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Effects of Heavy Metal Toxicity on Growth, Symbiosis, Seed yield and Metal Uptake in Pea Grown in Metal Amended Soil

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Abstract Soils contaminated with heavy metals present a major threat to sustainable agriculture. Understanding the effects of these metals on pea productivity will be useful. We studied the effects of cadmium, chromium and copper used both separately and as mixtures, on over all growth of pea plants inoculated with *Rhizobium* sp. Among the metals, copper was most toxic for pea plants and decreased the seed yield by 15% at 1,338 mg kg^{-1} compared to control plants whereas cadmium and chromium in general, increased the measured parameters. The metal accumulation in roots and shoots at 90 d and in grains at 120 d differed among treatments.

Keywords Heavy metals · *Rhizobium* · Pea · Phyto-accumulation

Accumulation of heavy metals in soils after its discharge from industrial sources has become a major threat to microbial diversity and crop yields. There are numerous studies where changes in rhizobial populations due to high concentration of heavy metals as well as effects of heavy metals on legume plants are reported (Heckman et al. 1987; Broos et al. 2005). For instance, the higher concentration of metals may induce interaction with sulphydryl groups leading to the inactivation of plant protein (Assche and Clijsters 1990). On the other hand, growth and plant growth promoting activities of microorganisms can be altered due to high concentration of metals (Wani et al. 2007a). In a study, only a single strain of *Rhizobium leguminosarum* survived in the metal contaminated plots and this strain failed to fix N_2 with white clover (*Trifolium repens* L.) although it fixed N_2 with *Trifolium subterraneum* (Hirsch et al. 1993). In a similar study, a pronounced metal toxicity on N_2 fixation in white clover was observed by Broos et al. (2005). Further studies on sludge field trials in Braunschweig showed that increasing sludge rates reduced the number of indigenous populations of *R. leguminosarum* bv. *trifolii* to low, or undetectable levels (Chaudri et al. 1993). Similarly, adverse effect of sludge application on N_2 fixation in faba bean (Chaudri et al. 2000) is reported. The reduction in growth and symbiosis in white clover were due to Cd, Pb and Zn, when plants were grown in soils highly contaminated with these metals (Rother et al. 1983). The effect of total metal concentrations on survival of *R. legumnosarum* however, did not occur in soils contaminated with Cd or with high Ni/Cd sludge. Though a large number of reports on the effects of sewage sludge having multiple metals on legumes are available, yet there is discrepancy in the reported results. And hence, a firm conclusion on the toxicity of heavy metals on legumes and their symbiotic partners can not be drawn. Moreover, majority of the adverse effects have been observed in sludge treated soils and possibly the factors other than metals could also lead to the increased toxicity. Due to lack of adequate data and conflicting reports on the effect of heavy metals on legumes and nodule bacteria, the present study was undertaken to assess the impact of varying levels of Cd, Cr and Cu on pea. The present study evaluates the effect of these metals when used separately and as mixtures, on growth, symbiosis, seed yield and grain protein. In addition, uptake of these metals by plant organs was also assessed.

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Materials and Methods

The soil samples were collected from Mathura road, 7 km away from Aligarh, Uttar Pradesh, India. There was consistent use of industrial sewage water on this soil. Heavy metals in soil samples were determined by the method of McGrath and Cunliffe (1985) using flame atomic absorption spectrophotometer. The metals determined included (mg kg^{-1}): Cd (12); Cr (68) and Cu (669). These metal concentrations were then used either alone or as mixtures to evaluate their effects on pea. *Rhizobium leguminosarum* RP 5 was grown in yeast extract mannitol broth at 28 ± 2°C for 6 d to a cell density of 6×10^8 cells ml^{-1}. Seeds of pea var. arkil were surface sterilized, rinsed six times with sterile water and dried. The sterilized seeds were inoculated with RP5 by soaking the seeds in liquid culture medium for 2 h using 10% gum arabic as adhesive to apply 10^8 cells to each seed. Metals (as chlorides of Cd and Cu and chromate of Cr) were dissolved in distilled water and applied to moist soil 15 d before sowing the inoculated seeds in 23 × 20 cm diameter clay pots. The effects of these metals were evaluated at half, normal and double the normal doses (mg kg^{-1} soil): Cd at 6, 12 and 24, Cr at 34, 68 and 136 and Cu at 334.5, 669 and 1338. The effects of some mixtures were also evaluated (mg kg^{-1} soil): Cd with Cr (6 and 34; 12 and 68; 24 and 136), Cd with Cu (6 and 334.5; 12 and 669; 24 and 1338), Cr with Cu (34 and 334.5; 68 and 669; 136 and 1338). Some pots without metals but inoculated with RP5 were used as control. Inoculated seeds were sown in pots containing 3 kg non-sterilized soil (organic C 0.4%, N 0.75 g kg^{-1}, P 16 mg kg^{-1}, pH 7.2 and WHC 0.44 ml g^{-1}, Cr 6.3, Cu 12.2, Cd 0.2 μg g^{-1} soil). Each treatment was replicated six times and was arranged in a complete randomized design. One week after emergence, the seedlings were thinned to three in each pot. The pots were maintained in an open field. All plants in three pots were removed 90 days after seeding (DAS), and were used for nodulation studies. Roots were washed and nodules were detached, counted, oven dried (80°C) and weighed. Plants uprooted at 90 DAS were oven-dried to measure the total biomass. The remaining three pots with three plants/pot were maintained until harvest. Total N content in roots and shoots were measured at 90 DAS (Iswaran and Marwah 1980). The leghaemoglobin was quantified at 90 DAS (Sadasivam and Manickam 1992). The leghaemoglobin was extracted with sodium phosphate buffer (pH 7.4). The extract was divided into two glass tubes (5 ml/tube) and equal amount of alkaline pyridine reagent was added to each tube. The haemochrome was read at 556 and 539 nm after adding a few crystals of potassium hexacyanoferrate and sodium dithionite, respectively. Plants were harvested at 120 DAS and seed yield and grain protein was estimated. Cadmium, Cr and Cu accumulation in roots and shoots was measured at 90 DAS while in seeds were determined at 120 DAS (Ouzounidou et al. 1992). Since the experiment was conducted consecutively for 2 years under the identical environmental conditions using the same treatments, and the data obtained were homogenous, data of measured parameters were pooled together and subjected to ANOVA. The difference among treatment means was compared by high range statistical domain (HSD) using Tukey test at 5% probability.

Results and Discussion

The effect of three concentrations of Cd, Cr and Cu on pea plant differed among treatments (Table 1). Among the single metal treatments, Cu was the most toxic and reduced the total dry matter significantly ($p \leq 0.05$) by 18% at 1,338 mg kg^{-1} soil. In contrast, Cd and Cr with all the three concentrations increased the dry matter, above the control. The dry matter accumulation was reduced even further when Cu was used in combination with Cd and Cr. The reduction in dry biomass of pea following mixtures of metals ranged between 6 (Cr with Cu at 34 and 334.5 mg kg^{-1} soil) to 20% (Cr with Cu at 136 and 1,338 mg kg^{-1}), above the control. In contrast, the mixture of Cd (24 mg kg^{-1} soil) and Cr (136 mg kg^{-1} soil) increased the dry matter by 16%, relative to the control. In the present study, Cd and Cr, when used singly, in general, did not reduce the pea growth. This study therefore, suggests that the toxicity of Cd or Cr might have been influenced by pea root exudates or pH changes of rhizosphere (Prasad 1999). Furthermore, the three levels of Cu in general, had the greatest toxic effects which could possibly due to the inactivation of proteins, enzymes and DNA through the generation of reactive oxygen intermediates. Though, all reactive oxygen intermediates are toxic, the ultimate damaging effect is, however, mainly by singlet oxygen ($^1O_2$) and hydroxyl radical (HO^*). These radicals in turn damage all classes of bio-molecules (Breen and Murphy 1995). In contrast to our findings, oxidative stress due to the Cd treatment was reported in pea leaves (McCarthy et al. 2001). Moreover, the reduction in growth could also be due to the decline in photosynthetic pigments (Bibi and Hussain 2005; Wani et al. 2006) "Rubisco activity" (Sheoran et al. 1990). Interestingly, the pea plants were, tolerant to Cd and Cr, which could probably be due to the synthesis of phytochelatins. The synthesis of phytochelatins is induced by most of the heavy metals in most of the higher plants and the phytochelatin synthetase involved in the synthesis of PCs requires metals for its activation (Grill et al. 1989). Since the phytochelatin synthetase activity has been detected largely in roots, and root is the first organ exposed to metal ions in the soil, the roots of the test plant might have restricted the

Table 1 Dry matter, nodulation, N contents, leghaemoglobin, seed yield and grain protein in pea as influenced by various concentrations of cadmium, chromium and copper added singly and in combination to sandy clay soil

Treatment	Concentration (mg kg^{-1}soil)	Dry weight (g $plant^{-1}$)	Nodulation		N content (mg g^{-1})		Leghaemoglobin content (m mol (g f.m)$^{-1}$)	Seed yield (g $plant^{-1}$)	Grain protein (mg g^{-1})
			No. $plant^{-1}$	Dry weight (g $plant^{-1}$)	Root	Shoot			
Cd	6	4.96a	110bc	0.217ab	38ab	47ab	0.14ab	9.2bc	231bc
	12	5.46ab	122a	0.229a	40a	50a	0.16a	9.8a	237a
	24	5.18a	115bc	0.210bc	37bc	46ab	0.14ab	9.5ab	232bc
Cr	34	4.46de	95de	0.184de	36bc	42bc	0.13ab	9.0bc	229cd
	68	4.77bc	101cd	0.202cd	38ab	46ab	0.16a	9.5ab	235ab
	136	4.02de	98cd	0.181de	35cd	44bc	0.13ab	9.1bc	230bc
Cu	334.5	3.23de	67ef	0.131de	33cd	38bc	0.11ab	8.1bc	225cd
	669	3.02de	62fg	0.124de	32cd	37bc	0.10ab	7.6bc	221de
	1,338	2.79de	56fg	0.109e	28cd	34c	0.08b	7.1bc	216de
Cd + Cr	6 + 34	4.54cd	103cd	0.183de	37bc	45bc	0.13ab	9.0bc	228cd
	12 + 68	4.39de	97cd	0.173de	35cd	42bc	0.12ab	8.5bc	226cd
	24 + 136	3.96de	92d	0.161de	32cd	40bc	0.10ab	8.1bc	224de
Cd + Cu	6 + 334.5	3.16de	62fg	0.115de	32cd	39bc	0.11ab	8.0bc	221de
	12 + 669	2.92de	58fg	0.107e	30cd	37bc	0.10ab	7.2bc	216de
	24 + 1,338	2.73e	50g	0.101e	26d	34c	0.08b	6.7c	211e
Cr + Cu	34 + 334.5	3.21de	65ef	0.118de	33cd	40bc	0.12ab	8.2bc	226cd
	68 + 669	3.04de	60fg	0.114de	31cd	38bc	0.10ab	7.5bc	223de
	136 + 1,338	2.86de	51g	0.105e	28cd	36ab	0.09b	7.0bc	220de
Control		3.41de	75e	0.139de	35cd	39bc	0.12ab	8.4bc	228cd
F value treatments (df = 18)		7.4*	84.9*	5.9*	4.4*	4.8*	4.1*	3.4*	7.0*

Each value is a mean of three replicates where each replicate constituted three plants/pot; Mean values followed by different letters in the same column are different at $p \leq 0.05$ according to Tukey test

* Significant at $p \leq 0.05$

uptake of Cd and Cr by forming a Cd or Cr–PC complex. The PC– metal complexes has been reported mostly for Cd but a few reports on PC forming complexes with Cu are also reported (Grill et al. 1987, 1989). We are however, not aware of such PC forming complexes and detoxification by pea plants.

Nodulation response to each metal at 90 DAS varied considerably (Table 1). Comparison between control and metal treatments, revealed a significant increase in the number of nodules per plant following Cd and Cr application to soil. Application of Cu to soil, however, reduced the number of nodules. Among the single metal treatments, 1,338 mg Cu kg^{-1} soil decreased the number of nodules by 25%, compared to control. In contrast, the number of nodules increased significantly ($p \leq 0.05$) by 53 and 31% with 24 mg Cd kg^{-1} and 136 mg Cr kg^{-1} soil, respectively. Similarly, mixtures of metals at all levels except Cd with Cr decreased the number of nodules compared to control. Among the metal combinations, Cd (24 mg kg^{-1}) with Cu (1,338 mg kg^{-1}) showed the largest adverse effect and significantly reduced the number of nodules by 33%, above the control. The reduction in nodulation was accompanied by significant decrease in dry mass of nodules. Generally, plants grown in soils amended with Cu had fewer nodules compared to other treatments. The reduction in the number of nodules is possibly due to the direct toxic effect of Cu either on the root hairs or rhizobia, as reported in greengram plants (Wani et al. 2007b). While comparing the sum of mean values of the effects of each metal, the order of toxicity on the symbiotic trait decreased in the following order: Cu < Cr < Cd. However, these concentrations were above the value found by McGrath et al. (1988) in Woburn for total Cu (99 mg kg^{-1}) and total Cd (10 mg kg^{-1}). Interestingly, like the effect of Cd and Cr on growth, no adverse effect of any of the three levels of these metals was observed on the symbiosis of the test plant. This observation was important because it raised the issue of whether or not the pea or *Rhizobium* used in this study can use multiple mechanisms of resistance to the same metal. In this context, several mechanism of resistance to metals in microorganisms is known. For instance, the polysaccharide layer surrounding the rhizobial cell can sequester metals and prevents their entry into the cell. The production of these polymeric layers often occurs without exposure to metal and is known to be involved in adhesion, nutrient storage and protection against desiccation and other environmental assaults. Moreover, some bacteria actively pump metal back out of cell once it crosses cell membrane. The average maximum decline in root N occurred at 1,338 mg Cu kg^{-1} (Table 1) that significantly reduced the root N by 20%, above the control. In comparison, Cd enhanced the root N by 6% at 24 mg kg^{-1} soil, compared to control. Among the dual metal treatments, Cd (24 mg kg^{-1} soil) when applied with Cu (1,338 mg kg^{-1} soil) significantly reduced the N content by 26% compared to the control. A trend similar to root N was observed for shoot N with three metals and their combinations. The average maximum increase in shoot N content at 24 mg Cd kg^{-1} was 18%, compared to control. The N content of roots was severely affected than shoot N with all levels of metals. The decrease in N content of plants might have been due to the reduction in pea- *Rhizobium* symbiosis, as indicated by a decline in the nodulation in this study. Moreover, the reduction in N was visible through the yellowing of leaves, which could possibly be due to the reduction of chlorophyll biosynthesis and depressive effect of these metals on nitrogenous bases (Sinha et al. 1988). A similar reduction in total root and shoot N in greengram was reported for Cd and Cr (Wani et al. 2007b). Furthermore, since many sites receiving sewage are often contaminated with a broad range of metal, it was therefore, decided to evaluate the performance of the pea plants in soil amended with multiple metals. Generally, when plants are exposed to unfavorable concentrations of more than one metal, various interactions can occur. Such effects could be independent, additive, synergistic or antagonistic. The antagonistic relationship among metals may result from the competition between the metals for common sites on the surface of the cell with the more efficient competitors preventing the uptake of other metal. In the present study, both synergistic and antagonistic effects were observed. For instance, the combination of Cd with Cu (24 and 1,338 mg kg^{-1}) showed a synergistic toxic effect on N content of plant organs than those observed for sole application of Cd or Cu. While Cd with Cr (136 and 1,338 mg kg^{-1}) exhibited a lesser effect on dry matter production, which could possibly be due to the antagonistic effect of Cd on Cr. A similar synergistic and additive effect on the growth of roots and shoots of bean with combination of Cd–Zn is reported (Chaoui et al. 1997).

The leghaemoglobin in nodules affect the entire system of nitrogen fixation. In this experiment, the nodules on the root system of pea plants raised in soil amended with Cu had considerably a lower concentration of leghaemoglobin. In contrast, the leghaemoglobin content was increased by 33% at 12 mg Cd kg^{-1} soil. In general, the leghaemoglobin in nodules of combined metal treatments were significantly decreased compared to control. A maximum reduction of 33% in leghaemoglobin was observed with Cd–Cu (at 24 and 1,338 mg kg^{-1} soil). Since Cd and Cr used either alone or in combination had no toxic effect on nodulation, we expected that nodules in the presence of these metals could contain leghaemoglobin at levels greater than the control. This study thus, suggested that the leghaemoglobin was not the target of Cd and Cr. Comparable observations on the effect of Cd, Ni, Cu and Zn on chickpea nodules is reported (Wani et al. 2007c). Seed yield decreased with increase in concentration of Cu used either separately or in combination. The average maximum increase of 13 and 8% was observed with Cd at 24 mg kg^{-1} soil and Cr at 136 mg kg^{-1}

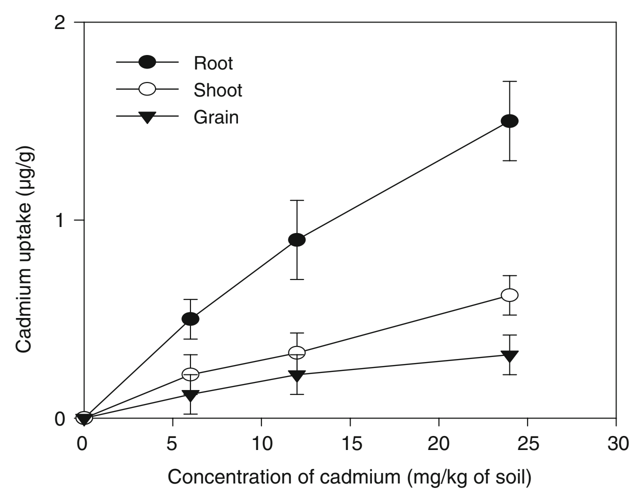

Fig. 1 Cadmium concentration in roots and shoots at 90 days and grains at 120 days after seeding the pea in cadmium amended soil

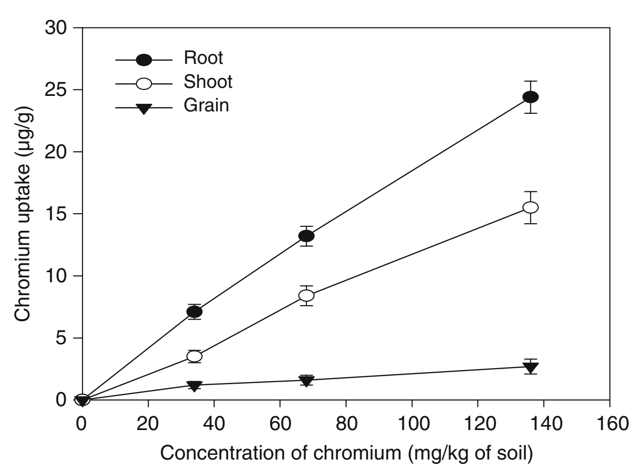

Fig. 2 Chromium concentration in roots and shoots at 90 days and grains at 120 days after seeding the pea in chromium amended soil

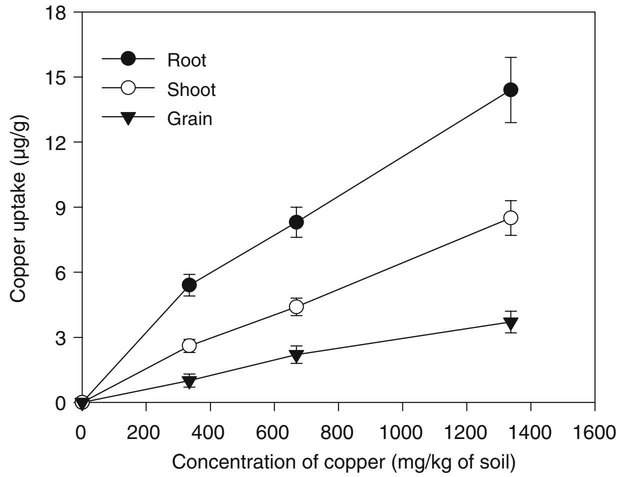

Fig. 3 Copper concentration in roots and shoots at 90 days and grains at 120 days after seeding the pea in copper amended soil

soil respectively, compared to control. In contrast, 1,338 mg Cu kg^{-1} soil significantly decreased the seed yield by 15%, above the control. The average maximum reduction in seed yield among combination treatments was 20% when 24 and 1,338 mg kg^{-1} of Cu was applied together, relative to the control. Indeed, the metals added to the soil showed the deleterious effect on the growth of plants that consequently reduced the seed yield. Among the metals used, Cu either alone or as mixture was the most toxic metal for seed production. Similar toxicity of Cu on lentil is reported (Wani et al. 2006). The reduction in seed yield following heavy metal application has been attributed to the effects of metals on the proliferation of roots and to shoots. The reduction in roots and shoots then led to the inhibitory effect on dry matter and consequently the seed yield (Wani et al. 2007c). Cadmium and Cr in general, progressively increased the grain protein (GP) with increasing concentrations. The average maximum GP was observed with 24 mg Cd kg^{-1} (232 mg g^{-1}) and 136 mg Cr kg^{-1} (230 mg g^{-1}). In comparison, Cu used either alone or as mixture decreased the GP consistently with increasing levels, relative to control. Cadmium (24 mg kg^{-1}) with Cu (1,338 mg g^{-1}) declined the GP by 7% compared to control. The mixtures of metals in general, had the greatest toxic effect on GP compared to single metal application. The reduction in GP in this study suggested that the enzymes and functional proteins are the main targets. Additionally, the indirect effect of metal on active metabolism of plants and perhaps their symbiotic partner and decreased availability of N to the seed in turn might have accounted for decreased GP. However, Cd and Cr in general, did not affect growth and symbiosis adversely, which could be the reason why seed production or GP increased with Cd and Cr. Moreover, since the soil used in this study was non- sterilized, there is every possibility of the presence of the Cr reducing bacteria that might have alleviated the toxicity of Cr.

The accumulation of Cd, Cr and Cu in roots and shoots (at 90 DAS) and grains (at 120 DAS) differed among treatments. The concentration of metals in plant organs were affected invariably by the dose of each metal applied. A higher amount of Cd (Fig. 1), Cr (Fig. 2) and Cu (Fig. 3) in roots, shoots and grains, were observed when these metals were used individually compared with dual metal application. The pea plants showed a maximum accumulation of Cd in roots (1.5 µg g^{-1}), shoots (0.62 µg g^{-1}) and grains (0.32 µg g^{-1}) with 24 mg kg^{-1} soil (Fig. 1). In comparison, the higher concentration of Cr in roots, shoots and grains was 24.4, 15.5 and 2.7 µg g^{-1}, respectively at 136 mg kg^{-1} soil (Fig. 2). The concentration of Cu was higher in roots (14.4 µg g^{-1}), shoots (8.5 µg g^{-1}) and grains (3.7) at 1,338 mg kg^{-1} soil (Fig. 3). The concentration of Cd, Cr and Cu in plant organs were however, reduced marginally when 24 mg kg^{-1} of Cd was applied with 136 mg kg^{-1} of Cr (Fig. 4) or 1,338 mg kg^{-1} of Cu (Fig. 5) and when 136 mg kg^{-1} of Cr was used with 1,338 mg kg^{-1} of Cu (Fig. 6). The phyto-accumulation of heavy metals was higher in roots compared with the shoots or grains at all levels of metals. It is also clear from this

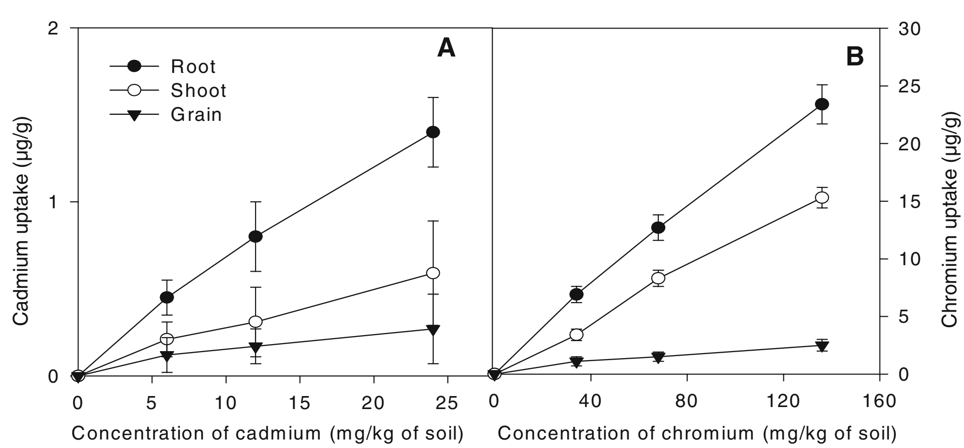

Fig. 4 Cadmium and chromium concentration in roots and shoots at 90 days and grains at 120 days after seeding the pea in cadmium and chromium amended soil

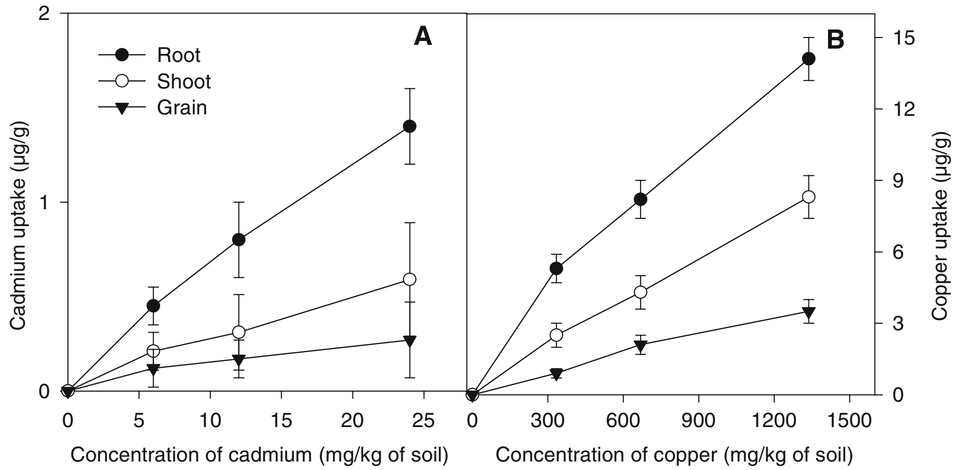

Fig. 5 Cadmium and copper concentration in roots and shoots at 90 days and grains at 120 days after seeding the pea in cadmium and copper amended soil

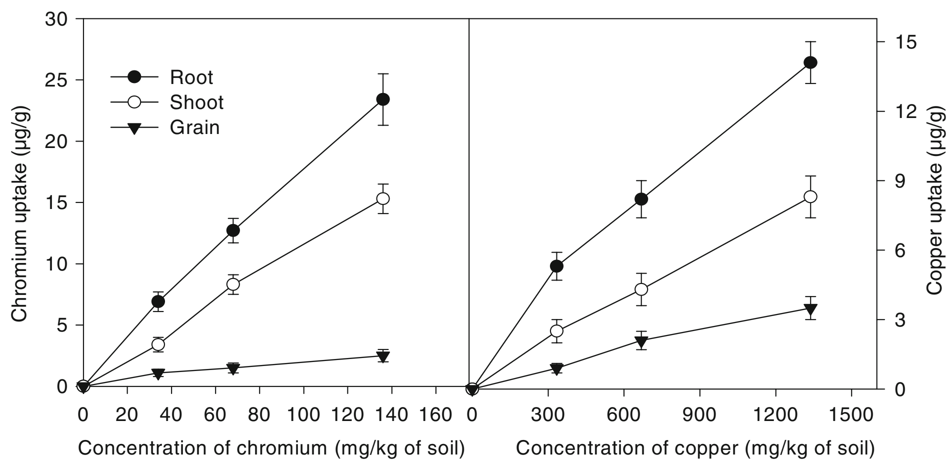

Fig. 6 Chromium and copper concentration in roots and shoots at 90 days and grains at 120 days after seeding the pea in chromium and copper amended soil

study that the simultaneous application of metals reduced the uptake by plant organs. The variation in the uptake of metals by the pea plants could be due to the antagonistic effect of one metal on the other. A second possibility could be the interaction between metals at the root surface for plant uptake. It could be concluded from the present study that the increasing metal levels reduced growth and nodulation efficiency of pea plants; leading eventually to the decreased seed yield. Further Cd, Cr and Cu were accumulated in grains, which when consumed could lead to human health problem. Understanding the mechanistic basis of metals with respect to their toxicity to pea plants and extent of accumulation will thus be required in better evaluating the full impact of metal contamination on the legume crops. Furthermore, since the effluents of many industries are contaminating the agronomic soils and making them unsuitable for sustainable agriculture, therefore, we suggest the growers (1) to avoid the use of sewage water for pea cultivation or (2) should not allow the metals showing toxicity in this study to accumulate to toxic level in the soil.

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