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Bangladesh J. Sci. Ind. Res. **48(4)**, 229-236, 2013

BANGLADESH JOURNAL OF SCIENTIFIC AND INDUSTRIAL RESEARCH

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Effects of heavy metals toxicity on the biochemical response in tomato plants grown in contaminated silt-soil

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Abstract

Toxic effects of Cu^{2+}, Zn^{2+} and Cd^{2+} in tomato plants were the reduction in vegetative growth of seedlings and also in flowering and fruiting. Toxicity of these heavy metals resulted in a reduction in chlorophyll a, chlorophyll b and soluble sugars, but in an increase in proline. Toxic metal stress caused a 71.71, 77.70 and 11.90% increase in the activities of peroxidase, catalase and ascorbic acid oxidase, respectively. With respect to the total uptake of Cd^{2+}, the biggest sink for the metal was provided by roots with 72.80% retention followed by 22% in the stem. Accumulation of Cd^{2+} and Zn^{2+} gradually increased in the order of root, stem and leaves, but for Cu^{2+} that was reversed, that is, it increased in the order of leaf, stem and root. A new consideration of metals bioavailability is discussed in terms of their respective solubility product constants, pH of precipitation, and metal complexation.

Keywords: Metal ions; Bioavailability ; Growth responses; Toxicology ; Contaminated silt-soil

Introduction

Several toxic metals have not only become ubiquitous, but have heavily loaded the environment, posing serious threats to humans and other forms of life (Wilson and Pyatt, 2007; Saeed *et al.* 2009). The lasting negative impact of these metals in the environment is due to their non-biodegradable nature, long biological half-lives ranging from 10 - 3,000 years, and the potential to accumulate in different body parts (Han, 2007). Excessive exposure to such metals may cause a variety of diseases, some of which may also have mutagenic and carcinogenic consequences (Gorsuch, 1991; Bo *et al*. 2009). Vegetables are also reported to have the potential to accumulate heavy metals when grown on contaminated soils, or when irrigated with sewage and wastewaters (Gorsuch, 1991; Bo *et al.* 2009). At higher levels, heavy metals cause growth inhibition and physio-biochemical disorders (Claire *et al.* 1991). Excess concentrations of some of these (Cd, Cr, Cu, Ni, Zn) in the soil have caused disruptionof ecosystems (Meagher, 2000). Cadmium is among the most serious metal toxicants (WHO, 1992). The metal is particularly dangerous as plants growing even in the low-level contaminated soils can absorb and accumulate Cd in their edible tissues in large quantities, without any visible indication, thereby becoming a part of the human food chain (Monteiro *et al.* 2008). A recent study in Europe noted the contents of Cd in vegetables to be higher than the known safe thresholds (Peris *et al.* 2007). Since cereals, potatoes and vegetables account for 70% of the dietary intake (Grawe, 1996), and Cd is reported to have high mobility from soil to plants (Jansson, 2002; Liu *et al.* 2007), the metal has been the subject of increasing interest of study (Yujing *et al*. 2008). Understanding the factors related with the uptake of heavy metals by plants, the resultant stress response, and thus the damage caused to the physiological functioning of plants, have been suggested to be critical to the long-term safety and conservation of agricultural resources and ecosystems (Monteiro *et al.* 2009). Consideration of the level of uptake, translocation, and accumulation of toxic metals in different plant parts is of significance, as it has been suggested that plants might react as the first line of defense against toxicity by reducing metal concentration in active organs or cells through selective exclusion during uptake, excretion, or retention in the roots (Wu *et al*. 2005). Metal toxicity causes interference with physiological functioning of plants, such as deleterious effects on the biosynthesis of chlorophylls (Qin, 1994; Hattab *et al.* 2009), inhibition of growth in plants grown in metals-contaminated soil (Hattab *et al.* 2009), and enhanced proteins content due to greater synthesis of metal-binding proteins, plant-metal chelators and antioxidant

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enzymes (Gratao *et al.* 2008; Nayek *et al.* 2010). Metal stress response causes altered levels of plant protective enzymes, including peroxidase, catalase and ascorbate peroxidase (Cheng, 2003; Israr *et al.* 2011), and accumulation of free proline (Cheng, 2003).

Tomato plant was selected as plant material as reports on the effect of heavy metals on physiological and biochemical parameters in tomato are rare. The aim of the present work is to study the accumulation of Cu^{2+}, Zn^{2+} and Cd^{2+} by various parts of the tomato plant at different stages of growth when grown in the silt-soil spiked with Cu^{2+}, Zn^{2+} and Cd^{2+} in a multimetal combination. Some physiological and biochemical parameters like their effects on proline and enzymes are also studied.

Materials and methods

Silt-soil preparation

Silt-soil used in the study was collected from a canal that bisects the city of Lahore which on its way receives flushing of domestic wastewater and run-off water from several points of inlet. The collected silt-soil was air-dried, homogenized, and passed through 1 mm sieve to remove stones and debris. To the silt-soil, a mixture of salts comprising 1000 mg $Cd(NO_3)_2$. $4H_2O$, 1000 mg $Cu(NO_3)_2$.$3H_2O$ and 1000 mg $ZnCl_3$ per kg silt-soil, which corresponded to 364.40 mg Cd^{2+}, 263.0 mg Cu^{2+} and 479.85 mg Zn^{2+}/Kg of the silt-soil was then added, well-mixed and transferred to PVC pots (33 cm^2). The pots were shifted inside a nylon-mesh net (18 m x 9 m x 4.8 m) which was routinely monitored for temperature, humidity and light. Tomato (*Lycopersicum esculentum*) seeds procured from the Seed Corporation of Pakistan, Lahore were sown for nursery seedlings directly to the PVC pots filled with the metals-contaminated silt-soil. Silt-soil without the added mixture of heavy metal salts was run as the control for raising seedling nursery and plant growth studies.

Physicochemical analysis of the silt-soil and tomato plants

Electrical conductivity and pH of the silt-soil were determined using the standard methods of soil analysis (Rhoades, 1982). Organic matter was calculated as the amount of carbon present in the silt-soil following the procedure of Walkey and Black (1934). Total heavy metal contents (Cu^{2+}, Zn^{2+}, Cd^{2+}) were determined in accordance with Rump (2008) using atomic absorption spectrophotometer (SOLAAR, Unicam 969, UK), and total cationic contents (Na^+, K^+) were analyzed by flame photometer (Jenway PFP7, UK). Bioavailable fractions of Cu^{2+}, Zn^{2+} and Cd^{2+} in the silt-soil were determined according to Novozamsky and coworkers (1993) by atomic absorption spectrophotometer. Cation exchange capacity of the silt-soil was determined as the concentration of exchangeable cations (Na^+, K^+) by flame photometer (Radojevic and Bashkin, 2007). Plants were analyzed for Cu^{2+}, Zn^{2+} and Cd^{2+} following the standard AOAC procedure (2005) which was done to determine their tendency of accumulation in root, stem, leaf, fruit of the tomato plant at seedling, vegetative, flowering-fruiting stage of growth, and their concentrations were calculated on fresh weight basis.

Determination of chlorophyll, sugars, protein and protine

Chlorophylls a and b were extracted from fresh leaf disks following the method of Lichtenthaler (1987). Soluble sugars were extracted from fresh plant material by the method of Yemm and Willis (1954). Total proteins were determined according to the standard AOAC-Kjeldahl procedure (2005). Proline content was determined according to Bates *et al.* (1973), and proline concentration was calculated by plotting a calibration curve using toluene as the blank.

Determination of antioxidant enzymes activities

Catalase activity was determined as the rate of decomposition of H_2O_2 (240 nm/min) against 0.036% H_2O_2 used as the blank and expressed as µmol of H_2O_2 decomposed min/g fresh weight (Aebi, 1984). The activity was calculated using molar extinction co-efficient of H_2O_2 at 240 nm (36 L/mol/cm). One unit of catalase activity catalyzes the conversion of 1 µmole of H_2O_2 to H_2O and O_2/min. Peroxidase activity was measured as the increase in absorbance at 420 nm/min due to oxidation of pyrogallol (Chance, 1955). Reaction mixture without the addition of crude enzyme extract was used as the blank. Peroxidase activity was calculated using molar extinction co-efficient of pyrogallol at 420 nm (19 L/mol/cm) and expressed as µmol of H_2O_2 decomposed/min/g fresh weight of leaves. A peroxidase unit is defined as the amount of enzyme that catalyzes the oxidation of 1 µmole of pyrogallol to purpurogallin/min. Ascorbate peroxidase assay was done by recording the decrease in absorbance at 290 nm due to the oxidation of ascorbate to dehydroascorbate by H_2O_2 (Nakano and Asada, 1981). Ascorbate peroxidase activity was calculated using molar extinction co-efficient of sodium ascorbate at 290 nm (2800 L/mol/cm) and expressed in µmol of ascorbate oxidized/min/g fresh weight of leaves. One unit of the enzyme activity is defined as the oxidation of 1 µmole of

ascorbate to dehydroascorbate/min.

Reproducibility and data analysis

Data presented in the study are the mean values of three separate experiments. Statistical analysis of the data was done using the Duncan's new multiple range test at $p \leq 0.05$ (Steel and Torrie, 1996).

Results and discussion

Physicochemical characteristics of the contaminated silt-soil

Physicochemical characteristics of the silt-soil spiked with Cu^{2+}, Zn^{2+} and Cd^{2+}, compared with the control (Table I). The silt-soil in the native state (without metals contamination) had 5.3% clay, 0.38% organic matter (OM), and the remainder being silt. These observations indicate that both the clay and OM fractions were low. pH of the native silt-soil was slightly alkaline, which fell from 7.9 to 7.7 on its spiking with Cu^{2+}, Zn^{2+} and Cd^{2+} (Table I). Similar small pH reduction was reported on soil contamination with heavy metals (Nayek *et al.* 2010). Cation exchange capacity (CEC) of both the metals-contaminated and control silt-soil was low, respectively, 14.5 and 15.5 meq/100 (Table I). The CEC of soil is a measure of its ability to bind cations, and is an indicator of the negatively charged binding sites on the soil surface where cations are adsorbed (Radojevic and Bashkin, 2007).

Table I. Physicochemical characteristics of the silt-soil contaminated with heavy metals (Cd^{2+}, Cu^{2+}, Zn^{2+})

Soil used	Physicochemical characteristics			
	pH (25 °C)	EC (µS/cm)	OM (%)	CEC (meq/100 g)
Contaminated silt-soil	7.7 ±0.02[a]	873.5±5.12[a]	0.41±0.04[a]	14.83±0.66[a]
Silt-soil as collected (control)	7.9±0.03[b]	646.0±4.17[b]	0.38±0.02[a]	15.50±0.10[a]

EC = electrical conductivity; OM = organic matter; CEC = cation exchange capacity; all values are mean of concurrent triplicate observations; ± standard deviation; values with different alphabets are significantly different from each other at p=0.05 (Duncan's multiple range test)

Bioavailability of metals

Bioavailability of metal ions in the spiked silt-soil was in the order of $Cd^{2+}>Cu^{2+}>Zn^{2+}$ (Table II), which was only 1.7%, 0.98% and 0.82% of their respective total contents. Similar trend of metal mobility ($Cd^{2+}>Cu^{2+}>Zn^{2+}$) has been earlier reported (Yobouet *et al.* 2010). The solubility of Cu^{2+}, Zn^{2+} and Cd^{2+}, calculated from their respective K_{sp} values as their hydroxides, carbonates and phosphates at the spiking concentrations of these metals used in the present studies were low (Table III). Since most cultivation soils are slightly alkaline, low solubility of metals may be regarded as a natural counter-mechanism to the survival of plants growing in contaminated soils. No previous study on metal stress and toxicity in plants has considered the solubility of metals as related to K_{sp}, concentration of the metals present, and the pH of their precipitation.

Effects of heavy metals on chlorophyll, soluble sugars, protein and prolines

Leaves of tomato plants grown in the silt-soil spiked with Cu^{2+}, Zn^{2+} and Cd^{2+} showed a significant decrease in 81.8% and 50.9% in chlorophylls a and b, respectively, accompanied by symptoms of chlorosis, browning of leaf margins, and stunted growth (Tables IV and V). Similar phytotoxic symptoms have been linked with Cd^{2+} and Cu^{2+} contamination (Hattab *et al.* 2009). Chlorosis from excess Cd^{2+} has been suggested to be due to interaction with foliar iron (Das *et al.* 1997), or suppressed iron uptake by plants (Haghiiri, 1974). Pea seedlings exposed to high Cd^{2+} and Cu^{2+} showed drastic decrease of chlorophyll a and chlorophyll b (Hattab *et al.* 2009). Reduction of chlorophylls a and b in lettuce exposed to Cd^{2+} contamination has also observed (Monteiro *et al.* 2009). Copper was reported to modify the physiological processes related with chlorophyll degradation due to the peroxidation of chloroplast membranes (Baszynski *et al.* 1988; Hattab *et al.* 2009). Cd^{2+} may substitute Mg^{2+} in the chlorophyll molecule, which might account for the decrease of chlorophylls a and b (Monteiro *et al.* 2009).

Soluble sugars in tomato plant leaves grown under the stress of Cu^{2+}, Zn^{2+} and Cd^{2+} was reduced by 57.5% (Table IV). Similar decrease in soluble sugars was observed in tomato, pea, and spinach plants irrigated with metals-contaminated (Cr, Cd, Pb, Zn, Cu) waste water (Nayek *et al.* 2010). The

Table II. Total metal contents (Cd^{2+}, Cu^{2+}, Zn^{2+}) and their bioavailable fractions in the artificially contaminated silt-soil

Soil used	Total metal fractions (mg/Kg)			Bioavailable metal fractions mg/Kg (%)*		
	Cd^{2+}	Cu^{2+}	Zn^{2+}	Cd^{2+}	Cu^{2+}	Zn^{2+}
Heavy metals contaminated silt-soil	364.40±7.32	263.0±5.87	479.74±9.21	6.20±1.26 (1.70)	2.59±0.05 (0.98)	3.92±0.07 (0.82)
Silt-soil as collected (control)	-	22.03±0.04	64.45±0.09	-	1.23±0.02 (5.58)	1.28±0.01 (1.99)

*within parenthesis are given percentage bioavailable fractions of the total metal contents; all values are mean of concurrent triplicate observations; ± standard deviation

Table III. Solubilities of Cd^{2+}, Cu^{2+}, and Zn^{2+} in the artificially contaminated silt-soil, calculated from their respective solubility product constants (K_{sp}), at the concentration* of metals added as contaminants, as the corresponding metal complexes hypothetically formed in the soil (Kolthoff *et al.* 1971)

Metal complex in soil	K_{sp} at 25 ^{o}C	pH of precipitation	Calculated solubilities at concentrations used in experimental soil
$Cu(OH)_2$	2.2×10^{-20}	5.36	2.29×10^{-9}
$CuCO_3$	1.4×10^{-10}	-	3.41×10^{-8}
$Cu_3(PO_4)_2$	1.39×10^{-37}	-	3.24×10^{-12}
$Zn(OH)_2$	1.2×10^{-17}	6.61	4.04×10^{-8}
$ZnCO_3$	1.19×10^{-10}	-	1.91×10^{-9}
$Zn_3(PO_4)_2$	9.0×10^{-33}	-	1.07×10^{-10}
$Cd(OH)_2$	5.27×10^{-15}	8.4	2.78×10^{-6}
$CdCO_3$	6.18×10^{-12}	-	1.60×10^{-9}
$Cd_3(PO_4)_2$	2.53×10^{-33}	-	9.25×10^{-11}

*Cu^{2+}, Zn^{2+} and Cd^{2+}, respectively, 263.0 mg, 479.85 mg and 364.40 mg/Kg silt-soil

decline of sugars content was related to the loss of photosynthetic pigments, and the high energy needs of plants due to oxidative stress and toxicity response (John *et al.* 2008; Nayek *et al.* 2010).

Proteins in tomato plant leaves grown in the silt-soil spiked with Cu^{2+}, Zn^{2+} and Cd^{2+} showed an increase of 28.1% (Table IV). This observation is consistent with the reported increase of proteins in beans, pea, spinach and tomato plants

Table IV. Chlorophylls, proline, soluble sugars, and total protein in leaves of tomato plants grown under Cd^{2+}, Cu^{2+}, and Zn^{2+} toxicity

Tomato plant growth conditions	Biochemical indicators				
	Chlorophyll a (µg/g)	Chlorophyll b (µg/g)	Proline (µg/g)	Soluble sugars (mg/g)	Total proteins (mg/g)
Plants grown under heavy metal stress	90.11±0.05^{a}	97.14±0.08^{a}	0.312±0.022^{a}	3.46±0.03^{a}	0.41±0.01^{a}
Control (plants grown in silt-soil not contaminated with heavy metals)	496.54±2.01^{b}	197.94±1.97^{b}	0.206±0.002^{a}	8.15±1.51^{b}	0.32±0.03^{b}
(%) Increase (+) / decrease (-)	(-) 81.85	(-) 50.92	(+) 51.46	(-) 57.55	(+) 28.13

All values are mean of concurrent triplicate observations; ± standard deviation; values with different alphabets are significantly different from each other at p=0.05 (Duncan's multiple range test)

(Nayek *et al.* 2010), wheat and Indian mustard (Chandra *et al*. 2009), and *Beta vulgaris* (Singh and Agarwal, 2007). The increase in protein content was suggested to be due to higher synthesis of metal binding proteins (Nayek *et al.* 2010) or plant-metal chelators and antioxidant enzymes in response to metal stress (Gratao *et al.* 2008). The content of proline, a proteinogenic amino acid essential for primary metabolism (Szabados and Savoure, 2009), in the tomato plants grown under the stress of Cu^{2+}, Zn^{2+} and Cd^{2+} showed an increase of 51.5% (Table IV). Similar observation of proline increase was reported in *Cicer arietinum* grown under Cd^{2+} stress (Tantrey and Agnihotri, 2010), and increase in tomato, pea, beans and spinach irrigated with metals-contaminated wastewater (Nayek *et al.* 2010). Proline accumulation is as an indicator of stress (Alia and Saradhi, 1991). It is also regarded as an abiotic stress marker against heavy metals (Tantrey and Agnihotri, 2010).

Response of antioxidant enzymes in tomato plants grown under metal stress

Leaves of tomato plants grown in silt-soil contaminated with metals showed an increase in the activities of peroxidase (µmol/min/g fresh weight of tomato leaves) was from 139.64 to 239.73, of catalse from 103.33 to 183.66, and of ascorbate peroxidase from 1.01 to 1.13 (Fig. 1). Hyperactivity of antioxidant enzymes, such as peroxidase, catalase and ascorbate peroxidase in plants irrigated with wastewater contaminated with metals was also observed (Nayek *et al.* 2010). Peroxidase activity in the leaves of *Raphanus sativus* increased proteins in response to Cd^{2+} to overcome oxidative stress of the metal (El-Beltagi *et al.* 2010). Increase in catalase activity in tomato plants was observed when Cd^{2+} was spiked in the soil (Gratao *et al*. 2008). Likewise, ascorbate peroxidase activity was induced due to Cd^{2+} stress in *Bechmeria nivea* (Liu *et al.* 2007). Production of protective enzymes in plants has been regarded as a defensive mechanism to reduce oxidative stress (Liu *et al.* 2007). Oxidative stress, induced on the accumulation of Cd^{2+} within plant cells, is evident from increased H_2O_2 accumulation (Liu *et al.* 2007; Israr *et al.* 2011). H_2O_2 acts as a stress response signal and stimulates activities of several radical scavenging enzymes, including peroxidase, catalase and ascorbate peroxidase (Grattao *et al.* 2006). Ascorbate peroxidase plays a role in the elimination of active oxygen species and maintenance of the redox status of cells (Foyer *et al.* 1997).

Effects of metal stress on the growth of tomato plants

The height of tomato plants under the metal stress was reduced by 24.6%, 43.8% and 58.7%, respectively, at the seedling, vegetative and flowering-fruiting stages (Table V). Cadmium caused retardation in the shoot growth was decreased in lettuce (Monteiro *et al.* 2009) and shoot of *Pisum sativum* (Hattab *et al.* 2009). Growth retardation has been regarded as one of the standard physical parameters for the evaluation of toxicity (Israr *et al.* 2011).

Accumulation of metals in different organs of tomato at different growth stages

The accumulation of Cu^{2+} and Zn^{2+} progressively increased in root, stem and leaf through the seedling, vegetative and

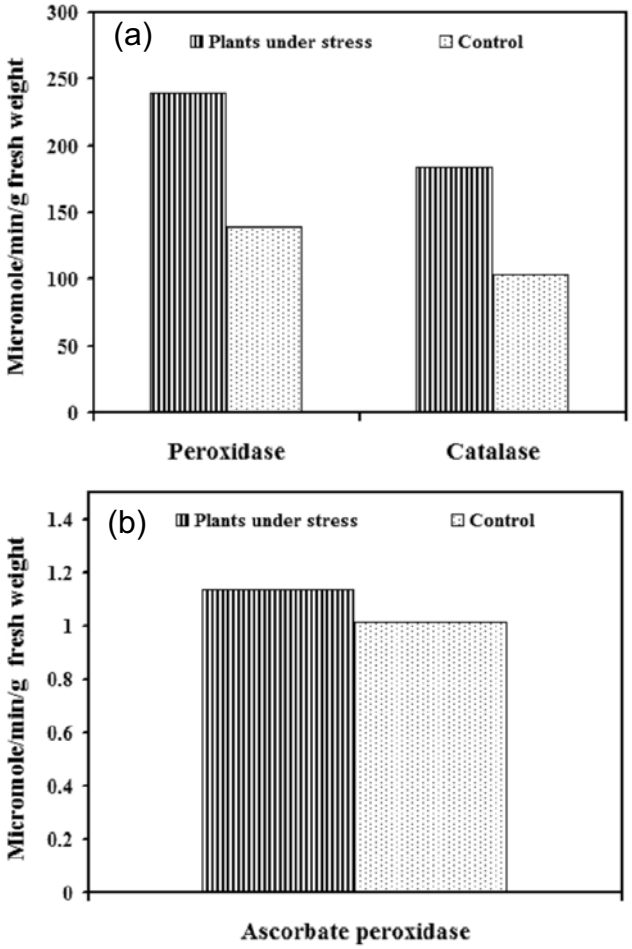

Fig. 1. Antioxidant enzyme activities of (a) peroxidase and catalase, and (b) ascorbate peroxidase in leaf of tomato plants grown under stress of heavy metals (Cd^{2+}, Cu^{2+}, Zn^{2+}).

Table V. Growth of tomato plants grown in silt-soil contaminated with Cd^{2+}, Cu^{2+}, and Zn^{2+} at different developmental stages

Stage of plant growth	Average height of plants (cm)		Growth reduction (%)
	Grown in metals contaminated silt-soil	Grown in silt-soil as collected (control)	
Seedling stage (days 1-10; nursery seedlings upto transplantation)	4.9±1.2	6.5±1.8	24.6
Vegetative stage (days 11-40; seedling transplantation to flowering)	7.7±2.2	13.7±2.6	43.8
Fruiting stage (days 41-75; flowering to fruit ripening	31.0±4.5	75.0±5.1	58.7

All values are mean of concurrent triplicate observations; ± standard deviation

flowering-fruiting stages, which corresponded with the enhanced needs and greater metabolic activities as the plants grew. Accumulation of only 1.01 and 1.82 µg/g Cu^{2+} and Zn^{2+} in the ripened tomato fruit, respectively, indicates less need to transfer these metals to the fruit pulp (Table VI). This observation agrees well with the report that whereas Cd^{2+} was transported in sufficient quantity to the aerial parts of tomato plants, it was not detected in fruits (Moral *et al.* 1994). Throughout the three stages of growth in the control silt-soil, the trend of accumulation was stem>leaf>root for Zn^{2+}, and stem>root>leaf for Cu^{2+}. In the metals-contaminated silt-soil, the trend of accumulation was root>stem>leaf for Cd^{2+} and Zn^{2+}, with the concentration of both, 2-4 times greater in root than in stem, and several times in both these parts than in leaf. These observations indicate that root and shoot functioned as toxic metal sinks as a mechanism to combat metal toxicity. No consistent trend of tissue-partitioning was noted for Cu^{2+} during the three stages of growth. Partitioning of metals in different plant parts is a common strategy to avoid toxicity (Monteiro *et al.* 2009). The biggest sink for Zn^{2+} with 52.6% was also in the root, followed by 24.4% and 23.1% in the stem and leaf, respectively. The pattern followed by Cu^{2+} was entirely different with 42.8% partitioning in the leaf, followed by 33.1% in the stem and 23.8% in the root. It is concluded that different plant tissues have the capacity to accommodate metals but are selective in terms of their need as micronutrients and their relative toxicities. Since Cd^{2+} is a toxic metal, it is immobilized principally in the root and the remaining bigger chunk in the stem. As both Cu^{2+} and Zn^{2+} are micronutrients, these are portioned

Table VI. Accumulation of Cd^{2+}, Cu^{2+} and Zn^{2+} in the root, stem and leaves at different stages of growth of tomato plants grown in metals-contaminated silt-soil

Stages of plant growth	Plant part	Heavy metals contaminated silt-soil			Silt-soil as collected (control)		
		Cd^{2+} (µg/g FW)	Cu^{2+} (µg/g FW)	Zn^{2+} (µg/g FW)	Cd^{2+} (µg/g FW)	Cu^{2+} (µg/g FW)	Zn^{2+} (µg/g FW)
Seedling stage (days 1-10;	Root	(-)	(-)	(-)	(-)	(-)	(-)
nursery seedlings upto	Stem	ND	10.12±2.1	103.19±4.8	ND	9.86±1.92	54.13±2.2
transplantation)	Leaf	ND	8.03±1.05	40.76±2.6	ND	5.02±0.97	10.01±0.9
Vegetative Stage	Root	96.53±3.19	19.74±1.9	120.33±3.6	ND	11.12±0.9	12.89±1.1
(days 11-40; seedling	Stem	19.82±1.72	30.52±2.2	50.72±2.6	ND	24.17±1.7	67.08±2.8
transplantation to flowering)	Leaf	4.65±0.92	33.90±2.5	51.01±2.3	ND	7.99±0.7	14.40±1.2
Fruiting Stage	Root	101.21±4.3	31.62±2.0	125.72±3.1	ND	14.23±1.2	16.23±1.2
(days 41-75; flowering to	Stem	30.62±2.19	44.31±2.6	58.27±2.7	ND	29.27±1.9	74.64±3.2
fruit ripening	Leaf	7.21±1.09	56.71±2.8	55.21±2.4	ND	10.12±0.6	18.30±1.2
	Fruit	-	-	-	-	1.01±0.07	1.82±0.06

All values are mean of concurrent triplicate observations; ± Standard deviation
(-) roots were not taken for analysis in seedling stage; ND = not detected; FW = fresh weight

in different tissues at different levels, reflecting partly their relative metabolic needs and partly as the immobilized fractions in the tissue sinks.

Acknowledgement

The authors M. Iqbal and A. Saeed, greatly acknowledge respectively, Alexander von Humboldt (AvH) Foundation, Bonn, Germany for the purchase of Atomic Absorption Spectrophotometer Unicam 969 (Oxford, UK) through their equipment subsidy grant programme to their Fellows, and International Foundation for Science (IFS), Stockholm, Sweden in collaboration with COMSTECH, Islamabad, Pakistan for the purchase of Shimadzu UV-1800 Spectrophotometer (Japan).

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Received: 05 July 2012; Revised: 04 July 2012; Accepted: 17 December 2013.