

Glutathione counteracts the inhibitory effect induced by cadmium on photosynthetic process in soybean

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Abstract

In soybean seedlings, Cd^{2+} affected growth and inhibited photosynthesis. Both the length and fresh mass decreased more in roots than in shoots. Cd^{2+} stress caused an increase in ratio of chlorophyll (Chl) $(a+b)/b$ by 1.3 fold and ratio of total xanthophylls/ β -carotene by 3 fold compared to the control. A reduced activity of photosystem 2 by about 85 % measured in Cd^{2+} -treated chloroplasts was associated with a dramatic quenching of fluorescence emission intensity, with a band shift of 4 nm. A major suppression of absorption was accompanied with shift in peaks in the visible region of the spectrum. In Cd^{2+} -treated chloroplasts a selective decline in linolenic acid (18:3), the most unsaturated fatty acid of chloroplasts, paralleled with the ten fold enhancement in ethylene production. A three fold increase in peroxidase activity was found in chloroplasts treated with Cd^{2+} compared to the control. Addition of 1 mM glutathione (GSH) counteracted all the retardation effects in soybean seedling growth induced by Cd^{2+} . Thus GSH may control the Cd^{2+} growth inhibition as it detoxifies Cd^{2+} by reducing its concentration in the cytoplasm and removing hydrogen peroxide generated in chloroplasts.

Additional key words: carotenoids; chlorophyll; ethylene; fluorescence emission spectra; fresh and dry mass of roots and shoots; *Glycine*; leaf and root dimensions; lipid peroxidation; photosystem 2.

Introduction

Heavy metal pollution affects the biological process in intact terrestrial plants (*e.g.*, Barcelo and Poschenrider 1990, Steffens 1990, Prasad 1995). Plants absorb and accumulate Cd^{2+} at a high rate, and consequently their growth is impeded. Cadmium causes a reduction in net photosynthetic rate (Krupa *et al.* 1993) as it inhibits the activity of photosystem 2 (PS2) by affecting the water-splitting system (Atal *et al.* 1991, Greger and Ögren 1991). Cadmium inhibits plant growth (Vassilev *et al.* 1997) and damages chloroplasts (Stoyanova and Tchakalova 1997). Membranes are considered a main target of Cd^{2+} pollution (Hendry *et al.* 1992). Cd^{2+} exerts its toxicity by inducing a membrane damage due to lipid peroxidation mediated by activated oxygen radicals that is quenched by antioxidant enzymes (Reddy and Prasad 1992). Glutathi-

one (GSH) and associated antioxidant system have been implicated in adaptation of plants to various oxidative stresses (Alscher 1989, Verkleij and Schat 1990), and therefore the GSH metabolism could be involved in Cd^{2+} tolerance if membrane peroxidation is one of the mechanisms of injury. Cd^{2+} tolerance may result also from the formation of phytochelatin complexes synthesized from GSH (Meuwly *et al.* 1993).

The present study tested the hypothesis that Cd^{2+} -induced retardation of soybean seedling growth involves peroxidation of membrane structure and alteration in pigment composition leading to suppression of photosynthesis, and the role of GSH in counteracting Cd^{2+} effects. To test this, pigment composition, activity of PS2, and peroxidation products (ethylene evolution, peroxidase activity, fatty acid contents) were determined in seedlings grown in different concentrations of Cd^{2+} and GSH.

Materials and methods

Plants: Seeds of soybean, *Glycine max* L., were surface sterilized with 1 % sodium hypochlorite for 20 min and thoroughly washed with distilled water. The seeds were germinated in darkness in Petri dishes containing distilled water with a definite concentration of GSH, L-cysteine, L-glutamic acid, ascorbic acid, or buthionine sulfoximine. After 24 h, seeds were germinated under irradiance of 26 W m^{-2} at a 10/14 h day/night photoperiod in sand for 9 d unless otherwise stated. Different concentrations of cadmium and test substances were applied to half-strength Hoagland solution (Epstein 1972) with minor modification by omitting EDTA as chelating compound. Length and fresh mass of roots and shoots were determined after 9 d in darkness. Each treatment was replicated 5 times and all experiments were repeated 3 times.

Chloroplast isolation: Thylakoids were isolated as described by Osman and El-Shintinawy (1988). Ten g of leaves were homogenized for 5 s in 60 cm^3 of isolation buffer containing 50 mM Tricine (pH 7.8), 50 mM NaCl, 3 mM $\text{MgCl}_2 \times 6 \text{ H}_2\text{O}$, and 0.5 mM EDTA. The homogenate was filtered through eight layers of cheese cloth and centrifuged for 2 min at $2000 \times g$. The resulting chloroplast pellet was resuspended in 200 cm^3 of suspension medium containing 400 mM sorbitol, 50 mM KH_2PO_4 (pH 7.2), 2 mM $\text{MgCl}_2 \times 6 \text{ H}_2\text{O}$, and 0.1 % bovine serum albumin. The suspension was centrifuged at $3000 \times g$ for 90 s. The pellet was suspended in 2 cm^2 of suspension buffer. Thylakoids containing 20 g(Chl) m^{-3} were used for measuring Hill reaction activity. Chloroplasts containing 5 g(Chl) m^{-3} were used for measuring absorption and fluorescence emission spectra. Chlorophyll (Chl) concentration was detected as described by Mackinney (1941).

Pigments: Extraction, separation, quantitative and qualitative analysis of pigments were done by thin layer chromatography according to the method of Cogdell (1988). Absorption and fluorescence emission spectra at room temperature were measured using the Perkin-Elmer LS50B fluorometer. The emitted radiation was collected at different wavelengths using a slit width of 2 mm. Samples were dark adapted for 15 min.

Hill reaction activity was measured as photoreduction of 2,6-dichlorophenol indophenol (DCPIP) according to Biswal and Mohanty (1976). Irradiance of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ was made for 5 min, and the electron transport rate was measured by recording the absorbance at 620 nm against the dark controls.

Ethylene evolution: Ten germinated plants were placed in a 5.5 cm^3 test tube, sealed with a rubber serum cap, and stored under "white light" (40 W m^{-2}) at room temperature for 15 min. Rate of ethylene production was measured using gas chromatography as described by El-Shintinawy and El-Shourbagy (1997).

Lipids were extracted from chloroplasts and fatty acids were analyzed using gas chromatography according to the modified procedures described by El-Shintinawy and Selim (1995).

Peroxidase activity: Leaves were frozen in liquid nitrogen, ground with a mortar, and mixed with 50 mM potassium phosphate buffer (pH 5.8). Peroxidase activity was measured according to Mac-Adam *et al.* (1992) following the change in absorbance at 470 nm of the assay medium containing 7.2 mM guaiacol, 11.8 mM H_2O_2 , and 0.1 cm^3 enzyme extract in a final assay volume of 3.0 cm^3 .

Results and discussion

A comparison of the effects of CdCl_2 and CdSO_4 on growth of 9-d-old soybean seedlings (Fig. 1) indicated two main points: (1) the inhibitory effect of Cd^{2+} was located in roots as judged by the more pronounced inhibition of their fresh mass and

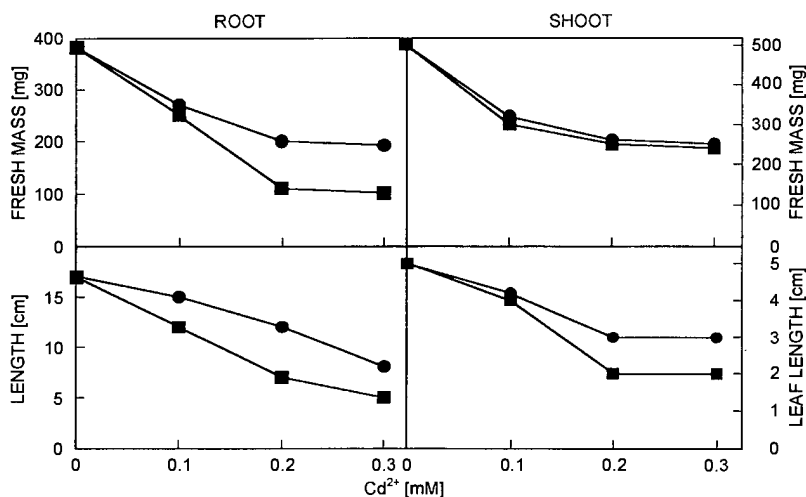


Fig. 1. Changes in fresh mass of roots and shoots and in lengths of roots and leaves of 9-d-old soybean seedlings germinated with 0, 0.1, 0.2, and 0.3 mM CdSO_4 (●) or CdCl_2 (■). Means calculated from five plants.

length compared to shoots induced by both CdCl_2 and CdSO_4 , (2) Cd^{2+} was more effective as CdCl_2 than CdSO_4 in inhibiting the seedling growth (of root fresh mass by 66 *versus* 50 %). Sulfate accumulates GSH and the reduced form of GSH constitutes phytochelatin complexes in many plant species under Cd^{2+} stress (De Kok *et al.* 1981). To elucidate the role of GSH in regulating the growth inhibition of roots induced by Cd^{2+} , different concentrations of GSH, buthionine sulfoximine (BSO - a potent inhibitor of the GSH system as recommended by Reese and Wagner 1987), and L-cysteine and L-glutamic acid (substrates of GSH synthesis as shown by Rennenberg 1982) were applied. Both the L-cysteine and L-glutamic acid reversed the inhibition of fresh mass of roots caused by CdCl_2 , L-cysteine being more effective; in contrast, BSO rendered this inhibition (Table 1). Also ascorbic acid did not improve the root growth inhibition caused by Cd^{2+} . These results show the effective role of GSH in counteracting the inhibition of growth induced by Cd^{2+} .

Table 1. Changes in fresh mass of roots excised from 9-d-old soybean seedlings with different treatments. Means calculated from five plants. BSO = buthionine sulfoximine; GSH = glutathione.

Cd^{2+} [μM]	Addition	Fresh mass [mg]
0	0	380 ± 0.005
200	0	110 ± 0.010
200	2 mM ascorbic acid	120 ± 0.050
200	5 mM ascorbic acid	125 ± 0.050
200	0.5 mM GSH	150 ± 0.002
200	1.0 mM GSH	250 ± 0.020
200	3.0 mM GSH	260 ± 0.040
200	0.5 mM L-cysteine	300 ± 0.040
200	1.0 mM L-cysteine	310 ± 0.040
200	1 mM L-glutamic acid	150 ± 0.005
200	3 mM L-glutamic acid	170 ± 0.003
200	0.1 mM BSO	100 ± 0.007
200	1.0 mM BSO	170 ± 0.001

Table 2 shows the change in pigment distribution of chloroplasts isolated from 9-d-old seedling leaves grown in absence of Cd^{2+} , with 200 μM CdCl_2 , or in presence of 1 mM GSH plus Cd^{2+} . The increase in Chl $(a+b)/b$ ratio in Cd^{2+} -treated chloroplasts was mainly attributed to the destruction of both Chl a and b , related to the changes in composition and structure of the light-harvesting Chl- a/b protein complex 2 induced by Cd^{2+} as reported by Krupa (1988). Cadmium treatment led to a significant degradation of β -carotene that raised the ratio of total xanthophylls/ β -carotene by about 3 fold accompanied by a decrease in Chl $(a+b)/\text{lutein}+\text{zeaxanthin}$ ratio by about 1.3 times. β -carotene and zeaxanthin are important accessory pigments involved in the protection against photoinhibition (Sharma and Hall 1992). However, GSH addition recovered all the above measured ratios near to the control values. These changes in pigment distribution may reflect the alterations in composition and structure of the light-harvesting complexes (Krupa 1988, Padmaja *et al.* 1990) affecting also chloroplast development (Ghoshroy and Nadakavukaren 1990).

Table 2. Pigment composition [$\mu\text{g kg}^{-1}(\text{f.m.})$] estimated in chloroplasts isolated from leaves of 9-d-old soybean seedlings grown under different treatments. Means calculated from three experiments. Chl = chlorophyll, GSH = glutathione.

Pigment	Control ($-\text{Cd}^{2+}$)	200 $\mu\text{M Cd}^{2+}$	200 $\mu\text{M Cd}^{2+}$ + 1 mM GSH
Chl <i>a</i>	1950.0 \pm 0.04	1003.0 \pm 0.05	1620.0 \pm 0.02
Chl <i>b</i>	711.0 \pm 0.03	320.0 \pm 0.05	572.0 \pm 0.02
Antheraxanthin	5.5 \pm 0.02	1.4 \pm 0.03	4.5 \pm 0.02
Neoxanthin	13.8 \pm 0.03	5.5 \pm 0.03	12.7 \pm 0.02
Violaxanthin	17.3 \pm 0.04	11.5 \pm 0.01	13.3 \pm 0.02
Lutein + zeaxanthin	32.5 \pm 0.03	26.0 \pm 0.02	30.0 \pm 0.03
β -carotene	23.0 \pm 0.05	4.6 \pm 0.05	19.5 \pm 0.03
Chl (<i>a+b</i>)	2661.0 \pm 0.07	1323.0 \pm 0.10	219.0 \pm 0.04
Total carotenoids	92.1 \pm 0.17	46.0 \pm 0.14	79.9 \pm 0.12
Total xanthophylls	69.1 \pm 0.12	41.4 \pm 0.09	60.5 \pm 0.09
Chl (<i>a+b</i>)/Chl <i>b</i>	3.7	4.2	3.8
Total xanthophylls/ β -carotene	3.0	9.0	3.1
Chl (<i>a+b</i>)/lutein+zeaxanthin	2.1	1.6	2.0

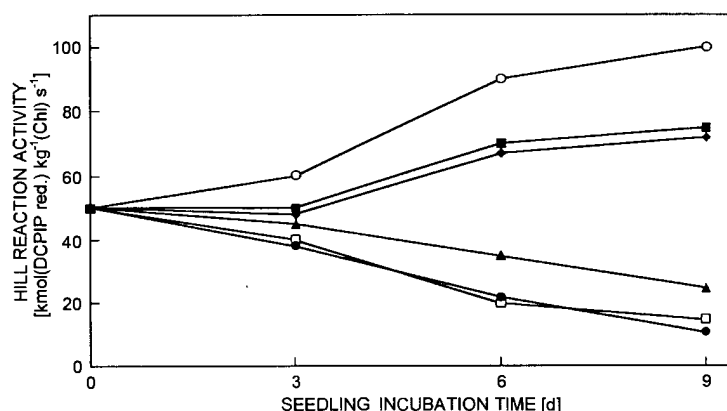


Fig. 2. Effects of 0 (○), 5 (■), 50 (▲), 100 (□), 200 (●) $\mu\text{M CdCl}_2$ or 200 $\mu\text{M CdCl}_2$ + 1 mM glutathione (◆) on photosystem 2 activity of chloroplasts isolated from 6-d-old soybean seedlings incubated for different intervals. Means calculated from three experiments.

To check if the alterations in photosynthetic pigment composition induced by Cd^{2+} impose the overall primary processes of photosynthesis, Hill reaction activity as well as absorption and fluorescence emission spectra were determined. Hill activity measured as DCPIP photoreduction decreased with increasing incubation time at different Cd^{2+} levels (Fig. 2). Treatment of 6-d-old seedlings with 5 $\mu\text{M CdCl}_2$ for 6 to 9 d stimulated the activity of PS2 by about 25 % whereas concentrations of 50 to 200 μM induced a gradual decline in the activity increasing with the incubation time;

maximum inhibition (about 85 %) occurred at 200 μM Cd^{2+} applied for 9 d. Addition of 1 mM GSH in the presence of 200 μM Cd^{2+} enhanced the activity by about 5 times. This indicates that Cd^{2+} may affect directly or indirectly the photochemical

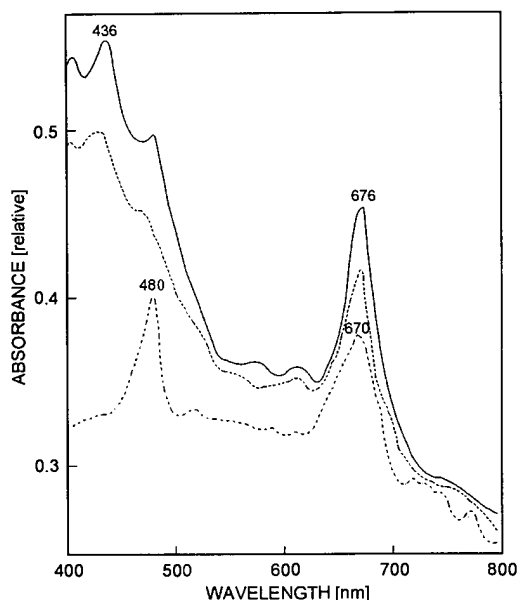


Fig. 3. Differences in absorption spectra of chloroplasts isolated from leaves of 9-d-old soybean seedlings: control (—), 200 μM Cd^{2+} (---, lower curve), and 200 μM Cd^{2+} + 1 mM glutathione (— · —, middle curve).

efficiency of PS2 which could be due to either the inefficient energy transfer from the light-harvesting complex to the reaction centre or the inability of the reaction centre to accept photons as a result of the structural alterations in the PS2 complex (Cao and Govindjee 1990). To examine these possibilities, I measured the absorption spectra of chloroplasts isolated from leaves treated with either 200 μM Cd^{2+} for 9 d with or without the addition of 1 mM GSH (Fig. 3): Cd^{2+} -treatment induced a major decrease in absorption accompanied with a disappearance of a peak at 436 nm, a presence of a sharp peak at 480 nm (both originated from carotenoids), and appearance of a peak at 670 nm (originated from Chl). GSH-treated chloroplasts showed similar absorption spectra as the control chloroplasts. Since Cd^{2+} affects the absorbance of Chl, fluorescence emission spectra were also recorded (Fig. 4). A quenching of fluorescence emission spectra at 686 nm accompanied with a peak shift of 4 nm was recorded in Cd^{2+} -treated chloroplasts. Again, GSH addition recovered the character of fluorescence emission. These results indicate that Cd^{2+} affects both the light-harvesting complex and the reaction centre of PS2.

For a better understanding of the nature of alterations of thylakoid membranes, peroxidase activity, lipid peroxidation, and ethylene production were measured (Table 3). Lipid peroxidation, measured in chloroplast membrane isolated from Cd^{2+} -

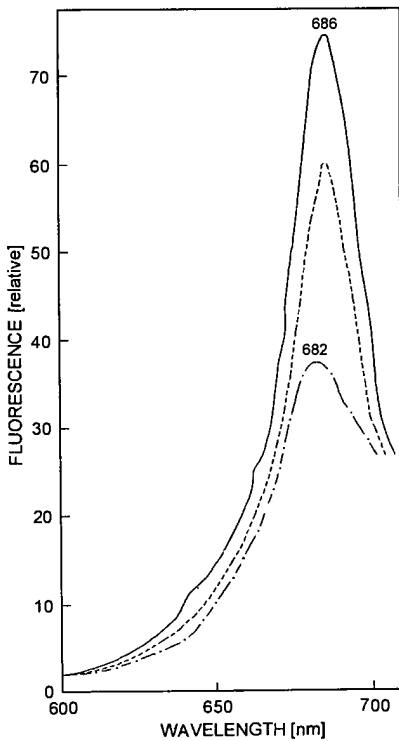


Fig. 4. Differences in fluorescence emission spectra at room temperature of chloroplasts isolated from leaves of 9-d-old soybean seedlings: control (—), 200 μM Cd^{2+} (- - -), and 200 μM Cd^{2+} + 1 mM glutathione (- · -).

Table 3. Effect of Cd^{2+} and glutathione (GSH) on ethylene evolution of whole seedling and on peroxidase activity, and the ratio of steric, oleic, linoleic, and linolenic acids in chloroplasts. Means calculated from three experiments.

	Control ($-\text{Cd}^{2+}$)	200 μM Cd^{2+}	200 μM Cd^{2+} + 1 mM GSH
Ethylene [$\mu\text{mol kg}^{-1} \text{ s}^{-1}$]	2333.3 \pm 3.3	23333.3 \pm 0.2	8666.7 \pm 0.8
Peroxidase [$\mu\text{mol kg}^{-1} \text{ s}^{-1}$]	166.7 \pm 0.3	500.0 \pm 0.2	250.0 \pm 0.5
Stearic acid, $\text{C}_{18:0}$ [% of d.m.]	2.50 \pm 0.02	4.50 \pm 0.01	3.50 \pm 0.01
Oleic acid, $\text{C}_{18:1}$ [% of d.m.]	7.80 \pm 0.02	5.30 \pm 0.03	9.30 \pm 0.02
Linoleic acid, $\text{C}_{18:2}$ [% of d.m.]	20.2 \pm 0.03	29.20 \pm 0.01	19.10 \pm 0.03
Linolenic acid, $\text{C}_{18:3}$ [% of d.m.]	52.0 \pm 0.02	30.00 \pm 0.01	47.00 \pm 0.01
Ratio $\text{C}_{18:0+1+2}/\text{C}_{18:3}$	0.6	1.6	0.7

treated leaves as a ratio of $(\text{C}_{18:0} + \text{C}_{18:1} + \text{C}_{18:2})/\text{C}_{18:3}$ was increased by about 2.6 times due to the dramatic decline in concentration of linolenic acid, the most unsaturated fatty acid in chloroplast membranes. In addition, ethylene evolution was increased by about 10 times and peroxidase activity by 3 times. GSH addition counteracted all the above peroxidation parameters: it lowered ethylene production by about 2.7 times, peroxidase activity by 2 times, and peroxidation index by 2.3 times. Increasing ethylene production and the specific inhibition of linolenic acid by

plants under environmental stress are regarded as a monitor of lipid peroxidation (El-Shintinawy and El-Shourbagy 1997). In addition, the increase in activity of peroxidase, one of the antioxidant enzymes, could be interpreted as an increase in cellular oxidative stress since H_2O_2 is one of the enzymatic reaction products (Chen and Kao 1995, Bhattacharjee 1998).

Cd^{2+} causes a failure of protection from oxidative damage as judged by increasing ethylene evolution and peroxidase activity. Furthermore, lipid peroxidation coupled with a reduction of carotenoid contents may act synergically to decrease the energy transfer to the reaction centre of PS2. Thus, Cd^{2+} induces a rate limitation in the overall processes of photosynthesis, by affecting primarily the contents of photosynthetic pigments and consequently the membrane structure. GSH has a dual function in counteracting Cd^{2+} growth retardation: first, as being an antioxidant and free radical scavenger, it protects chloroplasts from oxidative damage by trapping the hydroxyl radicals, secondly, as a substrate for phytochelatin synthesis that mainly sequesters and detoxifies excess Cd^{2+} ions. When both processes of detoxification, repairing membrane damage, and sequestration of Cd^{2+} are achieved by GSH, the electron transport rate as well as all photosynthetic characteristics are not perturbed and this results in a photosynthetic performance similar to that in the control.

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