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Photosynthetic Traits and Activities of Antioxidant Enzymes in Blackgram (*Vigna mungo* L. Hepper) Under Cadmium Stress

Sarvajeet Singh, Nafees A. Khan, Rahat Nazar and Naser A. Anjum
Plant Physiology and Biochemistry Division, Department of Botany,
Aligarh Muslim University, Aligarh-202 002, India

Abstract: Cadmium (Cd) is a non-essential heavy metal that does not have any metabolic use and can be harmful even at low concentrations. Blackgram (*Vigna mungo* L. Hepper cv. T9) plants were grown in pots containing a mixture of soil and compost treated with 0, 25, 50 and 100 mg Cd kg^{-1} soil as $CdCl_2$ for 30 days. The changes in total Chlorophyll (Chl), Chl a/b, net photosynthetic rate (P_N), stomatal conductance (gs), Water Use Efficiency (WUE) and Carbonic Anhydrase (CA) activity were noted. The activities of antioxidative enzymes in root and leaf were also assayed together with the content of Thiobarbituric Acid Reactive Substances (TBARS) and hydrogen peroxide (H_2O_2). The concentration of Cd in root and leaf increased with the increasing Cd concentrations. Greatest decrease in photosynthetic traits was observed with 100 mg Cd kg^{-1} soil. The activity of Superoxide Dismutase (SOD) increased in leaf but decreased in root, whereas the activity of catalase (CAT) decreased in both root and leaf. By contrast to CAT, the activity of ascorbate peroxidase (APX) increased in root and leaf. However, GR activity increased in root and decreased in leaf. The results suggest that the antioxidative enzymes showed differential pattern in root and leaf and the decrease in photosynthesis with 100 mg Cd kg^{-1} soil was associated with the accumulation of TBARS and H_2O_2 content and reduction in Chl content, stomatal conductance and CA activity.

Key words: Antioxidant enzymes, cadmium, carbonic anhydrase, oxidative stress, photosynthesis

INTRODUCTION

Cadmium (Cd) is a common metal pollutant introduced into the environment through industrial activities, sewage sludge application and commercial phosphorus fertilizers and subsequently become a part of the food chain (Wagner, 1993). It is easily taken up by plants and causes toxicity even at low concentrations (Sanita di Toppi and Gabbrielli, 1999). It reduces photosynthesis through inhibiting photosynthetic pigments synthesis (Somashekaraiah *et al*., 1992; Drazkiewicz *et al*., 2003; Mobin and Khan, 2007) and the enzymes involved in CO_2 fixation (Di Filippis and Ziegler, 1993; Seregin and Ivanov, 2001) and also reduces plant growth (Arduini *et al*., 2004; Wojcik and Tukiendorf, 2005; Khan *et al*., 2006). Plants activate antioxidative enzymes system to reduce the adverse effects of Cd stress (Dixit *et al*., 2001; Shah *et al*., 2001; Cho and Seo, 2005; Mobin and Khan, 2007), the response of which depends on plant species and the tissue analyzed (Gallego *et al*., 1999; Vitoria *et al*., 2001; Ferreira *et al*., 2002; Fornazier *et al*., 2002; Cardoso *et al*., 2002). It is assumed that differential activities of antioxidative enzymes in root and leaf may reduce the adverse effects of Cd stress and protect photosynthetic machinery from oxidative stress. The purpose of the present work was to evaluate the oxidative stress, response of antioxidative enzymes system in root and leaf and photosynthetic potential of blackgram (*Vigna mungo*) subjected to cadmium stress.

Corresponding Author: Nafees A. Khan, Plant Physiology and Biochemistry Division, Department of Botany, Aligarh Muslim University, Aligarh-202 002, India Tel: +91-571-2702016

MATERIALS AND METHODS

Plant Material and Growth Conditions

An experiment was conducted in the naturally illuminated greenhouse of the Department of Botany, Aligarh Muslim University, Aligarh, India. A mixture of soil and compost (3:1) with neutral in reaction (pH 7.1) was used for the study. The chemical properties of the soil were: organic carbon, 0.38%; CEC, 2.78 meq 100 g^{-1} soil; nitrogen, 88.4 mg kg^{-1} soil; phosphorus, 8.4 mg kg^{-1} soil; potassium, 110.6 mg kg^{-1} soil and cadmium, 0.31 mg kg^{-1} soil. Soil was mixed with appropriate amount of $CdCl_2$ to achieve 0, 25, 50 and 100 mg Cd kg^{-1} soil. Seeds of blackgram (*Vigna mungo* L. Hepper cv. T9) were sown in 23 cm diameter clay pots containing 4 kg soil on June 15, 2006. After germination, two plants per pot were maintained and watered with deionized water as and when required. Each Cd treatment as well as control was replicated three times. After 30 days of sowing, photosynthetic traits in leaves, activities of antioxidant enzymes and contents of Thiobarbituric Acid Reactive Substances (TBARS) and H_2O_2 were determined in root and leaf.

Determination of Cadmium

Cadmium content was determined in dried root and leaf samples digested in concentrated HNO_3-$HClO_4$ (3:1, v/v) and cadmium concentration was determined by atomic absorption spectrophotometer (GBG, 932 plus, Australia).

Measurement of Photosynthetic Traits

Leaf chlorophyll content was determined by its extraction in 90% acetone and the absorbance was read spectrophotometrically (Lichtenthaler, 1987).

The activity of Carbonic Anhydrase (CA) was determined in leaves used for photosynthetic measurement by the method of Dwivedi and Randhava (1974). Net photosynthetic rate (P_N), stomatal conductance (gs) and intercellular CO_2 concentration (Ci) were measured on fully expanded uppermost leaves on two plants per treatment using Li6200 portable photosynthesis system (LiCOR, Nebraska, USA) on a sunny day. During the measurements the air relative humidity, temperature and ambient CO_2 concentration were 68±5%, 24±2°C and 350±15 μmol mol^{-1}, respectively. Water Use Efficiency (WUE) was calculated by dividing the values of P_N with gs as described by Dudley (1996).

Determination of TBARS and H_2O_2

The content of TBARS in the root/leaf was determined as described by Dhindsa *et al*. (1981). The TBARS content was calculated using the extinction coefficient (155 mM^{-1} cm^{-1}). The content of H_2O_2 was measured in root/leaf by the method described by Jena and Choudhuri (1981). The H_2O_2 content was calculated using the extinction coefficient (0.28 μmol^{-1} cm^{-1}).

Enzyme Extraction and Assay

Root/leaf samples were homogenized with an extraction buffer containing 100 mM potassium phosphate buffer (pH 7.0), 0.5% Triton X-100 and 1% polyvinylpyrrolidone (PVP) using chilled mortar and pestle. The homogenate was centrifuged at 15000 x g for 20 min at 4°C. The supernatant obtained after centrifugation was used for the enzyme assays. For Ascorbate Peroxidase (APX), extraction buffer was supplemented with 2 mM ascorbate.

The activity of Superoxide Dismutase (SOD) was assayed by monitoring the inhibition of photochemical reduction of Nitroblue Tetrazolium (NBT) according to Dhindsa *et al*. (1981). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reaction of NBT.

The Activity of Catalase (CAT) was measured by the method of Aebi (1984) and was determined by monitoring the disappearance of H_2O_2 using the extinction coefficient 0.036 mM^{-1} cm^{-1}. One unit of enzyme was defined as the amount of enzyme necessary to decompose 1 µmol of H_2O_2 min^{-1} at 25°C.

The activity of APX was determined according to Nakano and Asada (1981). APX activity was calculated by using extinction coefficient 2.8 mM^{-1} cm^{-1}. One unit of enzyme is the amount necessary to decompose 1 µmol of substrate min^{-1} at 25°C.

The activity of Glutathione Reductase (GR) was determined as described by Foyer and Halliwell (1976) by monitoring the glutathione dependent oxidation of NADPH. The activity of GR was calculated by using extinction coefficient 6.2 mM^{-1} cm^{-1}. One unit of enzyme is the amount necessary to decompose 1 µmol of NADPH min^{-1} at 25°C.

The protein content in the samples was determined using Bovine Serum Albumin (BSA, Sigma) as standard (Bradford, 1976).

Statistical Analysis

The results are presented as means±standard error. Data were subjected to ANOVA test (SPSS ver. 11 Inc., Chicago, USA) and means were compared by using Duncan's Multiple Range Test, taking $p<0.05$ as significant.

RESULTS

Cadmium Accumulation

The accumulation of Cd in root and leaf increased with increasing Cd concentration in the soil (Fig. 1). However, for each Cd treatment its concentration was greater in roots than leaves. Cd concentration in the roots and leaves increased by 2.3 and 4.9 fold, respectively, with 100 mg Cd kg^{-1} soil compared to 25 mg Cd kg^{-1} soil.

Photosynthetic Traits

Increasing concentration of Cd significantly decreased the photosynthetic traits, P_N, gs, Ci, Chl and CA activity. Greatest significant reduction was observed with 100 mg Cd kg^{-1} soil. In plants treated with 100 mg Cd kg^{-1} soil, P_N and gs were lowered by 55 and 50%, respectively, in comparison to control. The Ci and CA activity remained unaffected with 25 mg Cd kg^{-1} soil, but significantly reduced with 50 and 100 mg Cd kg^{-1} soil. In comparison to control, maximum significant reduction in Ci and CA activity of 19 and 49% with 100 mg Cd kg^{-1} soil was observed. It was also noticed that 100 mg Cd kg^{-1} soil significantly reduced Chl content. Maximum reduction in Chl content of 43% was noted with 100 mg Cd kg^{-1} soil in comparison to control. The ratio of Chl a to Chl b increased with the increasing Cd concentration (Table 1).

Table 1: Changes in chlorophyll content (Chl), Chl a/b and net photosynthetic rate (P_N), stomatal conductance (g_S), Water Use Efficiency (WUE), intercellular CO_2 Concentration (C_i) and Carbonic Anhydrase (CA) activity of blackgram (*Vigna mungo* L. Hepper) exposed to Cadmium (Cd) after 30 days of sowing. Values are means of three replications±SE. Data followed by different letters within a row are significantly different at $p<0.05$

	Cadmium (Cd) concentrations (mg kg^{-1} soil)			
Parameters	0	25	50	100
Total Chl (mg g^{-1} FW)	1.730±0.02^a	1.680±0.09^a	1.240±0.05^b	0.980±0.01^c
Chl a/b	1.500±0.01^b	1.580±0.01^b	1.810±0.03^a	1.800±0.02^a
P_N (µmol CO_2 m^{-2} sec^{-1})	12.070±0.30^a	10.310±0.45^b	7.760±0.27^c	5.470±0.248^d
g_S (mol m^{-2} sec^{-1})	0.249±0.009^a	0.218±0.006^b	0.175±0.008^c	0.125±0.011^d
WUE (µmol mol)	48.470±0.66^b	47.290±0.36^b	44.340±0.49^a	43.760±0.44^a
C_i (µmol mol^{-1})	290±4.61^a	282±4.61^a	263±3.51^b	234±3.05^c
CA activity (m mol CO_2 g^{-1} FW)	1.620±0.047^a	1.400±0.125^a	1.170±0.018^b	0.820±0.005^c

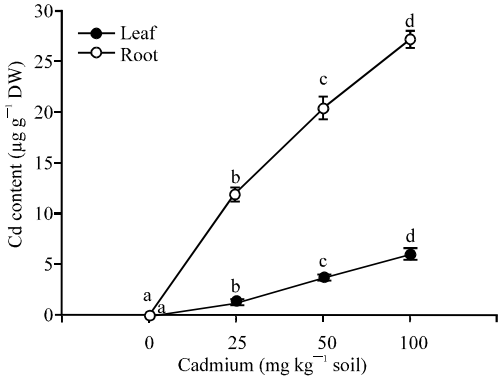

Fig. 1: Changes in cadmium accumulation in root and leaf of blackgram (*Vigna mungo* L. Hepper cv. T9) exposed to Cadmium (Cd) after 30 days of sowing. Values are means of three replications±SE. Data followed by different letter(s) in a graph line are significantly different at $p<0.05$

Contents of TBARS and H_2O_2

The contents of TBARS and H_2O_2 in root and leaf were measured to observe the involvement of oxidative stress (Fig. 2). Roots showed higher contents of TBARS and H_2O_2 than leaves. In roots and leaves, TBARS increased maximally by 57 and 73% with 50 mg Cd kg^{-1} soil in comparison to control. The effect of 50 and 100 mg Cd kg^{-1} soil was not significantly different. Leaves and roots exhibited an increase of 113 and 166% in H_2O_2 content with 100 mg Cd kg^{-1} soil in comparison to control (Fig. 1).

Antioxidant Enzyme Activities

Antioxidative enzymes responded differentially in roots and leaves to Cd treatments. Root SOD activity decreased significantly with 100 mg Cd kg^{-1} soil but remained statistically non-significant with 25 and 50 mg Cd kg^{-1} soil when compared with control. Maximum significant reduction in root SOD activity of 10% was observed with 100 mg Cd kg^{-1} soil in comparison to control. In leaves, SOD activity was increased with increasing Cd concentration. Leaf SOD activity was significantly increased by 23% with 100 mg Cd kg^{-1} soil in comparison to control, whereas, the effect of 25 and 50 mg Cd kg^{-1} soil remained non-significant (Fig. 3).

In comparison to control, 25 mg Cd kg^{-1} soil did not showed any change in root CAT activity but significant decrease in its activity was observed with 50 and 100 mg Cd kg^{-1} soil. Significant reduction in root CAT activity of 46% was observed with 100 mg Cd kg^{-1} soil. CAT activity in the leaves remained unchanged with 25 and 50 mg Cd kg^{-1} soil, whereas, 100 mg Cd kg^{-1} soil caused significant reduction of 25% in its activity when compared with control.

The activity of APX in roots and leaves increased with increasing Cd concentrations. Roots showed greater increase in APX activity than the leaves. In roots, 25 and 50 mg Cd kg^{-1} soil significantly increased the APX activity but it remained same as in 50 mg Cd kg^{-1} soil and 100 mg Cd kg^{-1} soil. Maximum significant increase of 271% in root APX activity was observed with 50 mg Cd kg^{-1} soil compared to control. APX activity in leaves was increased significantly with all Cd concentrations compared to control. Maximum increase in leaf APX activity of 113% was observed with 50 mg Cd kg^{-1} soil in comparison to control.

Cd treatments increased the GR activity in roots but decreased in leaves with 100 mg Cd kg^{-1} soil. In roots, it showed greatest significant increase of 55% with 50 mg Cd kg^{-1} soil, however, its effect was similar to 50 mg Cd kg^{-1} soil (Fig. 3).

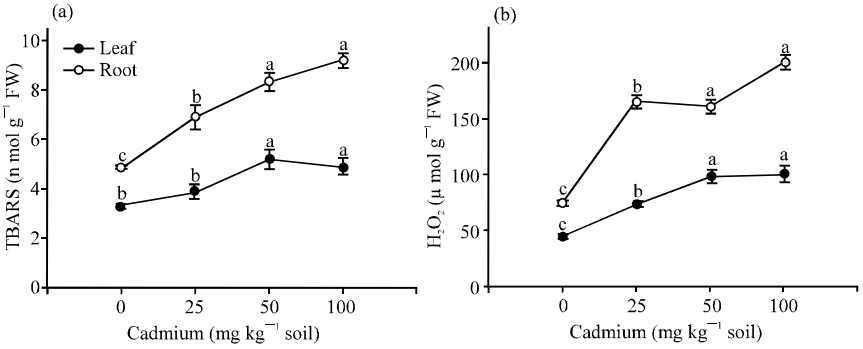

Fig. 2: Changes in (a) Thiobarbituric Acid Reactive Substances (TBARS) and (b) H_2O_2 content in root and leaf of blackgram (*Vigna mungo* L. Hepper cv. T9) exposed to Cadmium (Cd) after 30 days of sowing. Values are means of three replications±SE. Data followed by different letter(s) in a graph line are significantly different at $p<0.05$

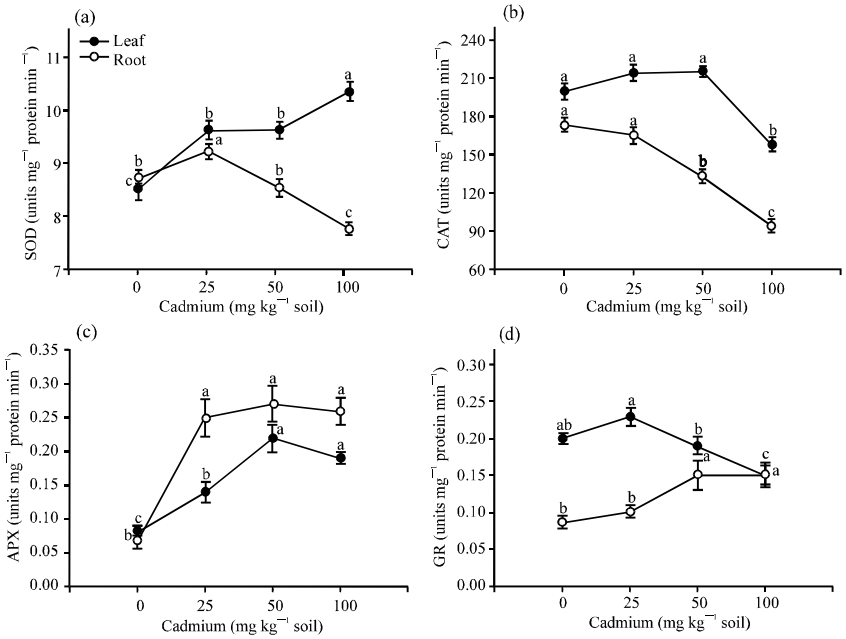

Fig. 3: Changes in (a) Superoxide Dismutase (SOD), (b) Catalase (CAT), (c) Ascorbate Peroxidase (APX) and (d) Glutathione Reductase (GR) activity in the root and leaf of blackgram (*Vigna mungo* L. Hepper cv. T9) exposed to Cadmium (Cd) after 30 days of sowing. Values are means of three replications±SE. Data followed by different letter(s) in a graph line are significantly different at $p<0.05$

DISCUSSION

The contents of TBARS are considered as an index of lipid peroxidation and Cd phytotoxicity (Pandolfini *et al*., 1992; De Vos *et al*., 1993; Lozano-Rodrigrez *et al*., 1997). An increased level of H_2O_2 content in root and leaf due to Cd stress caused elevated generation of reactive oxygen species and

lipid peroxidation. The activities of antioxidative enzymes showed a differential pattern in root and leaf and cooperated synergistically to protect photosynthetic machinery and maintain photosynthesis. Moreover, Cd accumulation differed in root and leaf and was translocated less to leaf. Superoxide dismutase constitutes the primary step of cellular defense. It dismutates $O_2 \cdot^-$ to H_2O_2 and O_2. Further, the accumulation of H_2O_2 is restricted through the action of catalase or by ascorbate-glutathione cycle, where ascorbate peroxidase reduces it to H_2O. Finally glutathione reductase catalyzes the NADPH-dependent reduction of oxidized glutathione to the reduced glutathione (Noctor *et al*., 2002). With the increasing Cd concentration the activity of SOD increased in leaf but decreased in root, whereas, the activity of CAT decreased in both root and leaf. Previous reports have also shown variable changes in the SOD activity in plants exposed to different metals including Cd (Chongpraditrum *et al*., 1992; Somashekaraiah *et al*., 1992; Luna *et al*., 1994; Gallego *et al*., 1996; Okamoto *et al*., 2001; Schickler and Caspi, 1999). The Cd-induced decline in CAT activity has also been reported by Somashekaraiah *et al*. (1992) and Gallego *et al*. (1996). The increase in APX activity in root and leaf indicates efficient conversion of H_2O_2 to H_2O. The activity of GR was also activated in root under Cd exposure, indicating operation of ascorbate-glutathione cycle at high rate to detoxify the ROS formed in the roots and to keep the glutathione in reduced form (Cobbett, 2000).

A greater reduction in Chl content and the decrease in stomatal conductance and CA activity due to Cd cumulatively contributed to the decrease in net photosynthetic rate. The decrease in stomatal conductance due to Cd and a parallel decrease in intercellular CO_2 concentration suggest the involvement of stomatal limitations to photosynthesis. Cadmium stress also produced disturbances in water balance and thus reduction in water use efficiency was observed with Cd treatments. This might be due to the inhibition of absorption and translocation of water, as previously observed by Barcelo and Poschenrieder (1990). The decrease in photosynthesis due to Cd has also been attributed to the increase in mesophyll resistance (Lamoreaux and Chaney, 1978) and decrease in the activity of ribulose 1,5 bisphosphate carboxylase by binding the SH group of the enzyme (Stiborova *et al*., 1986; Vassilev *et al*., 2003). Further, the observed higher decrease in Chl b than Chl a and the increase in Chl a to Chl b ratio may be linked to the reduction in Light Harvesting Chlorophyll Proteins (LHCPs) (Loggini *et al*., 1999) and decrease in photosynthesis due to Cd. The reduction in LHCPs content is an adaptive defence mechanism of chloroplast, which allows them to reduce the adverse condition (Asada *et al*., 1998). The decrease in Chl content has been also shown due to the inhibition of protochlorophyllide reductase and synthesis of 5-aminolevulinic acid (Stobart *et al*., 1985). The decrease in photosynthesis due to Cd toxicity has been reported in the literature (Sawhney *et al*., 1990; Sheoran *et al*., 1990; Khan *et al*., 2006; Mobin and Khan, 2007).

It is concluded that, blackgram (*Vigna mungo*) exhibited oxidative stress in root and leaf and plants maintained a highly integrated differential antioxidative enzymes system in root and leaf to protect photosynthetic apparatus and maintain photosynthesis against oxidative damage.

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