GLUTATHIONE-INDUCED RECOVERY IN *CHLORELLA* CELLS FROM METAL TOXICITY

Drora Kaplan[1], N.Tejo Prakash[2#], Aaron Abeliovich[1]

1. Environmental Microbiology Unit, Jacob Blaustein Desert Research Institute, Ben-Gurion University of the Negev, Sede-Boker Campus - 84990, Israel.
2. Biotechnology Division, Thapar Corporate Research and Development Centre, Patiala - 147 001, India.

SUMMARY

A number of ecotypes of plants and algae with increased tolerance to heavy metals have been isolated from sites that are polluted with metals leading to their application in bioremediation of contaminated ecosystems. Glutathione is a key component involved in development of resistance/tolerance. The present study was focused on identifying the inhibitory concentrations and effects of copper, nickel and zinc on the growth of *Chlorella* sp. and recovery of cells from metal toxicity in the presence of reduced glutathione (GSH). The observations made during the study showed that toxicity of copper, nickel and zinc is ameliorated in the presence of GSH. GSH was also observed to indirectly facilitate the recovery of functional viability (as indicated by chlorophyll content) of the cells.

INTRODUCTION

Metals are known to alter physiological functions of algae when the concentrations in the environment rises beyond the thereshold levels. Metals of interest in this study namely copper, nickel and zinc were known to be toxic to algae. Copper-exposed cells were observed to be disorganized resulting in irreversible damage to cell organelles, and cells failed to separate during division[1,2]. The toxic mechanisms of zinc includes alteration of the permeability of cell membrane, which leads to sharp reduction of potassium and sodium content of the cell, followed by inhibition of photosynthesis, nitrogen fixation and finally cell multiplications[2,3]. Though, the biological effects of nickel toxicity have not been elucidated, nickel was found to inhibit growth, affect flagellar movement and alter physiological properties in some species of algae[4,5].

With the result of natural processes and increasing heavy metal pollution due to

permissible, illegal and accidental discharges, plants have evolved efficient means of tolerance and detoxification of metals[6,7]. An important component in the mechanism of metal detoxification is glutathione (GSH). It is involved in defence against free radicals and reactive oxygen species[8]. GSH is also the substrate for synthesis of phytochelatins (metal-binding peptides) and the synthesis shares a common enzymology with GSH[9,10]. Phytochelatins synthesis induced by cadmium is associated with rapid depletion of total glutathione in plant cells[11] and whole plants[12]. Recent studies have also shown that addition of glutathione ameliorated growth inhibition caused by cadmium in algae and plant cell cultures and is presumed to be utilized in phytochelatin synthesis[13,14].

Based on the above observations, the present study was focused to:

(a) to determine the recovery pattern of *Chlorella* cells affected by sub-lethal concentrations of copper, zinc and nickel, in the presence of glutathione; and

(b) to estimate the accumulation of metals (copper and nickel) and variations in chlorophyll (for zinc) in affected and recovered cells.

MATERIALS AND METHODS

The *Chlorella* sp. used in this study was isolated from a stabililizing pond of domestic waste water treatment plant located at Beer-Sheva, Israel. Axenic cultures were grown in BG11 mineral medium[15], without EDTA with continous illumination of 150m mol. m^{-2} s^{-1}, at the surface of the flasks provided by cool white fluorescent lamps. All the experiments were performed in 250ml Erlenmeyer flasks containing 100ml of the medium, placed on a gyratory shaker (180 rev.min^{-1}) at 27^{0}C. Cells were counted using Neubauer haemocytometer and corresponding growth was monitored by measuring changes in turbidity at 540 nm. Cultures at exponential phase of growth were used for all the experiments.

All chemicals used were of analytical grade. Metals were added as copper sulphate ($CuSO_4.5H_2O$), zinc ($ZnSO_4$, H_2O) and nickel chloride ($NiCl_2.6H_2O$). The metal concentrations examined in this study were - control (0.32μM), 6.42μM and 12.84μM of $CuSO_4.5H_2O$ for copper; control (0.77μM), 15.32μM and 30.64μM $ZnSO_4.7H_2O$) for zinc; and control (no nickel) 20μM and 40μM of $NiCl_2.6H_2O$ for nickel.

One millilitre of cell suspension (~1 x 10^6) from cultures at exponential phase of growth

were inoculated into 100ml of the medium supplemented with the above mentioned concentrations of individual metals. Cell density of the samples (in duplicate) of each treatment was determined at the time of inoculation and each day thereafter by measuring the turbidity (540nm).

Growth rate was calculated by the following equation : $k=(\ln X_n - \ln X_1)/(T_n - T_1)$, where k is the specific growth rate of the algal culture, X_n is the number of cells on the day n (T_n) and X_1 is the number of cell on the first day after inoculation. The cell count was started from the first day after allowing the cells acclimatize.

For the recovery studies, glutathione (GSH) (from Sigma) was added to growth-inhibited cells of Cu, Zn and Ni exposure. The concentrations used were 50μM of GSH to the cells exposed to metals at low concentrations (Cu: 6.42μM; Zn: 15.32μM; Ni: 20μM) and 100μM to the cells exposed to high concentrations (Cu: 12.84μM; Zn: 30.64μM; Ni: 40μM). GSH was added after inhibition of growth was observed i.e., after 4-5 days of exposure, with an assumption that a significant amount of metals added is absorbed by the cells preventing extracellular sequestration of metals by GSH.

Block heater extraction method[16] was followed for extraction of metals. Cells were harvested by centrifugation and transferred to porcelain crucibles. The crucibles were ignited at 550°C in a muffle furnace for 60 min. On cooling, the contents were lightly scrapped from the walls of crucibles using teflon-coated blades and transferred to Corning tubes using 5ml of 3N HCl. The tubes were placed in an aluminium block heater and heated at 120°C for 2 hr. The tubes were then removed and the content of each of them was filtered through 0.45μm (Whatman) into a 25ml volumetric flask. The contents were brought to volume using double distilled water. The clarified media were also collected during centrifugation of cells and filtered through 0.45μm. Metal concentrations in cells, clarified medium and freshly prepared media were determined using Perkin-Elmer (AA 1100B) spectrophotometer after a pre-check on percent recovery of metals.

Pigments were extracted using N,N-dimethylformamide and total chlorophyll was calculated following the method given by Moran[17].

RESULTS AND DISCUSSION

The concentrations of copper, zinc and nickel were selected after testing a wide range of metal levels as indicated in the previous section. Growth rate of cells in the presence of increasing

concentrations of copper, nickel and zinc with and without exogenous GSH are presented in table I, II and III. In the present study, in general, for the cells exposed to the lower concentrations of metals (*viz.*, 6.42μM - copper; 20μM - nickel and 15.32μM - zinc) growth rate was 50-80% of the control cells. Growth rate of cells exposed to the higher concentrations (*viz.*, 12.84 μM- copper, 40μM - nickel and 30.64 μM zinc) were less that 10% of the control. Growth was completely inhibited in cells exposed to higher concentrations. These observations are supported by reports indicating prolonged lag phase and inhibition on exposure on sublethal doses of copper and nickel in *Chlorella vulgaris* and other *Chlorella* sp[18,19].

Variations in the chlorophyll content were estimated specifically for the cells exposed to zinc (lower concentrations of 15.32μM and higher concentrations of 30.64μM - zinc) as the alterations in the growth (on metal exposure and on glutathione addition) were not clearly evident. Chlorophyll content was taken as an indicator of functional viability of zinc-exposed cells.

Addition of glutathione (GSH) (on day 4-5) to the growth inhibited cells showed about 70-90% recovery (of the control) of growth upon addition of 50μM GSH for lower concentrations of metals (Cu and Ni) and 30-50% of the control upon addition of 100μM GSH to cells exposed to higher concentrations. In case of zinc-exposed cells (table III), the variations in total chlorophyll content between control and treated cells showed increase in the recovery cells when compared to the affected. The results (table I, II and III) are supported by evidences in the previous reports on GSH influence on growth inhibition by metals[13,14]. Mendum and co-workers[13] reported that exogenous glutathione increased the rate of phytochelatin (PC) accumulation in reponse to cadmium and also indicated that growth of tomato cells, in the presence of metals, depends upon ability of cells to synthesize PCs and similar observations were also noted in *Rubia tinctorum*[14]. Therefore, from the results obtained it may be assumed that exogenous GSH may be utilized by cells for phytochelatin synthesis.

The results have shown increased metal (Cu and Ni) uptake in the recovered cells (with exogenous GSH) when compared to affected cells (without exogenous GSH). Corresponding changes in metal levels of clarified media from Cu exposure could not be clearly indentified because of limitations in analytical sensitivity. In the case of nickel and zinc (table III), the low levels in clarified medium of glutathione-added cultures support the assumption of increased metal uptake by the cells in the presence of glutathione. Exogenous GSH may immobilize excess

intracellular metal ions by direct sequestration or thorough formation of phytochelatin, thus, resulting in further accumulation of metal ions from the ambient medium.

Table I: Growth rate, cell number and metal concentration in copper exposed cells with(+G) and without exogenous GSH.

Treatment (µM)	Growth rate	Growth rate (%)	Cell number (x 10^6)	Metal Concentration		
				Cells [µg (x 10^{-5}) /cell]	*Clarified medium (µg/L)*	*Fresh medium (µg/L)*
Control (0.32)	0.629	100.0	21	1.3	236	113
6.42	0.093	14.8	2	4.7	246	152
6.42 (+50G)	0.465	73.9	12	7.9	234	-
12.84	0	0	-	-	-	270
12.84(+100G)	0.201	31.9	3	40.8	430	-

Table II: Growth rate, cell number and metal concentration in nickel exposed cells with(+G) and without exogenous GSH.

Treatment (µM)	Growth rate	Growth rate (%)	Cell number (x 10^6)	Metal Concentration		
				Cells [µg (x 10^{-5}) /cell]	*Clarified medium (µg/L)*	*Fresh medium (µg/L)*
Control (0)	0.608	100	22	2.8	340	260
20	0.481	69	19	5.3	445	543
20 (+50G)	0.506	73	21	6.1	504	-
40	0	0	-	-	-	754
40 (+100G)	0.176	25	8	25.8	495	-

Table III: Cell number, chlorophyll content and metal concentrations in zinc-exposed cells with (+G) and without GSH.

Treatment (μM)	Cell number ($x 10^6$)	Total chlorophyll (mg/ml)	Metal concentration	
			clarified medium (μg/L)	*fresh medium (μg/L)*
Control (0.766)	10	22.1	65	38
15.32	1	4.9	166	544
15.32 (+50G)	2	6.5	84	-
30.64	0.5	0.4	411	934
30.64 (+100G)	8	5.2	94	-

The ability of GSH to restore the growth of metal exposed cells, observed in the present study, thus, reiterates the role of glutathione in the metal detoxification processes in plant cells.

ACKNOWLEDGEMENT

The corresponding author (NTP) acknowledges MASHAV-Centre for International Cooperation, Israel, for the award of post-doctoral visitorship. We thank the staff of Environmental Microbiology Unit, BIDR for excellent assistance and cooperation. NTP also thanks Dr. K.S.J.Rao, CFTRI, India, for valuble comments and encouragement.

REFERENCES

1. Ballan-Dufrancias, C., Marcaillou, C. and Amaird-Triquet, C. Biol. Cell., **72**: 103, 1991.
2. Knauer, K., Behra, R., Sigg, L. Environ. Toxicol. Chem., **16**: 220, 1997.
3. Vyamazal, J. Acta. Hydrochim. Hydrobiol., **14**: 83, 1986.
4. Spencer, D.F. In: Nickel in the Environment (ed. J.O.Nriagu), John-Wiley and Sons, p.339, 1980.
5. Rai, L.C., Rai, P.K., Mallick, N. Environ. Exp. Bot., **36**: 99, 1996.
6. Ernst, W.H.O., Verkeleij, J.A.C. and Schat, H. Acta. Bot. Neerl., **41**: 229, 1992.
7. Meharg, A.A. Plant Cell Environ., **17**: 989, 1994.
8. Alscher, R.G. Physiol. Plant., **77**: 457, 1989.
9. Grill, E., Winnacker, E.L. and Zenk, M.H. Proc. Natl. Acad. Sci. (USA)., **84**: 439, 1987.
10. Steffens, J.C. Ann. Rev. Plant Physiol. Plant Mol. Biol., **41**: 553, 1990.

11. Scheller, H.V., Huang, B., Hatch, E. and Goldsbrough, P.B. Plant Physiol., 85: 1031, 1987.
12. Tukendorf, A. and Rauser, W.E. Plant Sci., 70: 155, 1990.
13. Mendum, M.L., Gupta, S.C. and Goldsbrough, P.B. Plant Physiol., 93: 484, 1990.
14. Kubota, H., Sato, K., Yamada, T. and Maitani, T. Plant Sci., 106: 157, 1995.
15. Manual of Methods for General Bacteriology (eds. P. Gerhardt P. *et al*), ASM, 1981.
16. Diaz-Barrientos, E., Madrid, L., Cabrera, F. and Contreras, M.M. Comm. Soil Sci. Plant Anal., 22: 1559, 1991.
17. Moran, R. Plant Physiol., 69: 1376, 1982.
18. Butler, M., Haskew, A.E.J. and Young, M.M. Plant Cell Environ., 3: 119, 1980.
19. Uptis, V.V., Pakalne, D.S. and Nollendorf, A.F., Microbiology, 1974: 758, 1974.