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BRIEF COMMUNICATION

Cadmium-induced oxidative damage and antioxidant responses in *Brassica juncea* plants

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Abstract

Indian mustard (*Brassica juncea* L. cv. Vitasso) plants exposed to 10, 30, 50 and 100 μM of Cd for 5 d in hydroponic culture were analysed with reference to the distribution of Cd^{2+}, the accumulation of biomass and antioxidants and antioxidative enzymes in leaves. Cd induced a decrease in plant biomass. The maximum accumulation of Cd occurred in roots followed by stems and leaves. Cd induced a decrease in catalase (CAT) and guiacol peroxidase (GPX) activities but an increase in ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR) activities. Enhancement in dehydroascorbate reductase (DHAR) activity was also at 10 μM Cd. Glutathione reductase (GR) activity showed pronounced stimulation after all treatments, but glutathione S-transferase (GST) and glutathione peroxidase (GPOX) activities decreased. The effectiveness of ascorbate-glutathione cycle (AGC) was determined by the ratio of ascorbate to H_2O_2. This ratio decreased in the Cd-treated leaves which indicated that the cycle was disordered.

Additional key words: ascorbate-glutathione cycle, catalase, Indian mustard, guiacol peroxidase.

Contaminated soils with heavy metals pose a major environmental and human health problem, which may be solved by emerging phytoremediation methods. Plant properties important for metal phytoremediation are metal tolerance and accumulation. They are determined by metal uptake, root-shoot translocation, intracellular sequestration and chemical modification. Natural hyperaccumulators, such as *Brassica juncea* can accumulate exceptionally high concentrations of heavy metals in the roots (Reeves and Baker 2000, Simonova *et al.* 2007). They may be involved in phytoremediation directly as phytoremediation crops, or indirectly as sources of genes for improvement of other phytoremediation crops (Pollard *et al.* 2002). *Brassica juncea* L. cv. Vitasso possesses short vegetation period from 40 to 60 d, unpretentiousness to the soil type and forms high quantity of biomass. The plants accumulated heavy metals may be burned or processed.

Cd like other heavy metals produces oxidative stress possibly by generating free radicals and active oxygen species (Hendry *et al*. 1992). Understanding the biochemical detoxification strategies that plants (predominantly hyperaccumulators) adopt against oxidative stress induced by accumulated metal ions is a key to manipulate its heavy metal tolerance.

The aim of this study was to establish the possible antioxidative mechanism that could be operational in the leaves of *Brassica juncea* L. cv. Vitasso plants exposed for 5 d to environmentally relevant (10 μM) as well as to marginally acute (30, 50, 100 μM) of Cd in hydroponic culture.

The experiments were conducted on 25 d-old plants grown in a Knop solution in a chamber with 16-h photoperiod (PAR 200 μmol m^{-2} s^{-1}, temperature 25 ± 1 °C, relative air humidity 50 - 60 %). The nutrition solution was changed every two days and aerated every day. On the 20th day Cd (as $CdCl_2 . 2.5\ H_2O$) was added into nutrient solution: *1*) control - 0 μM Cd; *2*) 10 μM Cd; *3*) 30 μM Cd; *4*) 50 μM Cd; *5*) 100 μM Cd. The plants were treated for 5 d. Fresh mass (FM) of the roots, stems

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Abbreviations: AGC - ascorbate-glutathione cycle; APX - ascorbate peroxidase; CAT - catalase; DHAR - dehydroascorbate reductase; GPOX - glutathione peroxidase; GPX - guiacol peroxidase; GST - glutathione S-transferase; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase.
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and fully expanded leaves was determined immediately after harvesting. Dry mass (DM) of roots, stems and leaves was determined after drying at 55 - 60 °C till to constant mass. The roots, stems and leaves were digested in a HNO_3-$HClO_4$ (3:1, v:v) mixture and Cd concentrations were determined by atomic absorption spectrophotometry (*AAnalyst 300*, *Perkin-Elmer*, UK). In order to prepare crude extracts for the determination of the enzymes of ascorbate-glutathione cycle [glutathione peroxidase (GPOX), glutathione S-transferase (GST) and glutathione reductase (GR)], as well as catalase (CAT) and guiacol peroxidase (GPX), the fully expanded leaves were quenched in liquid nitrogen and grinded with 4 cm^3 of the extraction buffer [100 mM K_2HPO_4, pH 7.8, 5 mM EDTA, 2 % polyvinylpyrolidone (PVP)] that was added to 0.3 g (f.m.) of tissue powder. The extraction buffer for the determination of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) contained: 100 mM K_2HPO_4, pH 7.8, 1 mM ascorbate, 1 mM EDTA, 0.2 % PVP and was added to 0.15 g (f.m.) of tissue powder. After further grinding till thawing, the suspensions were centrifuged (16 000 *g*, 15 min, 4 °C). All enzymes were assayed spectrophotometrically (*Specord UV-VIS*, UK) by tracing the change in absorbance at 27 °C. GPOX (EC 1.15.1.1) was assayed according to the procedure of Edwards (1996); GST (EC 2.5.1.18) according to Li *et al*. (1995); GR (EC 1.6.4.2) according to Sherwin and Farrant (1998); CAT (EC 1.11.1.6) and GPX (EC 1.11.1.7) according to Chance and Maehly (1955); APX (EC 1.11.1.11) according to Nakano and Asada (1981); MDHAR (EC 1.6.5.4) according to Miyake and Asada (1992); DHAR (EC 1.8.5.1) according to Doulis *et al.* (1997). Total protein content was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standart. Leaf extracts for determination of low molecular antioxidants and hydrogen peroxide (H_2O_2) contents were made by grinding frozen tissue in $HClO_4$ as described Foyer *et al.* (1983). Reduced (GSH) and oxidized (GSSH) forms of glutathione were measured in extracts according to Doulis *et al.* (1997). Ascorbate and dehydroascorbate were assayed according to Foyer *et al.* (1983). H_2O_2 was determined by the method of Dhinsa *et al.* (1981). The level of lipid peroxidation was determined as 2-thio-barbituric acid (TBA) reactive substances chiefly malon-dialdehyde (MDA) as described Heath and Packer (1968).

All values were mean of at least five independent experiments. The significance of differences between control and each treatment was analysed using Student′s *t*-test

Indian mustard plants grown in the presence of 10, 30, 50 and 100 μM Cd showed accumulation of this metal in their roots, stems and leaves. Cd accumulation is more in plants exposed to 100 μM Cd than to 10 μM Cd. Maximum accumulation of Cd occurred in roots followed by stems and leaves (Fig. 1*B*). Low accumulation of Cd in leaves may be a strategy to protect photosynthetic function from Cd-induced oxidative stress (Chaoui *et al.* 1997). Treatment with increasing concentrations of Cd for 5 d significantly diminished the ratio fresh mass/dry mass (FM/DM) in the roots, stem and leaves (Fig. 1*A*).

The increased accumulation of lipid peroxides is indicative of enhanced production of toxic oxygen species. The content of MDA increased in the leaves of plants treated with increasing concentrations of Cd. H_2O_2 increased in the leaves of plants treated with 30 and 50 μM Cd with reference to the control (Table 1). Excessive contents of H_2O_2 could be minimized through the activities of CAT and peroxidases. Our results

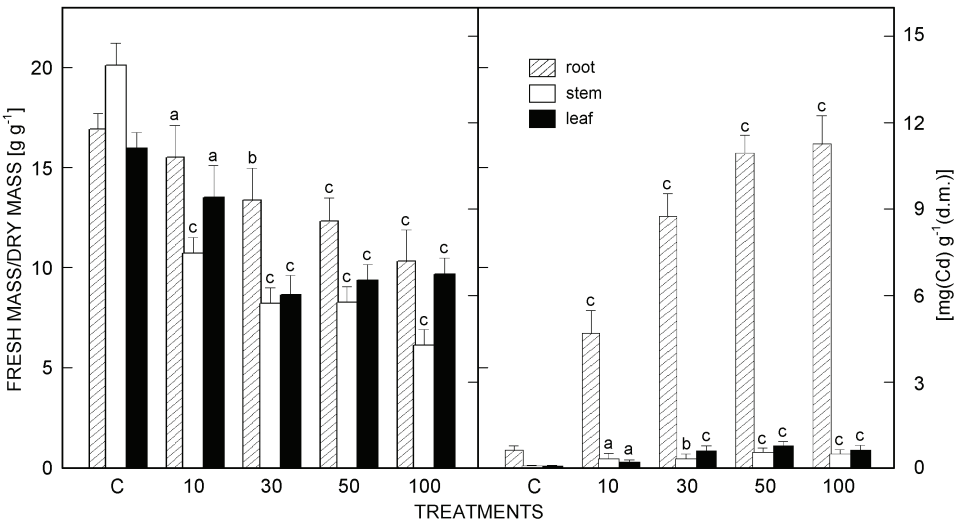

Fig. 1. Changes in the fresh/dry mass ratio and Cd content in roots, stems and leaves of *Brassica juncea* plants cultivated in a full nutrient solution with 0 (C), 10, 30, 50 or 100 μM Cd for 5 d. Data represent the means ± SD of five separate experiments. The significance of differences between control and each treatment is analysed using Student`s *t*-test at $P < 0.05$ (a), $P < 0.01$ (b) and $P < 0.001$ (c).

Table 1. Changes in the contents of MDA, H_2O_2, some low molecular antioxidants and activities of antioxidative enzymes in leaves of *Brassica juncea* plants cultivated in a full nutrient solution with 0, 10, 30, 50 or 100 μM Cd for 5 d. Means ± SD of five separate experiments. The significance of differences between control and each treatment was analysed using Student`s *t*-test at $P < 0.05$ (a), $P < 0.01$ (b) and $P < 0.001$ (c).

Parameters	0	10	30	50	100
MDA [nmol g^{-1}(f.m.)]	0.086 ± 0.005	0.085 ± 0.002[a]	0.066 ± 0.005[c]	0.086 ± 0.008[a]	0.088 ± 0.008[a]
H_2O_2 [μmol g^{-1}(f.m.)]	0.193 ± 0.015	0.529 ± 0.020[c]	1.827 ± 0.079[c]	1.858 ± 0.075[c]	1.365 ± 0.057[c]
CAT [mol g^{-1}(protein) min^{-1}]	0.14 ± 0.02	0.12 ± 0.01[a]	0.10 ± 0.008[a]	0.08 ± 0.01[a]	0.07 ± 0.01[a]
GPX [mol g^{-1}(protein) min^{-1}]	0.14 ± 0.02	0.15 ± 0.01[a]	0.06 ± 0.009[c]	0.12 ± 0.03[a]	0.13 ± 0.04[a]
APX [mol g^{-1}(protein) min^{-1}]	0.18 ± 0.02	0.23 ± 0.02[b]	0.12 ± 0.01[c]	0.37 ± 0.03[c]	0.25 ± 0.02[c]
MDHAR [mol g^{-1}(protein) min^{-1}]	0.20 ± 0.06	0.27 ± 0.04[a]	0.82 ± 0.09[c]	0.64 ± 0.09[c]	0.63 ± 0.06[c]
DHAR [mol g^{-1}(protein) min^{-1}]	0.13 ± 0.02	0.19 ± 0.02[b]	0.04 ± 0.004[c]	0.04 ± 0.01[c]	0.04 ± 0.02[c]
ascorbate [nmol g^{-1}(f.m.)]	13.72 ± 1.17	11.80 ± 0.73[a]	14.71 ± 1.13[a]	16.34 ± 1.31[a]	9.81 ± 1.31[b]
dehydroascorbate [nmol g^{-1}(f.m.)]	43.34 ± 7.39	26.67 ± 3.77[b]	70.83 ± 5.03[c]	54.17 ± 6.75[a]	35.42 ± 1.95[a]
GR [mol g^{-1}(protein) min^{-1}]	0.10 ± 0.007	0.14 ± 0.01[c]	0.14 ± 0.01[c]	0.13 ± 0.005[c]	0.11 ± 0.004[a]
GST [ΔE g^{-1}(protein) min^{-1}]	1.19 ± 0.10	1.15 ± 0.07[a]	0.73 ± 0.05[c]	0.98 ± 0.08[b]	0.79 ± 0.07[c]
GPOX [ΔE g^{-1}(protein) min^{-1}]	0.18 ± 0.02	0.17 ± 0.009[a]	0.04 ± 0.005[c]	0.04 ± 0.008[c]	0.03 ± 0.007[c]
GSH [nmol g^{-1}(f.m.)]	35.93 ± 3.54	36.16 ± 1.57[a]	49.58 ± 3.42[c]	32.67 ± 2.11[a]	22.46 ± 1.58[c]
GSSH [nmol g^{-1}(f.m.)]	33.67 ± 2.60	83.34 ± 8.85[c]	41.67 ± 4.08[b]	57.08 ± 10.31[b]	47.92 ± 5.46[c]
ascorbate/H_2O_2	0.071	0.022	0.008	0.009	0.007

showed that activities of CAT and GPX decreased progressively in the leaves of plants treated with increasing concentrations of Cd. APX activity is maximal in the leaves of plants exposed to 30 μM Cd, MDHAR activity is highest at 30 and 50 μM Cd and DHAR activity increased only after treatment with 10 μM Cd (Table 1). Increased activity of APX and decreased activities of CAT and GPX show that they are not competed to remove H_2O_2. The inadequate response of CAT and GPX activities to Cd in leaves is compensated by increasing activity of APX. Cd treatments lead to different changes of the ascorbate and dehydroascorbate contents (Table 1). Since ascorbate is the primary antioxidant and H_2O_2 is the major stable oxidant, the ratio of these redox components is indicative of the redox balance within the tissue. As Kingston-Smith *et al.* (1997) described the relative amounts of ascorbate and H_2O_2 may be used in determining the effectiveness of ascorbate-glutathione cycle (AGC). The measured ratios of ascorbate to H_2O_2 are always lower in the leaves of treated plants in comparison with the control (Table 1), which indicated that the cycle is not robust and it is disordered by Cd-stress.

Glutathione reductase, another enzyme of AGC, showed a significant increase in its activity after treatment with Cd. The increase with reference to the control was highest at 10 and 30 μM Cd (Table 1). GST and GPOX are known to be responsive to biotic and abiotic stresses, but they are not well characterized with respect to their antioxidative roles in plants. Cd treatment caused a decrease in GST activity and significant reduction in GPOX activity in leaves. Content of GSH, that maintains the cellular redox status and also serves as substrate for phytochelatin synthesis, increased in 10 and especially 30 μM Cd exposed plants (Table 1). Various levels of metal-induced depletion of GSH was reported in different plant species (De Vos *et al.* 1992, Gallego *et al.* 1996). The changes in the GSSH content was significant with respect to the control after treatment with Cd. The increase was highest at 10 μM Cd (Table 1). The stimulated GR activity in Cd-treated plants presumably did not allow an abrupt fall in the GSH content. The decline in the GSH content might be due to its transport from leaves to roots for the synthesis of phytochelatins or its utilization as a reducing substrate in the synthesis of ascorbate. GSH is also consumed and degraded in order to protect cellular membranes from lipid peroxidation.

The results suggest that the Cd-induced increase in some antioxidative enzymes in the leaves may represent a secondary defensive mechanism against oxidative stress that is not as direct as the primary defensive responses such as phytochelatins and vacuolar compartmentalization (Sanita de Toppi and Gabbrielli 1999). The induction of APX, MDHAR and GR provide additional defence against metal toxicity.

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