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Response of tolerant and wild type strains of *Chlorella vulgaris* to copper with special references to copper uptake system

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Summary

Copper tolerance in *Chlorella vulgaris* has been studied by comparing physiological properties and copper uptake in a wild type strain and a copper tolerant one. A concentration-dependent reduction in growth rate, dry mass, and content of chlorophyll a, protein, sugars and amino acids was noticed in both strains at 1.0 and 400 μg l^{-1} copper. The reduction in all parameters was higher in the wild type strain than in the tolerant one. As compared to the wild strain, the tolerant strain showed insignificant loss of potassium and sodium. The data on proline accumulation showed a positive relationship between copper toxicity and proline accumulation in both strains, more pronounced in the tolerant strain. The copper uptake was influenced by the copper concentrations the algae had been exposed to during their previous growth: the lower the copper concentration in the culture medium, the higher the activity of the uptake and the capacity of the cells to accumulate copper.

Key words: *Chlorella vulgaris*, copper, tolerance, uptake

Introduction

Tolerance to heavy metals in different organisms, including algae, may develop either during growth in metal-contaminated natural habitats or through successive cultivation at elevated doses of heavy metals in the laboratory (Rai et al., 1991). Cases of acquired resistance to copper in algae growing in Cu-contaminated freshwater habitats are numerous (Rai et al., 1991; Fathi and Falkner, 1997; Lombardi and Vieira 1998; Fathi and El-Shahed, 2000; Fathi, 2002; Backor et al., 2003; Bossuyt and Janssen, 2004).

Copper is an essential trace element for plants, because it participates in photosynthetic electron transport and plays a role as cofactor of several oxidizing enzymes. However, similarly to many trace elements, copper is toxic at higher concentrations, resulting in an inhibition of electron transport (Shioi et al., 1978), inhibition of photosynthetic pigments (Fathi et al., 2000), and decrease in intracellular potassium and

sodium concentrations (De Filippis, 1979). A lack of copper, on the other hand, can partially be compensated, e.g. by substitution of phycocyanin by cytochrome c (Sandmann and Boger, 1980).

In the present paper we investigate copper tolerance in the green alga *Chlorella vulgaris* (isolated from the River Nile) by comparing a wild type and a tolerant strain exposed to different copper concentrations with special references to their copper uptake systems.

Material and methods

Chlorella vulgaris Beyerinck was isolated from the River Nile at El-Minia (Egypt). The alga was grown in Kuhl's medium (Kuhl 1962), modified as follows: copper was eliminated, as were all known chelators (ferric citrate, citric acid, and Na-EDTA), and trace metal levels were reduced to 1/20th of the original amount proposed by Kuhl. The concentration of copper (provided to the medium as copper sulfate) was 0.5 μg l^{-1} (as in the river water). In order to simulate the natural situation, when phosphate needed for growth is not always available and therefore algae change between phosphate deficient and non-deficient states, cultivation was performed in a discontinuous mode using 200 ml medium that contained 10 μM phosphate. Every 24 hours, 100 ml of the algal cultures were removed and replenished by fresh medium again containing 10 μM phosphate. After dilution, phosphate was rapidly incorporated by the remaining algae and stored in form of polyphosphates, which served as an endogenous phosphorous source for growth. Cultures were grown at 27 ± 1.0° C at a 16 h light: 8 h dark cycle with a light intensity 70μ mol $m^{-2}s^{-1}$. Seven days cultures (optimal growth period) were diluted under aseptic conditions to provide stock cultures. All solutions were prepared using Millipore membrane filter 0.45μM (Schleicher and Schüll, Germany). All glassware was soaked for 24h in 10-15% nitric acid, rinsed in distilled water, and air-dried before use.

A copper tolerant strain of *Chlorella vulgaris* was isolated by successive culturing on agar plates (Kuhl's medium) containing different doses of copper (0.05 to 0.5 mg dm^{-3}), as described by Whitton and Shehata (1982). Colonies appearing on plates containing 0.4mg dm^{-3} copper were picked up and transferred repeatedly to agar plates with the same concentration of copper. Colonies growing successfully on such plates and also in liquid medium containing 0.4 mg l^{-1} copper were designated as the copper tolerant strain. When this tolerant strain was subcultured in the basal medium devoid of copper and then transferred to the medium with 0.4 mg l^{-1} copper, a gradual loss of tolerance in the strain was noticed after every successive generation (Rai et al., 1991). Hence, the strain isolated was not a spontaneous mutant but a physiologically adapted one.

A standard initial inoculum of the wild type and the tolerant strain was inoculated to culture flasks (250 ml each) that contained 50 ml of nutrient medium supplied with 1.0 and 400 μg l^{-1} copper and basal medium as a control. At the end of incubation period (7 d), cultures were filtered and washed several time with copper free medium for different measurements. At least three replicates for each sample and control were used.

The number of cells was determined using a Haematocytometer chamber. Pigments were estimated in acetone extract according to Metzner et al. (1965). Sugar content was determined by the anthrone method (Roe, 1955), using glucose as a standard. Total amino acid content was determined according to Moore and Stein (1948). Total protein was measured according to Lowry et al. (1951). Proline content of cells was estimated in aqueous extract using the acid ninhydrin method described by Bates et al. (1975). The K^+ and Na^+ present inside the cells were released by heating the washed pellets in boiling water bath. Cells were then removed by centrifugation and the amounts of K^+ and Na^+ were determined with the help of a Clinical flame photometer Corning 41° C (Corning Science Products, Halstead, England) according to Rai et al. (1991).

Copper uptake by algal cells was measured according to Fathi and Falkner (1997) by a Perkin-Elmer Atomic Absorption Spectrometer with graphite furnace (Model 4000, Norwalk, USA). The amount incorporated was calculated from the decrease of the copper concentration in the incubation medium, divided by the cell number and expressed in attograms $Cell^{-1} = 10^{-18}$ g $cell^{-1}$. The values calculated were the mean of triplicates, the standard deviation was less than 5% of these mean values. The copper content of the algae was determined after digestion of the washed and dried material for 15 min in a boiling mixture of HNO_3 and HCl (1:1 v/v).

Results and Discussion

The data in Table 1 show that copper supplementation severely reduced growth rate, dry mass, chlorophyll a, protein, sugars and amino acids content of the non-tolerant strain of *Chlorella vulgaris* in a concentration-dependent manner. However, the impact was insignificant in the tolerant strain. A concentration-dependent reduction in the parameters investigated (Table 1) is in good agreement with the previous findings (Fisher and Frood, 1980; Sato et al., 1986; Rai et al., 1991; Fathi and El-Shahed, 2000).

The results of this investigation clearly show that increased metal concentration depresses the photosynthetic activity of the two strains of *Chlorella vulgaris*, causing lower Chl-a content especially in the non-

Table 1. Effect of 1.0 and 400μg/l copper on growth rate, dry mass, the content of Chl-a, amino acids, protein, sugars and proline , as well as the potassium and sodium contents in the wild type and the copper-tolerant strain of *Chlorella vulgaris* measured after 7 days treatment. Mean of three replicates ± SE. Data in parentheses denote percentage of inhibition.

Strain	Non-tolerant			Tolerant		
Copper conc. (μg/l)	Cont.	1.0	400	Cont.	1.0	400
Growth rate (division /d)	0.51 ± 0.01	0.25 ± 0.00 (51.00)	0.02 + 0.00 (96.08)	0.65 ± 0.00	0.57 ± 0.01 (12.31)	0.55 ± 0.02 (15.38)
Dry mass (mg l^{-1} medium)	42.61 ± 0.10	20.51 ± 0.20 (52.00)	6.32 ± 0.01 (85.20)	45.33 ± 0.20	38.64 ± 0.10 (14.75)	38.00 ± 0.20 (16.17)
Chl-a (μg/ mg dry mass)	2.47 ± 0.02	1.66 ± 0.02 (32.80)	0.20 ± 0.00 (91.90)	5.56 ± 0.05	5.50 ± 0.04 (1.08)	5.50 ± 0.04 (1.08)
Amino acids (% dry mass)	34.31 ± 0.04	25.00 ± 0.01 (27.13)	10.61 ± 0.01 (69.07)	38.00 ± 0.02	36.82 ± 0.02 (3.10)	35.56 ± 0.02 (6.42)
Protein (% dry mass)	31.42 ± 0.10	15.33 ± 0.20 (51.21)	5.21 ± 0.01 (83.42)	31.02 ± 0.20	28.60 ± 0.02 (7.80)	27.75 ± 0.10 (10.54)
Sugars (% dry mass)	80.53 ± 0.05	33.00 ± 0.02 (52.81)	12.60 ± 0.01 (84.35)	82.20 ± 0.25	78.71 ± 0.02 (4.24)	78.06 ± 0.20 (5.04)
K^+ (g/g protein)	0.36 ± 0.03	0.12 ± 0.00 (66.70)	0.02 ± 0.00 (94.45)	0.39 ± 0.00	0.35 ± 0.00 (10.25)	0.36 ± 0.00 (7.69)
Na^+ (g/g protein)	0.25 ± 0.01	0.05 ± 0.00 (80.00)	0.02 ± 0.00 (92.00)	0.28 ± 0.00	0.25 ± 0.00 (10.71)	0.25 ± 0.20 (10.71)
Proline (x 10^{-8} ng/cell)	6.61 ± 0.02	8.22 ± 0.20	10.00 ± 0.02	21.53 ± 0.02	28.00 ± 0.02	30.12 ± 0.01

tolerant strain. This suppression can be attributed to inhibition of reductive steps in chlorophyll biosynthesis in algae or to the severe interaction interference with the transport chain, water splitting site and oxireductase being the most sensitive steps (De Filippis, 1981; Hofner et al., 1987). In addition, Singh and Singh (1992) reported that photoautotrophic growth of *Nostoc calcicola* was severely inhibited, with concurrent loss of photosynthetic pigments (Phycocyanin < Chlorophyll a < Carotenoids).

The data in Table 1 further show that copper treatment resulted in decreased Chl-a content in both strains, coupled with reduced sugar and protein content. The decrease in sugarcontent perhaps contributed to suppression of protein accumulation by shortage of carbon skeleton. However, the suppression of protein accumulation may be attributed to shortage of carbon skeleton resulting from low photosynthetic rate. Our results are in accordance with those of Khalil (1997), Fathi et al., (2000) and Okamoto et al., (2001).

As compared to Na^+, the loss of K^+ was more pronounced in both strains. However, the loss was more severe in the non-tolerant strain than in the tolerant one in case of Na^+. The low percentage of potassium and sodium in the cells of the tolerant strain after copper treatment supports the opinion that tolerance in the test alga is achieved through change in the permeability of the plasma membrane (De Filippis, 1979).

Table 1 shows a positive relationship between copper toxicity and proline accumulation in both strains, more pronounced in the tolerant strain. This relationship could be attributed to the protective role of proline against heavy metal toxicity. In this context, suggestions have been made that proline provides protection by maintaining the water balance, which is often disturbed by heavy metals (Schat et al., 1997), scavenging hydroxyl radicals (Smirnoff and Cumbes, 1989), chelating heavy metals in the cytoplasm (Farago and Mullen 1997), reducing metal uptake (Wu et al., 1998) and forming metal-proline complexes (Fathi and Zaki, 2003).

Table 2. Growth rate, copper content and uptake rate of the wild type and the tolerant strains of *Chlorella vulgaris* under steady-state growth conditions.

Parameters	Wild type	Tolerant strains
Growth rate (h^{-1})	0.021	0.027
Copper content (ag. Cell $^{-1}$)	1863	15500
Uptake rate (ag. Cell $^{-1}$)	39.12	418.5

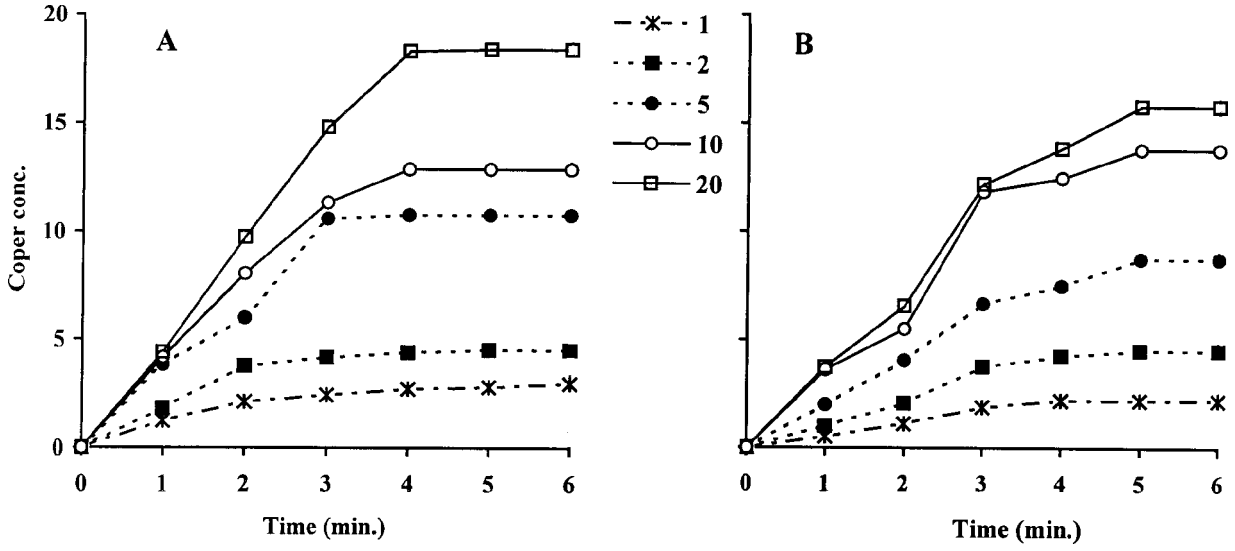

Fig. 1. Influence of the copper concentrations (1, 2, 5, 10 and 20 μg/l) on the time course of copper uptake (ag. Cell $^{-1}$) of wild type (A) and tolerant strain (B) of *Chlorella vulgaris*.

Table 2 shows that the copper content in the cells of the tolerant strain is much higher than in the cells of the wild type. Furthermore, under steady-state growth conditions the copper uptake rate (V_{Cu}) is higher in the tolerant strain than in the wild type one, which is proportional to the growth rate. The steady-state uptake rate was calculated according to the following equation (Droop, 1973): $V_{Cu} = \mu \times Q_{Cu}$ where μ is growth rate and Q_{Cu} is the copper content in the cells.

When algae were washed with a copper-free medium and then exposed to different copper con-centrations in the incubation medium employed for uptake experiments, both the initial velocities and the amount of copper absorbed during exposure to elevated copper concentration were related to the copper concentrations the organisms had experienced during preceding growth (Fig. 1). The higher the copper concentration in the growth medium, the lower the observed rate of net uptake under the experimental conditions. The simplest explanation for this dependence of uptake rates on the former copper levels in the culture medium is that a decrease of the copper concentration during growth leads to the activation of copper uptake system that catalyses the transport of this element into the cell. In this case, the uptake process should exhibit some type of saturation kinetics that is often analysed on the basis of a Michaelis-Menten hyperbola. For such an analysis, a double reciprocal plot of the concentration dependence of initial influx rates must follow a straight line. Our data, however, usually did not follow this function. The deviation from Michaelis-Menten equation observed is not surprising, since this equation adequately describes enzyme-catalysed processes only when initial unidirectional influx rates are measured and the product concentration is zero or when the free energy of product formation is very negative (Fathi and Falkner, 1997).

An alternative approach that is more suitable for the analysis of the concentration dependence of net fluxes of mineral salt uptake close to equilibrium was proposed by Thellier (1970; for a more elaborate presentation see Thellier et al., 1993). This approach, which is a force-flow description of cellular uptake derived from non-equilibrium thermodynamics, was also employed in the present study. A Thellier plot of uptake data obtained with the wild type and the tolerant strain followed straight lines that intercepted the log copper concentration axis at the concentrations 0.28 and 0.47 μg dm^{-3} for the wild type and tolerant strains, respectively (Fig. 2). The concentrations mentioned are those below which the net uptake of copper becomes negative and growth ceases. A Thellier plot of the concentration dependence on the rate of copper absorption showed that during the physiological adaptation to elevated copper concentrations the uptake system adopted characteristic properties, so that there was an extended range of validity of the flow-force relationship (Fathi and Falkner 1997; Fathi and El-Shahed, 2000). Generally, the data of the uptake experiments show that copper toxicity for algae depends on the environmental copper levels the algae have experienced during preceding growth. Therefore, a border line between toxic and non-toxic copper concentrations cannot be given in terms of fixed, species-dependent parameters, but is rather a function of the copper concentrations the algae are capable to adapt to during growth.

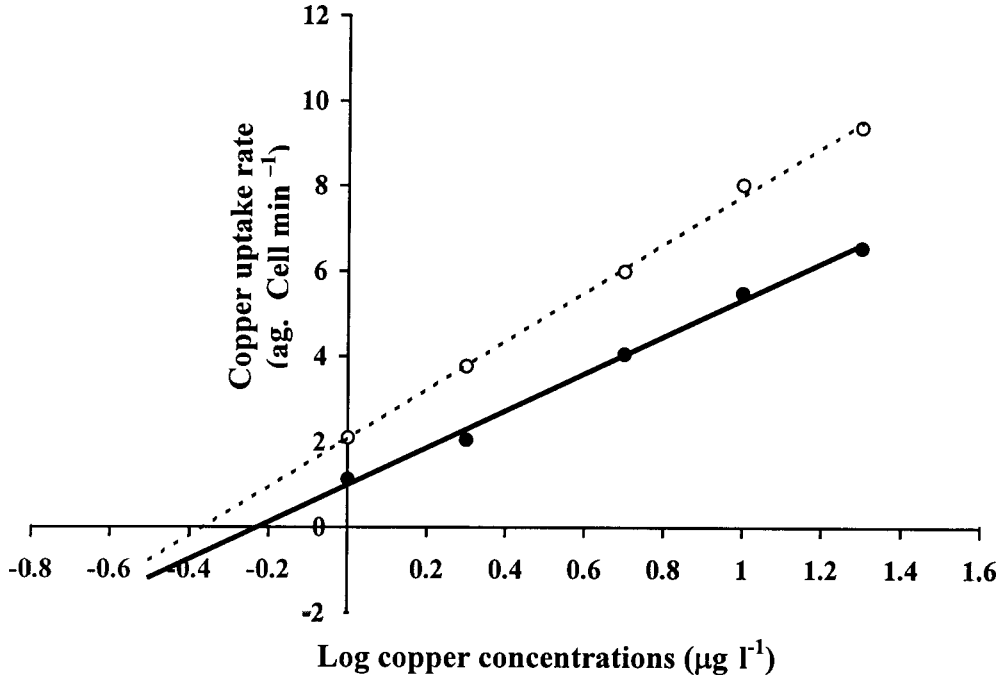

Fig. 2. Thellier plot of the initial velocities of copper influx, observed with the wild type (open circles) and tolerant strain (closed circles) of *Chlorella vulgaris*.

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