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Responses of *Abelmoschus esculentus* L. (lady's finger) to elevated levels of Zn and Cd

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Abstract: The paper deals with the accumulation of heavy metals and responses of lady's finger (*Abelmoschus esculentus* L.) plants grown to elevated levels of zinc (Zn) and cadmium (Cd), applied singly as well as in combination. Results showed that Zn + Cd application increased Zn accumulation in root, stem and leaves of the plant, but Cd accumulation decreased. The magnitude of adverse effects on all the measured parameters was lower under Zn + Cd treatment as compared to the sum of responses to individual treatments of Zn and Cd. Evidently, Zn and Cd exhibited antagonistic behaviour when applied in combination, resulting into reduced accumulation of Cd in plants.

Resumen: Este artículo trata sobre la acumulación de metales pesados y las respuestas las plantas de dedo de dama (*Abelmoschus esculentus* L.) que crecen en niveles altos de zinc (Zn) y cadmio (Cd), aplicados por separado o en combinación. Los resultados mostraron que la aplicación de Zn + Cd produjo un aumento en la concentración de Zn en la raíz, el tallo y las hojas de la planta, pero que la acumulación de Cd decreció. La magnitud de los efectos adversos sobre todos los parámetros medidos fue menor en el tratamiento Zn + Cd, en comparación con la suma de las respuestas a tratamientos individuales de Zn y Cd. Evidentmente, el Zn y el Cd mostraron un comportamiento antagonista cuando fueron aplicados en combinación, lo cual resultó en una acumulación reducida de Cd en las plantas.

Resumo: Este artigo aborda a acumulação de metais pesados e as respostas dos dedos de senhora (*Abelmoschus esculentus* L.), plantas que crescem com elevados níveis de zinco (Zn) e cádmio (Cd), aplicados separadamente bem como em combinação. Os resultados mostraram que a aplicação de Zn + Cd aumentou a acumulação de Zn na raiz, no tronco e nas folhas da planta, mas que a acumulação de Cd decresceu. A magnitude dos efeitos adversos em todos os parâmetros medidos foi menor sob tratamento Zn + Cd quando comparado com a soma das respostas aos tratamentos individuais com Zn e Cd. Evidentemente o Zn e o Cd exibiram comportamento antagonista quando aplicado em combinação, resultando numa acumulação reduzida de Cd nas plantas.

Key words: Antioxidant, cadmium, growth, lady's finger, metal accumulation, photosynthesis, zinc.

Introduction

Heavy metal contamination of agricultural soil around heavily industrialized areas is one of the growing environmental concerns throughout the world, particularly in the suburban areas of developing cities. Earlier studies have shown that application of industrial effluents, sewage sludge,

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phosphate fertilizers and wastewater for irrigation has increased the levels of heavy metals in the agricultural soils and also in edible portions of vegetable crops (Sharma *et al.* 2006, 2007; Singh & Kumar 2006).

Soil contamination by a single heavy metal is not a common phenomenon, whereas multi-metal contamination has been commonly reported. Sharma & Agrawal (2006) have shown that addition of Zn increased Cd uptake in carrot plants, but McKenna *et al.* (1993) and Morghan (1993) showed that addition of Zn significantly reduced Cd uptake in lettuce (*Lactuca sativa* L. var. Longifolia cv Paris Island) and spinach (*Spinacia oleracea* L. cv Vienna). Shute & Macfie (2006) reported that Zn increased the accumulation of Cd in soybean plants up to toxic levels. Oliver *et al.* (1994) also showed that *Triticum aestivum* plants grown in Zn deficient soil, when received sufficient levels of Zn, decreased Cd concentration in the grains. Similar effect was also reported for Cd in mustard (*Brassica juncea* (L.) Czern and Cosson) plants grown in Cd contaminated soil supplemented with Zn upto 340 mg kg^{-1}, whereas its higher dose (705 mg kg^{-1}) stimulated the accumulation of Cd (Podar *et al.* 2004). No interaction between these metals has been found for soybean (*Glycine max* var. Merr.) grown in Cd and Zn amended soils (White & Chaney 1980). Hart *et al.* (2002) have reported that Cd and Zn are transported by a common carrier protein of root plasma membrane, which has a greater affinity for Cd than Zn. Therefore, Cd and Zn experience a competitive inhibition during their uptake and transport in a soil-plant system.

The empirical data on the physiological, biochemical and growth responses due to heavy metal accumulation in lady's finger are rarely available for multimetal contaminated soil. Therefore, the present study was conducted with an aim to investigate the accumulation of heavy metals and consequent responses of lady's finger (*Abelmoschus esculentus* L.) plants grown in soil amended with elevated levels of Cd and Zn, singly as well as in combination. The objective of the study was to investigate (i) the accumulation of Cd and Zn in different plant parts, and (ii) to assess the growth, physiological and biochemical responses of the test plant to the accumulation of Cd and Zn.

Materials and methods

Experimental setup

The present study was carried out in the Botanical garden of the Banaras Hindu University, Varanasi, India (latitude 25° 18' N, longitude 83° 01' E, altitude 76.19 m above mean sea level) during January to March, 2007. Eighty kg of surface soil (0-15 cm) was collected from the agricultural field, air dried and uniformly mixed with recommended doses of nitrogen (69 kg ha^{-1}), phosphorus (20 kg ha^{-1}) and potassium (78 kg ha^{-1}) as urea, single super phosphate and muriate of potash, respectively. The physico-chemical properties of the experimental soil are given in Table 1. The soil was divided into three heaps. The first heap was used as control (20 kg soil) i.e. no Cd or Zn was added to the soil. The second (30 kg soil) and third (30 kg soil) heaps were mixed properly with Cd and Zn @ 2 and 20 mg kg^{-1} of dry soil, respectively. A total of ten pots were filled @ 2 kg pot^{-1} for each group. The remaining soils from the second and the third heaps were mixed properly for Cd + Zn treatment (2 mg kg^{-1} Cd + 20 mg kg^{-1} Zn) and ten pots were filled. Five genetically uniform seeds (soaked in water for 24 h to identify the healthy seeds) of lady's finger were hand sown at 2 cm depth in each pot. To maintain the uniform moisture content, known volume of water was added to each pot.

Table 1. Physico-chemical characteristics of the soil used during experiment.

Parameters	Units	
Soil type		Alluvial
pH		7.80 ± 0.01
Electrical conductivity	d Sm^{-1}	0.21 ± 0.01
Organic carbon	%	0.60 ± 0.02
Total P	%	0.17 ± 0.003
Total N	%	0.05 ± 0.003
Available N	kg ha^{-1}	201.30 ± 5.83
Available P	kg ha^{-1}	39.07 ± 0.59
Available K	kg ha^{-1}	310.04 ± 5.78
Total Cd	mg kg^{-1}	1.50 ± 0.05
Total Zn	mg kg^{-1}	25.05 ± 1.20
Available Cd	mg kg^{-1}	0.04 ± 0.002
Available Zn	mg kg^{-1}	0.94 ± 0.002

Values are mean of three replicates ± 1S.E.

Measurement of physiological parameters

The physiological parameters such as photosynthetic rate (Pn), transpiration rate (E) and stomatal conductance (Gs) were measured on second leaf from top at ambient climatic conditions using a Portable Photosynthetic System (LI-6200,

LI-COR, INC, Lincoln, NE, USA) at 30 and 40 days after germination (DAG). During measurements, photosynthetically active radiation (PAR) varied between 1000 - 1200 µmol m^{-2} s^{-1}. These measurements were made on three plants from each treatment.

Biochemical analysis

Three plants were randomly sampled from each group of pots for various biochemical analyses (photosynthetic pigments, protein content and antioxidant enzyme activities) at 30 and 40 DAG. Chlorophylls and carotenoids were quantified on dry weight basis according to Maclachlan & Zalik (1963) and Duxbury & Yentsch (1956), respectively. Peroxidase (POD), superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities in leaf tissues were assessed using the methodology of Britton & Mehley (1955), Beauchamp & Fridovich (1971) and Nakano & Asada (1981), respectively. Total soluble protein content in leaf tissues was quantified by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Growth and biomass measurements

Growth parameters, such as number of leaves, leaf area and root and shoot lengths were measured at 30 and 45 DAG. Root and shoot lengths were added to obtain total plant length. Leaf area was measured using a portable Leaf Area Meter (Model LI-COR-3000, LI-COR, Inc., Lincoln, NE, USA). For biomass determination, root and shoot portions were separated and oven dried at 80 ^{0}C to constant weight. Dry weight of each plant part was measured.

Analysis of Cd and Zn in plant and soil samples

For analysis of Cd and Zn, air dried soil and oven dried plant parts collected at 50 DAG were digested according to the method of Allen *et al.* (1986). The concentrations of Cd and Zn in the filtrate were determined by using Atomic Absorption Spectrophotometer (Model 2380, Perkin - Elmer, INC., Norwalk, CT, USA). Precision and accuracy of analyses were ensured through replicate analyses of samples against Standard Reference Material (SRM-1570) of National Institute of Standard and Technology for all the heavy metals.

Statistical analysis

The statistical significance of differences between treatment means was determined by using Duncan's multiple range test ($P<0.05$). ANOVA was used to see the effects of variables such as treatment, age and their interaction on the measured parameters. All the statistical analysis was performed with the help of SPSS software version 12.

Results

Cd and Zn accumulation

Significant differences in Cd and Zn accumulation in root, stem and leaf were found due to Zn and Cd treatments, singly and in combination as compared to the control (Fig. 1A & 1B). Zn accu-

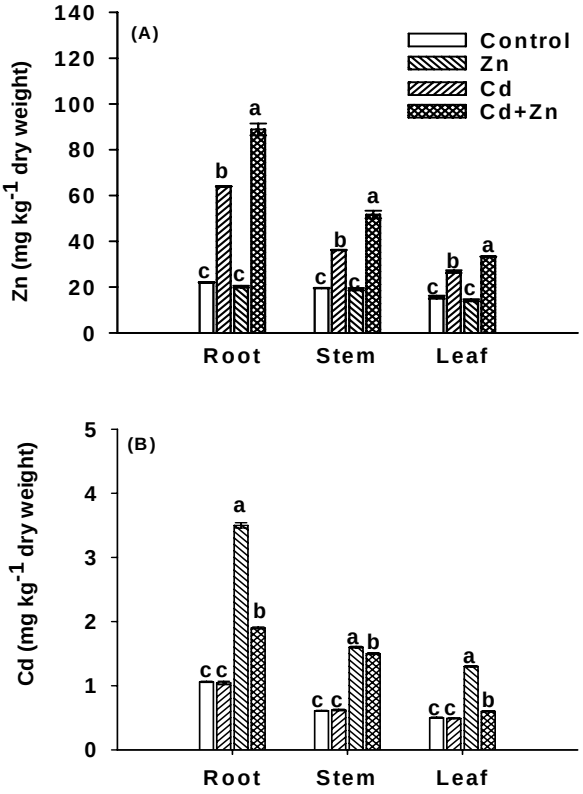

Fig. 1. Zn (A) and Cd (B) accumulation in root, stem and leaf of lady's finger (*A. esculentus* L.) plants grown in soil treated with Cd and Zn at 50 DAG. Bars (mean of three replicates ± 1S.E.) followed by similar letters are not significantly different at $P \leq 0.05$ (Duncan's multiple range test).

Table 2. Age wise effects of Cd and Zn, individually and in combination on photosynthetic pigments and selected physiological parameters of lady's finger (*A. esculentus* L.) plants.

Age/ Parameter	Treatment				ANOVA test		
	Control	Zn	Cd	Zn + Cd	Age (A)	Treatment (T)	A × T
30 DAG							
Total Chlorophyll (mg g^{-1} DW)	1.14 ± 0.02^{a}	0.86 ± 0.02^{b}	0.81 ± 0.01^{b}	0.70 ± 0.02^{c}			
Carotenoids (mg g^{-1} DW)	0.42 ± 0.01^{a}	0.35 ± 0.01^{b}	0.33 ± 0.01^{b}	0.30 ± 0.01^{d}			
Photosynthesis [µMol (CO_2) m^{-2} s^{-1}]	7.28 ± 0.06^{a}	5.44 ± 0.12^{b}	4.35 ± 0.06^{c}	3.95 ± 0.10^{d}			
Transpiration [Mol (H_2O) m^{-2} s^{-1}]	7.18 ± 0.01^{a}	6.56 ± 0.02^{b}	5.57 ± 0.02^{c}	3.69 ± 0.01^{d}			
Stomatal conductance (cm s^{-1})	1.08 ± 0.12^{a}	0.80 ± 0.02^{b}	0.62 ± 0.02^{b}	0.60 ± 0.02^{b}			
40 DAG							
Total Chlorophyll (mg g^{-1} DW)	1.92 ± 0.02^{a}	1.48 ± 0.03^{b}	1.47 ± 0.03	1.36 ± 0.04^{c}	***	***	**
Carotenoids (mg g^{-1} DW)	0.63 ± 0.01^{a}	0.52 ± 0.01^{b}	0.50 ± 0.01^{bc}	0.49 ± 0.01^{c}	***	***	**
Photosynthesis [µMol (CO_2) m^{-2} s^{-1}]	5.43 ± 0.14^{a}	3.77 ± 0.05^{b}	2.33 ± 0.06^{c}	1.46 ± 0.04^{d}	***	***	***
Transpiration [Mol (H_2O) m^{-2} s^{-1}]	6.36 ± 0.14^{a}	5.10 ± 0.12^{b}	4.89 ± 0.12^{b}	2.62 ± 0.05^{c}	***	***	*
Stomatal conductance (cm s^{-1})	0.62 ± 0.05^{a}	0.25 ± 0.01^{b}	0.16 ± 0.02^{bc}	0.14 ± 0.02^{c}	***	***	***

Values (mean of three replicates ± 1S.E.) in each row followed by different letters are significantly different at P≤0.05 (Duncan's multiple range test). Levels of significance: *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.

mulation in all plant parts increased after Zn + Cd application as compared to individual Zn treatment, whereas Cd accumulation decreased under Zn + Cd application compared to Cd treatment. The maximum accumulation of both Cd and Zn was recorded in root, followed by stem and then leaves of the test plant.

Physiological and biochemical parameters

The responses of plants under single and combined treatments of Zn and Cd with respect to physiological (photosynthetic rate: Pn, transpiration rate: E, stomatal conductance: Gs) and biochemical (total chlorophyll: Tc, carotenoids: Car, protein content: Pc, superoxicde dismutase: SOD, peroxidase: POD and ascorbate peroxidase: APX) parameters are presented in Tables 2 & 3, respectively.

Photosynthetic rate declined significantly over control due to application of Cd and Zn, singly and in combination at both the ages of observations (Table 2). The combined effects of Cd and Zn on physiological parameters were higher as compared to individual treatments of Zn and Cd. Total chlorophyll and carotenoid contents were lower in plants treated with Zn and Cd singly and in combination as compared to control, the reductions being higher at Zn + Cd treatment (Table 2). Pn, E and Gs declined by 46, 49 and 48 %, respectively under Zn + Cd treatment as compared to control plants at 30 DAG. One way ANOVA showed significant effects of age, treatment and their interaction on photosynthetic pigments, transpiration and stomatal conductance (Table 2).

As compared to control plants, higher activities were observed for SOD, POD and APX in plants treated with Zn and Cd (Table 3). Combined treatment of Zn + Cd, however, led to higher increments in SOD, POD and APX activities in leaf tissues by 132, 92 and 84 % as compared to Zn applied singly and 26, 90 and 46 % as compared to Cd applied singly, respectively at 30 DAG (Table 3). As compared to control, protein content declined by 31, 35 and 47 % under Zn, Cd, and Zn + Cd

Table 3. Age wise effects of Cd and Zn, individually and in combination on protein content and antioxidative enzyme activities in foliage of lady's finger (*A. esculentus* L.) plants.

Age/ Parameter	Treatment				ANOVA test		
	Control	Zn	Cd	Zn + Cd	Age (A)	Treatment (T)	A × T
30 DAG							
Protein (mg g^{-1} DW)	7.07 ± 0.12[a]	4.91 ± 0.12[b]	4.58 ± 0.12[c]	3.72 ± 0.51[d]			
Superoxide dismutase (unit g^{-1} FW)	2.60 ± 0.12[d]	4.70 ± 0.12[c]	8.60 ± 0.23[b]	10.90 ± 0.17[a]			
Peroxidase (μM pur. min^{-1} g^{-1} FW)	36.63 ± 1.39[c]	49.89 ± 0.65[b]	50.30 ± 1.21[b]	95.62 ± 0.81[a]			
Ascorbate peroxidase (nM min^{-1} g^{-1} FW)	15.00 ± 0.41[d]	30.00 ± 0.82[c]	38.00 ± 0.41[b]	55.33 ± 0.62[a]			
40 DAG							
Protein (mg g^{-1} DW)	10.47 ± 0.23[a]	8.30 ± 0.20[b]	7.89 ± 0.08[b]	5.71 ± 0.11[c]	***	***	***
Superoxide dismutase (unit g^{-1} FW)	8.70 ± 0.17[d]	10.40 ± 0.17[c]	11.50 ± 0.40[b]	12.50 ± 0.29[a]	***	***	***
Peroxidase (μM pur. min^{-1} g^{-1} FW)	93.28 ± 0.58[d]	113.20 ± 0.66[c]	118.90 ± 0.75[b]	121.00 ± 0.81[a]	***	***	***
Ascorbate peroxidase (nM min^{-1} g^{-1} FW)	37.00 ± 0.82[d]	43.00 ± 0.82[c]	58.67 ± 0.85[b]	86.00 ± 1.08[a]	***	***	***

Values (mean of three replicates ± 1S.E.) in each row followed by different letters are significantly different at P≤0.05 (Duncan's multiple range test). Level of significance: *** = $p \leq 0.001$.

Table 4. Age wise effects of Cd and Zn, individually and in combination on growth characteristics of lady's finger (*A. esculentus* L.) plants.

Age/ Parameter	Treatment				ANOVA test		
	Control	Zn	Cd	Zn + Cd	Age (A)	Treatment (T)	A × T
30 DAG							
Number of leaves ($plant^{-1}$)	3.60 ± 0.08[a]	3.32 ± 0.01[b]	3.30 ± 0.08[b]	3.30 ± 0.01[b]			
Leaf area (cm^2 $plant^{-1}$)	2036.00 ± 5.02[a]	1744.66 ± 6.98[b]	1368.00 ± 5.02[c]	1263.33 ± 6.12[d]			
Root length (cm $plant^{-1}$)	5.80 ± 0.04[a]	5.30 ± 0.16[b]	4.80 ± 0.12[c]	4.50 ± 0.12[c]			
Shoot length (cm $plant^{-1}$)	10.60 ± 0.12[a]	10.10 ± 0.08[a]	8.60 ± 0.16[b]	8.40 ± 0.29[b]			
Total plant length (cm $plant^{-1}$)	16.40 ± 0.08[a]	15.40 ± 0.08[b]	13.40 ± 0.04[c]	12.90 ± 0.41[c]			
45 DAG							
Number of leaves ($plant^{-1}$)	3.80 ± 0.08[a]	3.40 ± 0.08[b]	3.36 ± 0.01[b]	3.33 ± 0.01[b]	*	***	NS
Leaf area (cm^2 $plant^{-1}$)	2530.00 ± 7.76[a]	2490.00 ± 6.53[b]	2178.30 ± 6.57[c]	2066.00 ± 6.16[d]	***	***	***
Root length (cm $plant^{-1}$)	8.80 ± 0.12[a]	7.70 ± 0.24[b]	6.50 ± 0.20[c]	6.13 ± 0.20[c]	***	***	**
Shoot length (cm $plant^{-1}$)	13.70 ± 0.33[a]	13.00 ± 0.16[b]	12.80 ± 0.20[b]	10.70 ± 0.04[c]	***	***	**
Total plant length (cm $plant^{-1}$)	22.50 ± 0.45[a]	20.70 ± 0.41[b]	19.30 ± 0.20[c]	16.83 ± 0.24[d]	***	***	*

Values (mean of three replicates ± 1S.E.) in each row followed by different letters are significantly different at P≤0.05 (Duncan's multiple range test). Levels of significance: *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$, NS = Not Significant.

Table 5. Age wise effects of Cd and Zn, individually and in combination on biomass accumulation in lady's finger (*A. esculentus* L.) plants.

Age/ Parameter	Treatment				ANOVA test		
	Control	Zn	Cd	Zn + Cd	Age (A)	Treatment (T)	A × T
30 DAG							
Root (g plant^{-1})	0.15 ± 0.01^{a}	0.10 ± 0.01^{b}	0.09 ± 0.002^{b}	0.06 ± 0.003^{c}			
Shoot (g plant^{-1})	0.46 ± 0.02^{a}	0.31 ± 0.01^{b}	0.19 ± 0.01^{c}	0.16 ± 0.01^{c}			
Total (g plant^{-1})	0.61 ± 0.03^{a}	0.41 ± 0.02^{b}	0.28 ± 0.01^{c}	0.23 ± 0.02^{c}			
45 DAG							
Root (g plant^{-1})	0.20 ± 0.01^{a}	0.16 ± 0.01^{b}	0.12 ± 0.01^{c}	0.08 ± 0.01^{d}	***	***	*
Shoot (g plant^{-1})	0.64 ± 0.01^{a}	0.52 ± 0.01^{b}	0.50 ± 0.01^{b}	0.39 ± 0.06^{c}	***	***	NS
Total (g plant^{-1})	0.84 ± 0.02^{a}	0.68 ± 0.01^{b}	0.62 ± 0.01^{c}	0.47 ± 0.01^{d}	***	***	***

Values (mean of three replicates ± 1S.E.) in each row followed by different letters are significantly different at P≤0.05 (Duncan's multiple range test. Levels of significance: ***= p ≤ 0.001, ** = p ≤ 0.01, * = p ≤ 0.05, NS = Not Significant.

treatments, respectively (Table 3). One way ANOVA showed that age, treatment and age × treatment had significant effects on all the biochemical parameters (Table 3).

Plant growth and biomass

Significant differences in number of leaves and plant height were found between metal treated and control plants (Table 4). Metal application in combination had less negative effects on growth parameters as compared to the sum of individual treatments. As compared to control, number of leaves and total height of plants decreased by 8 and 21 %, respectively under Zn + Cd treatment at 30 DAG. Significant effects of age, treatment and their interaction were recorded for all the growth parameters except for number of leaves (Table 4).

The root, shoot and total biomass of the plants were also affected significantly due to age, treatment and their interaction (Table 5). Cd treatment caused more reduction in biomass compared to Zn, while combined treatment of both the metals exhibited lower reduction in biomass as compared to the sum of their individual treatment (Table 5).

Discussion

In the present study, no symptoms of visible injury on above ground parts of test plant were recorded. The concentrations of both Cd and Zn increased in all the plant parts as compared to control when treated singly or in combination. Under combined application, concentration of Zn increased whereas that of Cd decreased in all the plant parts. These results are consistent with the earlier observation of Shute & Macfie (2006). Zn applications retarded root and shoot elongations in *Vigna radiata* (green gram) (Veer & Lata 1989), *Phaseolus vulgaris* (Chaoui *et al.* 1997), *Vigna radiata* and *Sorghum bicolor* (Balashouri 1995), and *Bacopa monniera* (Ali *et al.* 1999). Exposure of plants to excessive amounts of heavy metals in soil may induce many alterations in plants, such as reduction of growth, especially root growth (Weigel & Jager 1980) and disturbance in mineral nutrition and carbohydrate metabolism (Moya *et al.* 1993), which may strongly reduce the plant biomass. Inhibition in growth under metal toxicity leads to reduction in biomass production (Balashouri 1995; Quariti *et al.* 1997). The results of the present study suggest that the magnitude of biomass reduction depends upon the concentrations of heavy metals accumulated in the tissues. The reduction in biomass may be a consequence of reduction in chlorophyll biosynthesis and photosynthesis as observed in the present study.

The decrease in Pn may be ascribed to inhibition of various reaction steps in the Calvin cycle, Hill reaction and CO_2 fixation (Prasad & Strzalka 1999). Zn accumulation in leaves was found to inhibit the electron transport by acting at the oxidizing site of PSII (Van Assche & Clijsters 1986). Baker *et al.* (1982) proposed a site for Zn action between PSII and PSI in the electron transport chain. Rubisco activity was also found to decrease under Zn treatment (Van Assche *et al.* 1980). Reductions in stomatal conductance were observed under Cd and Zn treatments during the

present study. Van Assche *et al.* (1979, 1980) showed reduction in stomatal conductance and retarded activities of chloroplastic and peroxisomal enzymes at supra optimal concentration of Zn compared to plants receiving optimal Zn concentration. Higher magnitude of reductions in measured parameters under combined treatments of Cd and Zn could be a consequence of their interactive interference with essential metabolic activities (Alia *et al.* 1995; Van Assche & Clijsters 1990).

Significantly lower foliar protein content was recorded in plants treated with Zn and Cd, singly and in combination. Reductions in protein content under heavy metal stress have been reported (Bhattacharya & Choudhuri 1994). Heavy metals are known to produce free radicals, which induce senescence through enhancing catabolism of some metabolites, such as chlorophyll, proteins and RNA (Khudsar *et al.* 2004). In response to higher production of ROS under Cd and Zn stress, antioxidative enzyme activities such as SOD, POD and APX enhanced to counteract against oxidative damage in metal stressed plants. Cd and Zn have been shown to stimulate the activity of several antioxidative enzymes in different plants (Hasan *et al.* 2009; Khudsar *et al.* 2004).

Conclusions

Cd accumulation in root, stem and leaves decreased when Zn was applied in combination with Cd, though it increased the accumulation of Zn. The bioaccumulation of both Zn and Cd adversely affected the growth, biochemical and physiological characteristics that resulted in reduced biomass production of lady's finger. The combined treatment, however, had lower magnitude of negative effects as compared to the sum of individual effects. Thus, it can be suggested that Cd accumulation in plants commonly grown in Cd contaminated soil can be prevented through application of Zn fertilizers, but the dose of Zn application needs to be standardized at different concentrations of Cd.

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