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Effect of Cadmium Treatment on the Total Thiol Groups, Glutathione and Phytochelatin Contents in *Oenanthe javanica*

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Abstract : In order to evaluate the usefulness of *O. javanica* for the phytoremediation, it was grown for 1, 3, 7, 14, 21 days and was exposed to 50 μM of $CdCl_2$ in hydroponic medium after 3 weeks. Its biomass and contents of chlorophylls were analyzed. The growth of *O. javanica* showed little difference between cadmium treated and non-treated groups, while its contents of chlorophylls of Cd-treated group decreased up to 50% compared to the case of non-treated group. Its accumulated cadmium concentrations were 2.1, 7.3 and 113 μmoles Cd/g dry weight in the leaf, stem and root, respectively. The total contents of thiol increased 0.5, 1 and 7 times in the leaf, stem and root, respectively, while the contents of glutathione tended to decrease by 43%, 70% and 47% in the leaf, stem and root, respectively. Using HPLC analysis, the reasonable peaks of thiol compounds in shoot and root of Cd-treated sample were compared to those of non-treated sample in *O. javanica*, and found to be phytochelatins. In case of *Nicotiana tabacum cv.* Xanthi tested as control plant, the cadmium treatment for 3 weeks resulted in the decrease of both biomass and chlorophyll up to 70% and 75%, respectively. The roots of tobacco became rotten and eventually died. These results suggested that *Oenanthe javanica* is cadmium-tolerant hyperaccumulator.(Received December 20, 1996; accepted March 17, 1997)

Introduction

Pollution of the biospere with toxic metals has been dramatically accelerated since the begining of the industrial revolution. The primary sources of this pollution are the burning of fossil fuels, minimg and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides and sewage. Heavy metals are toxic to most organisms and a variety of mechanisms have evolved for coping with the the toxic effects of these elements. In mammals and some fungi, proteins that bind heavy metals within the cell are synthsized. These metallothioneins are characterized by their low molecular weight, induction by heavy metals, high cysteine content, and their ability to bind a number of heavy metals. There have been a number of reports in recent years that both differentiated plants and plant cells grown in cultures produce heavy metal binding complexes when exposed to metal ions.[1,2] It has been shown recently that the most abundant heavy metal binding complex in a number of higher plants and a *Schizosaccharomyces pombe* is comprised of a family of peptides that are structurally related to GSH.[2-4] These peptides, termed phytochelatins, have the structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ where n=2 to 10. Phytochelatins are analogous to metallothioneins in that they are induced by heavy metal and bound with heavy metal. The mechanism of action of these two groups of compounds appears to be similar in their use of cysteine SH group for binding heavy metals. Several fungi and plants which have these mechanisms have been applied to remove the contaminated heavy metal from environment.[5-8] Recently, the value of heavy metal accumulating plants for environmental remediation has been realized.[8] Several heavy metal accumulating plants such as water hyacinth[9] and duckweed[5] were isolated.

The use of specially selected and engineered metal-accumulating plants for environmental clean-up is a technology called phytoremediation. *Oenanthe javanica* grows well in wastewater including excess nitrate and phosphate. In order to evaluate the usefulness of *O. javanica* for the phytoremediation, it was exposed to 50 μM of $CdCl_2$ in hydroponic medium, its biomass and content of chlorophyll were analyzed. As a control, *Nicotiana tabacum cv.* Xanthi was also exposed and investigated. The relationship between cadmium, thiol, glutathione and phy-

Key words : phytoremediation, phytochelatin, glutathione, cadmium, *Oenanthe javanica*
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tochelatin in *O. javanica* was analyzed.

Materials and Methods

Plant material

O. javanica was propagated by cutting method in soil. Two weeks after planting, seedlings were transferred to 25 cm×20 cm×15 cm styroform box containing 2.5 L of Hoagland solution.[10] One week after transferring to nutrient solution, $CdCl_2$ solution was added into it to make 50 μM. The nutrient solution was changed weekly. Seedlings were cultured at 25℃ under 16h/8h (light/dark) condition. Then the leaves, stems and roots from bulked sample were sequentially collected at 1, 3, 7, 14 and 21 days after cadmium treatment. Tobacco, used as a control plant, was cultured under the same Cd treatment condition after germination.

Assay for the concentration of chlorophylls

The chlorophyll contents were determined following the method of Goodwin.[13] Five leaf disks obtained from each second shoot using cork borer (diameter: 5 mm) were grounded and extracted with 1 m*l* of 80% acetone. Extractants were centrifuged at 12,000 rpm at 4℃. The supernatants were analyzed with spectrophotometer at 645 and 663 nm. Contents of chlorophylls were calculated with the following equation:

$$\text{Chlorophyll a (mg/g)} = 12.7 \times A_{663} - 2.69 \times A_{645}$$
$$\text{Chlorophyll b (mg/g)} = 22.9 \times A_{645} - 4.68 \times A_{663}$$

Preparation of protein free extracts

The frozen tissues were lyophilized. Two hundred miligram tissue were extracted in 1 m*l* 5% (w/v) 5-sulfosalicylic acid to remain acid condition at 4℃ using a mortar and a pestle.[11,12] The homogenates were stored in Eppendorf tubes. They were centrifuged at 15,000 g for 5 min and their supernatant was immediately analyzed for Cd, total non-protein thiol group, glutathione and phytochelatins.

Assay for the contents of Cd, total non-protein thiol group and glutathione

Cadmium concentration was determined by atomic absorption spectrophotometer.[12] Total contents of non-protein thiol groups were determined by the method of Schat and Kalff.[14] Glutathione was analyzed by DTNB-GSSG reductase recycling assay method proposed by Tietze:[15] 700 μ*l* of 2 mM NADPH in 143 mM sodium phosphate buffer (pH 7.5) and 5 mM Na_2EDTA, 100 μ*l* of 6 mM DTNB in 143 mM Sodium phosphate buffer (pH 7.5) and D.W were mixed. The mixed solutions were warmed at 30℃ in a water bath for 15 min. The sample was added with mixing in a final volume of 1 m*l*. Two units of GSSG reductase in 143 mM Sodium phosphate buffer (pH 7.5) was added with mixing for the initiation of the assay. Absorbances at 412 nm were determined for 15min at every minute. The rate of reaction was usually expressed as the change of absorbance in every minute at 412 nm. The amount of GSH was calculated using a standard curve, in which the GSH equivalents were plotted against the rate of change of absorbance at 412 nm.

HPLC analysis for Cd-induced thiol compounds

Phytochelatins were separated by HPLC with a Delta-Pak C_{18} column(100Å, 4 μm, 7.8×300 mm, id: Waters), using the following modification in the method of de Knecht *et. al.*.[12] The flow rate through the column was 3 m*l*/min. The sample volume was 300 μ*l*. The gradient program was 0.1% (v/v) TFA for 2 min, followed by a gradient of 0 to 20% (v/v) acetonitrile in 0.1% TFA for 60 min. Then, the column was washed with 50% acetonitrile in 0.1% TFA for 10 min and equilibrated in water with 0.1% TFA for 15 min for the regeneration. The eluent was derivatized with 1.8 mM DTNB in 0.3 M potassium phosphate buffer and 15 mM Na_2EDTA (pH 7.8), which flowed at a rate of 1.5 m*l*/min by an postcolumn pump. The mixture passed through a 5 m*l* reaction coil with a residence time of 67 sec. The absorbance of derivatized material was measured at 412 nm.

Results

Effect of Cd on plant growth and chlorophyll concentration

For the determination of tolerence concentration against cadmium on *O. javanica*, preliminary experiment was carried out at various cadmium concentrations for 1 week (Table 1). The highest cadmium concentration that did not affect the growth was 100 μM. New leaves were not developed at 500 μm. *O. javanica* died at 1 mM cadmium. All experiments were carried out at 50 μM cadmium to make sure to have long term tolerance.

The effect of Cd on the growth of *O. javanica* and *N. tabacum* was investigated (Fig. 1). The growth of *O. javanica* showed little difference between cadmium-treated and non-treated groups after 3 weeks of cul-

Table 1. Growth of *O. javanica* after 1 week at several concentrations of cadmium treatment.

	Concentrations of cadmium (μM)					
	10	50	100	500	1000	5000
Growth of *O. javanica*	++	++	++	+	−	−

(++), not affect; (+), inhibit development of new leaves; (−), completely inhibit.

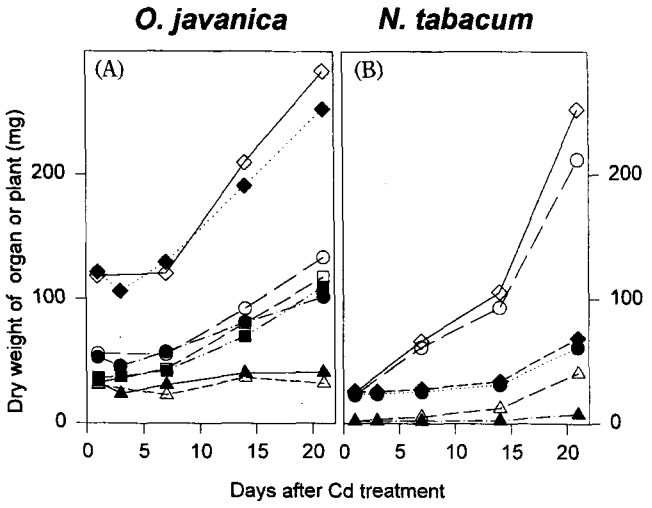

Fig. 1. Changes in the biomass of *O. javanica* and *N. tabacum* after 50 μM cadmium treatment. Open symbol, non-treated samples; closed symbol, cadmium-treated samples. A: ◇—◆, total biomass; ○—●, leaf; □—■, stem; △—▲, root. B: ◇—◆, total biomass; ○—●, shoot; △—▲, root. Each point is the mean of two independent experiments which were carried out with four samples of two pooled plants each.

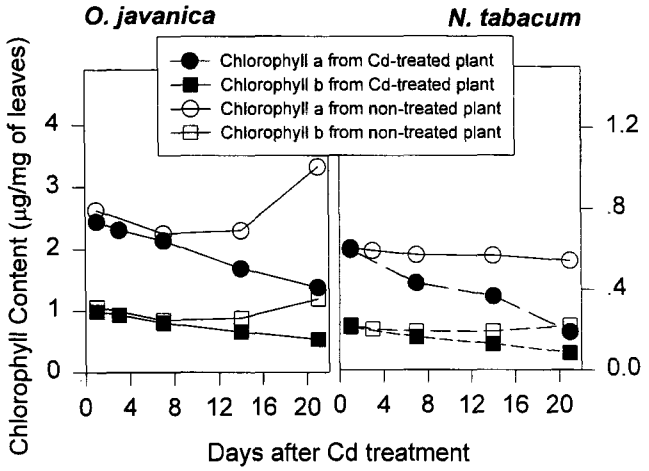

Fig. 2. Chlorophyll concentrations in the secondary leaves of *O. javanica* and *N. tabacum* after 50 μM cadmium treatment. Each point is the mean of two independent experiments which were carried out with four samples of two pooled plants each.

turing. However, in case of *Nicotiana tabacum cv.* Xanthi tested as control plant, cadmium treatment for 3 weeks resulted in decrease of biomass upto 70%. The effect of Cd on chlorophyll concentration of *O. javanica* and *N. tabaccum* is shown in Fig. 2. In case of *O. javanica*, both chlorophyll a and b did not show difference between cadmium-treated and non-treated groups after 1 week. After 3 weeks concentrations of chlorophyll a and b in cadmium-treated group were 50% less than those in non-treated group. However, in case of tobacco, treatment of cadmium resulted in continuous decrease of chlorophyll concentration upto 75% during 3 week culture. The roots of tobacco became rotten and eventually died. The growth difference between Cd-treated and non-treated group in *O. javanica* and *N. tabacum* after 3 week Cd treatment is shown in Fig. 3.

Cd concentrations in *O. javanica*

The accumulated cadmium concentrations in the leaf, stem and root of *O. javanica* after 1 day Cd-treatment were 0, 0.3 and 23 μmoles Cd/g dry weight, respectively (Fig. 4). While after 3 days they were 0.65, 2.4 and 63 μmoles/g, respectively. During intial 3 days, *O. javanica* dramatically took up cadmium. After that, cadmium-uptake in root continuously increased to 113 μmoles/g. Cadmium-uptake rates in stem and leaf were also slightly decreased while cadmium-uptake in stem and leaf appeared to increase to 2.1 and 7.3 μmoles/g, respectively. Total contents of cadmium in roots, stems and leaves of *O. javanica* at 3 weeks after 50 μM cadmium treatment were 0.204 μmoles/97 mg, 0.767 μmoles/105 mg and 3.955 μmoles/35 mg, respectively.

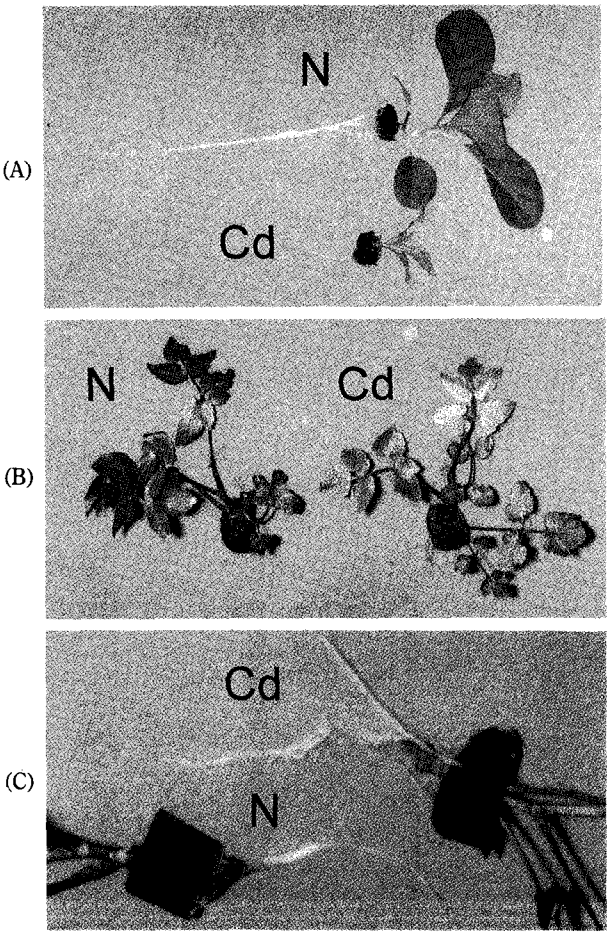

Fig. 3. Photographs of *N. tabacum* and *O. javanica* at 3 weeks after 50 μM cadmium-treatment. N, non-treated samples; Cd, cadmium-treated samples; A, *N. tabacum*; B, *O. javanica*; C, the roots of *O. javanica*.

Total thiol and glutathione concentrations in *O. javanica*

Total thiol and glutathione concentrations in organs as

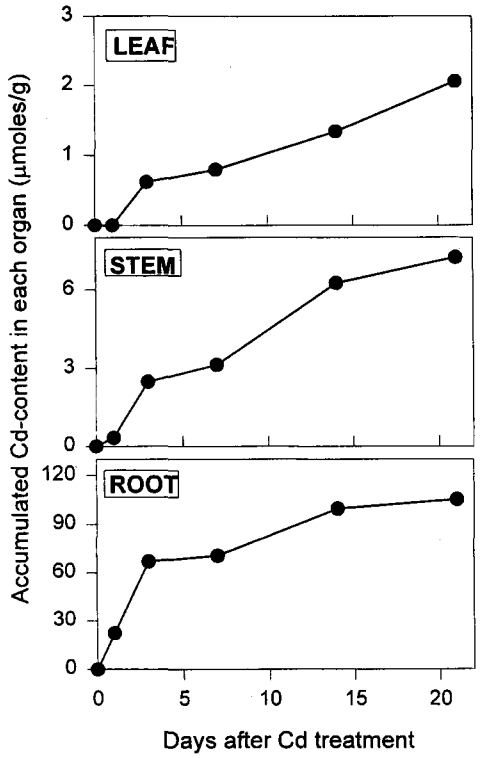

Fig. 4. Accumulation of cadmium in each organ of *O. javanica* treated with 50 μM cadmium. Each point is the mean of two independent experiments which were carried out with four samples of two pooled plants each.

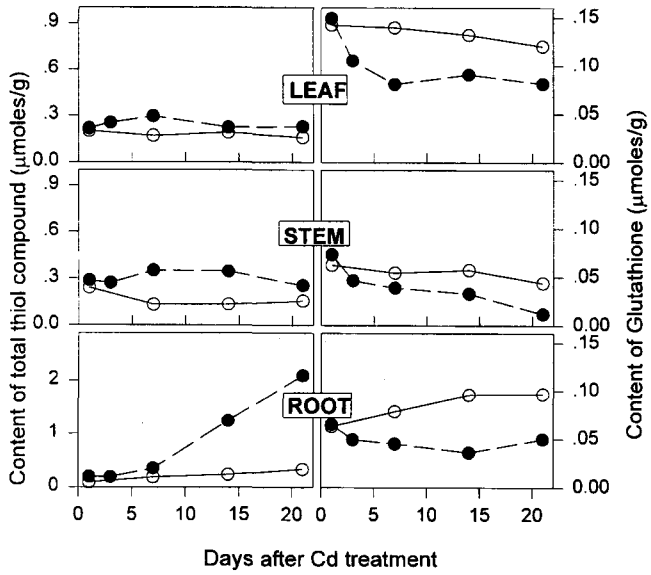

Fig. 5. Changes in the total thiol compound and glutathione concentrations of each organ from the *O. javanica* treated with 50 μM cadmium. ○—○, non-treated samples; ●—●, cadmium-treated samples; Each point is the mean of two independent experiments which were carried out with four samples of two pooled plants each.

a function of Cd treatment time are shown in Fig. 5. Total thiol concentrations of leaf and stem were slightly increased after cadmium treatment, while total thiol concentration of root was dramatically increased 2, 4.5 and

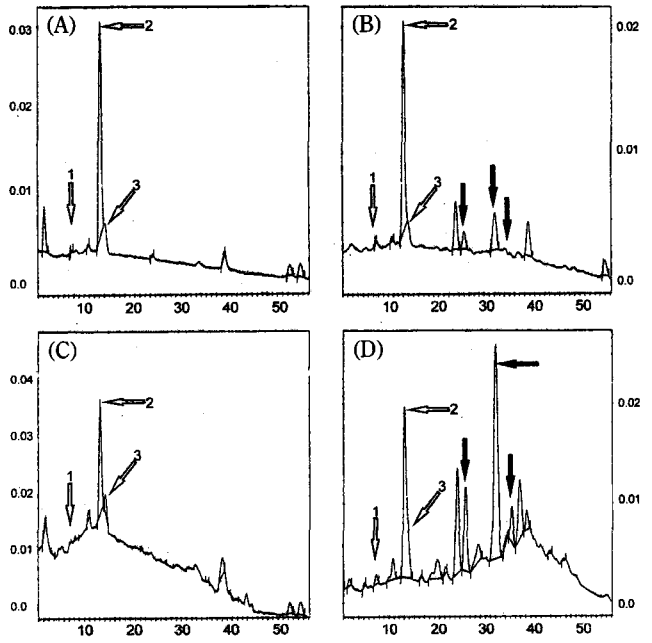

Fig. 6. HPLC profiles for Cd-induced thiol compounds in *O. javanica* at 3 weeks after 50 μM cadmium treatment. A, shoot (non-treated); B, shoot (cadmium-treated); C, root (non-treated); D, root (cadmium-treated). Open arrow: 1 (cysteine), 2 (glutachione), 3 (γ-glutamylcysteine). Closed arrow: cadmium-induced thiol compounds.

7.1 times at 1, 2 and 3 weeks after cadmium-treatment, respectively. The concentration of glutathione was decreased upto 43%, 70% and 47% in the leaf, stem and root, respectively. By the Cd treatment total thiol contents were increased, while glutathione contents were decreased. It implied that the other thiol compounds increased.

HPLC analysis for Cd-induced thiol compounds

Phytochelatins were separated by HPLC on a Delta-Pak C_{18} column using the method of de Knecht *et. al.*.[12] The eluent was derivatized with 1.8 mM DTNB in 0.3 M potassium phosphate buffer and 15 mM Na_2EDTA (pH 7.8), which was added at a flow rate of 1.5 *ml* min^{-1} by an postcolumn pump. The profiles of Cd-treated and non-treated samples had cysteine, glutathione and γ-glutamylcysteine peaks at 6.5, 12.2 and 13.0 min, respectively (Fig. 6). The reasonable peaks of thiol compounds in shoot and root of Cd-treated sample were compared to non-treated shoot and root of *O. javanica*, and shown to be phytochelatins.

Discussion

In order to evaluate the usefulness of *O. javanica* for the phytoremediation, its Cd-uptake, Cd-translocation and the resistance against cadmium were investgated by the measuring physiological and biochemical parameters. In order to investigate the long term torelence against

cadmium, *O. javanica* was treated to cadmium 3 weeks long in this study and this plant did not show much growth inhibition during this period. In the previous studies, some plants such as *Brassica juncea, Thlaspi* caerulescens and Silene vulgaris which have been introduced as very effective plants for phytoremediation were tested the cadmium torelance only 1 week long.[8,12]

The roots of *O. javanica* absorbed cadmium from hydroponic medium. The absorbed cadmium was transferred to stems and leaves through xylem. Transport of cadmium from roots to stems and leaves was affected by the uptake-rate of cadmium in roots (Fig. 4). And the capacity for cadmium accumulation of root reached almost the saturation at the 3rd day. A rate limiting step of cadmium translocation from the roots to the stems and leaves might be the transport from epidermis to endomermis in roots. Cadmium ions entered the root via symplastic and apoplastic pathway.[8] Once cadmium ions have entered the root they are either stored or exported to the stems and leaves. Cadmium transport to the stems and leaves takes place in the xylem. At the same time cadmium redistributes in the stem and leaves via the phloem.[8] In order to enter the xylem vessels the rest cadmium ions must first cross the casparian strip which divides the endodermis and the epidermis. To cross this strip of water impermeable cell wall cadmium ions must move symplastically, as apoplastic transport is blocked.[8] In this experiment, it took one day to cross this strip for cadmium ion in *O. javanica.*

As the cadmium treatment time extended, total thiol concentrations gradually increased, while glutathione contents were decreased (Fig. 5). It implied that any thiol compounds increased. The increased thiol compounds were shown in HPLC profiles of Fig. 6 (B,D). The DTNB react on thiol residue of cysthein, glutathione, phytochelatin and other thiol compounds stochiometrically. The profiles of Cd-treated and non-treated samples had cystein, glutathione and γ-glutamylcysteine peak at 6.5, 12.2 and 13.0 min, respectively. The peaks of cysteine and γ-glutamylcysteine were very small. The most of thiol compounds was glutathione in cadmium non-treated samples. Knecht *et. al.*[12] obtained similar HPLC profiles after analysis of extracts from Cd-treated plant material. The induction of phytochelatins in response to Cd exposure has previously been reported for many other whole plants and plant cells in cultures.[12,16] Therefore, the peaks for phytochelains were identified based on the retention time. The peaks of thiol compounds in root were higher than in shoot of Cd-treated sample. The precusor of phytochelatin is glutachione which is synthesized from γ-glutamylcysteine. In the HPLC profiles, the peak of glutathione was much higher than the that of γ-glutamylcystein. According to the stochiometrical detection of thiol compounds by postcolumn modification method with DTNB, the conversion of γ-glutamylcysteine into glutathione was not proportional to that of glutathione into phytochelatin. Therefore the synthesis of γ-glutamylcysteine might be a rate-limiting step for synthesis of phytochelatin. In order to improve the strategy for the breeding of tolerent plants against cadmium more effectively, the synthesis step of γ-glutamylcysteine and/or cysteine shoud be considered.

These results suggested that *Oenanthe javanica* was cadmium-tolerant hyperaccumulator. The relationship between cadmium, thiol, glutathione and phytochelatine indicates that cadmium induces the synthesis of phytochelatines, and the increased synthesis of phytochelatines is responsible for the accumulation and detoxification of cadmium.

Acknowledgements

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카드늄을 처리한 미나리 (*Oenanthe javanica*)에서 전체 Thiol 잔기, 글루타치온, Phytochelatin의 농도 변화

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초록 : 미나리 (*O. javanica*)는 오염된 하수 등에서도 매우 잘 자라는 식물체이다. 이런 미나리를 phytoremediation에 응용하기 위하여 50 μM 카드늄을 처리하여 카드늄에 대한 저항성 및 식물체에 축적된 카드늄을 정량하고 생리적, 생화학적 변화를 조사하였다. 카드늄을 처리하고 3주 뒤에 미나리는 거의 성장저해를 받지 않았으나 대조구로 사용한 담배 (*N. tabacum* Xanthi)는 75% 성장저해를 받았다. 미나리는 뿌리, 줄기, 잎에 건중량 1 그램당 각각 2.1, 7.3, 113 μmoles의 카드늄을 축적하였다. 카드늄을 처리한 미나리의 경우 전체 thiol 잔기의 농도가 카드늄을 처리하지 않은 미나리에 비해 뿌리, 줄기, 잎에서 각각 0.5, 1, 7배 증가하였으나 글루타치온 농도의 경우는 대조구에 비해 각각 57%, 30%, 53% 까지 감소하였다. 이를 HPLC로 조사해본 결과 증가된 thiol의 함량은 새로운 thiol 잔기를 지닌 물질의 생성으로 기인된 것이고, retention time을 기초로 이는 phytochelatin인 것으로 보여진다.

찾는말 : phytoremediation, phytochelatin, glutathione, 카드뮴, 미나리
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