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Synthesis of phytochelatins in *Helianthus annuus* is enhanced by cadmium nitrate

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Phytochelatins are the principal heavy metal-detoxifying components in plants. To investigate phytochelatin (PHC) production and the importance of these compounds for heavy metal tolerance, sunflower (*Helianthus annuus*) was exposed to cadmium. The leaves and roots of sunflower plants cultivated in the presence of 15, 25 and 50 M $Cd(NO_3)_2$ for 3 and 9 days showed increased tolerance to cadmium and contained higher concentrations of phytochelatins. The phytochelatin level was assayed by using HPLC and the Cd level was determined by atomic spectrum analysis.

Key words: Phytochelatin, Helianthus annuus, cadmium nitrate, phytoremediation

Introduction

Heavy metals such as Cd, Pb, Hg, As, and Se are environmental pollutants of increasing importance in the world (ZHU et al. 1999). Heavy metal toxicity poses major environmental and health problems. Cadmium, for example, is a non-essential heavy metal which is toxic to living cells at very low concentrations. Cd ions displace Ca or Zn in proteins and can cause oxidative stress (CLEMENS et al. 1999). Plants under heavy metal stress synthesize phytochelatin by a -glutamyl-cysteinyl-dipeptidyl trans-peptidase using glutathione as a substrate (GRILL et al. 1985). Metal-binding phytochelatins may be universal in the plant kingdom (GRILL et.al. 1985). Plants can be used to remove heavy metals from the soil by accumulating, stabilizing, or biochemically transforming them. This cost-effective and environment-friendly technology has been called phytoremediation (ZHU et al. 1999). Heavy metals, unlike organic pollutants, cannot be chemically degraded or biodegraded by microorganisms. An alternative biological approach is to use plants to clean up polluted waters and soils.

The detoxification mechanisms of plants in response to heavy metals have been widely studied, especially where heavy metals in contaminated soil could be removed by plants through bioremediation processes. Plants produce metal-binding peptides under heavy--metal stress (YAN et al. 2000). (-Glu-Cys)$_n$Gly peptides (n = 2–11) were found in a Cd-,

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Ag-, Bi-, Pb-, Hg-binding complex produced by higher plants and named phytochelatins (PHCs) (LOEFFLER et al.1989, KLAPHECK et al.1995, INOUHE et al.2000). PHCs can be viewed as linear polymers of the -glutamylcysteine (-Glu-Cys) portion of glutathione. These peptides could be enzymatically produced by stepwise condensation of -Glu-Cys moieties to glutathione itself and the growing phytochelatin chain (GRILL et al.1989).

In the present study, we examined the effect of $Cd(NO_3)_2$ on PHC synthesis and determined Cd levels in the leaves and roots of sunflower plants. The aim of the study was to evaluate whether sunflower plants can be used in bioremediation.

Materials and Methods

Plant material: Sunflower (*Helianthus annuus L.* cv. B 950) seeds were sterilized in 2% hypochlorite solution for 15 min. After being thoroughly washed, the seeds were kept between two pads of moistened cotton, for 3 days, and transferred to Hoagland's solution with/without 15, 25 50 M $Cd(NO_3)_2$ for 15 days in a growth chamber with 250 mol m^{-2} s^{-1} light intensity and a 16 h/8h light/dark light cycle. Both control and Cd treated leaves and roots were frozen in liquid nitrogen immediately after harvesting and stored at –20 °C.

Cd Analysis: Total Cd in tissues was determined using the method of HAN and LEE (1996) employing atomic absorption spectroscopy. Frozen tissues (1.0 g) were ground in a mill and then stirred in a solution containing 5 mL sulfuric acid at 4 °C for 10 min before centrifugation at 13 000 g for 15 min. A one-mL aliquot of the reaction mixture was subjected to atomic absorption spectroscopy analysis.

HPLC Analysis: Analysis of the PHCs was performed according to INOUHE et al. (2000) and modified by KLAPHECK et al. (1995) as follows. Frozen tissues were extracted with (1 mL g^{-1} FW) 10% (w/v) 5-sulfosalicylic acid at 0 °C. Extracts were centrifuged at 8.000 g for 5 min and supernatants were kept at 0 °C for 30 min immediately before HPLC. 20 L samples were injected to a reversed-phase LC18 column (250 4.6 mm, 5 m) (Supelco) connected to a HPLC pump (Cecil 1100, Cambridge, UK) and the column was eluted with linear gradient of acetonitrile in 0.1% (w/v) aqueous trifluoroacetic acid at a flow rate of 1 mL min^{-1}. The column eluent was derivatized with 75 M 5,5'-dithio--*bis*-(2-nitrobenzoic acid) (NBA) in 50 mM potassium phosphate (pH 7.6) at a flow rate of 1 mL min^{-1} and monitored 410 nm. PHC concentrations were expressed as mol^{-1} g FW, based on peak areas of the glutathion (GSH) standard.

Results

The levels of phytochelatins and Cd assayed in roots and leaves of sunflower were determined to be time course and $Cd(NO_3)_2$ concentration. Phytochelatin and Cd levels are presented in Table 1–2. Addition of 25 M and 50 m $Cd(NO_3)_2$ to the nutrient solution leads to a significant enhancement of PHCs in the leaves and roots of sunflower plants. The PHC content changes during the growth of the seedlings, reaching different concentrations after 9 d (nearly 45 M g^{-1} FW and 30 M g^{-1} FW) in roots and leaves respectively (Tab. 1). The highest phytochelatin level (49.84 mol g^{-1} FW) was observed in the roots by day 9 of incubation with 50 M $Cd(NO_3)_2$ (Fig. 1). The level of phytochelatin was determined to be higher (1.3–1.8 fold) in the roots than the leaves at different $Cd(NO_3)_2$ concentrations. Incubation with $Cd(NO_3)_2$ leads to synthesis of PHCs in roots and leaves.

Tab. 1. Phytochelatin levels in the roots and leaves of sunflower plants after exposure to $Cd(NO_3)_2$

Phytochelatin Levels (M g^{-1} FW)								
Cd(NO3)2	Roots (3d)		Roots (9d)		Leaves (3d)		Leaves (9d)	
15 M	24.60	1.30	43.14	2.28	13.10	0.78	17.35	1.80
25 M	27.20	1.84	47.16	2.08	17.22	0.88	31.42	2.40
50 M	32.40	3.62	49.84	3.14	21.42	1.66	37.10	1.78

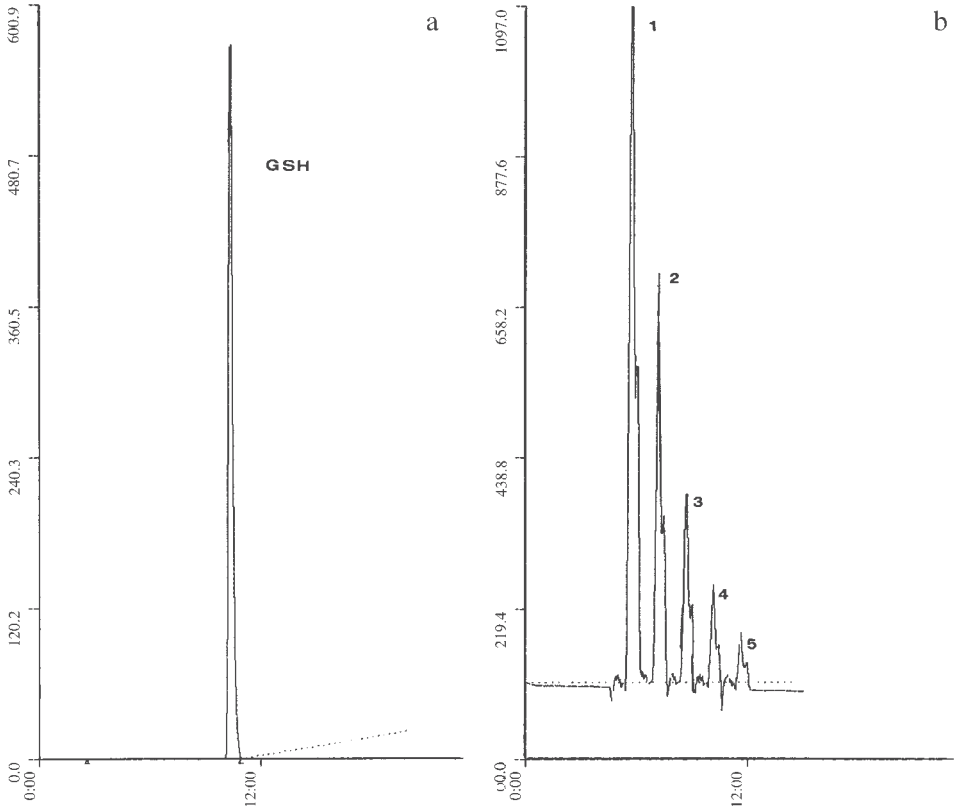

Fig. 1. HPLC analysis of reduced glutathion (GSH) (a) and phytochelatins (b). Peaks 1–5 represent phytochelatins in the extract of roots of sunflower plants exposed to $Cd(NO_3)_2$.

To investigate distribution of Cd in roots and leaves we conducted a study on the time-dependent accumulation of Cd. Remarkable amounts of Cd had accumulated in the roots of the sunflower (Tab. 2). Also as shown in Table 2, more Cd accumulated in the root than in the leaves.

In the present study, we found that sunflower (*Helianthus annuus* Lc 950) leaves and root tissues showed increasing PHC levels after exposure to Cd; here we examine the effects of Cd on PHC synthesis with regards to $Cd(NO_3)_2$ concentration and time course.

Tab. 2. Cadmium levels in roots and leaves of sunflower plants after exposure to $Cd(NO_3)_2$.

Cadmium levels (M g^{-1} FW)								
$Cd(NO_3)_2$	Roots (3d)		Roots (9d)		Leaves (3d)		Leaves (9d)	
15 M	0.08	0.01	0.11	0.005	0.02	0.00	0.04	0.00
25 M	0.17	0.02	0.29	0.08	0.07	0.00	0.11	0.001
50 M	0.24	?.02	0.36	0.01	0.17	0.001	0.20	0.00

Discussion

Heavy metals, unlike organic pollutants, cannot be chemically degraded or biodegraded by microorganisms. An alternative biological approach to deal with this problem is phytoremediation, i.e. the use of plants to clean up polluted waters and soils. Heavy metals can be removed from polluted sites by phytoreaction (ZHU et al. 1999). PHCs can be detected in plant tissues in liquid cultures exposed to heavy metals and the level of PHCs observed in plant tissues correlates with the depletion of metal ions from the medium. PHCs were induced to varying levels by a wide range of metal ion tested. The most effective appeared to be Cd, Ag, arsenate, Cu, Hg, and Pb ions. PHCs appeared to play an important role in Cd and arsenate detoxification in *Arabidopsi*s and yeast (COBBET et al. 2000). Previous studies have demonstrated a phytochelatin response to heavy metal exposure. Evidence suggests that during exposure, phytochelatins accumulate in root tissues and leaves. The present study established the formation of PHCs in the root tissues and leaves of sunflower plants. Several analyses in PHC occurrence studies have documented a ligation of Cd^{+2}, Ag^{+2}, Hg^{+2} and Pb^{+2} by thiolate coordination, as is known for investigated hyperaccumulator species. Evidence for the hyperaccumulating species derives from PHC analysis studies. GALLEGO et al. (1996) have reported that exposure to Cd^{+2} increased PHCs in sunflower and noted that PHCs protected the plant against its harmful effects.

The documented study of the binding of the metalloid to PHCs presented here indicates a detoxifying role for PHCs in an economically important species. In the light of this evidence we emphasize the concept of metal complexation and detoxification by the PHC peptides during the Cd exposure.

The results presented suggest that $Cd(NO_3)_2$ exposure induces the formation of phytochelatins in the root tissues and leaves. As seen in Table 1 and Table 2 Cd and PHC levels increased in the roots and leaves. Although a high level phytochelatin exists in the root tissue, the leaves accumulate a remarkable quantity of Cd and synthesis phytochelatins. From a comparison of Table 1 and Table 2, it can be seen that cadmium probably stimulates very significant phytochelatin synthesis. It would seem that there is a positive feedback in the synthesis of phytochelatins after exposure to $Cd(NO_3)_2$. It is expected that phytochelatins may be found to play an important role in removing heavy metals from polluted environments and in bioremediation.

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