الطحلب و كمية خضاب البناء الضوئى (يخضور أ ، يخضور ب والكاروتينيدات) و المركبات النيتروجينية (الأحماض الأمينية ، البرولين والبروتينات (ذائبة ، وغير ذائبة وكلية) بالإضافة إلي الأنماط البروتينية و نشاط انزيم مختزل النترات ومحتوى الأسكوربات والمركبات الفينولية.

وتمت تنمية هذا الطحلب في وسط غذائي يحتوي علي تركيزات مختلفة من كبريتات النحاس (٥و٠-١٠٠-١٥٠-٢٠٠-٢٥٠ مللي مول). ولقد أدى تركيز ٥و٠ ملليمول الى زيادة النمو ممثلة فى الوزن الجاف من الخلايا ومحتوي خضاب البناء الضوئى). كما انخفض الوزن الجاف ومحتوى هذه الخضاب بزيادة تركيزات النحاس فى المزارع المعاملة. ولوحظ أيضا انخفاض نشاط إنزيم مختزل النترات ومحتوي المواد الفينولية بزيادة تركيز كبريتات النحاس فى المزارع المعاملة. و من ناحية أخري أدت زيادة تركيز كبريتات النحاس الي زيادة أكسدة الليبيدات وزيادة محتوي المالوندای الديهيد بنسبة ١٨٦%. و ازداد محتوى البروتينات الذائبة بنسبة ١٦٦% بالنسبة للعينة غير المعامله عند اعلى تركيز للنحاس فى الوسط ، كما أدت زيادة تركيز كبريتات النحاس خصوصاً تركيز ١٥٠ مللي مول إلي ظهور عدد من البيبتيدات الجديدة و ظهور عدد من الببتيدات ذات الاوزان الجزيئية المنخفضة مثل٢٥، ٢٦،٢٨،٧٠ والمعتقد أنها تزيد من مقاومة الخلية للاجهاد.