

## TOLERANCE TO CADMIUM AND PHYTOCHELATINS SYNTHESIS IN *AGROSTIS TENUIS*

### TOLERANȚA LA CADMIU ȘI SINTEZA FITOCHELATINELOR ÎN *AGROSTIS TENUIS*

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**Abstract:** The paper has as aim the cellular and biochemical identification of some proteins (phytochelatin and iso-phytochelatin) able to bind the cadmium ions. For this, cell suspensions of root callus proceeded from an *Agrostis tenuis* ecotype (harvested from a metalliferous area, polluted with heavy) were obtained. The cell suspensions were treated with different cadmium concentrations. One can ascertain that the *Agrostis* ecotype, from polluted area, has the ability to synthesize phytochelatin and, it manifests an increased tolerance to cadmium ions (solution concentrations of 100  $\mu\text{M}$ ). One can conclude that phytochelatin synthesis has an important role into plant tolerance to cadmium.

**Rezumat:** Prin prezenta lucrare s-a urmarit identificarea celulara si biochimica a unor proteine (fitocheletine si izo-fitocheletine) capabile de a lega ionii de Cd. Pentru aceasta s-au obtinut suspensii celulare din calus radicular provenit de la un ecotip de *Agrostis tenuis* (recoltat dintr-o zona metalifera, poluata cu metale grele). Suspensiile celulare au fost tratate cu diferite concentratii de Cd. S-a constatat ca ecotipul de *Agrostis* provenit din zona poluata are capacitatea de a sintetiza fitocheletine si, astfel, manifesta o toleranta crescuta fata de ionii de Cd (concentratii ale solutiei de 100  $\mu\text{M}$ ). Se poate concluziona ca sinteza de fitocheletine are un rol important in toleranta plantelor la Cd.

**Key words:** cadmium tolerance, suspension-cultured cells, phytochelatin, *Agrostis tenuis*

**Cuvinte cheie:** toleranța la cadmiu, suspensii de culturi celulare, fitocheletine, *Agrostis tenuis*

#### INTRODUCTION

Hayashi et al. (Murasugi et al., 1981; Kondo et al., 1984) were the first who discovered into yeast cells (*Schizosaccharomyces pombe*) treated with cadmium salts, cadmium-binding peptides. These peptides were named “cadystin” and the identification of their structure emphasized the fact that these peptides are composed from glutathione repetitive units (n) ( $\gamma\text{EC}$ )<sub>n</sub>G (n=2,3). Similar peptides ( $\gamma\text{EC}$ )<sub>n</sub>G, with glutathione repetitive number units ranges between 2 and 11 were also found in superior plant (Grill et al., 1985). These peptides, named phytochelatin, contribute to increasing of superior plant tolerance to heavy metal harmful action.

The phytochelatin synthesis starting from GSH and mediated by the phytochelatin-synthetase was demonstrated by Grill et al., 1989, using suspension-cultured cells of *Silene cucubalus*. As results of these *in vitro* experiments, the  $\gamma\text{EC}$  dypeptidil-transpeptidase enzyme was isolated and characterized. This enzyme binds the GSH units one of another in order to form phytochelatin (Grill et al., 1989; Loeffler et al., 1989). This enzyme expression is more efficient when is activated by the cadmium ion presence (Grill et al., 1989). Similar enzymatic activities were also reported in other plants (Howden et al., 1995a, 1995b; Chen et al., 1997). Relative recently, the genes for the synthesis of phytochelatin-synthetase from *Arabidopsis* plants (Ha et al., 1999) and from *Triticum aestivum* (Clemens et al., 1999) were isolated. Homeologous genes were also identified in the yeast cells as well as in nematode *Caenorhabditis elegans*. These discoveries suggest the fact that the phytochelatin-synthetase

could has important functions as part of a large variety of organisms (yeasts, plants and invertebrates).

The present paper presents the effect of cadmium on *Agrostis tenuis* root cell growth. It was also tested the effect of cadmium on phytochelatin synthesis in both suspension-cultured cells and root tissues of *Agrostis tenuis*.

## **MATERIALS AND METHODS**

### **Plant samples**

Plant samples (*Agrostis tenuis*) were harvested from a North geographical area of the country: Baia Mare, metalliferous area, strongly and long-term affected by the heavy metal pollution.

To achieve the experiments, suspension-cultured cells from root callus were used.

Suspension-cultured cells were obtained from root callus according to standard protocols (Dodds and Roberts, 1985). Cell suspensions were maintained into sub-cultures in 90 ml liquid Murashige-Skoog medium, into flasks of 300 ml. According to working protocol, a tenth of culture volume was inoculated into a new medium, and the suspension-cultured cell was grown on an agitator at 80 rpm, under conditions of 28 °C and neon light.

### **Growth experiments**

The CdSO<sub>4</sub> solutions (10-100 µM) were sterilized and added into flasks of 100 ml, at 30 ml liquid Murashige-Skoog medium.

To determine the cadmium influence on cell growth, the adding of CdSO<sub>4</sub> solutions was done before to achieve the cell inoculum. The cell suspensions (about 100 mg fresh raw) were inoculated into culture media and grown during 12 days at the temperature of 27°C.

To determine the phytochelatin content, the CdSO<sub>4</sub> solutions were added over the cell suspensions after 3 days of growing into liquid Murashige-Skoog medium, free of Cd ions.

The cell growth was determined by the weighting of fresh raw and dry one at an interval of 2 days, during experiment (12 days).

### **HPLC analysis to determine the phytochelatin (PCs) and glutathione (GSH) contents**

The cell suspensions were extracted at 0°C, with 10% sulfosalicylic acid (SSA), using an acid volume equal to the fresh weight of cell suspension (after method described by Mendum et al., 1990). The extracts were centrifuged at 10,000 g during 1 minute, and the supernatant was maintained 30 minutes at 0°C, before HPLC analysis. The phytochelatin separation was performed in sample 20-µL. The sample was injected in a reverse-phase column and connected at a HPLC pump. The column was eluted with acetonitril linear gradient in 0.1% (w/v) trifluoroacetic acid at a flowing rate of 0.5 ml/minute. The acetonitril gradient was programmed at 0% during 4 minutes, from 0% to 10% in 4 minutes and from 10% to 20% (v/v) in 40 minutes. The elution from a column was derivatized with 75 µM 5.5'-ditiobis (2-nitrobenzoic acid) in 50 mM potassium phosphate (pH=7.6), at a flowing rate of 1 ml/minute and monitored at 412 nm (Grill et al., 1987), using an UV detector. The phytochelatin retention time was identified as adequate to peptides (γEC)<sub>n</sub>G (n=2-5)(Matsumoto et al., 1990). The phytochelatin content was expressed as millimoles of sulfhydryl equivalent per kilogram fresh

weight of cells, using GSH as standard. The total glutathione content from cell extract was measured by method proposed by Anderson, 1985.

## RESULTS AND DISCUSSIONS

### Cd ion effect on cells growth

As follows of the performed analyses, one can emphasize the fact that at high concentrations of CdSO<sub>4</sub> (100 µM) the root cell growth is inhibited. It was examined the Cd effect on cell growth from the suspension-cultured cells realized by root callus of the *Agrostis tenuis* ecotype. In the liquid Murashige-Skoog medium culture were added, immediately after the cell inoculation, different Cd ion concentrations. The concentrations of CdSO<sub>4</sub> between 10 and 20 µM (which represent 5.3 and 10.6 ppm Cd respectively) do not inhibit the cell growth, during the 12 days of experiment (Fig.1). This reveals the fact that the root cells from the suspension-cultured cells presents tolerance to effect induced by Cd ions. At higher CdSO<sub>4</sub> concentrations, of 50 µM and 100 µM respectively (which correspond to 26.5 and 53 ppm Cd respectively), the cell growth is initially inhibited. One can ascertain that after 4 days and 8 days respectively (depending on the Cd concentration) the growth rate establishes itself (Fig.1).

The Cd ion effect on the cells growth was compared with a check (*Agrostis* cells from polluted environment, inoculated in culture medium without Cd ion addition).

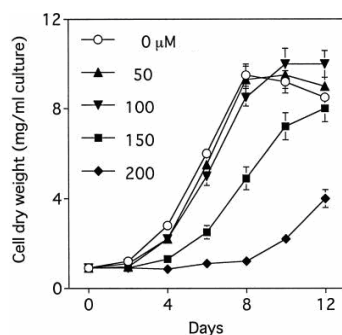


Figure 1. Effect of Cd on growth of suspension-cultured cells of tolerant *Agrostis* (from Baia Mare area). *Agrostis* cells (100 mg) were inoculated in 30 mL of liquid Murashige-Skoog medium and treated with 10 to 100 µM CdSO<sub>4</sub> for 12 days. Cells were collected at a 2-d interval and the dry weights were determined. Data are expressed as means ± SE (n = 3).

### Phytochelatin synthesis into cells

Phytochelatin are heavy- metals complexing peptides which contribute to detoxification processes and confer tolerance to plants, vs. harmful action of metallic ions (Inouhe et al., 1994; Rauser, 1995).

The cells were pre-cultured, during three days, on culture medium without Cd ions. After that, during three days, Cd solutions in different concentrations were added. Under the effect of this treatment, the *Agrostis* cells began to produce phytochelatin (n=2-4)

proportionally with the Cd ion concentration into medium (Figure 2). The phytochelatin presence (n=2-4) into tolerant ecotype cells was detected in high quantities (Figure 3).

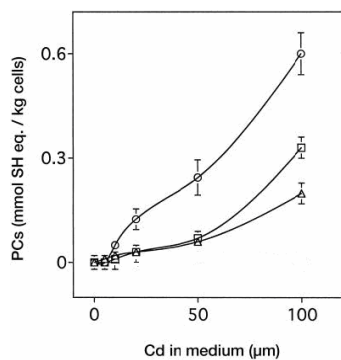


Figure 2. Effects of Cd on the levels of phytochelatin peptides in tolerant *Agrostis* cells. The suspension cells were precultured for 3 d in 30 mL of liquid Murashige-Skoog medium and then treated with various concentrations of CdSO<sub>4</sub> for 3 days. PC peptides (n = 2-4) were determined by the post-column HPLC method. Data are expressed as means ± SE (n = 3).

△, (TEC)<sub>2</sub>G; ○, (TEC)<sub>3</sub>G; □, (TEC)<sub>4</sub>G in tolerant *Agrostis* cells.

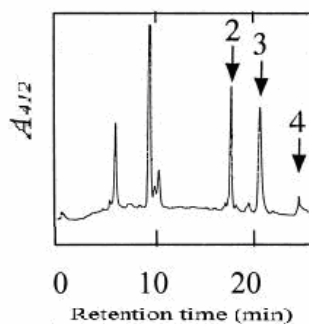


Figure 3. Formations of phytochelatin peptides in suspension cells of tolerant *Agrostis*. The suspension cells of *Agrostis* were precultured for 3 d in 30 mL of liquid Murashige-Skoog medium and then treated with 10 µM CdSO<sub>4</sub> for 3 days. The extracts were analyzed by the post-column HPLC method. Arrows with numbers (n = 2-4) represent the elution times of (TEC)<sub>n</sub>G peptides (n = 2-4).

### GSH level into cells

GSH represents the substrate for the phytochelatin synthesis. To confirm the fact that the glutathione biosynthesis is involved into phytochelatin synthesis, the GSH total content was determined (Figure 4).

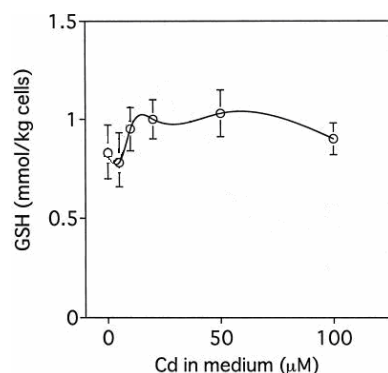


Figure 4. Effects of Cd on the total glutathione contents in tolerant and non-tolerant *Agrostis* cells. The suspension cells were precultured for 3 d in 30 mL of liquid Murashige-Skoog medium and then treated with various concentrations of CdSO<sub>4</sub> for 3 d in the same medium. The total GSH contents in the cells were determined as described in "Materials and Methods." Data are expressed as means  $\pm$  SE (n = 3).

In the *Agrostis* cells the Cd presence determines an increase of GSH content, but this increase is not proportionally with the Cd concentration increase, because a part of synthesized GSH is consumed to synthesize phytochelations. It is well known the fact that the Cd ions have a dual effect on GSH metabolism; on one hand, they lead to the GSH level increasing by GSH synthesis increasing, but, on the other hand, they lead to the GSH level diminution by its consumption for phytochelatin synthesis. Other Cd ion effect also occurs in this experiment.

## CONCLUSIONS

The suspension-cultured cells of *Agrostis* plants harvested from a polluted area present a high tolerance to Cd ions (Figure 1). These cells develop a high tolerance to 100  $\mu$ M Cd solution concentrations. Based on results, one can underline that, the plants grown under strong and long-term pollution (as in Baia Mare area) could develop some defense mechanisms and become tolerant ecotypes.

The biochemical and genetic bases of plant tolerance/resistance to Cd involve both phytochelatin synthesis dependent processes and independent ones (Wagner and Krotz, 1989). This study demonstrated that the *Agrostis* cells from polluted area present efficiency in phytochelatin synthetase activity (Figures 2, 3). These results sustain the idea that phytochelatin synthetase is an essential enzyme which confer to plants tolerance to Cd ion toxic action (Howden et al., 1995b).

The experiments showed that besides phytochelatin, GSH is also involved in plant tolerance to Cd. One can know the fact that GSH could protect plants against heavy-metal toxic action, having anti-oxidative or metal acceptor role (Xiang and Oliver 1998). This study also underlines that the GSH has an important role into detoxification processes of plants contaminated with Cd (Figure 4).

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