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Thiamine and Pyridoxine Alleviate Oxidative Damage by Copper Stress in Green Alga *Chlorella vulgaris*

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THIAMINE and pyridoxine were investigated for their capacity to alleviate oxidative damage by copper stress on a local alga (*C. vulgaris*). Lower levels of Cu^{+2} induced a slight stimulation in growth criteria (growth rate & dry weight), photosynthetic pigments (0.5µM) and O_2-evolution (0.5, 10 µM) of *C. vulgaris* which were inhibited by high Cu^{+2} concentrations. In contrast, O_2-uptake was retarded at the lower Cu^{+2} levels then significantly increased by increasing the Cu^{+2} in algal cultures. Proline, MDA contents, and antioxidant enzyme activity of *C. vulgaris* markedly increased under Cu^{+2} stress. On the other hand, addition of thiamine or pyridoxine alleviated the oxidative damage of Cu^{+2} on *C. vulgaris* growth and enhanced growth, pigment contents, O_2- evolution, and antioxidant enzyme activity in the algal cultures compared to reference controls. While O_2-uptake, proline content, and lipid peroxidation levels were decreased, thiamine or pyridoxine scavenger systems might be important for supporting the ability of *C. vulgaris* to resist copper toxicity.

Keywords: Copper sulfate, Green alga, Thiamine, Pyridoxine, Antioxidant enzyme, Lipid peroxidation, Proline.

Microalgae are sensitive indicators of environmental change and, as the basis of most freshwater and marine ecosystems, are widely used in the assessment of risk and development of environmental regulations for metals (Levy *et al*., 2007). Cu^{+2} is essential for macroalgae, and at low concentrations participates in important biological reactions as an enzymatic cofactor and electron carrier in photosynthetic and respiratory processes. For example, Cu^{+2} is required as a cofactor of superoxide dismutase (EC 1.1.5.1.1) **(**Andrade *et al.*, 2004). Copper can interfere with numerous physiological processes and is considered to be potentially cytotoxic when applied in higher amounts, and its toxicity varies among different macroalgae (Chang & Sibley, 1993). The toxicity of copper is mainly related to free ions and is it a potent inhibitor of photosynthesis in microalgae (Küpper *et al.*, 2002). Excess Cu^{+2} in plant cells may activate molecular oxygen and generate reactive oxygen species (ROS) (Wang *et al*., 2004 and Li *et al*., 2010). Cu-induced generation of hydrogen peroxide, hydroxyl radicals, and other ROS has been directly correlated with damage to membrane lipids and proteins (Gupta & Kalra, 2006). This toxic effect coming from the cellular oxidative state may be allayed by several antioxidative systems such as superoxide dismutase (SOD), catalase and peroxidase (POD) (Joseph & Jini, 2010).

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Peroxidases may remove excess H_2O_2 caused by metal stress. They are involved in several physiological and biochemical processes, such as cell growth and expansion (Fang & Kao, 2000), auxin catabolism (Passsardi *et al*., 2004), and lignification (Brownleader *et al*., 2000).

Proline accumulates in several plants under stress, providing the plants with protection against damage by ROS. Proline plays important roles in osmoregulation (Laliberte & Hellebust, 1989), protection of enzymes (Nikolopoulos & Manetas, 1991), stabilization of the machinery of protein synthesis (Kadpal & Rao, 1985), regulation of cytosolic acidity (Venekemp, 1989), and scavenging of free radicals (Smirnoff & Cumbes, 1989). It also acts as an effective singlet oxygen quencher (Alia *et al*., 2001). Malondialdehyde (MDA) is a cytotoxic product of lipid peroxidation and an indicator of free radical production and consequent tissue damage (Ohkawa *et al*., 1979).

Vitamins are organic compounds that are required in trace amounts to maintain normal growth and proper development of organisms. These compounds act as coenzymes and thus take essential part in the regulation of metabolism. Vitamins, can be limiting factors in plant development (Hassanein *et al*., 2009). Thiamine (vitamin B1) is a necessary ingredient for the biosynthesis of the coenzyme Thiamine pyrophosphate, so it plays an important role in carbohydrate metabolism. It is an essential nutrient for both plants and animals (Kawasaki, 1992). Pyridoxine (vitamin B6) is a water-soluble vitamin and is part of the vitamin B complex group. Several forms of the vitamin are known, but pyridoxal phosphate (PLP) is the active form and is a cofactor in many reactions of amino acid metabolism, including transamination, deamination, and decarboxylation. PLP also is necessary for the enzymatic reaction governing the release of glucose from glycogen.

Materials and Methods

Organism and culture conditions

Axenic cultures of *Chlorella vulgaris* (a unicellular and non-motile green microalga) were isolated from soil of Qena Governorate, Egypt. All experiments were carried out in 500 ml Erlenmeyer flasks containing 100 ml Bold′s basal medium (Bischoff & Bold, 1963),and incubated at temperature 25± 1C°, 30 µE/ m^2/ s (cool white fluorescent light), with 16hr light /8hr dark period. The cultures were supplied with sterilized dry air and CO_2 (97 %:3 %, v/v) for 7 days**.**

Treatments

Chlorella vulgaris Beijer cultured was amended to 200 mg L^{-1} of both thiamine and pyridoxine individually in the absence or presence of various levels of Cu^{+2}: 0.5, 10, 50, 100 and 200 µM in the form of copper sulfate. The control (o) was absolutely Cu^{+2} and vitamin free (only media).

Measurements

Dry weight of cells was taken after filtering and drying overnight at 105ºC.

Determination of the optical density of the green algal suspension was at 560 nm (Wetherel, 1961). The photosynthetic pigments (Chl.a,Chl.b and carotenoids) were determined using the spectrophotometric method recommended by Metzner *et al*. (1965). Oxygen evolution was determined using an oxygen meter (G867 with O_2 electrode). Dark respiration (dark oxygen uptake) was determined as oxygen uptake in the dark, the system mentioned above for the estimating of oxygen evolution was used for estimating dark respiration. At the end of oxygen evolution measurements, all the lights were switched off and the flasks were wrapped tightly in aluminum foil for complete darkness. Proline content was determined according to Bates *et al*. (1973).

Determination of antioxidant enzymes activity

Algae samples were prepared as described in Mukherjee & Choudhuri (1983). A fresh sample (500 mg) was frozen in liquid nitrogen and finely ground with a chilled pestle and mortar, the frozen powder was added to 10 ml 100 mM phosphate buffer (KH_2PO4/K_2HPO_4, pH 7.0) containing 0.1 mM Na_2EDTA and 0.1 g polyvinylpyrrolidone (PVP), the homogenate was filtered through cheese cloth then centrifuged at 15,000 g for 10 min, the supernatant was recentrifuged at 18,000 g for 10 min, and the resulted supernatant collected and stored at 4˚C for assay of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX). 0.5 ml of crude extract used for each enzyme assay.

SOD (EC 1.15.1.1) activity was measured according to Dhindsa & Matowe (1981). CAT (EC 1.11.1.6) activity was measured as described in Aebi (1984) as the decrease of absorbance at 240 nm as a consequence of H_2O_2 consumption, and expressed according to Havir & Mellate (1987). POD (EC 1.11.1.7) activity was determined according to Maehly & Chance (1954). APX (EC 1.11.1.11) activity was determined as the decrease in absorbance of ascorbate at 290 nm as oxidised ascorbic acid (Asada & Chen, 1992).

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using the method of Heath & Packer (1968) as follows: to 2.0ml aliquot of the supernatant 4.0 ml of 0.5% thiobarbituric acid (TBA) in 20 % Trichloroacetic acid (TCA) was added. The mixture was heated at 95˚C for 30min and quickly cooled in an ice bath then centrifuged at 10000 g for 10 min. The absorbance of supernatant was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The MDA content was calculated using its absorption coefficient of 155 nmol cm^{-1} and expressed as nmol (MDA) g^{-1} fresh matter.

Statistical analysis

The data were subjected to analysis by the least significant differences test (L.S.D) using PC-STATE program version 1A, coded by Rao,M: Blane, K and Zannenberg, M, University of Georgia. In each case the data were obtained from triplicate cultures and represented as means ±SD.

Results

Effects of thiamine and pyridoxine on growth rate and dry weight

Table 1 shows that, there are variable results according to the concentration of the Cu^{+2} ions. There were no significant results in the growth rate (as optical density) and dry weight of *C. vulgaris* cultures at 0.5µM Cu^{+2}. Thereafter, increasing the Cu^{+2} concentration in the algal cultures medium reduced growth rate and dry weight about 73% and 71%, respectively, compared to the control, when the level was 200 µM Cu^{+2}.

Thiamine (vit.B1) or pyridoxine (vit.B6) treatments enhanced the growth and dry weight of *C. vulgaris* up to10µM Cu^{+2} and still alleviated the inhibitory effect of Cu^{+2} at 50 µM Cu^{+2}, relative to the reference controls.

TABLE 1. Effects of thiamine and pyridoxine on growth rate and dry weight of *C. vulgaris* grown under copper (Cu^{+2}) stress.

Treatments	Cu^{+2} (µM)	Optical density	%	dry weight µg ml^{-1}	%
Reference control	0	1.35±0.02	100.00	170.67±0.67	100
	0.5	1.38±0.16	102.22	175.79±0.17	103.00
	10	1.17**±0.04	86.66	145.00**±0.37	84.96
	50	0.96**±0.06	71.11	121.84**±0.97	71.39
	100	0.66**±0.05	48.88	95.97**±0.36	56.23
	200	0.37**±0.06	27.40	49.67**±1.42	29.10
Cu^{+2} +200 mg L^{-1} thiamine	0	1.78**±0.04	131.85	210.85**±0.79	123.54
	0.5	1.60**±0.09	118.52	203.16**±0.42	119.04
	10	1.50*±0.06	111.11	171.72±0.63	100.62
	50	1.24±0.12	91.85	161.38±0.88	94.56
	100	0.98**±0.11	72.59	140.73**±0.37	82.46
	200	0.90**±0.01	66.66	119.20**±1.14	69.84
Cu^{+2} +200 mg L^{-1} pyridoxine	0	1.92**±0.05	142.22	221.00**±0.84	129.49
	0.5	1.66**±0.06	122.96	203.89**±0.37	119.46
	10	1.52**±0.15	112.59	173.44±0.62	101.62
	50	1.30±0.04	96.30	162.72±0.36	95.34
	100	1.06**±0.06	78.52	142.77**±0.47	83.65
	200	0.92**±0.08	68.81	116.08**±0.72	68.01
L.S.D	5%	0.12		9.85	
	1%	0.16		13.27	

*Significant differences at (p=0.05) **highly significant differences from control at (p=0.01). ± SD, n=3

Effects of thiamine and pyridoxine on photosynthetic pigments

Table 2 shows that, 0.5µM Cu^{+2} slightly increased in the concentrations of Chl.a, Chl.b, carotenoids and the total pigments. Above 0.5µM Cu^{+2} these values decreased significantly as the concentration of Cu^{+2} increased. The reduction in the Chl.a, Chl.b, carotenoids, and the total pigments were about 78%, 78%, 66 % and76 %, respectively compared to the absolute control.

TABLE 2. Effects of thiamine and pyridoxine on photosynthetic pigments (Chl.a, Chl.b and carotenoids) of *C. vulgaris* grown under copper (Cu^{+2}) stress.

Treatment	Cu^{+2} (µM)	Chlo.a µg ml^{-1}	%	Chlo.b µg ml^{-1}	%	Car. µg ml^{-1}	%	Total µg ml^{-1}	%
Reference control	0	6.04±0.06	100.00	3.11±0.12	100.00	1.91±0.11	100.00	11.06±0.16	100.00
	0.5	6.35±0.05	105.13	3.26±0.32	104.82	2.1±0.17	109.95	11.71±0.19	106.88
	10	5.21**±0.17	86.26	2.64**±0.27	84.89	1.75±0.32	91.62	9.60**±0.38	86.80
	50	4.37**±0.17	72.35	2.12**±0.22	68.17	1.50*±0.15	78.53	7.99**±0.17	72.24
	100	2.59**±0.40	42.88	1.25**±0.12	40.19	1.10**±0.18	57.59	4.94**±1.54	44.67
	200	1.34**±0.16	22.19	0.68**±0.14	21.86	0.65**±0.01	34.03	2.67**±0.15	24.14
Cu^{+2} +200 mg L^{-1} thiamine	0	7.32**±0.09	121.19	4.21**±0.18	135.37	2.72**±0.48	142.41	14.25**±0.58	128.84
	0.5	7.16**±0.37	118.54	3.81**±0.28	122.51	2.55**±0.57	133.51	13.52**±0.95	122.24
	10	6.67*±0.18	110.43	3.51*±0.10	112.86	2.21±0.20	115.71	12.39*±0.44	112.02
	50	5.38*±0.12	89.07	2.70*±0.19	86.82	1.63±0.17	85.34	9.71*±0.23	87.27
	100	4.47**±0.54	74.03	2.13**±0.20	68.49	1.46±0.17	76.44	8.06**±0.29	72.88
	200	2.63**±0.10	43.54	1.21**±0.10	38.91	0.98**±0.07	51.31	4.82**±0.07	43.58
Cu^{+2} +200 mg L^{-1} pyridoxine	0	8.14**±0.13	134.77	4.41**±0.18	141.80	2.66**±0.38	139.27	15.21**±0.53	137.52
	0.5	7.59**±0.31	125.66	4.23**±0.15	136.01	2.37*±0.93	124.08	14.19**±0.56	128.30
	10	7.24**±0.34	119. 87	3.84*±0.28	123.47	2.31±0.15	120.94	13.39**±0.70	121.07
	50	5.78±0.27	95.69	2.91±0.09	93.80	1.84±0.22	96.34	10.53±0.38	95.21
	100	4.73**±0.22	78.31	2.32**±0.29	74.60	1.59±0.08	83.25	8.64**±0.09	72.75
	200	2.95**±0.70	48.84	1.42**±0.10	45.66	1.17**±0.05	61.26	5.54**±0.73	50.09
L.S.D	5%	0.57		0.35		0.46		1.03	
	1%	0.76		0.48		0.62		1.39	

*Significant differences at (p= 0.05) ** highly significant differences from control at (p=0.01). ± SD, n=3

Thiamine (vit.B1) enhanced pigment contents in the algal cultures by about 21%, 35% and 42% for Chl. a, Chl.b and carrotenoids, respectively compared to the reference controls. It could alleviate Cu^{+2} toxicity and enhanced total pigment contents in the algal cultures by 22% and 12% at 0.5 and 10 µM Cu^{+2} , respectively compared to the reference control.

Pyridoxine (vit.B6) treatments alleviated the inhibitory effect of Cu^{+2} and enhanced total pigment contents in the algal cultures by about 28 and 21% at 0.5 and 10 µM Cu^{+2}, respectively, compared to the reference control.

Effects of thiamine and pyridoxine on O_2- evolution, O_2- uptake, proline and MDA contents

Data presented in Table 3 show the changes occurred photosynthetic rate (oxygen evolution), respiration (dark oxygen uptake), proline content and malondialdehyde (MDA**)** content.

Photosynthetic rate (oxygen evolution)

The copper treatment induced insignificant changes in the photosynthetic oxygen evolution up to 10 µM Cu^{+2} and then a highly significant decrease (>60%) by increasing the concentration of Cu^{+2} at 200 µM Cu^{+2}.

TABLE 3. Effects of thiamine and pyridoxine on O_2- evolution, O_2- uptake, proline and MDA of *C. vulgaris* grown under copper (Cu^{+2}) stress.

Treatment	Cu^{+2} (µM)	O_2-evolution mg L^{-1}	%	O_2-uptake mg L^{-1}	%	Proline µg mg^{-1} D.W.	%	MDA nmol g^{-1} F.W.	%
Reference control	0	5.17±0.55	100.00	3.26±0.25	100.0	0.53±0.07	100.00	45.67±0.60	100.00
	0.5	5.84±0.61	112.96	3.17±0.02	97.24	0.55±0.01	103.77	45.98±0.11	100.68
	10	5.22±0.05	100.97	3.12±0.19	95.71	0.60±0.01	113.21	46.81±0.46	102.50
	50	4.01**±0.10	77.56	4.20**±0.15	128.83	0.70**±0.01	132.08	57.07**±0.75	124.96
	100	3.26**±0.06	63.06	4.32**±0.06	132.52	0.85**±0.04	160.38	66.59**±0.56	145.81
	200	1.62**±0.11	31.33	4.61**±0.12	141.41	1.04**±0.06	196.23	78.12**±0.84	171.05
Cu^{+2} +200 mg L^{-1} thiamine	0	6.52**±0.65	126.11	3.25±0.02	99.69	0.51±0.01	96.23	48.01*±0.36	105.12
	0.5	6.11*±0.04	118.08	3.15±0.16	96.63	0.53±0.03	100.00	40.93**±0.72	89.62
	10	5.27±0.33	101.93	2.97**±0.14	91.10	0.49±0.08	92.45	44.32±0.98	97.04
	50	4.70±0.41	90.91	3.21±0.24	98.47	0.58±0.02	109.43	47.53±0.80	104.07
	100	3.84**±0.22	74.27	3.56**±0.05	109.20	0.67*±0.03	126.42	54.52**±1.09	119.38
	200	3.37**±0.16	65.18	3.67**±0.11	112.58	0.74**±0.02	139.62	59.73**±0.35	130.79
Cu^{+2} +200 mg L^{-1} pyridoxine	0	6.76**±0.66	130.75	3.19±0.23	97.87	0.44±0.01	83.02	45.69±0.57	100.04
	0.5	6.18*±0.07	119.54	3.01**±0.01	92.33	0.42*±0.01	79.25	38.48**±0.58	84.26
	10	5.81±0.21	112.38	3.03*±0.22	92.94	0.47±0.01	88.68	44.94±0.57	98.40
	50	4.71±0.40	91.10	3.15±0.27	96.63	0.53±0.01	100.00	45.61±0.58	99.87
	100	4.01**±0.01	77.56	3.00**±0.06	92.02	0.61±0.01	115.09	55.17**±0.85	120.80
	200	3.48**±0.02	67.31	3.60**±0.04	110.43	0.70**±0.03	132.08	60.95**±0.63	133.46
L.S.D	5%	0.76		0.18		0.11		1.91	
	1%	1.02		0.24		0.15		2.57	

*Significant differences at (p=0.05) ** highly significant differences from control at (p=0.01). ± SD, n=3

Respiration (dark oxygen uptake)

In contrast, at lower levels of copper stress, the dark O_2-uptake gradual decreased, thereafter, it progressively increased to be about 41% over control at 200 µM Cu^{+2}.

Application of both vitamins induce a marked stimulation in the O_2-evolution rate till 10 µM Cu^{+2} with advantage to pyridoxine application compared to the corresponding stressed cultures. In contrast, O_2-uptake inhibited at 0.5 -10 µM Cu^{+2} levels, above there was gradual increase in O_2-uptake in *C. vulgaris* cultures compared to the absolute control.

Proline conten

The proline content increased slightly up to 10 µM Cu^{+2}, then a sharply increased (about 97%) as the concentration of Cu^{+2} increased at the level of 200µM Cu^{+2} in relation to the control.

Malondialdehyde (MDA) content

On the other hand, a level of 10 µM Cu^{+2} induced insignificant changes in MDA content of *C. vulgaris* cultures, and then a highly significant accumulation. The highest increase was 71 % over the control value at 200 µM Cu^{+2}.

The supplementary two vitamins resulted in pronounced inhibition in the accumulation of proline and MDA contents compared to algae treated with only Cu^{+2}, whatever the Cu^{+2} level used. Moreover the amount of proline and MDA remained mostly around those of control algae at the level of 50 µM Cu^{+2}. The retarding effect was more pronounced in pyridoxine than in thiamine treated alga in the case of proline.

Effects of thiamine and pyridoxine on antioxidant enzymes activity (SOD, CAT, POD & APX):

Activity of SOD, CAT and POD was markedly and progressively increased by increasing the concentration of Cu^{+2} in the algal cultures. SOD, CAT and POD activities reached maximum values about 179%, 200%, and 173 % of the absolute control, respectively in *C. vulgaris* treated with 200 µM Cu^{+2}. On the other hand, the APX activity remained around the absolute control value at the all levels of Cu^{+2} (Table 4).

TABLE 4. Effects of thiamine and pyridoxine on antioxidant enzymes activity of *C. vulgaris* grown under copper (Cu^{+2}) stress.

Treatments	Cu^{+2} (µM)	SOD unit min^{-1} g^{-1} F.W.	%	CAT unit min^{-1} g^{-1} F.W.	%	POD unit min^{-1} g^{-1} F.W.	%	APX unit min^{-1} g^{-1} F.W.	%
Reference control	0	2.24±0.08	100.00	3.07±0.05	100.0	1.21±0.09	100.00	0.82±0.01	100
	0.5	2.49*±0.12	111.16	3.25±0.04	105.86	1.29±0.06	106.61	0.86±0.01	104.88
	10	2.77**±0.36	123.66	3.43*±0.08	111.73	1.48*±0.03	122.31	0.81±0.01	98.78
	50	3.26**±0.05	145.54	3.81**±0.06	124.10	1.52*±0.01	125.62	0.75*±0.03	91.46
	100	3.53**±0.08	157.59	4.58**±0.05	149.19	1.57*±0.04	129.75	0.77±0.03	93.90
	200	4.00**±0.11	178.57	6.15**±0.04	200.33	2.10**±0.04	173.33	0.81±0.01	98.78
Cu^{+2} +200 mg L^{-1} thiamine	0	2.33±0.07	104.02	3.01±0.09	97.05	1.11±0.10	91.74	0.77±0.01	93.90
	0.5	2.79**±0.18	124.55	3.41*±0.09	111.07	1.39±0.03	114.88	0.89*±0.01	108.54
	10	3.12**±0.13	139.29	3.72**±0.09	121.17	1.64**±0.05	135.54	0.96**±0.03	117.07
	50	3.54**±0.11	158.04	4.54**±0.27	147.88	1.79**±0.03	147.93	1.05**±0.06	128.05
	100	4.01**±0.09	179.02	5.70**±0.13	185.67	1.89**±0.03	156.20	1.28**±0.02	156.10
	200	4.21**±0.15	187.45	6.63**±0.41	215.96	2.07**±0.04	171.07	1.45**±0.02	176.83
Cu^{+2} +200 mg L^{-1} pyridoxine	0	2.27±0.06	101.34	3.11±0.10	101.30	1.19±0.07	98.35	0.78±0.01	95.12
	0.5	3.05**±0.06	136.16	3.73**±0.11	121.50	1.58**±0.05	130.58	0.96**±0.02	117.07
	10	3.31**±0.20	147.77	4.32**±0.21	140.72	1.79**±0.09	147.93	1.07**±0.05	130.49
	50	3.70**±0.19	165.52	4.96**±0.29	161.56	1.92**±0.12	158.68	1.20**±0.02	146.34
	100	4.36**±0.15	194.64	6.03**±0.21	196.42	2.15**±0.05	177.69	1.44**±0.07	175.61
	200	4.60**±0.09	205.36	6.97**±0.06	227.04	2.39**±0.02	197.52	1.59**±0.02	193.90
L.S.D	5%	0.24		0.28		0.27		0.06	
	1%	0.33		0.37		0.36		0.08	

*Significant differences at (p=0.05) level ** highly significant differences from control at (p=0.01). ± SD, n=3

A pronounced additional stimulation in the activities of SOD, CAT, POD and APX was observed as a result of vitamin treatments; especially at high levels of Cu^{+2}. The stimulation effect was more pronounced in pyridoxine than in thiamine treated algae (Table 4).

Discussion

The present study indicated that application of low Cu^{+2} concentration (0.5 µM) to *C. vulgaris* led to slight increases in growth criteria (growth rate & dry weight) and pigment contents. On the other hand, progressive increases in Cu^{+2} concentration caused gradual reduction in these values. Such biphase response to copper was also revealed by other investigators (Deef, 2007 and Gao *et al*., 2008).

The reduction in growth could be due to inhibition of normal cell division by the metal, as has been reported for *Spirulina platensis*-S5.exposed to copper (Choudhary *et al*., 2007). The decrease in the rate of cell division caused by metals is primarily attributed to their binding to sulfhydryl groups which are important for regulating the plant cell division (Visviki & Rachlin, 1991).

Three reasons may be responsible for the inhibitory effect on Chl. a, Chl. b and carotenoids seen in excess Cu^{2+}. First, Cu^{2+} probably induces production of reactive oxygen species and inhibits the reductive steps in the biosynthesis pathway of these pigments (Clijsters *et al*., 1999). Second, Cu^{2+}can directly destroy the structure and function of chloroplast by binding with SH group of enzymes and overall chlorophyll biosynthesis (Singh, 1995). Third, it may activate pigment enzyme and accelerate the decomposition of pigment (Hou *et al*., 2007). Moreover, carotenoids appeared to be more resistant to Cu^{2+} phytotoxicity than Chl.a and Chl. b (Li *et al*., 2010).

Supplementary thiamine or pyridoxine resulted in a considerable increased in growth criteria (rate & dry weight) and pigment contents of the tested alga and thus partially alleviated the toxic effects of Cu^{+2} as compared to the reference controls (Hamada, 2001 and Desouky *et al*., 2011).

In the present study the effect of different concentrations of Cu^{2+} on photosynthetic O_2 evolution showed a tendency towards reducing the amount of O_2 evolved by test alga in response to Cu^{2+}. However, an increase in O_2 evolution by *C. vulgaris* was observed at lower Cu^{2+} concentrations. The magnitude of the inhibitory action was found to increase with higher metal concentrations.

It is clear that the photosynthetic process depends on the content of pigments, which had been inhibited at the higher concentrations of Cu^{2+}. Moreover, increased generation of reactive oxygen species induced by this metal can induce membrane lipid peroxidation and increase unstacking of thylakoids in *Scenedesmus incrassatulus* (Perales-vela *et al*., 2007).

Respiration (dark O_2 -uptake) of *C. vulgaris* cultures gradual decreased at lower Cu^{+2} levels, then a highly significant increased by increasing the Cu^{2+} in alga cultures (Andrade *et al*., 2004).

Application of both vitamins (either thiamine or pyridoxine) induces a marked stimulation in O_2-evolution compared to the reference controls. In contrast, O_2-uptake was retarded at the lower and moderate Cu^{+2} levels, and then activated at the higher levels compared to the absolute control (Desouky, 2011).

The result suggested that accumulation of proline responds to Cu^{+2} and it varies with respect to the toxicity caused by Cu^{+2} treatment. Proline accumulation prevents membrane distortion and acts as a hydroxyl radical scavenger. Mehta & Gaur (1999) also note a protective role of proline in mitigating metal-induced lipid peroxidation in *C. vulgaris*. Thus greater accumulation of proline under high Cu^{+2} level of the present study suggested the protective role of proline to the alga to survive Cu^{+2} stress (Choudhary *et al*., 2007).

Our results indicate that concentrations of Cu^{2+} increases oxidative damage as is evident from increased lipid peroxidation. Thus, the increased level of MDA suggests that metal ion stimulate free radical generating capacity of the microorganism. It is accordance with the previous findings (Thounaojam *et al*., 2012) that MDA accumulated greatly after the exposure of Cu^{+2} and cell membrane is the primary site of Cu^{+2} toxicity. It might be due to the overproduction of ROS under Cu^{+2} stress, which is highly destructive to cell membrane.

Increase in both proline and MDA contents with increasing Cu^{+2} concentrations are indicative of a correlation between free radical generation and proline accumulation. Our study also depicted an inverse relationship between growth criteria and proline accumulation in the test alga under Cu^{+2} oxidative stress (Fig.1a, b). This might involve reduction in cell division or delay of exponential growth due to proline accumulation (Maggio *et al*., 2002).

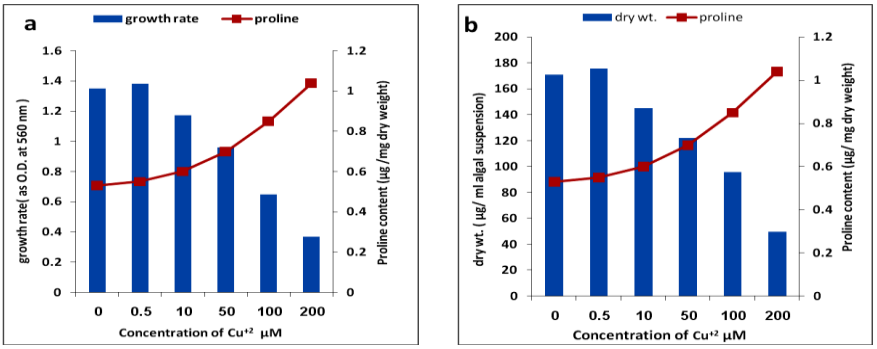

Fig.1. Correlation between proline accumulation and growth criteria (growth rate a and dry weight b) of *C.vulgaris* under Cu^{+2} stress.

Antioxidant enzymes play important roles in defense mechanisms may provide a strategy to enhance oxidative stress tolerance. In the present study Cu^{+2}

treatment resulted in a marked and progressive increase in the activities of SOD, CAT & POD, which can be considered as indicators for evidence of enhanced production of reactive oxygen species, such as the superoxide radical (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-) under Cu^{+2} stress (Verma *et al*., 2011 and Thounaojam *et al*., 2012). On the other hand, the APX activity remained around the absolute control value at the all levels of Cu^{+2}.

Stimulation of antioxidant enzymes reflects the ability of the *C. vulgaris* to withstand the Cu^{+2} induced oxidative stress. Proline accumulation also appears to be an additional defense against Cu^{+2}oxidative stress.

Thiamine or pyridoxine treatments induced pronounced inhibition in the accumulation of proline and MDA content when compared to algae treated with only Cu^{+2} (Al-Hakimi & Hamada, 2011). This confirmed the alleviating capacity of these vitamins on the algal growth. In contrast, antioxidant enzymes activities increased markedly, thus improving alga resistance.

Conclusion

The growth of *C. vulgaris* appears biphase response to copper and exogenous thiamine or pyridoxine partially alleviated the toxic effects of Cu^{+2} in the growth criteria by promoting photosynthetic rate and antioxidant enzymes activities (SOD, CTA, POD & APX) which are associated with a marked retardation in proline and MDA contents, and consequently stimulate the alga growth.

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استخدام الثيامين و البيريدوكسين لتخفيف ضرر التأكسد الناتج من زيادة تركيز أيون النحاس في مزارع طحلب *الكلوريلا فولجاريس*

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يهدف البحث الى دراسة مدى قدرة الثيامين او البيريدوكسين (فيتامين ب١وب٦ ٢٠٠ مللى جرام فى اللتر) لتخفيف ضرر تأكسد أيون النحاس في مزارع طحلب *الكلوريلا فولجاريس* المعزولة محليا . أوضحت الدراسة زيادة طفيفة فى معدل النمو والوزن الجاف و المحتوى الصبغي (كلوروفيل أ , كلوروفيل ب والكاروتين) وكمية الأكسجين المتصاعد لطحلب الكلوريلا عند مستوى ٥, ميكرو مول من أيون النحاس, كما تأثرت هذه القيم بشدة أعلى من هذا المستوى, أوضحت الدراسة انخفاض كمية الأكسجين المستهلك في الظلام تحت المستوى المنخفض على العكس من المستويات المرتفعة التى أدت إلى زيادة كمية الأكسجين المستهلك. وهناك زيادة واضحة فى محتوى الحمض الأميني برولين ومحتوى المالوندای الدهيد وأنشطة إنزيمات مضادات الأكسدة مع زيادة أيون النحاس فى الوسط الغذائى. أدت المعاملة بفيتامين ب١وب٦ إلى إزالة او تخفيف الاثر السام لأيوان النحاس مع زيادة واضحة فى النمو و المحتوى الصبغي وكمية الأكسجين المتصاعد ونشاط إنزيمات مضادات الأكسدة, بينما انخفضت كمية الأكسجين المستهلك ومحتوى كلا من الحمض الأميني برولين و المالوندای الدهيد مقارنة بمثيلاتها المعاملة فقط بالنحاس.