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# Alterations in Chloroplast Ultrastructure of Suspension Cultured *Nicotiana tabaccum* Cells by Cadmium

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**ABSTRACT** Cadmium (Cd) is one of several heavy metals which are potentially harmful to human health. Tobacco smoke contaminated with Cd constitutes one of the main sources of the toxic cadmium found in humans. Cd has been shown to inhibit growth, photosynthesis, cell division and cause ultrastructural changes in many plant species. In this study, the effect of Cd on the ultrastructure of suspension cultured *Nicotiana tabaccum* cells was determined. *N. tabaccum* cell suspension culture exposed to 100 µM Cd exhibits growth retardation and alteration of chloroplast ultrastructure. *N. tabaccum* cells from suspension cultures were selected for their ability to grow and to be green in normally lethal concentrations of Cd. Cells resistant to 100 and 250 µM cadmium (added as cadmium chloride) were isolated. The Cd-tolerant cell lines showed normal growth and normal chloroplast ultrastructure when they were exposed to lethal concentrations of Cd.

KEYWORDS: cadmium, chloroplast ultrastructure, *Nicotiana tabaccum*.

## INTRODUCTION

Tobacco smoke and vegetative food contaminated with cadmium (Cd) constitute two main sources of the toxic heavy metal found in humans. Smoking tobacco of 20 cigarettes per day will cause the inhalation of 2-4 µg of Cd.[2] Long-term exposure to low concentrations of Cd resulted in the accumulation of Cd in liver and kidneys of smokers.[11] High levels of Cd have also been correlated with an increased incidence of lung cancer.[16]

Tobacco plants take up Cd from soils contaminated with Cd from industrial mining and sewage disposal operations. Cd is translocated and accumulated in significant amounts in leaves.[6,9] Suspension-cultured tobacco cells accumulated Cd to at least twice the Cd concentration in the culture medium. The accumulation of Cd has been shown to inhibit growth,[8] photosynthesis, cell division and to cause ultrastructural changes.[13,14] In suspension cultured tobacco cells, Cd caused no changes in the mitochondria, cell membranes or cytoplasmic density, but high concentrations of Cd increased cell volume and caused some changes in nuclei vesicles. However, the effects of Cd on chloroplast ultrastructure have not been reported.

Plants have evolved a variety of mechanisms to provide cellular protection against the adverse effects of Cd. One mechanism involves the detoxification and homeostasis of heavy metal ions by synthesis of Cd binding peptides (Cd-Bps) which consist of a mixture of (γ-glu-cys) 4 gly and (γ-glu-cys) 5-gly. A Cd-inducible enzyme is responsible for the main production of Cd-BPs[6] in *Datura innoxia*[7] and in *Lycopersicon esculentam* (tomato).[10] Cd-resistant and Cd-tolerant phenotypes are the result of the cells overproduction of Cd-BPs. However, the exact mechanism of Cd-detoxification by Cd-BPs in Cd-tolerant tobacco cells, the mechanisms by which Cd affects chloroplast structure and the responses of Cd-sensitive and Cd-tolerant cells to the damage caused by Cd are still not well understood. Therefore, the aims of this study are to assess the sensitivity and the responses of chloroplast structure to Cd in Cd-sensitive and Cd-tolerant tobacco cells.

Cd has been chosen as the principal metal in this study because it is widely distributed in the biosphere. It causes serious environmental pollution more than other heavy metals due to its availability in the soil. Tobacco was chosen as a model plant since tobacco smoking is one of the main sources of Cd exposure to man. Suspension cultured tobacco cells, rather than whole plants, were used because they allow more uniform exposure to the stress condition, greater flexibility, shorter generation times and they respond to Cd in a manner similar to that reported for whole plants.

## MATERIALS AND METHODS

### Cell culture, growth conditions and Cd treatments

Unselected tobacco cell suspension cultures (*Nicotiana tabaccum* Wisconsin 38) derived from leaf protoplasts were kindly provided by Dr R A Bressan (Horticulture Department, Purdue University, USA). Cells were grown in liquid MS (Murashige and Skoog, 1962) medium, containing 0.4 mg/l thiamine HCl, 0.1 g/l I-inositol, 3 mg/1 2, 4–dichlorophenoxy-acetic acid, 0.5 mg/l Kinetin and 3% (w/v) sucrose. All cultures were grown at 22-24°C on a gyrator shaker (100 rpm) with continuous illumination of cool white fluorescent and 4 bulb lamps, 67W at 1000 foot candles. Cells were subcultured every 2 weeks. Calli were subcultured every 4 weeks in a solidified MS medium with 0.75% (w/v) agar. Cd was added to cultures on day 0 from sterile 0.5 M $CdCl_2$ of stock solution to an appropriate final concentration. All cell lines were maintained as both suspension and callus culture in medium containing appropriate concentrations of Cd.

### Selections of Cd-tolerant cell lines

Cd-tolerant cell lines were selected by progressively elevating the Cd concentration in the culture medium from 10 to 25, 50, 100, 200 and 250 µM. Cells that were still green were subcultured and maintained in the media containing 100 or 250 µM of Cd. These cell lines were designated CdR100 and CdR250, respectively.

### Growth study

*N. tabaccum* Cd-sensitive cell were grown in the MS media with and without 100 µM of Cd. CdR100 cell lines were grown in the media containing 100 µM of Cd. Cd-sensitive and CdR100 cells from suspension cultures were inoculated into 250 ml of fresh medium at a density of 11 mg /ml fresh weight of culture. Two replicates at each concentration were used. Growth was determined from fresh weight by vacuum filtration of 2 ml of collected cells. The samples were taken every 2 days for 60 days.

### Chlorophyll measurement

Chlorophyll from Cd-sensitive cells grown in media with and without 100 µM Cd and CdR100 cells grown in media containing 100 µM Cd was extracted with 80 % acetone. Samples were taken every 3 days for 21 days. Total chlorophyll was determined spectophotometically according to Arnon.[1] The concentration in mg/ml was calculated from the 652 nm absorbance ($A_{652}$) multiplied by 5.6.

### Electron microscopy

Cd-sensitive cells grown in MS media with and without 100 µM Cd were collected and examined on days 1, 2, 3, and 4, after culturing and CdR100 grown in media containing 100 µM Cd were also collected and examined. At least 2 replicate cultures were prepared and micrographed each day. Cells were harvested by centrifugation (1000 g, 5 min) and fixed with phosphate-buffered 2.5% (v/v) glutaraldehyde for 30 min. After 30 min, the cells were centrifuged (1000 g, 5 min). The pelleted cells were mixed with 1% melted agar and the agar was allowed to solidify. The agar was then cut into approximately 1 $mm^3$ pieces and fixed for an additional 30 min in phosphate-buffered 2.5% (v/v) glutaraldehyde. The agar pieces were rinsed three times with phosphate-buffered 1% (w/v) osmium tetroxide for 1 h. All fixations were carried out at 22-24 °C. After dehydration in a graded series of ethyl alcohol and two changes in propylene oxide, the agar pieces containing tobacco cells were embedded in Epon 812 (Ernest F Fullam, Inc, Schenectady, NY). Thin sections stained with uranyl acetate and lead citrate were examined in a Zeiss 10-C transmission electron microscope operating at 60 kV.

## RESULTS AND DISCUSSION

### The effect of Cd on growth of Cd-sensitive and Cd-tolerant suspension culture cells

There was no growth of Cd-sensitive cells when they were treated with 100 µM Cd (Fig 1). Cd-sensitive cultured cells were challenged with progressively higher levels of Cd. Tolerant cells were isolated from these unadulterated populations by selecting groups of cells that were still green and growing. Various Cd tolerant cell lines were es-

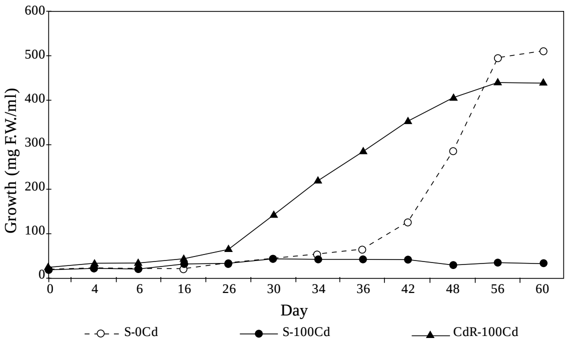


Fig 1. Growth curve of cadmium sensitive and cadmium tolerant cell lines grown in MS media with and without cadmium. S-0Cd = Cd-sensitive cells grown in 0 Cd, S-100Cd = Cd-sensitive cells grown in 100 µM Cd, CdR-100Cd= Cd-tolerant cells grown in 100µM Cd. Vertical bars represent standard errors.

tablished. To date, cells tolerant to 250 μM Cd have been selected. There was no significant difference in the growth between Cd-sensitive cells when grown in the absence of Cd and CdR100 when grown in the presence of 100 μM Cd (Fig 1). There was significant difference in the growth between Cd-sensitive and Cd-tolerant cells when they were grown in the presence of 100 μM Cd (Fig 1).

## The effect of Cd on chlorophyll content

In the absence of Cd, the chlorophyll content of Cd-sensitive cells increased and reached the highest peak on day 9. In the presence of 100 μM Cd, the chlorophyll content of Cd-sensitive cells decreased during the first 3 days, then started to increase and reached the highest peak on day 9. In the presence of 100 μM Cd, chlorophyll content of Cd-tolerant cells increased and reached the highest peak on day 15, which was later than Cd-sensitive cells. Overall, there were no clear differences between the chlorophyll content of Cd-sensitive, Cd treated-sensitive and CdR100 except that the chlorophyll content of CdR100 increased a little bit later (Fig 2). This was principally due to the reduction in growth since chlorophyll content was calculated per unit fresh weight.

## The effect of Cd on chloroplast ultrastructure

The chloroplasts of Cd-sensitive cells grown in the absence of Cd showed normal chloroplast structure, containing numerous well compartmentalized grana stacks (Fig 3 A and B). The chloropasts of Cd-sensitive cells treated with 100 μM Cd showed fewer layers of grana stacks (Fig 3 C and D). Cd at 100 μM concentration had an effect on the number of layers of grana stacks but no effect on the general

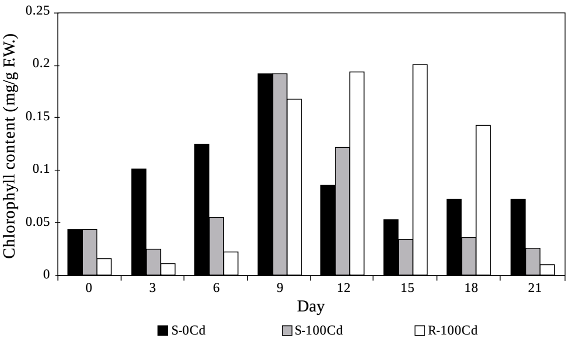


Fig 2. Chlorophyll content of Cd-sensitive and Cd tolerant cells when they are grown in the presence and absence of Cd. S-0Cd = Cd-sensitive cells grown in 0 Cd, S-100Cd = Cd-sensitive cells grown in 100 μM Cd, R-100Cd= Cd-tolerant cells grown in 100 μM Cd. Vertical bars represent standard errors.

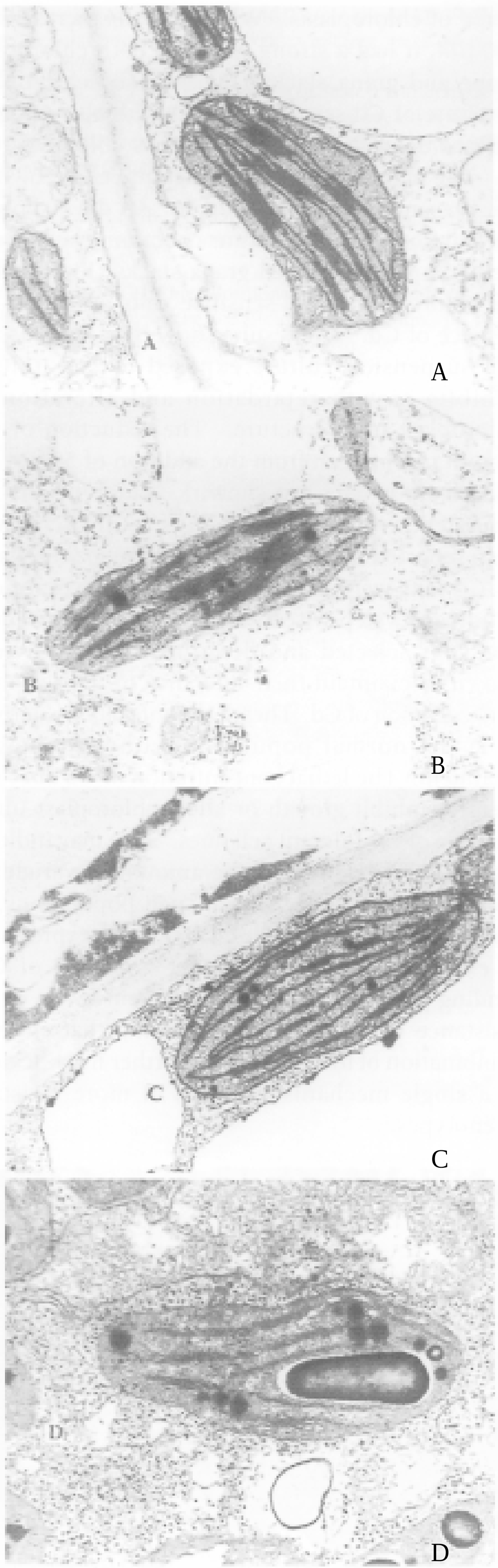


Fig 3. Chloroplast ultrastructure of N. tabaccum cultured cells (A, B) Cd-sensitive cells 1 day old (A) and 4 days old (B) grown without Cd (C, D) Cd-sensitives cells 1 day old (C) and 3 days old (D) grown in 100 μM Cd.

shape of chloroplasts. When Cd was increased to 400 µM, it had a strong effect on both chloroplast shape and grana stacks (Fig 4). The chloroplast structure of Cd-sensitive cell treated with 400 µM Cd was deformed and grana stacks collapsed (Fig 4). The chloroplasts of Cd-tolerant cells grown in the presence of 100 µM Cd for 48 days showed normal shape and structure containing numerous well compartmentalized grana stacks (Fig 5) as in the chloroplast of Cd-sensitive cells grown in the absence of Cd. The results show that *N. tabaccum* cell suspension culture exposed to 100 µM Cd exhibits growth retardation and alteration of chloroplast ultrastructure. The reduction of the growth can be seen from the addition of 50 µM Cd and onward (data not shown). These results are consistent with previous findings.[12] Cd ion has also been reported to be toxic to *Datura innoxia* cells.[8]

Cd ions were utilized to select Cd-resistant cell cultures by a step-wise increase in Cd concentration. The cells selected and described here have been shown to maintain their tolerance to the selection concentration of Cd. These tolerant cells are derived from the normal population without mutagen treatment. The lethal concentration of Cd does not seem to inhibit growth or affect chloroplast ultrastucture of Cd-tolerant cell lines. So, it may indicate that either a relatively large amount of variability occurs spontaneously within such populations, or that Cd induces a heritable change in the expresssion of existing genes which direct synthesis of Cd-binding proteins (Cd-BPs).[7] Different levels of Cd resistance were obtained, suggesting that either a combination of mechanisms or further modification of a single mechanism results in more resistant phenotypes.

The data presented here clearly show a difference in the development of chloroplasts between the sensitive and tolerant cells. Cadmium has a pronounced negative influence on grana thylakoids in the sensitive cells. Ghoshroy and Nadakavukaren[4] have reported similar data in soybean seedlings grown in Hogland's solution containing 100 µM of cadmium chloride. They also observed retardation of chloroplast development and severe disruption of grana thylakoids. They concluded that cadmium must interfere with membrane synthesis in chloroplasts. Our chlorophyll data are somewhat inconclusive. However, Ghoshroy[3] also reported no reduction in chlorophyll ratio in soybean plants grown in Hogland's solution containing 100 µM of cadmium chloride. She suggested either a reduction of Cd uptake or triggering of Cd chelation above 50 µM as possible reasons why decreased chlorophyll a/b ratio, as well as a reduction of PSI and PSII polypeptides, was observed at 30 and 50 µM Cd chloride, but not at 100 µM.

The well developed grana stacking of the chloroplasts of the Cd-tolerant cells suggests that they are overexpressing some mechanism of detoxification of Cd. It seems unlikely that the ability to synthesize Cd-binding proteins arose *de novo*, within resistant

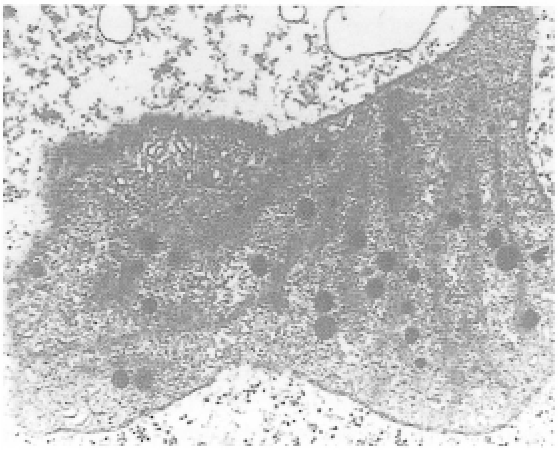

Fig 4. Chloroplast ultrastructure of Cd-sensitive cells grown in 400 µM Cd for 4 days.

Fig 5. Chloroplast ultrastructure of Cd-tolerant cells grown in 100 µM Cd for 46 days.

cells. Therefore, resistance to Cd may be the result of the cells' capacity to overproduce metal-binding proteins and peptides, relative to sensitive cells. Such proteins and peptides may play some role in trace metal metabolism.[5, 12, 15, 17] Further work should be done to determine the exact mechanisms of Cd tolerance of these cells and what genes are involved in these mechanisms.

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