

## BRIEF COMMUNICATION

## Effects of cadmium stress upon activities of antioxidative enzymes, photosynthetic rate, and production of phytochelatins in leaves and chloroplasts of wheat cultivars differing in yield potential

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### Abstract

We tested the mode of action of Cd on photosynthesis and activities of ATP-sulfurylase (ATP-S), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and on contents of phytochelatins (PCs) and glutathione (GSH) in two cultivars of wheat (*Triticum aestivum* L.) PBW-343 and WH-542 differing in yield potential. Cd treatment increased Cd content and photosynthetic activity in PBW-343 more than in WH-542. The activities of APX, GR, ATP-S, and synthesis of PCs and GSH were also increased by Cd, but the CAT and SOD activities were inhibited in both the cultivars. The efficient functioning of antioxidative enzymes, production of PCs and GSH, helped in counteracting the effects of Cd namely in PBW-343, protected photosynthetic ability, and increased the tolerance to Cd.

**Additional key words:** ascorbate peroxidase; ATP-sulfurylase; catalase; glutathione; glutathione reductase; stomatal conductance; superoxide dismutase; *Triticum*; water use efficiency.

**Abbreviations:** AOS – active oxygen species; APX – ascorbate peroxidase; ATP-S – ATP-sulfurylase; CAT – catalase; Chl – chlorophyll; GR – glutathione reductase,  $g_s$ , stomatal conductance; GSH – glutathione; GSSG – oxidized glutathione;  $P_N$  – net photosynthetic rate; PCs – phytochelatins; SOD – superoxide dismutase.

Cadmium is one of the most toxic amongst heavy metals (Wagner 1993). Although present in traces in soils, its level has increased enormously in recent times through the use of sewage sludge and city refuse. Cd entries into the food chain, and thus become harmful to humans (Pence et al. 2000, Stolt et al. 2006). High contents of Cd in plants inhibit the chlorophyll (Chl) synthesis, photosynthesis, growth, yield, and induce even plant death (Sanita di Toppi and Gabbriellini 1999, Zhang et al. 2003, Hassan et al. 2005, Wojcik et al. 2005, Burzyński and Żurek 2007, Ebbs and Uchil 2008, He et al. 2008). It induces the formation of active oxygen species (AOS), superoxide anion ( $O_2^{\cdot-}$ ), hydroxyl ( $OH^{\cdot}$ ) radicals and  $H_2O_2$ , which cause oxidative stress and damage the photosynthetic apparatus and membrane permeability through the production of thiobarbituric acid reactive substances (Mobin and Khan 2007). Plants adopt different strategies to reduce Cd-induced oxidative damage. One of them is the increase of activities of antioxidative enzymes (Gratao et al. 2006). The antioxidative enzyme system

constitutes superoxide dismutase (SOD) as the primary step of cellular defence. It dismutates  $O_2^{\cdot-}$  to  $H_2O_2$  and  $O_2$ . Further, the accumulation of  $H_2O_2$  is restricted by the action of the ascorbate-glutathione cycle, where ascorbate peroxidase (APX) reduces it to  $H_2O_2$  or by the action of catalase (CAT) in peroxisomes. In the ascorbate-glutathione cycle, the final step is catalysed by glutathione reductase (GR) which in turn catalyses the NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) (Noctor et al. 2002). Glutathione is related to the sequestration of heavy metals and is also an essential component of the cellular antioxidative defence system, which keeps AOS under control (Noctor and Foyer 1998). Glutathione is used in the removal of AOS. The first step of S assimilation is the activation of S by the enzyme ATP-sulfurylase (ATP-S), and in a cascade of enzymatic steps the inorganic S is converted to the important non-protein tripeptide, glutathione (Tausz et al. 2004).

Phytochelatins (PCs) form various complexes with

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Cd, due to presence of thiolic groups of Cys, which chelates the Cd, and prevents it from circulating as free Cd inside the cytosol (Grill *et al.* 1985). The PCs synthesis in cells is related to relevant contents of metals in the environment. They are synthesized from glutathione, by means of the enzyme PCs synthase (Grill *et al.* 1989). Thus, PCs can be used as a biomarker for the actual metal stress in plants and their synthesis is induced because plants suffer from excess internal heavy metals, such as Cu, Cd, or Zn (Grill *et al.* 1989, Cobbett 2000). We conducted a pot culture experiment to check the localization of antioxidative enzymes, PCs, ATP-S, and Cd in leaves and chloroplasts, and its implication on photosynthetic characteristics in wheat cultivars differing in yield potential.

Two cultivars of wheat (*Triticum aestivum* L.), PBW-343 (Cd-tolerant) and WH-542 (Cd-non-tolerant), were grown during 2005–2006 in earthen pots of 9-cm diameter. The pots were filled with thoroughly mixed soil and organic manure. The pots were watered with potassium phosphate buffer to maintain pH 7.1. Cd was given as  $\text{Cd}(\text{NO}_3)_2$  and the nitrate content in  $\text{Cd}(\text{NO}_3)_2$  was balanced with urea. Phosphate and potash were added in sufficient amounts. The high Cd concentration was selected on the basis of studies of the effects of 25, 50, 75, and 100 mg Cd  $\text{kg}^{-1}$  soil: 100 mg(Cd)  $\text{kg}^{-1}$  ( $\text{Cd}^{100}$ ) soil treatment caused maximum reduction in the characteristics in both the cultivars (Khan *et al.* 2006). Seeds were treated with  $\text{Cd}^0$  or  $\text{Cd}^{100}$  in completely randomized block design. Each treatment was replicated five times. The plants were grown under natural irradiation till 30 d after sowing.

Net photosynthetic rate ( $P_N$ ) and stomatal conductance ( $g_s$ ) were measured in fully expanded leaves using an infra red gas analyzer model Li 6200 (Li-Cor, Lincoln, NE, USA) placed in a 1-l leaf chamber at light saturation intensity between 11–12 h. The atmospheric conditions during measurement were photosynthetically active radiation (PAR) =  $1016 \pm 6 \text{ l } \mu\text{mol m}^{-2} \text{ s}^{-1}$ , relative humidity =  $60 \pm 3\%$ , atmospheric temperature =  $22 \pm 1^\circ\text{C}$  and atmospheric  $\text{CO}_2$  =  $360 \mu\text{mol mol}^{-1}$ . The ratio of atmospheric  $\text{CO}_2$  to intercellular  $\text{CO}_2$  concentration was constant. Photosynthetic water use efficiency (WUE) was calculated as  $P_N/g_s$  (Dudley 1996). For determining the content of Cd and antioxidative enzymes activities isolated chloroplasts were used. Fresh leaf samples were ground in an ice-cold isolation medium containing 0.33 M sorbitol, 50 mM HEPES-KOH, 2 mM EDTA, 2 mM EGTA, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{MnCl}_2$ , and 0.2 % (m/v) bovine serum albumin at pH 7.3. The homogenate was centrifuged at  $2\,000 \times g$  for 10 min and the pellet was suspended in a second aliquot of the isolation medium and centrifuged at  $6\,500 \times g$  for 20 min by using centrifuge (CPR 24 Remi, New Delhi, India) Temperature of centrifugation was  $4^\circ\text{C}$ . The lower dark green band containing the intact chloroplasts was collected after careful removing of the upper part, and then diluted with

an ice-cold buffer containing 0.33 M sorbitol and 50 mM HEPES-KOH, pH 7.3 (Cline *et al.* 1985). The activity of APX (EC, 1.11.1.11) was determined according to Nakano and Asada (1981) by measuring the decrease in absorbance at 290 nm. The assay mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM  $\text{H}_2\text{O}_2$ , and enzyme extract. APX activity was calculated by using extinction coefficient of  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ . The activity of GR was determined as described by Foyer and Halliwell (1976) by monitoring the glutathione dependent oxidation of NADPH at 340 nm. The assay mixture contained 25 mM phosphate buffer (pH 7.8), 0.5 mM oxidized glutathione (GSSG), 0.2 mM NADPH, and enzyme extract. GR activity was calculated by using extinction coefficient of  $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ . For determining ATP-S, fresh leaves were rapidly ground at  $4^\circ\text{C}$  in buffer consisting of 10 mM  $\text{Na}_2\text{EDTA}$ , 20 mM Tris-HCl (pH 8.0), 2 mM DTT, and *ca.*  $0.01 \text{ g cm}^{-3}$  insoluble polyvinylpyrrolidone, using 1 : 4 (m/v) tissue to buffer ratio. The homogenate was strained through gauze and centrifuged at  $20\,000 \times g$  for 10 min at  $4^\circ\text{C}$ .

ATP-S activity was measured using Mo-dependent formation of pyrophosphate. The reaction was started by adding  $0.1 \text{ cm}^3$  of crude extract to  $0.5 \text{ cm}^3$  of the reaction mixture, which contained 7 mM  $\text{MgCl}_2$ , 5 mM  $\text{Na}_2\text{MoO}_4$ , 2 mM  $\text{Na}_2\text{ATP}$ , and 0.032 units per  $\text{cm}^3$  of sulfate-free inorganic pyrophosphate in 80 mM Tris-HCl buffer (pH 8.0). After incubation at  $37^\circ\text{C}$  for 15 min, the phosphate content was determined calorimetrically (cf. Lappartient and Tourine 1996). Proteins were determined using the method of Lowry *et al.* (1951).

Content of total GSH was determined according to the methods of Gupta *et al.* (1998) and Hissin and Hilf (1976). Plants were homogenized in 0.1 M sodium phosphate, 0.005 M EDTA buffer (pH 8.0), and 25 % metaphosphoric acid (used as protein precipitant). The homogenate was centrifuged at  $12\,000 \times g$  at  $4^\circ\text{C}$  for 15 min. The supernatant was used for total GSH determination according to fluorescence (Fluorescence spectrophotometer, model no. 850, Hitachi, Tokyo, Japan, 5 nm spectral slit-widths) at 420 nm after excitation at 350 nm. Total PCs were determined by the methods of de Knecht *et al.* (1992) and Gupta *et al.* (1998).

Cd content was determined by atomic absorption spectrophotometer (Perkin-Elmer A, Analyst, 300; Norwalk, CT, USA) in nitric : perchloric acid (3 : 1, v/v) digested samples. Cd content was expressed on Chl basis. Chl was estimated spectrophotometrically after  $0.1\text{--}0.2 \text{ cm}^3$  of chloroplast suspension was diluted with 80 % acetone to a total volume of  $4 \text{ cm}^3$ .

Chlorophyll was estimated following the method of Mac Kinney (1941). Fresh leaves (100mg) were homogenised in mortar with sufficient quantity of 80% acetone. The extract was filtered and the supernatant collected in the volumetric flask. The process was repeated three times and each time the supernatant was

collected in the same flask. Finally the volume was made upto 10ml with 80% acetone. 5ml sample of chlorophyll extract was transferred to a cuvette and the absorbance was read at 645 nm and 663 nm by spectrophotometer. The following equation given by Arnon (1949) was adopted to calculate the total chlorophyll content.

$$\text{Total chlorophyll content (mg/g)} = [20.2 (\text{OD}_{645}) + 8.02(\text{OD}_{663})] \times V/W \times 1000,$$

where V = total volume of the solution (ml), W = weight of the tissue (g) used for the extraction of the pigments.

All results are shown in Table 1.  $P_N$ ,  $g_s$ , and WUE were inhibited by  $\text{Cd}^{100}$  in both cultivars in comparison to

$\text{Cd}^0$ . In PBW-343, the reductions were less than in WH-542. Due to the Cd treatment, the activity of CAT was lower in the leaves by 24.32 % in PBW-343 and 36.84 % in WH-542. No activity of CAT was detected in chloroplasts of both the cultivars. In PBW-343 the decrease in SOD activity induced by  $\text{Cd}^{100}$  was 21.01% in chloroplasts and 9.97 % in leaves, whereas in WH-542 it was 41.54 % in chloroplasts and 28.03 % in leaves. The activity of APX increased due to  $\text{Cd}^{100}$  in chloroplasts of PBW-343 by 140 % and in leaves by 75 %, in WH-542 by 65 and 45 %. In leaves of both cultivars the activity of GR increased by  $\text{Cd}^{100}$  treatment in leaves (by 75.26 % in PBW-343 and by 37.74 % in WH-542), but declined

Table 1. Differences in photosynthetic characteristics, enzyme activities and substance contents in chloroplasts (Chl) and leaves (L) of two cultivars (tolerant PBW-343, non-tolerant WH-542) of wheat grown at  $\text{Cd}^0$  (control) or  $\text{Cd}^{100}$  and determined at 30 d after sowing. APX – ascorbate peroxidase; ATP-S – ATP-sulphurylase; CAT = catalase; Chl = chlorophyll; FM = fresh mass; GR – glutathione reductase;  $g_s$  – stomatal conductance; GSH – total glutathione;  $P_N$  – net photosynthetic rate; PCs – phytochelatin; SOD = superoxide dismutase; WUE – water use efficiency. The same letters in each column show no significant difference at  $p < 0.05$  determined by Duncan's multiple range test.  $n = 5$ , ND = not detected.

		PBW-343 $\text{Cd}^0$	$\text{Cd}^{100}$	WH-542 $\text{Cd}^0$	$\text{Cd}^{100}$
$P_N$ [ $\mu\text{mol}(\text{CO}_2) \text{ m}^{-2} \text{ s}^{-1}$ ]		19.42±0.058a	12.62±0.098c	15.87±0.098b	5.53±0.075d
$g_s$ [ $\text{mmol m}^{-2} \text{ s}^{-1}$ ]		429±5.19a	349±6.35c	369±6.34b	234±5.20d
WUE [ $\text{mol mol}^{-1}$ ]		45.23±0.075a	36.18±0.069c	43.01±0.092b	23.66±0.092d
CAT [ $\mu\text{mol}(\text{H}_2\text{O}_2) \text{ kg}^{-1}(\text{protein}) \text{ s}^{-1}$ ]		2.22×10 <sup>-5</sup> ±0.040 a	1.68×10 <sup>-5</sup> ±0.034b	2.28×10 <sup>-5</sup> ±0.040a	1.44×10 <sup>-5</sup> ±0.052c
SOD [ $\text{U mg}^{-1}(\text{protein})$ ]	Chl	26.36±0.054c	20.82±0.046a	26.67±0.052c	15.59±0.052b
	L	13.24±0.063c	11.92±0.017a	13.26±0.035c	9.54±0.052b
APX [ $\text{mmol}(\text{AA}) \text{ kg}^{-1}(\text{protein}) \text{ s}^{-1}$ ]	Chl	42.0×10 <sup>-5</sup> ±0.117c	100.9×10 <sup>-5</sup> ±0.116a	22.3×10 <sup>-5</sup> ±0.058d	36.8×10 <sup>-5</sup> ±0.116b
	L	7.8×10 <sup>-6</sup> ±0.029c	1.38×10 <sup>-6</sup> ±0.023a	4.62×10 <sup>-6</sup> ±0.058d	6.6×10 <sup>-6</sup> ±0.116b
GR [ $\text{U g}^{-1}(\text{protein}) \text{ s}^{-1}$ ]	Chl	4.50×10 <sup>-3</sup> ±0.058a	1.80×10 <sup>-3</sup> ±0.058c	2.16×10 <sup>-3</sup> ±0.052b	1.38×10 <sup>-3</sup> ±0.046d
	L	5.82×10 <sup>-3</sup> ±0.069c	10.20×10 <sup>-3</sup> ±0.029a	3.18×10 <sup>-3</sup> ±0.046d	4.38×10 <sup>-3</sup> ±0.046b
ATP-S [ $\text{mmol kg}^{-1}(\text{protein}) \text{ s}^{-1}$ ]	Chl	94.8×10 <sup>-14</sup> ±0.116c	133.4×10 <sup>-14</sup> ±0.230a	83.8×10 <sup>-14</sup> ±0.116d	101.7×10 <sup>-13</sup> ±0.173b
	L	124.8×10 <sup>-13</sup> ±0.231c	176.6×10 <sup>-14</sup> ±0.116a	105.6×10 <sup>-13</sup> ±0.173d	126.1×10 <sup>-13</sup> ±0.145b
GSH [ $\text{mol kg}^{-1}(\text{FM})$ ]	Chl	0.13×10 <sup>-15</sup> ±0.0080c	0.24×10 <sup>-15</sup> ±0.0029a	0.12×10 <sup>-15</sup> ±0.0029d	0.21×10 <sup>-15</sup> ±0.0037b
	L	0.11×10 <sup>-15</sup> ±0.0056c	0.19×10 <sup>-15</sup> ±0.0057a	0.10×10 <sup>-15</sup> ±0.0061d	0.164×10 <sup>-15</sup> ±0.0057b
PCs [ $\text{mmol kg}^{-1}(\text{FM})$ ]	Chl	1.00×10 <sup>-9</sup> ±0.0006c	1.65×10 <sup>-9</sup> ±0.0046a	0.90×10 <sup>-9</sup> ±0.0006d	1.45×10 <sup>-9</sup> ±0.006b
	L	0.80×10 <sup>-9</sup> ±0.0012c	1.29×10 <sup>-9</sup> ±0.0057a	0.80×10 <sup>-9</sup> ±0.0086c	1.26×10 <sup>-9</sup> ±0.0057b
Cd [ $\text{mmol kg}^{-1}(\text{Chl})$ ]	Chl	ND	3.24×10 <sup>-14</sup> ±0.046a	ND	2.19×10 <sup>-14</sup> ±0.040b
	L	ND	2.143×10 <sup>-14</sup> ±0.029a	ND	1.50×10 <sup>-14</sup> ±0.057b

in chloroplasts (by 60.00 % in PBW-343 and by 35.00 % in WH-542). The activity of ATP-S was higher in  $\text{Cd}^{100}$  plants than in the  $\text{Cd}^0$  ones in both PBW-343 and WH-542: in PBW-343 it was by 40.00 % in both chloroplasts and leaves, while in WH-542 by 18.00 % in chloroplasts and by 20.00 % in leaves. After  $\text{Cd}^{100}$  treatment the content of leaf GSH was higher by 72.7 % in PBW-343 and by 64.2 % in WH-542. In chloroplasts these increments were 84.6 % in PBW-343 and 75.0 % in WH-542. Content of PCs in leaves and chloroplasts of PBW-343 was higher than in WH-542. The increments of content of PCs induced by  $\text{Cd}^{100}$  in leaves and chloroplasts were 61.2 and 65.0 % in PBW-343 and 57.5 and 61.1 % in WH-542, respectively. Cd content was not detected in both cultivars at  $\text{Cd}^0$ , but in  $\text{Cd}^{100}$  plants it was

3.24 and 2.19 mmol(Cd)  $\text{kg}^{-1}(\text{Chl})$  in PBW-343 and WH-542, respectively.

According to Foyer and Noctor (2005), Cd toxicity in plants is due to alteration in oxidant levels. Cd enhances lipid peroxidation and generates oxidative stress in plants leading to growth inhibition and even plant death (Somashekaraiah *et al.* 1992). We found that Cd was more accumulated in leaves and chloroplasts of PBW-343 than in WH-542. This caused greater damage to the photosynthetic apparatus and a larger decrease in  $P_N$ ,  $g_s$ , and WUE in WH-542. Also the Calvin cycle enzymes are inhibited by Cd (Siedlecka and Krupa 1999, Di Cagno *et al.* 2001).

The decrease in CAT activity in both the cultivars under Cd stress we found is in agreement with the

findings of Shah *et al.* (2001) and Sandalio *et al.* (2001) who studied rice and pea plants. CAT is present in peroxisomes and mitochondria but not in chloroplasts (Mishra *et al.* 2006). CAT is sensitive to  $O_2^-$  radicals and thus its increasing content under Cd stress may result in inactivation of the enzyme (Cakmak 2000). The decrease may also be associated with degradation caused by induced peroxisomal proteases or may be due to photoinactivation of enzyme (Sandalio *et al.* 2001). Apparently the decreased activity of CAT was compensated by increased activity of  $H_2O_2$  degrading enzymes, *i.e.* APX.

A major safeguarding mechanism against free radicals is provided by SOD, which catalyzes the conversion of  $O_2^-$  to  $H_2O_2$  and induces damage of membranes and photosynthetic apparatus. The  $H_2O_2$  is then decomposed in the presence of CAT (Hassan *et al.* 2005). In our study, SOD activity was inhibited in  $Cd^{100}$  plants, more in WH-542 than in PBW-343. SOD activity in plants changes with Cd concentration, plant species, plant age, and repeated stress periods (Piquery *et al.* 2000, Sandalio *et al.* 2001, Shah *et al.* 2001).

The activities of APX and GR were higher in PBW-343 showing efficiency of the antioxidative enzyme mechanism that led to the protection of the photosynthetic machinery and lesser reduction in photosynthesis. Our finding of increase in the activity of APX under  $Cd^{100}$  stress is similar to the findings of Pietrini *et al.* (2003) and Mishra *et al.* (2006).  $H_2O_2$  destruction in chloroplasts requires the induction of APX through the ascorbate-glutathione cycle, where APX uses ascorbate as hydrogen donor and GR catalyses the NADPH dependent reduction of GSSG to GSH and maintains high ratio of GSH/GSSG and activation of  $CO_2$ -fixing enzymes (Noctor and Foyer 1998).

The increase in GR activity in  $Cd^{100}$  leaves and its decline in chloroplasts under  $Cd^{100}$  may be related to plant defence. GR responses to Cd stress by maintaining GSSG prior to the incorporation into PCs, and/or by the activation of the ascorbate-glutathione cycle for the removal of  $H_2O_2$ . The increased activity of GR could be explained by transcriptional or translational modification to keep an adequate GR content (Xiang and Oliver 1998, Romero-Puertas *et al.* 2002). Higher GR activity in leaf could maintain a higher GSH/GSSG ratio in leaves than in roots, which results in more oxidative damage to roots

at high Cd concentration (Mishra *et al.* 2006). We found a decrease in GR activity in chloroplasts, which is in agreement with results of Zhang *et al.* (2003) in cucumber chloroplasts and of Pietrini *et al.* (2003) in *Phragmites australis* chloroplasts.

When Cd enters the cytosol, a system strictly related to S metabolism is promptly activated, finally resulting in the production of important complexing agents termed PCs (Sanita di Toppi and Gabbriellini 1999), since biosynthesis of PCs is closely dependent on S metabolism (Leustek *et al.* 2000). The accumulation of PCs in leaves and chloroplasts we found may contribute to transport of PCs from roots through vascular tissues of the plant. Increase in PCs contents in both leaves and chloroplasts may be due to induction of PC synthase gene. The general alteration of the S metabolic pathways induced by Cd is a possible consequence of an increase in the GSH demand driven by PCs biosynthesis. The production of glutathione, a low molecular mass compound, is important as it is the precursor for the synthesis of PCs (Inouhe 2005). Cd generates the expression of genes encoding enzymes involved in GSH synthesis (Schafer *et al.* 1998). May *et al.* (1998) reported that elevated contents of GSH correlate with tolerance to environmental stress, but enhanced GSH synthesis is an intrinsic response of plants to stress, which may be involved in signal transduction and environmental stress. Thus GSH may serve as a biomarker for metal toxicity in plants, especially for Cd.

The activity of ATP-S in leaves and chloroplasts increased in both the cultivars under Cd stress. The first effects of Cd are on ATP-S due to its high affinity to metabolic processes of S metabolism (De Knecht *et al.* 1995). The activation step of sulfate is mediated by ATP-S because sulfate is metabolically inert (Leustek 2002). A high ATP-S activity may provide tolerance to plants against stress. ATP-S expression and activity is weakly induced upon S depletion but repressed through glutathione (Teuveny and Filner 1997). In PBW-343, the ATP-S activity was higher and thus showed greater tolerance to Cd stress resulting in lesser reduction of photosynthesis. Exposure to heavy metals induces the accumulation of ATP-S and other enzymes of S-assimilation and increases tolerance to heavy metals (Lee and Leustek 1999, Pilon-Smits *et al.* 1999, Dominguez-Solis *et al.* 2001).

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