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ASIAN JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN: 0976-3376

Asian Journal of Science and Technology
Vol. 08, Issue, 09, pp.5450-5462, September, 2017

RESEARCH ARTICLE

POLYPHENOL OXIDASE, ACID PHOSPHATASE AND PHOTOSYNTHETIC ENZYMES ACTIVITY IN *CAJANUS CAJAN* SEEDLINGS UNDER CADMIUM STRESS

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ARTICLE INFO

Article History:
Received 19th June, 2017
Received in revised form
26th July, 2017
Accepted 04th August, 2017
Published online 15th September, 2017

Key words:
Acid phosphatase activity,
Cadmium, Hill reaction,
PEP carboxylase,
Polyphenol oxidase,
Pigeonpea cultivars.

ABSTRACT

Role of polyphenolic compounds in different biological functions like biotic and abiotic stress resistance make them important for plants. In the present investigation different cadmium (Cd) concentrations representing 0, 0.02, 0.04 and 0.06 mM were used in three pigeonpea cultivars (LRG30, LRG41 and ICPL85063) on various enzymatic activities. Polyphenol oxidase and acid phosphatase activity of the different parts of the three pigeonpea cultivars increased with increasing seedling growth and with increasing concentration of Cd supplied. However, the acid phosphatase activity of the cotyledons increased only up to 4 days of seedling growth. The greater activities of these enzymes were observed in cv. LRG30 in response to Cd treatment. The δ-amino levulinic acid dehydratase, phosphoenolpyruvate carboxylase, ribulose bisphosphate carboxylase, CO_2 fixation, carbonic anhydrase and hill reaction activities of the three pigeonpea cultivars decreased with increasing concentrations of externally supplied Cd ions. These activities were conspicuously affected in cv. LRG41 and ICPL85063 and appeared more sensitive to Cd treatment.

INTRODUCTION

The unprecedented increase in heavy metal pollution has become a matter of major concern over the globe (Jamali *et al*., 2007). Cadmium (Cd) stands 7th out of the 20 toxins and has no known biological function (Morel, 2008). It has been estimated that major source of Cd release into the air are the production of nonferrous metals followed by iron and steel production, fossil fuel combustion, cement production and waste incineration (Pacyna and Pacyna, 2001). Cd is constantly added and gets accumulated to the plough layer of soil through various natural and anthropogenic activities such as volcanic eruptions, mining, smelting, mismanagement of industrial waste and use of phosphate fertilizers (Grant, 2011) and its addition to the arable land is a widely recognized problem. Cd is potentially toxic to all organisms including plants, animals and humans as well. Plant polyphenols have shown wide variety of properties including plant resistance against microbial pathogens, solar radiation and metal stress. They play role in protecting plants not only by participating in constitutive agents but also by accumulating in plants under various environmental stresses. It has been found that phenol metabolism stimulates in response of metal stress in plants for the protection of plants and recovery from metal injury. Polyphenol oxidase (PPO) a copper-containing enzyme catalyzes the oxidation of phenols to quinones. It is synthesized in cytoplasm and found in the chloroplast of healthy plant cells. The role of PPO as antioxidative enzyme under heavy metal stress has been established (Sharma *et al*., 2014; Devi Chinmayee *et al*., 2014). Increase in the total phenolic content in *Brassica juncea* plants under cadmium stress has been reported (Kapoor *et al*., 2014). Phenolic compounds act as antioxidants and help in scavenging free radicals or chelation of metals. Despite the reported role of polyphenols as effective antioxidant there is very scanty of information about the involvement of polyphenols in cellular responses of ROS generated under metal stress. Thus, by noticing the change in polyphenols under metal stress can help to understand the role of polyphenols in defensive mechanism to avoid metal poisoning (Santana-Casiano *et al*., 2014). Acid phosphatase (AP), one of the abundant hydrolases in the plant cell is believed to have a role in plant adaptation to heavy metal contamination (Johnson and Proctor, 1984; Gabbrielli *et al*., 1989). Acid phosphatase unspecifically catalyzes the hydrolysis of a variety of phosphate esters in an acidic environment. These enzymes are proposed to act in the maintenance of the phosphorus status of the plant, particularly with respect to a role in accessing phosphorus from the soil. Several factors have been shown to influence the activity of

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AP (Duff *et al*., 1994), but the effects of heavy metals on AP are poorly understood (Tabaldi *et al*., 2007). Increase in AP activity has also been demonstrated in Cd treated crop plants (Ernst, 1980; Grunhage and Jager, 1982).

Inhibition of chlorophyll biosynthesis enzymes and activation of its enzymatic degradation plays crucial role in net loss in chlorophyll content (Somashekaraiah, 1992). Aminolevulinate (ALA) is a crucial compound in chlorophyll biosynthesis and its synthesis is the rate-limiting and regulatory step. Cd inhibits ALA synthesis at the site of availability of glutamate for ALA synthesis and interferes by interacting with SH group of enzymes, δ-aminolevulinic acid dehydratase (Mysliwa-Kurdziel and Strzalka, 2002) and porphobilinogen deaminase (Skrebsky *et al*., 2008), leading to the accumulation of chlorophyll biosynthesis intermediates like ALA and porphyrins. In fact ALA accumulation is considered to be a reason for generation of reactive oxygen species which alters redox status of plants and thus disturbing plant homeostasis as reported in Soybean and *Cucumis* (Noriega *et al*., 2007; Goncalves *et al*., 2009). Additionally Cd reacts with protochlorophillide reductase, which causes photoreduction of protochlorophillide into chlorophyllide thus diminishing the raw material for chlorophyll synthesis (Stobart *et al*., 1985).

One of the common changes noted in plants exposed to Cd stress is the modulation of carbon metabolism enzymes (Gouia *et al*., 2003; Chiraz *et al*., 2008). Phosphoenolpyruvate carboxylase (PEPC) plays an anaplerotic role in the provision of C skeletons for amino acid synthesis (Champigny and Foyer, 1992). Under stress condition, including that induced by Cd, PEPC activity increases (Foyer *et al*., 2003; Gouia *et al*., 2003). The increase in PEPC activity enhances the C flow through the anaplerotic pathways by providing C skeletons for the tricarboxylic acid cycle and for amino acid synthesis. It is known that the activities of PEPC enzyme responsible of L-ketoglutarate synthesis, co-ordinate C and N metabolism (Scheible *et al*., 2000). Ribulose 1,5-bisphosphate carboxylase, a bifunctional enzyme with the capacity to competitively use CO_2 or O_2 is the key enzyme responsible for overall CO_2 fixation during photosynthesis. Some studies have shown the existence of an inhibitive effect of cadmium upon the content of soluble proteins as well as upon enzyme activities such as Rubisco (ribulose-1,5-biphosphate carboxylase/ oxygenase (Siedlecka *et al*., 1997). Plants exposed to stress exhibit enhanced photorespiration that is believed to be a waste full process involved in release of CO_2 (Azam and Farooq, 2003; Bota *et al*., 2004). In plants, it results in many toxic symptoms such as inhibition of growth and photosynthesis, activation or inhibition of enzymes, disturbances in plant-water relations and ion metabolism, and formation of free radicals (Wahid *et al*., 2009; Valentoviova *et al*., 2010). Cd is taken up by roots through plasma membrane transporters such as ZIP (ZRT-IRT like protein; Zinc regulated transporter, Iron-regulated transporter) and NRAMP (natural resistance associated macrophage protein) in competition to the essential nutrients of plants (Kim *et al*., 2002) and consequently it is translocated to shoots thereby leading to growth diminution which in due part emanates from disturbed photosynthesis (Bazzaz and Govindjee, 1974).

Photosynthesis inhibition may be attributed to diminished chlorophyll biosynthesis (Shukla *et al*., 2008), interrupted O_2 - evolving reactions of PSII and altered electron flow around PSI and PSII (Mallick and Mohn, 2003). Cd hampers calvin cycle by slowing down activity of various enzymes hence resulting in decreased photosynthesis (Ying *et al*., 2010). Cd has also been known to show inhibitory effect on various enzymes such as ribulose-1,5-biphosphate carboxylase oxygenase (Mobin and Khan, 2007), phosphoenolpyruvate carboxylase (Latif, 2008), and carbonic anhydrase (Mobin and Khan, 2007). In intact plants, Hill reaction action was affected adversely by Cd^{2+} ions (Sheoran *et al*., 1990b). Cd toxicity may be attributed to both acceptor and donor side of PSII thus preventing photoactivation (Sigfridsson *et al*., 2004). On the donor side due to high affinity, Cd exchanges with Ca^{++} in Mn^{++}/Ca^{++} cofactor present in oxygen evolving complex (Faller *et al*., 2005; Pagliano *et al*., 2006), the exchange leads to reduced kinetics of Hill reaction. On acceptor side Cd decreased the rate of electron transfer from Q_A to Q_B due to interaction with nonheme Fe and conformational modification of Q_B pocket (Geiken *et al*., 1998). The aim of the present study is to determine the effect of various enzymes on pigeonpea cultivars in the absence of metal and in the presence of different cadmium concentrations, and to look deeper into the heavy metal tolerance among cultivars of *Cajanus cajan*, an important pulse crop of India.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of three cultivars of pigeonpea (*Cajanus cajan* (L.) Millspaugh) namely LRG30 (Long duration, 180-300 days), LRG41 (Medium duration, 150-180 days), and ICPL85063 (Short duration, 100-150 days) obtained from ICRISAT, Patancheru and LAM, Guntur, Andhra Pradesh, India were used for the present investigation. These varieties are grown around the Visakhapatnam and its surrounding villages. The seeds of healthy and uniform size were selected and surface sterilized with 0.001 M mercuric chloride for 2 min, washed thoroughly with glass-distilled water and then soaked in distilled water for 2 h. The soaked seeds were then spread over plastic trays (approximately 50 seeds per tray) lined with two-layered whatman No.1 filter paper containing different concentrations of cadmium. Cadmium as cadmium chloride: $CdCl_2$ H_2O was used in three concentrations of metal representing 0.02, 0.04 and 0.06 mM for cadmium. These concentrations were selected on the basis of preliminary experiments in which the concentrations less than 0.02 mM for cadmium. The seeds raised in distilled water served as controls. Twenty five ml of each test solution was added separately to each tray and the filter papers were replaced on every alternate day during the study period. The seeds of the three cultivars were allowed to germinate at 30 ± 2°C for 8 days under a photoperiod of 12 h and at a photosynthetic photon flux density (PPFD) of 195 µmol $m^{-2}s^{-1}$. The analyses were made in different parts of the seedling viz. root, shoot and cotyledons separated prior to start of each experiment. Five replicates were used for each treatment.

Polyphenol oxidase activity

Polyphenol oxidase activity was determined according to the method of Kar and Mishra (1976). The procedure similar to peroxidase was followed for obtaining the enzyme extract of polyphenol oxidase. Five ml of the assay mixture for the polyphenol oxidase activity comprised: 125 µ moles of

phosphate buffer, pH 6.8, 50 µ moles of pyrogallol, and 1 ml of the 20 times diluted enzyme extract. This was incubated for 5 min at 25 °C. The reaction was stopped by adding 0.5 ml of 5% H_2SO_4 (v/v). The amount of purpurogallin formed was determined by taking the absorbance at 420 nm on 150-200 UV-VIS spectrophotometer (Hitachi, Japan). The enzyme activity was expressed in absorbance unit. The 0.1 difference was taken as one unit of enzyme activity.

Acid phosphatase activity

Acid phosphatase activity was assayed based on the method of Shinshi *et al*. (1976) by spectrophotometric measurement of the rate of formation of p-nitrophenol (Pnp) produced by enzyme catalysis of p-nitrophenyl phosphate (Pnpp) in the presence of sodium hydroxide, -nitrophenol forms a yellow phenolate ion and could be quantified readily.

Extraction: Five hundred mg of fresh plant tissue was ground with 1 g of acid washed and 4 ml of cold 0.1 M sodium acetate buffer (pH 5.0) in a precooled glass mortar. The homogenate was filtered through two layers of cheese cloth and the filtrate was centrifuged at 20,000 g for 20 min at 2 ºC. The collected supernatant was served as enzyme extract.

Enzyme assay: Acid phosphatase activity was assayed in 2 ml of sodium acetate buffer (pH 5.0) containing 0.1 mg/ml of p-nitrophenyl phosphate and 0.1 ml of enzyme extract after 5 min the reaction was stopped by adding the 1 ml of 0.2 M NaOH and the absorbance was read at 405 nm by using systronics 112 spectrophotometer. The enzyme activity was then calculated by employing the extinction coefficient of mm A^{405}=18.0 (Melani and Guerritore, 1964) and the results were expressed as µmole (pNP) per minute per gram fresh weight of the sample.

δ-Amino levulinic acid dehydratase (δ-ALAD) activity

δ-Amino levulinic acid dehydratase (δ-ALAD) activity was determined as per the method described by Schneider (1970) by assaying the porphobilinogen (Pbg) formed by the condensation of two molecules of δ-aminolevulinic acid and the amount of Pbg thus formed was estimated based on its reaction with p-dimethylaminobenzaldehyde (DMAB) in acid solution to form a red compound.

Extraction: Crude enzyme extract was prepared by homogenization of shoots in 5 ml of 0.05 M Tris-HCl buffer (pH 8.2) containing 0.1 M dithiothreitol. The homogenate was filtered through four layers of cheese cloth and centrifuged for 15 min at 15,000 g at 4 ºC. The supernatant was used for the enzyme assay. One ml aliquot of the enzyme extract was used for the estimation of protein content by following the method of Lowry *et al.* (1951).

Enzyme assay: One ml of enzyme extract was incubated with 0.27 ml of l mg/ml δ-aminolevulinic acid (sigma), 1.35 ml of 0.05 M tris-HCl buffer (pH 8.2) with 0.1 M dithiothreitol and 0.08 ml of 0.02 M $MgCl_2$ for 2.5 hours at 37 ºC. After incubation the reaction was stopped with 0.3 ml 3 M trichloroacetic acid and centrifuged at 200 g for 15 min. To the supernatant Echrlich reagent (1:1) was added and the absorbance was read at 555 nm after 15 min on a systronics 112 spectrophotometer. The amount of porphobilinogen formed was calculated by using molar extinction coefficient of 6.1 X 10^4 and the enzyme activity was expressed as moles PBG formed per 2.5 hours per mg protein.

Preparation of Ehrlich reagent

One gram of DMAB (P-dimethyl-aminobenzaldehyde) was dissolved in a mixture of 30 µ glacial acetic acid and 8 ml 70% perchloric acid. The final volume of the solution was diluted to 50 ml with acetic acid. This reagent was somewhat unstable and should be used on the day it was made. Any remainder was discarded (Mauzerall and Granick, 1956).

Ribulose bisphosphate carboxylase activity (EC 4.1.1.39) and Phosphoenolpyruvate carboxylase (EC 4.1.1.31): The extracts containing ribulose bisphosphate carboxylase phosphoenolpyruvate carboxylase were obtained and their activities were determined separately by the method of Van *et al*. (1976) as modified by Jana and Choudhuri (1982). One gram of fresh leaves were homogenized in a chilled mortar using 10 ml of cold extraction buffer 200 mM Tricine, (pH 8.6) containing 10 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 0.2 mM EDTA, 1.6% polyvinyl pyrrolidone (PVP). The homogenate was filtered through two layers of cheese cloth. The filtrate was centrifuged for 25 minutes at 18,000 x g at 4 °C. The supernatant was used for the estimation of ribulose bisphosphate carboxylase and phosphenolpyruvate carboxylase activities. The activation of ribulose bisphosphate carboxylase and phosphoenolpyruvate carboxylase were done using 0.5 ml of activating medium consisting of 20 mM $MgC1_2$ and 10 mM $NaHCO_3$ (pH 8.6) for 10 minutes before assaying the enzyme activity (Gezeilus and Hallgren, 1980).

Assay of Ribulose bisphosphate carboxylase

The one ml of assay mixture of ribulose bisphosphate carboxylase consists of 150 mM Tricine (pH 8.6), 50 mM $MgC1_2$, 40 mM 2-mercaptoethanol, 6.4 mM ribulose bisphosphate (Sigma), 10 mM $NaH^{14}CO_3$ (5µ Ci/m mole). After a period of temperature equilibrium for 10 minutes the reaction was initiated by the addition of 0.25 ml activated enzyme extract to 1.0 ml of assay mixture and stopped it after 2 minutes at 30 °C with the addition of 0.5 ml of 6 N HCl saturated with 2, 4-dinitrophenylhydrazine. The aliquots were placed in scintillation vials and after processing the radioactivity was determined by Automatic Liquid Scintillation System (ECIL LSS 34). The values were expressed in cpm/mg chl and cpm/leaf.

Assay of phosphoenolpyruvate carboxylase

One ml of assay mixture of phosphoenolpyruvate carboxylase contained 150 mM ricine (pH 8.6), 50 mM $MgC1_2$, 40 mM 2-mercaptoethanol and 5 mM phosphoenolpyruvate (Sigma), 5 mM sodium glutamate and 10 mM $NaH^{14}CO_3$. The reaction was initiated by adding 0.2 ml of activated enzyme extract to 1.0 ml of assay mixture. The reaction was stopped by the addition of 0.5 ml of 6 N HCl saturated with 2, 4-dinitrophenylhydrazine. The aliquots were placed in scintillation vials and after proper processing the radioactivity was determined by Automatic Liquid Scintillation System (ECIL LSS 34). The values were expressed in cpm/mg chl and cpm/leaf.

Photosynthetic rate

Photosynthetic rates of the leaves of different pigeonpea genotypes were determined by feeding leaf discs with $NaH^{14}CO_3$. The $^{14}CO_2$ fixation rate was determined by the method Jones and Osmond (1973) as modified by Rao and Ghildiyal (1985). Four leaf discs (1cm diameter) were placed in petridishes (5.0 cm diameter) having 5.7 ml water. Feeding was initiated by adding 0.3 ml of aqueous medium containing 5.0 µci $NaH^{14}CO_3$ (1.0 mci/m mole) to each petri dish. Feeding period maintained was 30 minutes kept in sunlight. After 30 minutes, 6 ml of 4 N HCl was added to stop the reaction. Insoluble material was hydrolysed in 2 N HCl at 80 °C for 2 hours. Final volume was adjusted to 25 ml. One ml of this solution was placed in scintillation vial dried at 65 °C and counted for ^{14}C activity in a liquid scintillation counter (ECIL LSS 34). The counts obtained represent total ^{14}C incorporation into soluble and insoluble fraction and provides a measure of the rate of photosynthesis.

Carbonic Anhydrase (EC 4.2.1.1) activity

Carbonic anhydrase activity in shoots was determined according to the method adopted by Dwivedi and Randhawa (1974). Five hundred mg of fresh plant tissue was cut into small pieces in 10 ml of 0.2 M cysteine at 0-4 °C. Immediately after blotting the tissue, they were transferred to a test tube containing 4 ml of 0.2 M phosphate buffer (pH 6.8), 4 ml of 0.2 M sodium bicarbonate in 0.02 M NaOH solution and 0.2 ml of 0.002% bromothymol blue indicator. The tubes were incubated at 0-4 °C for 10 min. Carbondioxide liberate during catalytic action of carbonic anhydrase on $NaHCO_3$ was estimated by titrating the reaction mixture with 0.05 M HCl using methyl red indicator. The activity of carbonic anhydrase was expressed as mg of CO_2 released per minute per gram fresh weight.

Hill reaction activity

Hill reaction activity of the isolated chloroplasts was determined according to the method of Trebst (1972). Five grams of washed and surface moisture blotted leaves were cut into small pieces. These pieces were ground in a chilled mortar with about 60 ml of chilled 0.4 M sucrose, 0.05 M KH_2PO_4-Na_2HPO_4 buffer (pH 7.2) and 0.01 M KCl. The homogenate was filtered through eight layered cheese cloth, and centrifuged at about 200 x g for 2 min. The supernatant was collected and centrifuged at about 1000 x g for 10 min. The supernatant was discarded and the pellet (chloroplast) was resuspended in about 10 ml of the buffer solution. All the preparations were carried out at 0 ºC. The reduction of 2,6-DCIP was measured by adding 5 ml of the chloroplast suspension and 0.5 ml of $5x10^{-4}$ M DCIP (0.145 mg DCIP/ml water) enough of buffer solution was used to bring the final volume to 10 ml. The test tubes containing the above reaction mixture were exposed to 1000 watt bulb at a distance of 15 cm. To serve as the dark control, one tube was covered with aluminum foil to avoid light. All the tubes were put in a large beaker of water to maintain the desired temperature (usually about 20 ºC). The disappearance of the blue colour is measured as a change in absorbance at 600 nm as a consequence of the reduction of DCIP by the electrons that are obtained from the oxidation of water by the operation of photosystem II of photosynthesis was determined using 150-200 UV-VIS spectrophotometer (Hitachi, Japan). The concentration of chlorophyll was determined by the method of Arnon (1949). The Hill reaction activity of chloroplasts was expressed as micromoles of DCIP reduced per milligram chlorophyll per hour.

Statistical analysis

The data presented in this work are the average of at least five replicates per treatment; means ± standard error (S.E) are given in the figures. Each experiment was carried out in duplicate. According to the Tukey test, values ± 0.05 were considered significantly different.

RESULTS

Polyphenol oxidase activity

The polyphenol oxidase activity of the roots of Cd treated germinating seeds of pigeonpea cultivars exhibited a continuous increase from 2 to 8 days of seedling growth. The polyphenol oxidase activity of the roots of the pigeonpea seedlings grown in different concentrations of Cd always registered greater values when compared to their controls and their activity increased with increasing concentration of externally supplied metal ions (Fig.1 A,B,C). The polyphenol oxidase activity of the shoots and cotyledons of the respective Cd treated germinating seeds of pigeonpea exhibited a trend similar to that observed for roots (Figs.1D,E,F,G,H,I). The polyphenol oxidase activity of the roots of 6-day old pigeonpea seedlings germinated and grown in 0.02, 0.04 and 0.06 mM Cd concentration showed an increase of 1.4, 2.1 and 2.3 folds in cv. LRG30; 1.44, 2.22 and 2.44 in cv. LRG41 and 1.33, 1.44 and 1.55 folds in cv. ICPL85063 respectively over their corresponding controls. The increase in enzyme activity of the shoots of respective Cd treated germinating seeds registered 1.12, 1.64 and 2.0 folds in cv. LRG30; 1.12 , 1.56 and 2.0 in cv. LRG41 and 1.47, 1.6 and 1.67 folds in cv. ICPL85063 over their corresponding controls. Further, the polyphenol oxidase activity of the cotyledons of the respective Cd treated germinating seeds also showed an increase of 1.06, 1.23 and 1.34 folds in cv. LRG30; 1.06, 1.23 and 1.35 in cv. LRG41 and 1.23, 1.27 and 1.5 folds in cv. ICPL85063 over their controls. Among the three cultivars of pigeonpea seedlings studied, LRG30 exhibited greater polyphenol oxidase activity than in cv. LRG41 and ICPL85063 in response to Cd treatment.

Acid phosphatase activity

The acid phosphatase activity of the pigeonpea cultivars, LRG30, LRG41 and ICPL85063 differed in their response to Cd treatment. The variation in acid phosphatase activity of the different parts of the pigeonpea seedlings in response to increasing concentrations of externally supplied Cd is shown in Figure 2. The acid phosphatase activity of the roots of the three pigeonpea cultivars grown in different concentrations of Cd showed a tendency to increase with increasing age of the seedlings (Fig. 2 A,B,C). The enzyme activity of the roots, very similar in the control. Seedlings of the three pigeonpea cultivars were slightly enhanced by Cd in cv. LRG41 and ICPL85063, while it was rapidly enhanced in LRG30. However, the acid phosphatase activity of the roots registered lower values in 0.06 mM Cd-treated germinating seeds of pigeonpea cultivars, LRG30 in 0.04 and 0.06 mM in Cd-treated germinating seeds of pigeonpea cultivars LRG41 and

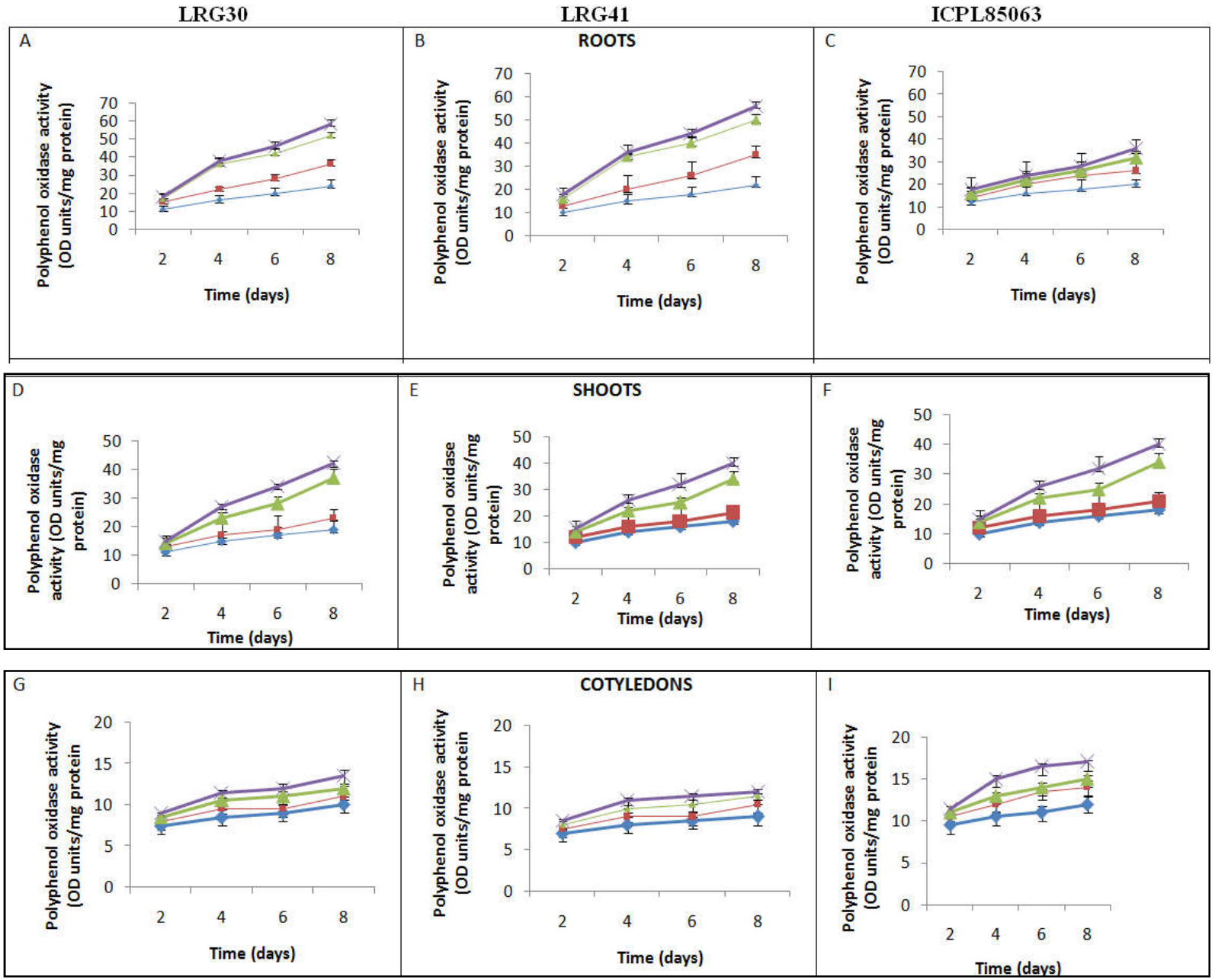

Fig. 1. Polyphenol oxidase activity of roots (A,B,C), shoots (D,E,F) and cotyledons (G,H,I) of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.)

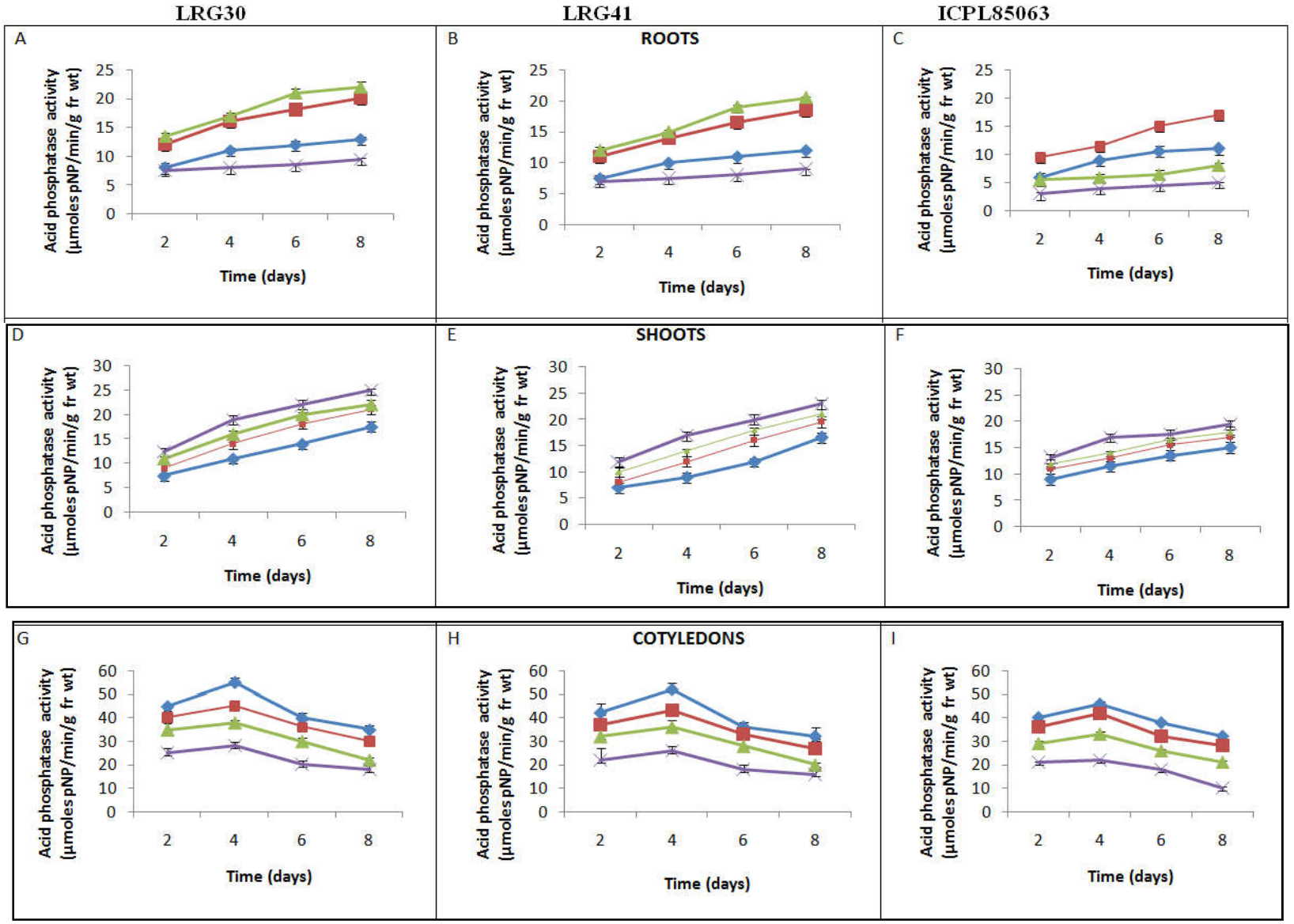

Fig. 2. Acid phosphatase activity of roots (A,B,C), shoots (D,E,F) and cotyledons (G,H,I) of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.)

ICPL85063 when compared to their respective controls but the acid phosphatase activity of the shoots of the three pigeonpea cultivars was enhanced by all the three Cd treatments over their respective controls. Moreover, the acid phosphatase activity of the shoots increased continuously with increasing seedling age. On the other hand, the acid phosphatase activity increased up to 4 days of seedling growth followed by a declining trend. In addition, the acid phosphatase activity of the cotyledons decreased with increasing concentrations of Cd used in the study (Fig. 2 D,E,F,G,H,I). The per cent increase (+) or decrease (-) in the acid phosphatase activity of the roots of 6-day old pigeonpea seedlings grown in 0.02, 0.04 and 0.06 mM Cd concentrations were +50.0, +75.0 and -29.17 in cv.LRG30; 50, 72.72, -27.28 in LRG41 and +42.85, -38.1 and -57.15 in cv.ICPL85063 respectively in relation to their controls.

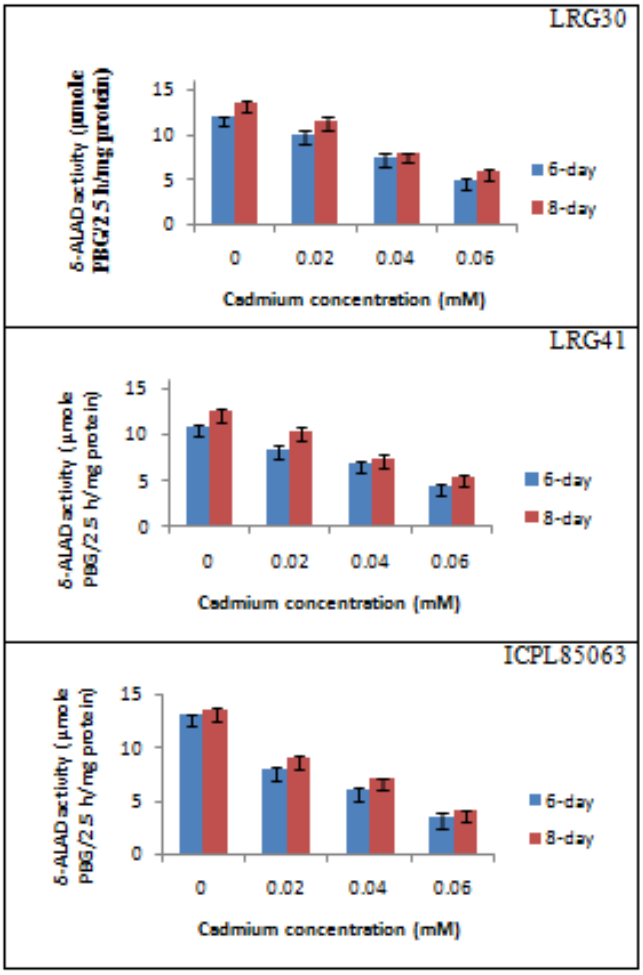

Fig. 3. δ-Amino levulinic acid dehydratase activity of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.)

The per cent increase in the acid phosphatase activity of the shoots of respective Cd-treated germinating seeds were 28.57, 42.85 and 57.14 in cv.LRG30; 33.34, 50.0, 66.67 in LRG41 and 14.81, 22.23 and 29.63 in cv. ICPL85063 over their corresponding controls. On the 6th day of the study, the acid phosphatase activity of the cotyledons of respective Cd treatments showed an inhibition of 10.0, 25.0 and 50.0% in cv. LRG30; 8.4, 22.23, 50.0 in LRG41 and 15.8, 31.60 and 52.64% in cv. ICPL85063 in relation to their appropriate controls. Among the three cultivars of pigeonpea the maximum activity of acid phosphatase was observed in LRG30 in response to Cd treatment.

δ-Aminolevulinic acid dehydratase activity

The δ-Aminolevulinic acid dehydratase activity was estimated in terms of µmoles of porphobilinogen formed per mg protein. In all the Cd treatments the δ-ALAD activity was increased from 6 to 8 days of seedling growth and decreased with increasing concentrations of externally supplied Cd (Fig. 3). The per cent decrease in the δ-ALAD activity of the shoots of the 6-day old pigeonpea seedlings germinated and grown in 0.02, 0.04 and 0.06 mM Cd concentrations were 16.7, 25.0 and 58.34 in cv.LRG30; 22.73, 36.37 and 59.1 in LRG41 and 38.47, 53.85 and 73.08 in cv. ICPL85063 respectively when compared to their controls.

PEP carboxylase activity

The Phosphoenolpyruvate (PEP) carboxylase activity measured in the 6- and 8-day old seedlings of three pigeonpea cultivars with response to Cd treatment is shown in Figure 4.

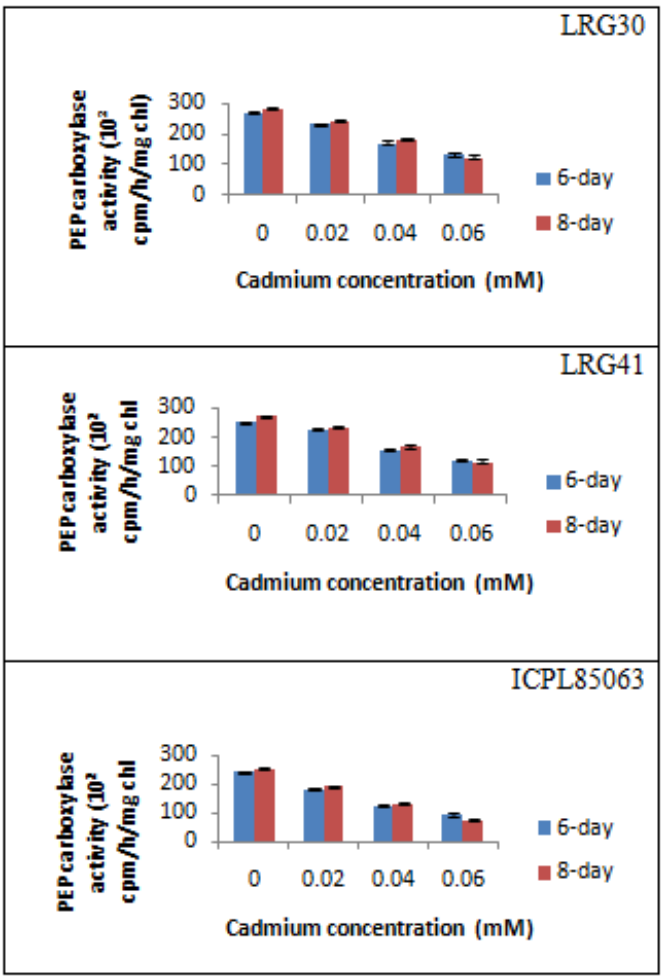

Fig. 4. PEP case activity of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.)

Among the Cd treatment, the seedlings of three cultivars LRG30, LRG41 and ICPL85063 grown in 0.02 and 0.04 mM Cd concentration exhibited a slight increase in the PEP carboxylase activity from 6 to 8 days of growth. However, in three cultivars of pigeonpea, the 0.06 mM Cd treatment

showed a declining trend in the enzyme activity from 6 to 8 days of seedling growth. At any given stage of study the PEP carboxylase activity decreased with increasing concentrations of externally supplied Cd and registered lower values when compared to their controls (Fig. 4). The per cent reduction in the PEP carboxylase activity of the shoots of 6-day old pigeonpea seedlings grown in 0.02, 0.04 and 0.06 mM Cd concentrations were 14.82, 37.04 and 51.86 in cv.LRG30; 10.0, 38.0 and 52.0 in LRG41 and 25.0, 50.0 and 62.5 in cv. ICPL85063 respectively in relation to their controls. Of the three cultivars of pigeonpea studied, the cv. ICPL85063 registered lower levels of PEP carboxylase activity in response to Cd treatment.

RUBP carboxylase activity

The Ribulose 1,5-bisphosphate carboxylase activity of the shoots of the control and 0.02 mM Cd treated germinating seeds exhibited an increasing trend from 6 to 8 days of seedlings growth. However, during this period the RuBp carboxylase activity of the shoots of 0.04 and 0.06 mM Cd treated germinating seeds decreased in cv.LRG30, LRG41 and ICPL85063. In addition, all the Cd concentrations employed in the study reduced the RuBp carboxylase activity of the shoots of three pigeonpea cultivars when compared to their respective controls and the reduction becomes more conspicuous with increasing concentrations of externally supplied Cd (Fig.5).

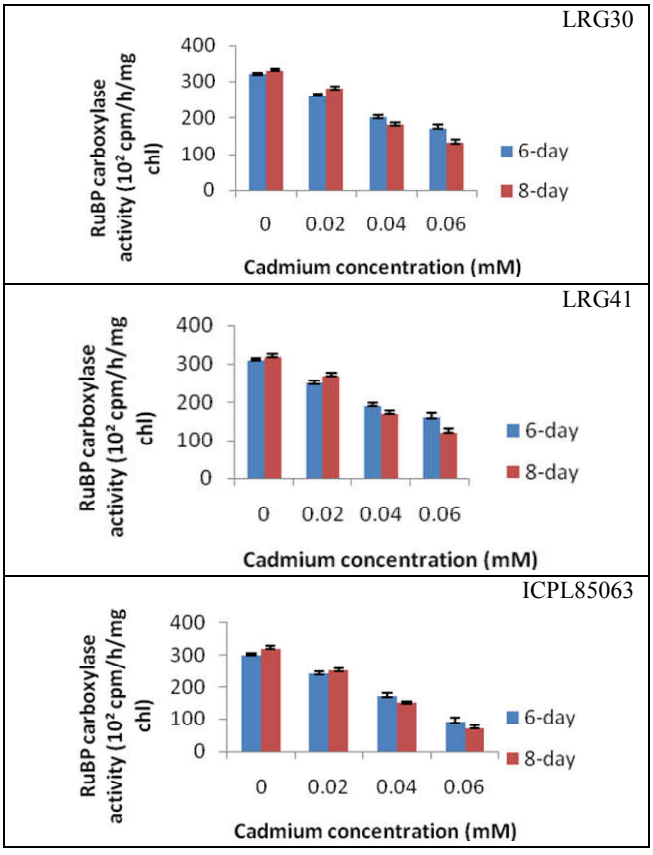

Fig. 5. RuBP case activity of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.)

The activity of RuBp carboxylase assayed from the shoots of 6-day old pigeonpea seedlings showed an inhibition of 18.75% in cv.LRG30, 19.36% in LRG41 and 20% in cv. ICPL85063 in the Cd concentrations of 0.02 mM with respect to their controls. At 0.04 and 0.06 mM Cd concentrations, the inhibition was 37.5 and 46.88% in cv.LRG30, 38.7 and 48.4% in LRG41 and 43.34 and 70% in cv. ICPL85063 respectively with reference to their controls. Among the three cultivars of pigeonpea studied, the RuBp carboxylase activity was affected more in LRG41 and ICPL85063 than in LRG30 in response to Cd treatment.

Photosynthetic rate

The rate of photosynthesis as measured in terms of $^{14}CO_2$ fixation by the shoots of the pigeonpea cultivar in response to Cd treatment is shown in Figure 6. The photosynthetic rate increased from 6 to 8 days in the control seedlings of three cultivars, LRG30, LRG41 and ICPL85063. In the Cd treatments the rate of photosynthesis decreased from 6 to 8 days of seedlings growth. The rate of photosynthesis decreased with increasing concentrations of externally supplied Cd (Fig.6).

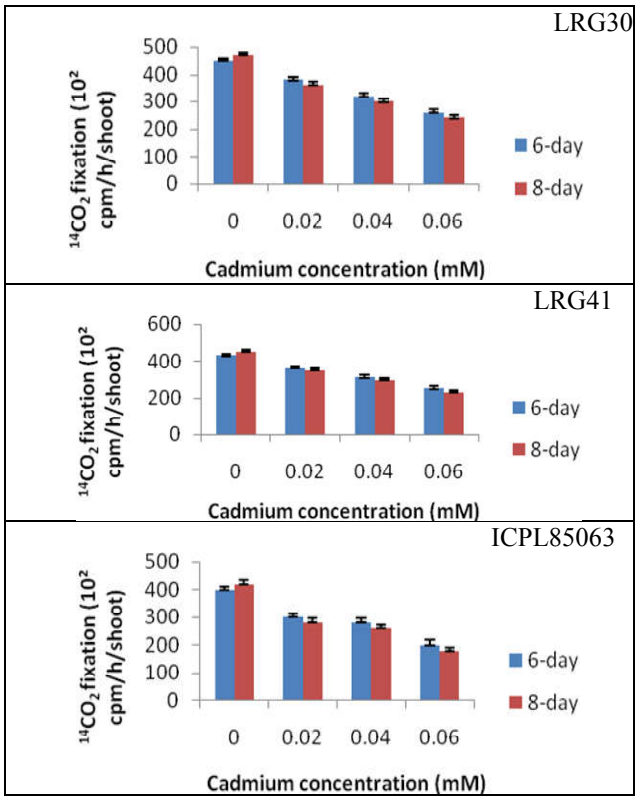

Fig. 6. $^{14}CO_2$ Photosynthetic rate of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.)

The incorporation of $^{14}CO_2$ in the shoots of pigeonpea seedlings germinated and grown for 6 days in 0.02, 0.04 and 0.06 mM Cd concentrations exhibited a reduction of 15.56, 28.89 and 42.23% in cv. LRG30; 16.28, 27.9 and 41.87% in cv. LRG41 and 25.0, 30.0 and 50.0% in cv. ICPL85063 respectively with reference to their controls. The incorporation of $^{14}CO_2$ was affected more in cv. LRG41 and ICPL85063 than in cv.LRG30 in response to Cd stress.

Carbonic anhydrase activity

The carbonic anhydrase activity of the shoots of Cd treated germinating seeds increased from 6 to 8 days of seedling growth. However, the carbonic anhydrase activity of the three

pigeonpea cultivars decreased in the order of increasing concentration of externally supplied Cd (Fig. 7).

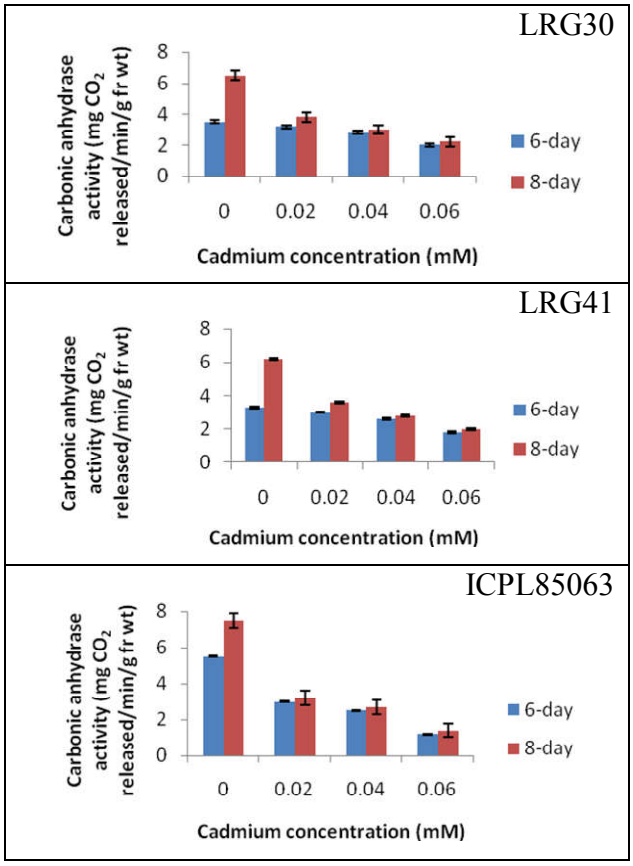

Fig. 7. Carbonic anhydrase activity of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.)

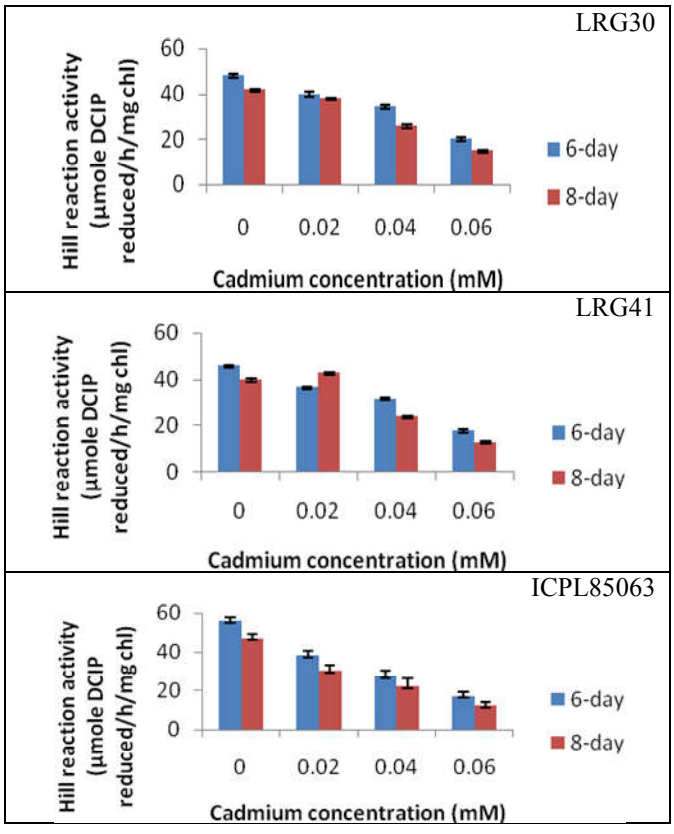

Fig. 8. Hill reaction activity of shoots of seedlings of the three pigeonpea cultivars LRG30, LRG41 and ICPL85063 in response to cadmium stress (Vertical lines represent S.E.)

The carbonic anhydrase activity of the shoots of the 6-day old pigeonpea seedlings showed a reduction of 8.6, 20.0 and 42.86% in cv.LRG30, 9.1, 21.22, and 45.46% in LRG41 and 45.5, 54.55 and 78.2% in cv. ICPL85063 in response to 0.02, 0.04 and 0.06 mM Cd concentrations respectively with reference to their controls. Among the three cultivars of pigeonpea studied, LRG30 recorded greater level of enzyme activity than LRG41 and ICPL85063.

Hill Reaction activity

The hill reaction activity as measured in terms of photochemical reduction of DCIP by the isolated chloroplasts of the three pigeonpea cultivars grown in different concentrations of Cd is shown in Figure 8. The hill reaction activity decreased conspicuously from 6 to 8 days of seedling growth in all the Cd treated germinating seeds of pigeonpea. In addition, the hill reaction activity of the shoots of the three pigeonpea cultivars also decreased with increasing concentration of externally supplied Cd (Fig. 8). The hill reaction activity of the shoots of 6-day old pigeonpea seedlings grown in 0.02, 0.04 and 0.06 mM Cd concentrations showed a reduction of 16.7, 29.17 and 58.34% in cv. LRG30; 19.57, 30.44 and 60.87% in cv. LRG41 and 18.4, 51.8 and 69.65% in cv. ICPL85063 respectively in relation to their controls. The hill reaction activity was much affected in cv. LRG41 and ICPL85063 than in LRG30 in response to both Cd treatments.

DISCUSSION

Polyphenol oxidase activity may be considered as an index of stress level in plants (Godzik, 1967). The activity of PPO enhanced under stress conditions caused by the presence of toxic heavy metals, is responsible for binding and detoxification of heavy metals. Therefore, the mechanism of Cd detoxification is the formation and trapping of Cd-Ca crystals during the process of phenol polymerization by phenol oxidases. The polyphenol oxidase activities of the different parts of Cd treated germinating seeds of three pigeonpea cultivars exhibited continuous increase from 2 to 8 days of seedling growth moreover, the enzyme activity increased with increasing concentrations of externally supplied Cd ions and registered greater levels than their controls (Fig. 1 A,B,C,D,E,F,G,H,I). The induction of PPO activity might be due to its role in phenolic compound synthesis, which plays an important role in detoxification of heavy metals in tobacco (Edreva and Apostolova, 1989; Ruiz *et al*., 1999), maize (Baccouch *et al*., 1998) and pine, etc. (Giertych *et al*., 1999; Kováčik and Klejdus, 2008; Saffar *et al*., 2009). The total PPO activity in licorice plants increased significantly exposed to cadmium stress (Zheng *et al*., 2010). Among the three cultivars of pigeonpea, LRG30 exhibited greater polyphenol oxidase activity than LRG41 and ICPL85063 in response to Cd treatment. The increased activity of polyphenol oxidase in cv.LRG30 indicated it's relatively less sensitivity to Cd stress. Our results indicated that changes of PPO activity might participate in the defense mechanism of pigeonpea plants against cadmium toxicity. Acid phosphatase is ubiquitously distributed enzyme in plant kingdom and non-specifically catalyses the hydrolysis of a variety of phosphate esters in acid environment which enable the plant to maintain an adequate phosphorus level. Increases in the acid phosphatase activity with high Cd treatments might be due to the decline of phosphate (P) level in the cell of P starvation and that

intracellular and extracellular acid phosphatases are integral components of plants response to P deficiency (Johnson and Proctor, 1984; Gabbrielli *et al.*, 1989; Duff *et al*., 1993). The various toxic metals interfere with plant metabolism, inactivating some enzymes or increasing the activity of others (Lee *et al*., 1976a). The acid phosphatase activity of the Cd treated germinating seeds of the three pigeonpea cultivars showed a tendency to increase with increasing age of the seedlings. The acid phosphatase activity of the roots of the three pigeonpea cultivars increased at the lower concentrations and decreased at the higher concentrations of Cd treatments. However, the enzyme activity of the roots of the pigeonpea cultivars, LRG41 and ICPL85063 decreased in all the Cd concentrations employed in the study (Fig. 2 A,B,C). On the other hand, the acid phosphatase activity of the shoots of the three pigeonpea cultivars increased with increasing concentrations of externally supplied metal ions (Fig. 2 D,E,F). Increase in acid phosphatase activity has also been demonstrated in cadmium-treated crop plants (Ernst, 1980; Grunhage and Jager, 1982). This enzyme has also been reported to increase in ageing Rheo leaf (Leo and Sacher, 1970). It was also suggested that increased activity of acid phosphatase in Cd^{2+} treated crop plants may be an indication of senescence induced by the heavy metal ions (Lee *et al*., 1976a). The acid phosphatase activity of the pigeonpea cultivars, LRG30 and LRG41 and ICPL85063 differed in their response to Cd treatments. Nevertheless, among the three cultivars, greater activity of acid phosphatase activity was observed in LRG30 in response to Cd treatment. The acid phosphatase activity of the cotyledons of the three pigeonpea cultivars increased up to 4 days of seedling growth followed by a decline. In addition, the acid phosphatase activity of the cotyledons of the three pigeonpea cultivars decreased with increasing concentrations of Cd supplied (Fig. 2 G,H,I).

The biosynthesis of chlorophyll in higher plants is highly sensitive to heavy metals (Hampp *et al*., 1976; Baszynski *et al*., 1982; Stobart *et al*., 1985; Padmaja *et al*., 1990). Carotenoids play a pivotal role in photoprotection of chlorophylls against photooxidative damage by quenching reactive oxygen species (ROS) such as singlet oxygen (Behera *et al*., 2002). Mishra *et al*. (2006) reported that decrease in carotenoid content in Cd-treated plants, which might be interpreted as an overproduction of ROS. In addition, ALA-D inhibition could have led to ALA accumulation that within the cell might contribute to enhance ROS production (Noriega *et al*., 2007). The δ-Aminolevulinic acid dehydratase activity of the shoots of the three pigeonpea cultivars increased from 6 to 8 days of seedling growth and decreased with increasing concentrations of externally supplied Cd (Fig. 3). ALA-D activity was reduced with increasing Cd levels was observed in plants of *Pfaffia glomerata* (Skrebsky *et al*., 2008). Altered ALA-D activity concomitantly with reduced chlorophyll contents has been reported in many plants exposed to various metals (Pereira *et al*., 2006). Phosphoenolpyruvate carboxylase activity is the primary cytosolic enzyme replenishing oxaloacetate in the tricarboxylic acid cycle. The accumulation of ammonia in Cd-treated plants is rather a consequence of protein proteolysis and amino acid hydrolysis. Therefore, the ability of cells to switch form one development state to another or to adapt the new environmental condition often requires the rapid dismantlement of existing regulatory networks through proteolysis. In addition, Cd stress induced PEPC activity and the induction was partially due to PEPC protein proteolysis (Champigny and Foyer, 1992). In both 6- and 8-day old seedlings of pigeonpea cultivars, the PEP carboxylase activity decreased with increasing concentration of externally supplied Cd and registered lower values when compared to their respective controls (Fig. 4). Under Cd stress, CO_2 assimilation rate is limited since the amount and activity of Rubisco was decreased. More that, RuBPcase activity is regulated to maintain a balance between the capacities of the photosynthetic apparatus to produce and consume RuBP and triose phosphates (Stiborova, 1988). Targeting the accumulation of Cd in leaves enabled us to investigate its effects on the most abundant enzyme, Rubisco. Both the carboxylase and the oxygenase activities of Rubisco are known to be susceptible to abiotic stresses. The RUBP carboxylase activity of the three pigeonpea cultivars decreased in all the Cd treatments and registered lower values when compared to their respective controls. The reduction becomes more conspicuous with increasing concentration of externally supplied metal ions (Fig. 5). The activity of R

UBISCO strictly depends on the formation of a ternary complex between the enzyme, an activating CO_2 molecule and Mg^{2+} (Lorimer, 1981). Substitution between bivalent cations, is common in metalloproteins when the relative concentration of the competing ions changes markedly in the tissues (Clarkson and Hanson, 1980). Moreover, the inhibition of RuBp carboxylase activity with different metal ions such as Cd^{2+}, Cu^{2+}, CO^{2+}, and Zn^{2+} has been explained by metal interaction with the functional SH groups of the enzyme (Stiborova, 1988; Stiborova *et al*., 1988). Among the three cultivars of pigeonpea, the RuBp carboxylase activity was affected more in cv.LRG41 and ICPL85063 than in LRG30. The rate of photosynthesis in the three pigeonpea cultivars decreased with increasing concentrations of externally supplied Cd (Fig. 6). Photosynthetic CO_2 fixation is a highly sensitive process and is significantly affected by the heavy metal (Mohanty and Mohanty, 1988; Sheoran *et al*., 1990a). Impairment of photosynthetic activity of plants exposed to heavy metal cannot be ascribed exclusively to reduce chlorophyll content only. The primary inhibitory effect of cadmium on photosynthetic process of excised leaves was suggested to be metal-induced stomatal closure (Lamoreaux and Chaney, 1978). Further several enzymes of calvin cycle are directly affected by metals (Azeez *et al*., 1986; Van Assche and Clijsters, 1990). Sheoran *et al*. (1990a,b) proposed that the reduction in photosynthesis of pigeonpea under Cd^{2+} and Ni^{2+} treatment might be due to the decreased availability of water, which by lowering water potential causes closure of stomata. This was associated with increased content of abscisic acid, which was believed to affect the process of photosynthesis (Bishnoi *et al*., 1993). The carbonic anhydrase activity of the shoots of Cd treated germinating seeds increased from 6 to 8 days of seedling growth. However, the carbonic anhydrase activity of the three pigeonpea cultivars decreased in the order increasing concentrations of externally supplied Cd ions (Fig. 7). Lee *et al.* (1976a) reported that decreased carbonic anhydrase activity in soya bean seedlings grown in Cd containing solution. Among the three cultivars of pigeonpea studied, LRG30 recorded greater levels of enzyme activity than LRG41 and ICPL85063. Photosynthesis particularly photosynthetic electron transport process is sensitive to heavy metal ions (Van Assche and Clijsters, 1983; Clijsters and Van Assche, 1985). Both cyclic and noncyclic photophosphorylations were proven to be inhibited by excess

of Cd (Lucero *et al*., 1976). The non cyclic photophosphorylation was very sensitive to heavy metals (Honey Cutt and Krogmann, 1972; Hampp *et al*., 1973). The reduction in photosynthetic electron transport capacity in higher plants grown in heavy metal contaminated medium was attributed to the inhibition of both the water splitting system at the oxidizing side of PSII and the NADPH oxidoreductase of the reducing side of PSI (Teije *et al*., 1990; Siedlecka and Baszynski, 1993). In the present study the Cd treatments decreased the hill reaction activity of the shoots of the three pigeonpea cultivars. The hill reaction activity was much effected in LRG41 and cv. ICPL85063 than in cv.LRG30 in response to Cd treatment (Fig. 8).

Conclusions

The polyphenol oxidase and acid phosphatase activity exhibited lower values in the pigeonpea cultivars, LRG41 and ICPL85063 than in LRG30 in response to Cd treatments. The effects were more conspicuous under Cd treatment. The δ-ALAD, PEP case, RuBP Case, $^{14}CO_2$ fixation rate, carbonic anhydrase and hill reaction activity were affected more in the pigeonpea cultivars LRG41 and ICPL85063 in response to Cd treatments. Enhanced polyphenol oxidase activity help in the activation of defense system and reduced toxic effects of Cd was observed.

Acknowledgement

The author is grateful to the University Grants Commission (UGC), New Delhi, India for providing post doctoral fellowship.

REFERENCES

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts polyphenoloxidase in *Beta vulgaris*. *Plant Physiol*., 24: 1-15.

Austenfeld, F.A., 1979. The effect of Ni, Co, and Cr on net photosynthesis of primary and secondary leaves of *Phaseolus vulgaris* L. *Photosynthetica*, 13: 434-438.

Azam, F and S. Farooq. 2003. Elevated CO_2 and stress tolerance in crop plants with particular reference to agro-climatic conditions of Pakistan. *Pak. J. Biol. Sci*., 6:1096-1107.

Azeez, P.A., A.K. Tripathi and D.K. Banerjee. 1986. Influence of heavy metals on carbon assimilation of Blue-green algae (Cyanobacteria). Abstract. 7th International congress on photosynthesis, providence, 301-309.

Baccouch, S., A. Chaoui and E. El-Ferjani. 1998. Nickel toxicity: Effects on growth and metabolism of maize. *J. Plant Nutr*., 21: 577-588.

Baszynski, T., M. Krol, Z. Krupa, M. Ruszkowska, U. Wojcieska and D. Wolinska. 1982. Photosynthetic apparatus of spinach exposed to excess copper. Z. *Pflanzen Physiol*., 108: 385-395.

Bazzaz, M. and B. Govindjee. 1974 Effects of cadmium nitrate on spectral characteristics and light reactions of chloroplasts. *Environ Lett*., 6**:** 1-2.

Behera, R.K., P.C. Mishra and N.K. Choudhury 2002. High irradiance and water stress induced alterations in pigment composition and chloroplast activities of primary wheat leaves. *J. Plant Physiol*., 159: 967-973.

Bishnoi, N.R., I.S. Sheroran and R. Singh. 1993. Effect of cadmium and nickel on mobilization of food reserves and activities of hydrolytic enzymes in germinating pigeonpea seeds. *Biol. Plant*., 35: 583-589.

Bota, J., H. Medrano and J. Flexas. 2004. Is photosynthesis limited by decreased Rubisco activity and RuBP content under progressive water stress? *New Phytol*., 162: 671-681.

Carlson, R., W. Bazzaz and G.L. Rolfe. 1975. The effect of heavy metals on plants II. Net photosynthesis and transpiration of whole corn and sunflower plants treated with the Pb, Cd, Ni and Ti. *Environ. Res*., 10: 113-120.

Champigny, M.L and C.H. Foyer. 1992. Nitrate activation of cytosolic protein kinases diverts photosynthetic carbon from sucrose to amino acid biosynthesis: basis for a new concept. *Plant Physiol*., 100: 7-12.

Chiraz, C., G. Houda, D. Mohamed and GM. Habib. 2008. Differential toxicological response to cadmium stress of bean seedlings grown with NO_3^- or NH_4^+ as nitrogen source. *Int. J. Bot*., 4:14-23.

Clarkson, D.T., J.B. Hanson. 1980. The mineral nutrition of higher plants. *Annual Review of Plant Physiology*, 31: 239-298.

Clijsters, H and F. Van Assche. 1985. Inhibition of photosynthesis by heavy metals. *Photosyn. Res*., **7**: 31-40.

Devi Chinmayee, M., M. Anu, B. Mahesh, S. Mary, I. Mini and T.S. Swapna. 2014. A comparative study of heavy metal accumulation antioxidant responses in *Jatropha curcas*. *IOSR-JESTFT,* 8(7): 58-67.

Duff, S.M.G., G. Sarath and W.C. Plaxton. 1994. The role of acid phosphatase in plant phosphorus metabolism. *Physiol. Plant*, 90: 791-800.

Duff, S.M.G., W.C. Plaxton and D.D. Lefebvre. 1993. Phosphate starvation response in plant cells: De novo synthesis and degradation of acid phosphatase. *Proc. Natl. Acad. Sci*. USA., 88: 9538-9542.

Dwivedi, R.S. and N.S. Randhawa. 1974. Evolution of a rapid test for the hidden hunger of zinc in plants. *Plant Soil*, 40: 445-451.

Edreva, A. and E. Apostolova. 1989. Manganese toxicity in tobacco. A biochemical investigation. *Agrochimica*, 33: 441-451.

Ernst, WHO., 1980. Biochemical aspects of cadmium in plants. In: Nriagu, J.O. (ed.): Cadmium in the Environment, Part I: Ecological Cycling. pp.639-653. InterScience Publ., John Wiley & Sons, New York-Chichester-Brisbane-Toronto.

Faller, P, K. Kienzler, A. Krieger-Liszkay. 2005. Mechanism of Cd^{2+} toxicity: Cd^{2+} inhibits photoactivation of photosystem II by competitive binding to the essential Ca^{2+} site. *Biochim. Biophys Acta*, 1706: 158-164.

Foyer, C.H., M. Parry and G. Noctor. 2003. Markers and signals associated with nitrogen assimilation in higher plants. *J. Exp. Bot*., 54 (382): 585-593.

Gabbrielli, R., L. Grossi and O. Vergnano. 1989. The effects of nickel, calcium and magnesium on the acid phosphatase activity of two *Alyssum* species. *New Phytol*., 111: 631-636.

Geiken, B., J. Masojidek, M. Rizzuto, M.L. Pompili and M.T. Giardi. 1998. Incorporation of [^{35}S] methionine in higher plants reveals that stimulation of the D1 reaction center II protein turnover accompanies tolerance to heavy metal stress. *Plant, Cell and Environ*., 21: 1265-1273.

Gezeilus, K and J.E. Hallgren. 1980. Effect of SO_3^{2-} on the activity of ribulose bisphosphate carboxylase from seedlings of *Pinus silvestris*. *Physiol. Plant*, 49: 354-358.

Giertych, M.J., P. Karolewski and L.O. de Tommerman. 1999. Foliage age and pollution alter content of phenolic compounds and chemical elements in *Pinus niger* needles. *Water Air Soil Pollut*., 110: 363-377.

Godzik, S., 1967. Polyphenol oxidase activity in vegetation injured by industrial air pollution. *Biuletyn zakladu Badan Nauk*, 10: 103-113.

Goncalves, J.F, F.T. Nicoloso, A.G. Becker, L.B. Pereira, L.A. Tabaldi, D. Cargnelutti, C.M.G. dePelegrin, V.L. Dressler, J.B.T. da Rocha and M.R.C. Schetinger. 2009. Photosynthetic pigments content, δ-aminolevulinic acid dehydratase and acid

phosphatase activities and mineral nutrients concentration in cadmium-exposed *Cucumis sativus* L. *Biologia*, 64: 310-318.

Gouia, H., A. Suzuki, J. Brulfert and M.H. Ghorbel. 2003. Effects of cadmium on the co-ordination of nitrogen and carbon metabolism in bean seedlings. *J. Plant Physiol*., 160: 367-376.

Grant, CA., 2011. Influence of phosphate fertilizer on cadmium in agricultural soils and crops. *Agric. Agri. Food Canada*, 54:143-155.

Grunhage, L. and H.J. Jager. 1982. Combinations wirkungen von SO_2 und cadmium auf *Pisum sativum* L.2. Enzyme, free amino sauren organische sauren and zucker. *Angew. Bot*., 56: 167-178.

Hampp, R., K. Beulich and H. Ziegler. 1973. Der Einflu B Von Bleiionen auf Enzyme des reducktiven pentosephosphatcyclus. *Biochem. Physiol. Pflanzen*., 164: 588-595.

Hampp, R., K. Beulich and H. Ziegler. 1976. Effects of zinc and cadmium on photosynthetic CO_2-fixation and hill activity of isolated spinach chloroplasts. *Z. Pflanzen Physiol*., 77: 336-344.

Honey Cutt, R.C. and D.W. Krogmann. 1972. Inhibition of chloroplast reactions with phenyl mercuric acetate. *Plant Physiol*., 49: 376-380.

Jamali, M.K., T.G. Kazi, M.B. Arain, H.I. Afridi, N. Jalbani and A.R. Memon. 2007. Heavy metal contents of vegetables grown in soil, irrigated with mixtures of waste water and sewage sludge in Pakistan, using ultrasonic-assisted pseudo-digestion. *J. Agron. Crop Sci*., 193: 218-228.

Jana, S. and M.A. Choudhuri. 1982. Changes in the activities of ribulose 1,5-bisphosphate and phosphoenolpyruvate carboxylases in submersed aquatic angiosperms during aging. *Plant Physiol*., 70: 1125-1127.

Johnson, W.R. and J. Proctor. 1984. The effects of magnesium, nickel, calcium and micronutrients on the root surface phosphatase activity of a serpentine and non serpentine clone of *Festuca rubra* L. *New Phytol*., 96: 95-101.

Jones, H.G. and C.B. Osmond. 1973. Photosynthesis by thin leaf slices in solution 1. Properties of leaf slices and comparison with whole leaves. *Aust. J. Biol. Sci*., 26: 15-24.

Kapoor, D., S. Kaur and R. Bhardwaj. 2014. Physiological and biochemical changes in *Brassica juncea* plants under Cd induced stress. *Biomed. Res. Int*., 2014: 726070.

Kar, M and D. Mishra. 1976. Catalase, peroxidase and polyphenol oxidase activities during rice leaf senescence. *Plant Physiol*., 57: 315-319.

Kim, Y.Y., Y.Y. Yang and Y. Lee. 2002. Pb and Cd uptake in rice roots. *Physiol. Plant*, 116: 368-372.

Kováčik, J. and B. Klejdus. 2008. Dynamics of phenolic acids and lignin accumulation in metal-treated *Matricaria chamomilla* roots. *Plant Cell Reports*. 27: 605-615.

Lamoreaux, R.J. and Chaney. W.R. 1978. The effect of cadmium on net photosynthesis, transpiration and dark respiration of excised silver maple leaves. *Physiol. Plant*. 43: 231-236.

Latif, A.A. 2008. Cadmium induced changes in pigment content, ion uptake, proline content and phosphoenolpyruvate carboxylase activity in *Triticum aestivum* seedlings. *Aust. J. Bas. App. Sci*., 2: 57-62.

Lee, K.C., B.A. Cunningham, G.M. Paulsen, G.H. Liang, and R.B. Moore. 1976a. Effects of cadmium on respiration rate and activities of several enzymes in soybean seedlings. *Physiol. Plant*. 36: 4-6.

Leo, P De and J.A. Sacher. 1970. Control of ribonuclease and acid phosphatase by auxin and abscisic acid during senescence of Rheo leaf section. *Plant Physiol*., 46: 806-811.

Lorimer, G.H. 1981. The carboxylation and oxygenation of ribulose 1,5-bisphosphate the primary events in photosynthesis and photorespiration. *Annu. Rev. Plant Physiol*., 32: 349-383.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R. Randall. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem*. 193: 265-275.

Lucero, H.A., C.S. Andreo and R.H. Vallejos. 1976. Sulphydryl groups in photosynthetic energy conservation. iii. Inhibition of photophosphorylation in spinach chloroplasts by $CdCl_2$. *Plant Sci. lett*., 6: 309-313.

Mallick, N., F.H. Mohn. 2003. Use of chlorophyll fluorescence in metal-stress research: a case study with the green microalga Scenedesmus. *Ecotoxicol. Environ. Saf*., 55: 64-69.

Mauzerall, D and S. Granick. 1956. The occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urine. *J. Biol.Chem*., 219: 435-446.

Melani, F and A. Guerritore. 1964. Continuous optical determination of alkaline phosphatase. *Experientia*, 20: 464-466.

Mishra, S., S. Srivastava, R.D. Tripathi, R. Govindarajan, S.V. Kuriakose, M.N.V. Prasad. 2006. Phytochelatin synthesis and response of antioxidants during cadmium stress in *Bacopa monnieri* L. *Plant Physiol. Biochem*., 44: 25-37.

Mobin, M and N.A. Khan. 2007. Photosynthetic activity, pigment composition and antioxidative response of two mustard (*Brassica juncea*) cultivars differing in photosynthetic capacity subjected to cadmium stress. *J. Plant Physiol*. 164: 601-610.

Mohanty, N and P. Mohanty. 1988. Cation effects on primary processes of photosynthesis. In.advances in forntier areas of plant biochemistry (Eds.R.Singh and S.K.Sawhney). Prentice Hall, New Delhi, pp. 1-18.

Morel, F.M.M. 2008. The co-evolution of phytoplankton and trace element cycles in the oceans. *Geobiology*, 6: 318-324.

Mysliwa-Kurdziel, B and Strzalka, K. 2002. Influence of metals on the biosynthesis of photosynthetic pigments. In: Prasad MNV, Strzalka K (eds) Physiology and biochemistry of metal toxicity and tolerance in plants. Springer, Netherlands, pp 201-228.

Noriega, G.O., K.B. Balestrasse, A. Batlle, M.L. Tomaro. 2007. Cadmium induced oxidative stress in soybean plants also by the accumulation of δ-aminolevulinic acid. *Biometals*, 20: 841-851.

Pacyna, J.M. and E.G. Pacyna. 2001. An assessment of global and regional emissions of trace metals to the atmosphere sources worldwide. *Environ. Rev*., 9: 269-298.

Padmaja, K., D.D.K. Prasad and A.R.K. Prasad. 1990. Inhibition of chlorophyll synthesis in *Phaseolus vulgaris* L. seedlings by cadmium acetate. *Photosynthetica*, 24: 399-405.

Pagliano, C., M. Raviolo, F.D. Vecchia, R. Gabbrielli, C. Gonnelli, N. Rascio, R. Barbato, N.L. Rocca. 2006. Evidence for PSII donor-side damage and photoinhibition induced by cadmium treatment on rice (*Oryza sativa* L.). *J. Photochem. Photobiol. Biol*. 84: 70-78.

Pereira, L.B., L.A. Tabaldi, J.F. Goncalves, G.O. Jucoski, M.M. Pauletto, S.N. Weis, F.T. Nicoloso, D. Borher, J.B.T. Rocha, M.R.C. Schetinger. 2006. Effect of aluminum on δ-aminolevulinic acid dehydratase (ALA-D) and the development of cucumber (*Cucumis sativus*). *Environ. Exp. Bot*, 57: 106-115.

Rao, T.R.K and M.C. Ghildiyal. 1985. Analysis of photosynthetic source and sink relationship in mungbean (*Vigna radiata* L. (Widczek). *Indian J. Plant Physiol*., 28: 135-144.

Ruiz, J.M., P.C. García, R.M. Rivero and L. Romero. 1999. Response of phenolic metabolism to the application of carbendazim plus boron in tobacco. *Physiol. Plant*, 106(2): 151-157.

Saffar, A., M.B. Bagherieh Najjar and M. Mianabadi. 2009. Activity of antioxidant enzymes in response to cadmium in *Arabidopsis thaliana*. *J. Biol. Sciences*, 9: 44-50.

Santana-Casiano, J.M., M. González-Dávila, A.G. González, M. Rico, A. López, A. Martel. 2014. Characterization of polyphenol exudates from *Phaeodactylum tricornutum* and their effects on the chemistry of Fe (II)-Fe(III). *Mar. Chem*., 158: 10-16.

Scheible, W.R., A. Krapp, and M. Stitt. 2000. Reciprocal diurnal changes of phosphoenolpyruvate and NADP-isocitrate dehydrogenase expression regulate organic acid metabolism during nitrate assimilation in tobacco leaves. *Plant Cell Environ*., 23: 1155-1167.

Schneider, H.A.W. 1970. Activity and properties of δ-amino levulinate dehydratase in greening tissue cultures of *Nicotiana tabacum* cv.Samsum. Z. *Pflanzen Physiol*., 62: 133-145.

Sharma, N., G.S. Hundal, I. Sharma, R. Bhardwaj. 2014. 28-Homobrassinolide alters protein content and activities of glutathione-S-transferase and polyphenol oxidase in *Raphanus sativus* L. plants under heavy metal stress. *Toxicol. Int*., 21(1): 44-50.

Sheoran, S., H.R. Singal and R. Singh 1990b. Effect of cadmium and nickel on photosynthesis and the enzymes of the photosynthetic carbon reduction cycle in pigeonpea (*Cajanus cajan* L.). *Photosynth. Res*., 23: 345-351.

Sheoran, S., N. Agarwal and R. Singh 1990a. Effects of cadmium and nickel on *in vivo* carbondioxide exchange rate of pigeonpea (*Cajanus cajan* L.). *Plant soil*, 129: 243-249.

Shinshi, H., M. Miwa, K. Kato, M. Noguchi, T. Matsushima and T. Sugimura 1976. A novel phosphodiesterase from cultured tobacco cells. *Biochemistry*, 15: 2185-2190.

Shukla, U.C., R.C. Murthy, P. Kakkar. 2008. Combined effect of ultraviolet-β radiation and cadmium contamination on nutrient uptake and photosynthetic pigments in *Brassica campestris* L. seedlings. *Environ. Toxicol.*, 23: 712-719.

Siedlecka, A., T. Baszyński. 1993. Inhibition of electron flow around photosystem I in chloroplasts of Cd-treated maize plants is due to Cd-induced iron deficiency. *Physiol. Plant*, 87: 199-202.

Siedlecka, A., Z. Krupa, G. Samuelsson, G. Quist and P. Gardestrom. 1997. Primary carbon metabolism in *Phaseolus vulgaris* plants under Cd/Fe interaction. *Plant Physiol. Biochem*., 3: 951-957.

Sigfridsson, K.G.V., G. Bernat, F. Mamedoy and S. Styring. 2004. Molecular interference of Cd^{2+} with photosystem II. *Biochim. Biophys. Acta*, 1659: 19-31.

Skrebsky, E.C., L.A. Tabaldi, L.B. Pereira, R. Rauber, J. Maldaner, D. Cargnelutti, J.F. Goncalves, G.Y. Castro, M.R.C. Schetinger, F.T. Nicoloso. 2008. Effect of cadmium on growth, micronutrient concentration, and δ-aminolevulinic acid dehydratase and acid phosphatase activities in plants of *Pfaffia glomerata*. *Braz. J. Plant Physiol*., 20: 285-294.

Somashekaraiah, B, K. Padmaja, A. Prasad. 1992. Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseolus mungo*): Involvement of lipid peroxides in chlorophyll degradation. *Physiol. Plant*, 85: 85-89.

Stiborova, M. 1988. Cd^{2+} ions affect the quaternary structure of ribulose-l,5-bisphosphate carboxylase from barley leaves. *Biochem. Physiol. Pflanzen*, 183: 371-378.

Stiborova, M., 1988. Cd ions affect the quaternary structure of ribulose-1,5-bisphosphate carboxylase from barley leaves. *Biochem. Phys. Pflanzen*., 183: 371-378.

Stiborova, M., M. Ditrichova and A. Benzinova. 1988. Mechanism of action of Cu^{2+}, Co^{2+} and Zn^{2+} on ribulose-l,5-bisphosphate carboxylase from barley (*Hordeum vulgare* L.). *Photosynthetica*, 22: 161-167.

Stobart, A.K., W.T. Griffith, J. Ameen-Bukhari, R.P. Sherwood. 1985. The efffect of Cd^{2+} on the biosynthesis of chlorophyll in leaves of barley. *Physiol Plant*, 63: 293-298.

Tabaldi L.A., R. Ruppenthal, D. Cargnelutti, V.M. Morsh, L.B. Pereira, M.R.C. Schetinger. 2007. Effects of metal elements on acid phosphate activity in cucumber (*Cucumis sativus* L.) seedlings. *Environ. Exp. Bot*., 59: 43-48.

Teige, M., B. Huchzermeyer and G. Schultz. 1990. Inhibition of chloroplast ATP synthase/ATP as is a primary effect of heavy metal toxicity in spinach plants. *Biochem. Physiol. Pflanzen*, 186: 165-168.

Trebst, A. 1972. Measurement of hill reaction and photo-reduction methods. *Enzymol*., 24: 146-165.

Valentoviová, K., L. Halušková, J. Huttová, I. Mistrík, L. Tamás. 2010. Effect of cadmium on diaphorase activity and nitric oxide production in barley root tips. *J. Plant Physiol*., 167: 10-14.

Van Assche, F. and Clijsters H., 1990. Effects of metals on enzyme activity in plants. *Plant, Cell and Environment*, 13: 195-206.

Van Assche, F. and Clijsters, H. 1983. Multiple effects of heavy metals on photosynthesis. In: Marcelle R (ed) Effects of stress on photosynthesis. Nijhoff/Junk, The Hague 7, pp 371-382.

Van, T.K., W.T. Haller and G. Bowes. 1976. Comparison of the photosynthetic characteristics of three submerged aquatic plants. *Plant Physiol*., 568: 761-768.

Wahid, A., M. Arshad and M. Farooq. 2009. Cadmium phytotoxicity: Responses, mechanisms and mitigation strategies. In: Lichtfouse E (ed) Advances in sustainable agriculture, vol 1. Springer, Netherlands, pp 371-403.

Weigel, H.J. 1985. The effect of Cd^{2+} on photosynthetic reactions of mesophyll protoplasts. *Physiol. Plant*, 63: 192-200.

Willet, I.R. and T. Batey. 1977. The effects of metal ions on the root surface phosphatase activity of grasses differing intolerance to serpentine soil. *Plant Soil*, 48: 213-221.

Ying, R.R., R.L. Qiu, Y.T. Tang, P. J. Hu, H. Qiu, H.R. Chen, T.H. Shi, J.L. Morel. 2010. Cadmium tolerance of carbon assimilation enzymes and chloroplast in Zn/Cd hyperaccumulator *Picris divaricata*. *J. Plant Physiol*., 167: 81-87.

Zheng, G., H.P. Lv, S. Gao and S.R. Wang. 2010. Effects of cadmium on growth and antioxidant responses in *Glycyrrhiza uralensis* seedlings. *Plant Soil Environ*., 56(11): 508-515.

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