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Cadmium, chromium and copper in greengram plants

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Abstract – Soils contaminated with heavy metals including cadmium, chromium and copper present a major concern for sustainable agriculture. We studied the effects of cadmium, chromium and copper used both separately and as mixtures, on plant growth, nodulation, leghaemoglobin, seed yield and grain protein in seeds, in greengram inoculated with *Bradyrhizobium* sp. (*Vigna*). Cadmium at 24 mg kg⁻¹ of soil reduced the dry matter accumulation and number of nodules by 27 and 38%, respectively. Chromium at 136 mg kg⁻¹ of soil increased the dry phytomass and nodule numbers by 133 and 100%, respectively. The average maximum increase of 74% in seed yield occurred at 136 mg Cr kg⁻¹ of soil. Cadmium and copper at 24 and 1338 mg kg⁻¹ soil decreased the seed yield by 40 and 26%, respectively. Chromium at 136 kg⁻¹ of soil increased the root and shoot N and leghaemoglobin content by 42, 31% and 50%, respectively. In contrast, the root and shoot N decreased by 22% at 24 mg Cd kg⁻¹ of soil, while a maximum decrease of 50% in leghaemoglobin content occurred at 12 and 669 and 24 and 1338 mg Cd with Cu kg⁻¹ of soil, relative to the control. The average maximum grain protein (283 mg g⁻¹) was observed at 136 mg Cr kg⁻¹ of soil, while minimum grain protein (231 mg g⁻¹) was recorded at 24 and 1338 mg kg⁻¹ of cadmium with copper. The metal accumulation in roots and shoots at 50 days after sowing and in grains 80 days after seeding differed among treatments. The degree of toxicity of heavy metals to the measured parameters decreased in the order Cd > Cu > Cr.

heavy metals / *Bradyrhizobium* / greengram / phyto-accumulation

1. INTRODUCTION

Greengram [*Vigna radiata* L. wilczek] is a major grain legume and is grown widely in tropical countries. In India, greengram occupies an area of three million hectares, accounting for 14% of the total pulses area and 7% of total production (Singh et al., 2004). Meanwhile, for production of greengram, symbiotic nodule bacterium is used either for coating the seeds or can be directly incorporated into soils in order to enhance the yield. Although reports of metals on rhizobia are contradictory, several studies have demonstrated that some of these metals are incompatible with rhizobia (Broos et al., 2005) and legumes (Broos et al., 2004).

The toxicity of heavy metals to nitrogen-fixing rhizobia and the process mediated by them has been the subject of intense research. Changes in rhizobial populations due to high concentration of heavy metals, as well as effects of heavy metals on legume plants, have been documented. The toxicity of heavy metals can cause multiple effects on plants. For instance, a higher concentration of metals may induce interaction with sulfhydryl groups, leading to the inactivation of plant protein (Assche and Clijsters, 1990). On the other hand, the growth and plant growth-promoting activities of microorganisms can be altered because of a high concentration of metals (Broos et al., 2004).

In one study, only a single strain of *Rhizobium leguminosarum* survived in the metal-contaminated plots, and this strain failed to fix N₂ with white clover (*Trifolium repens*

L.), although it fixed N₂ with *Trifolium subterraneum* (Hirsch et al., 1993). 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). The adverse effects of sludge application on rhizobial species and the concomitant effect on N₂ fixation in faba bean (Chaudri et al., 2000) and chickpea (Yadav and Shukla, 1983) have been reported. There is evidence that suggests that reduction in plant growth, nodule size and nitrogenase activity in white clover was due to Cd, Pb and Zn, when plants were grown in soils highly contaminated with these metals (Rother et al., 1983). In a similar study, a pronounced metal toxicity to white clover was confirmed in a sludge-treated soil where N₂ fixation was halved by increasing metal concentrations in soil (Broos et al., 2005). The effect of total metal concentrations on the survival of *R. leguminosarum*, however, did not occur in soils contaminated with cadmium salts or with high Ni/Cd sewage sludge. Similarly, the sewage sludge, containing a higher concentration of Zn, adversely affected the survival of *R. leguminosarum* bv. *trifolii* (Broos et al., 2005).

Reports on the toxicity of heavy metals to biological nitrogen fixation (BNF) are, however, conflicting. Earlier studies demonstrated that acetylene reduction activity (ARA) was strongly affected by heavy metals in mine spoils or in sludge-amended soils (Heckman, 1987). In contrast, no significant adverse effect of metal-contaminated sludge on N₂ fixation in white clover was detected (Ibekwe et al., 1995). In a field study, Heckman et al. (1987) failed to detect adverse effects on soybean plant growth and BNF in biosolid-amended soils.

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Though a large number of reports on the effects of sewage sludge containing multiple metals are available, there is discrepancy in the reported results. Hence, a firm conclusion on the toxicity of heavy metals to legumes and their symbiotic partners cannot be drawn. Moreover, the majority of the adverse effects have been observed in sludge-treated soils and possibly factors other than metals (e.g. contaminants, excess N supply) could lead to the increased toxicity. Considering the lack of adequate data and conflicting reports on the effect of heavy metals on legumes and nodule bacteria, and the possibility of damage to the crop due to the deposition of heavy metals in the soil, the current study was initiated to examine the effects of varying levels of cadmium, chromium and copper on greengram. The present study evaluates the effect of these metals when used separately and as mixtures, on growth, symbiosis, seed yield and grain protein of greengram. In addition, the uptake of these metals by plant tissues and grains was also assessed, when greengram was grown in sandy clay loam soils.

2. MATERIALS AND METHODS

2.1. Soil analysis for heavy metals

The soil samples were collected from Mathura road, 7 km from Aligarh, Uttar Pradesh, India. There was consistent use of industrial sewage water on this soil. The soil samples were collected in polythene bags and were used for heavy metal determination by a flame atomic absorption spectrophotometer (Model GBC 932B Plus Atomic Absorption Spectrophotometer). The soil samples were finely ground and digested with nitric acid and perchloric acid (3:1) and the heavy metals were analyzed (McGrath and Cunliffe, 1985). The normal concentration of metals determined in the soils included (mg kg^{-1}): - Cd (12); Cr (68) and Cu (669). These metal concentrations were then applied either separately or as mixtures to evaluate their effects on greengram.

2.2. Microbial inoculation, metal treatments and plant culture

Bradyrhizobium sp. (vigna) was grown in yeast extract mannitol broth in flasks at 28°C for six days to a cell density of 6×10^8 cells ml^{-1} . Seeds of greengram (cv. K-851) were surface-sterilized, rinsed six times with sterile water and shade-dried. The sterilized seeds were inoculated with *Bradyrhizobium* sp. (vigna) by dipping the seeds in the liquid culture medium for two hours using 10% gum arabic as an adhesive to apply approximately 10^8 cells to each seed. Metals (as chlorides of cadmium and copper and chromate of chromium) were dissolved in distilled water and applied to the moist soil 15 days 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): cadmium at 6, 12 and 24, chromium at 34, 68 and 136 and copper at 334.5, 669 and 1338. The normal concentrations were comparable with those detected in

sewage-treated soils used in greengram production. The effects of some mixtures were also evaluated (mg kg^{-1} soil): cadmium with chromium (6 and 34; 12 and 68; 24 and 136), cadmium with copper (6 and 334.5; 12 and 669; 24 and 1338), and chromium with copper (34 and 334.5; 68 and 669; 136 and 1338). Some pots without metals but inoculated with *Bradyrhizobium* sp. (vigna) were used as control for comparison. Fertilizer at 20:40:40 mg kg^{-1} soil with N as urea, P as diammonium phosphate and K as potash was dissolved in 500 mL water for each pot and added to the soil surface at the time of sowing in March 2005, and this experiment was repeated with the same treatments in March 2006. Ten inoculated seeds were sown in each pot containing 10 kg non-sterilized sandy clay loam soil (organic carbon 0.4%, Kjeldahl N 0.75 g kg^{-1} , Olsen P 16 mg kg^{-1} , pH 7.2 and water-holding capacity 0.44 mL g^{-1} , Cr 6.3, Cu 12.2, Cd 0.2 $\mu\text{g g}^{-1}$ of soil). Six pots used for each treatment were arranged in a complete randomized design. One week after emergence, the seedlings were thinned to three in each pot. The pots were watered with tap water daily and were maintained in open field conditions.

2.3. Plant growth, nodulation and N content

All plants in three pots for each treatment were removed at 50 days after seeding (DAS), and were used for destructive plant analysis to record nodulation. The roots were carefully washed and nodules were detached, counted, oven-dried at 80°C and weighed. Plants uprooted at 50 DAS were oven-dried at 80°C to measure the total plant biomass. The remaining pots (three pots) for each treatment with three plants per pot were maintained until harvest. Total nitrogen content in roots and shoots was measured at 50 days after seeding by the micro-Kjeldahl method of Iswaran and Marwah (1980).

2.4. Leghaemoglobin content, seed yield and grain protein

The leghaemoglobin content in fresh nodules recovered from the root system of plants raised under metal stress and free of metals (control) was quantified at 50 days after seeding (Sadasivam and Manickam, 1992). The leghaemoglobin was extracted with sodium phosphate buffer (pH 7.4). The extract was divided equally into two glass tubes (5 mL/tube) and an equal amount of alkaline pyridine reagent was added to each tube. The haemochrome formed was read at 556 and 539 nm after adding a few crystals of potassium hexacyanoferrate and sodium dithionite, respectively. Plants were finally harvested at 80 days after seeding and seed yield and grain protein (Lowrey et al., 1951) were estimated.

2.5. Cadmium, chromium and copper uptake

Cadmium, chromium and copper uptake by the roots and shoots of greengram was measured at 50 days after seeding, while accumulation of these metals in seeds was determined at 80 DAS. The plant samples were digested in nitric acid and perchloric acid (4:1) following the method of Ouzounidou

et al. (1992), and metal concentrations were determined using flame atomic absorption spectrophotometry.

2.6. Statistical analysis

Each pot in this study was considered as a replicate and each individual treatment was replicated six times. Since the experiment was conducted consecutively for two years under identical environmental conditions using the same single and combination treatments, and the data obtained were homogeneous, the data of the measured parameters were pooled together and subjected to analysis of variance. The difference among treatment means was compared by high-range statistical domain (HSD) using Tukey's test at the 5% level of probability.

3. RESULTS AND DISCUSSION

3.1. Plant growth

The effect of three concentrations of cadmium, chromium and copper, applied separately and in combination on greengram plants, differed among treatments (Tab. I). Among the single metal treatments, cadmium was found to be the most phytotoxic and reduced the total dry matter accumulation significantly ($P \leq 0.05$) by 18, 22 and 27% at 6, 12 and 24 mg kg⁻¹ soil, respectively. In contrast, chromium at 34, 68 and 136 mg kg⁻¹ soil increased the dry matter production 0.6, 1.0 and 1.3 times, respectively, relative to the control. The dry matter accumulation was reduced even further when cadmium was used in combination with chromium and copper at all three concentrations. The reduction in dry biomass of greengram following application of mixtures of metals ranged between 24 (Cd with Cr at 6 and 34 mg kg⁻¹ soil) and 41% (Cd with Cu at 24 and 1338 mg kg⁻¹) relative to the control. In contrast, the combination of chromium and copper increased the dry matter by 31% at 136 and 1338 mg kg⁻¹ soil, relative to the control.

The toxicity of metals to nodule bacteria in vitro or legume plants varies widely. Heavy metals, therefore, affect the viability of *Rhizobium* (Broos et al., 2005), and consequently the legume – *Rhizobium* symbiosis (Broos et al., 2004). In the present study, the lower rates of metals except cadmium when used singly, in general, did not affect the plant growth negatively. This study therefore suggests that the lower rates of the tested heavy metals might have been influenced by root exudates, such as organic acid (Jackson et al., 1990), or pH changes in the rhizosphere and the metals involved (Prasad, 1999). In contrast, when metal concentrations become too high, the plant barrier loses its function, probably due to toxic action by the metal, and the uptake massively increases. Moreover, as a result of increased uptake, these metals interact with many cellular components, thereby interfering with the normal metabolic functions, causing cellular injuries and, in extreme cases, death of the plants. In the present study, the higher concentrations of cadmium and copper, in general, had the greatest phytotoxic effect on greengram plants, possibly due to the

inhibition or damage of all classes of biomolecules including proteins, enzymes and DNA (Asada, 1994) through the generation of reactive oxygen intermediates. Although all reactive oxygen intermediates are more or less highly reactive and are toxic to living organisms, the ultimate damaging effect is, however, mainly by singlet oxygen (¹O₂) and hydroxyl radicals (HO^{*}). The rapid and specific reaction of these radicals in turn damages all classes of bio-molecules (Breen and Murphy, 1995). Oxidative stress due to cadmium treatment has been reported in pea (*Pisum sativum* L.) leaves (McCarthy et al., 2001), while copper is known to interfere with oxidative enzymes in bean (*Phaseolus vulgaris*) leaves (Shainberg et al., 2001). Moreover, the reduction in plant growth could also be due to the decline in photosynthetic pigments (Bibi and Hussain, 2005; Wani et al., 2006) and Rubisco activity (Sheoran et al., 1990).

In the present study, the greengram plants were, however, tolerant to the three concentrations of chromium, probably due to the synthesis of phytochelatin (PC); a simple γ – glutamyl peptide (Grill et al., 1985). The synthesis of phytochelatin is induced by most of the heavy metals including the multi-atomic anions (Maitani et al., 1996) in most of the higher plants (Gekeler et al., 1989), and phytochelatin synthetase involving the synthesis of phytochelatin requires metals for its activation (Grill et al., 1989). Since phytochelatin synthetase activity has been detected largely in roots (Steffens, 1990), and the root is the first organ exposed to the metal ions in the soil, the roots of the test plant might have provided an effective means of restricting the uptake of chromium by forming a chromium – phytochelatin complex. The phytochelatin – metal complex has been reported mostly for cadmium but a report of phytochelatin forming complexes with copper is also available (Grill et al., 1987). The authors are, however, not aware of such phytochelatin forming complexes with chromium and detoxification effects by greengram plants. Further, the metals were used to evaluate their effects on symbiotic traits of greengram plants.

3.2. Symbiotic traits

Nodulation response to the three concentrations of cadmium, chromium and copper at 50 days after sowing varied considerably (Tab. I). Comparison between the control and metal treatments revealed a significant increase in the number of nodules per plant following 34, 68 and 136 mg Cr kg⁻¹ of soil at 50 days after sowing, while cadmium at 6, 12 and 24 mg kg⁻¹ of soil and 334.5, 669 and 1338 mg Cu kg⁻¹ of soil reduced the number of nodules. Among the single metal treatments, cadmium and copper decreased the number of nodules by 38 and 23% at 24 and 1338 mg kg⁻¹ soil, respectively, compared with control. In contrast, the number of nodules increased significantly ($P \leq 0.05$) by 100% at 136 mg Cr kg⁻¹ of soil. Similarly, mixed heavy metals at all concentrations except Cr with Cu (at 34 and 334.5 mg kg⁻¹ of soil) decreased the number of nodules compared with control. Among the metal combinations, Cd with Cu showed the largest adverse effect and significantly reduced the number of nodules by 62% (at 24

Table I. Dry matter, nodulation, N contents, leghaemoglobin, seed yield and grain protein in chickpea 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 (mg plant ⁻¹)	Nodulation		N content (mg g ⁻¹)		Leg haemoglobin content [m mol (g f.m) ⁻¹]	Seed yield (g plant ⁻¹)	Grain Protein (mg g ⁻¹)
			No. plant ⁻¹	Dry weight (mg plant ⁻¹)	Root	Shoot			
Cd	6	225gh	11de	8b	40de	50cd	0.07cd	3.5ef	245de
	12	212h	10ef	7b	38ef	47ef	0.06de	3.1gh	240fg
	24	198hi	8fg	6b	35fg	43g	0.05ef	2.5ij	235gh
Cr	34	444c	19b	13b	58b	67b	0.11ab	4.5c	270b
	68	549b	21ab	25a	60ab	70ab	0.11ab	5.6b	275b
	136	636a	26a	30a	64a	72a	0.12a	7.3a	283a
Cu	334.5	265ef	12de	9b	42cd	51c	0.08bc	3.8de	247d
	669	249g	11de	8b	40de	49de	0.07cd	3.6ef	243ef
	1338	224gh	10ef	7b	36fg	46fg	0.06de	3.1gh	240fg
Cd + Cr	6+34	207hi	10ef	7b	40de	49de	0.06de	3.3gh	242ef
	12+68	192ij	9fg	6b	38ef	46fg	0.06de	2.9h	238fg
	24+136	176jk	7gh	6b	35fg	45fg	0.05ef	2.1jk	235gh
Cd + Cu	6+334.5	188jk	9fg	7b	38ef	49de	0.05ef	2.0k	238fg
	12+669	180jk	6gh	8b	35fg	46fg	0.04f	1.9k	235gh
	24+1338	161k	5h	5b	32g	43g	0.04f	1.7k	231h
Cr + Cu	34+334.5	289e	15c	8b	40de	50cd	0.09ab	3.5ef	246d
	68+669	298e	13de	10b	39ef	48de	0.09ab	3.2gh	245de
	136+1338	358d	12de	6b	37ef	48de	0.10ab	2.6i	243ef
Control		273ef	13de	9b	45c	55fg	0.08bc	4.2cd	256c
F value treatments (df = 18)		142.5*	23.0*	12.9*	93.8*	140.9*	18.0*	281*	167.4*

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's test. * Significant at $P \leq 0.05$.

and 1338 mg kg⁻¹ of soil), relative to the control. The reduction in nodulation was accompanied by a significant decrease in dry mass of nodules.

Greengram plants grown in sandy clay soils amended with cadmium, chromium and copper had fewer nodules compared with control. The reduction in the number of nodules is possibly due to the direct toxic effect of these metals either on the root hairs or rhizobia, as observed in zinc- and cadmium-treated alfalfa plants (Ibekwe et al., 1996). In general, cadmium had the greatest adverse effect on symbiosis when it was applied to the soil either singly or in combination. While comparing the sum of the mean values of the effects of each metal, the order of toxicity to the symbiotic trait decreased in the following order: cadmium < copper < chromium. In a similar study, the nitrogen fixation in white clover was halved at soil total metal concentrations of 428 mg Cu kg⁻¹ and 10 mg Cd kg⁻¹ (Broos et al., 2004). However, these concentrations were above the value found by McGrath et al. (1988) in Woburn for total copper (99 mg kg⁻¹) and equal for total cadmium (10 mg kg⁻¹). Interestingly, like the effect of chromium on plant growth, no adverse effect of any of the three concentrations of this metal was observed on the symbiotic properties of the test plant. This observation was important because

it raised the issue of whether or not the greengram plants or *Bradyrhizobium* used in this study can use multiple mechanisms of resistance to the same metal. In this context, several mechanisms of resistance to metals in microorganisms are known. For instance: the production of a secreted polysaccharide layer which surrounds the cell and can ionically sequester metals, preventing their entry into the cell. Interestingly, 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 the metal back out of the cell once it has crossed the cell membrane with the use of energy-dependent efflux pumps. Roane and Kellogg (1996), in a study of lead resistance in soil communities from lead-contaminated soils, also observed increasing resistance with increasing metal concentration. The effect of metal treatments on N contents in plant tissues was also assessed.

3.3. N content

The effects of three concentrations of cadmium, chromium and copper on N content in roots and shoots at 50 days after

seeding was variable (Tab. I). The average maximum decline in root N following single metal applications occurred at 24 mg Cd kg⁻¹ (35 mg N g⁻¹) and 1338 (36 mg N g⁻¹) mg Cu kg⁻¹ (Tab. I) and significantly ($P \leq 0.05$) reduced the root N by 22 and 20%, respectively, relative to the control. In comparison, chromium progressively enhanced the root N by 29, 33 and 42% at 34, 68 and 136 mg kg⁻¹ of soil, compared with control. Among the dual metal treatments, cadmium with copper (at 24 and 1338 mg kg⁻¹ soil) significantly reduced the N content by 29% compared with the control. A trend similar to root N was observed for shoot N with the three metals and their combinations. The average maximum increase in shoot N content with chromium ranged between 22 (34 mg Cr kg⁻¹ soil) and 31% (136 mg Cr kg⁻¹ soil), compared with control. The N content of the roots was more severely affected than the shoot N at all the concentrations of tested metals.

The decrease in N content of greengram plants might have been due to the reduction in the greengram – *Bradyrhizobium* 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 the depressive effect of these metals on nitrogenous bases (Sinha et al., 1988). A similar reduction in total root and shoot N in alfalfa (Ibekwe et al., 1996) has been reported with zinc and cadmium. Furthermore, since many sites that are irrigated with sewage are often contaminated with a broad range of metals, it was therefore important to examine the effectiveness of the test plants when cadmium, chromium and copper were present simultaneously in the soil and to compare the results with those of treatments where the same metals were applied separately. Normally, when plants are exposed to unfavorable concentrations of more than one metal, various interactions can occur. Such combination effects could be independent, additive, synergistic or antagonistic (Berry and Wallace, 1981). Antagonistic interaction 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 metals. In the present study, both synergistic and antagonistic effects were observed. For instance, the combination of cadmium with copper (24 and 1338 mg kg⁻¹) showed a greater synergistic toxic effect on N contents of plant tissues than that observed for single application of cadmium and copper; while chromium with copper (136 and 1338 mg kg⁻¹) exhibited a lesser effect on dry matter production, which could possibly be due to the antagonistic effect of chromium on copper (Bewley and Stofzky, 1983). A similar synergistic and additive effect on the growth of roots and shoots of bean with a combination of cadmium-zinc has been reported (Chaoui et al., 1997). The effects of cadmium, chromium and copper on leghaemoglobin, seed yield and grain protein are discussed in the following section.

3.4. Leghaemoglobin

The important role of the leghaemoglobin in the nodule suggests that changes in its concentration could affect the entire system of nitrogen fixation. In this experiment, the nodules

on the root system of greengram plants raised in soil amended with cadmium and copper had a considerably lower concentration of leghaemoglobin. In contrast, the leghaemoglobin content was increased by 50% at 136 mg Cr kg⁻¹ soil. Levels of leghaemoglobin in combined metal treatments were significantly decreased compared with control. A maximum reduction of 50% in leghaemoglobin was observed with cadmium-chromium (at 12 and 669; 24 and 1338 mg kg⁻¹ soil). Since chromium used either singly or in combination treatments had no toxic effect on nodulation, we expected that nodules in the presence of this metal could contain leghaemoglobin at levels greater than the control. This study thus suggested that the leghaemoglobin was not the target of chromium. Comparable observations on the effect of cadmium, nickel, copper and zinc on soybean nodules have been reported (Stephen and Weidensaul, 1978).

3.5. Seed yield

The effect of heavy metals on seed yield was variable (Tab. I). Seed yield decreased consistently for each metal with increasing concentration, used either separately (except the three concentrations of chromium) or in combination. The average maximum increase of 7, 33 and 74% was observed with chromium at 34, 68 and 136 mg kg⁻¹ soil, respectively, compared with control. In contrast, cadmium at 24 mg kg⁻¹ soil significantly ($P \leq 0.05$) decreased the seed yield by 40%, relative to the control. The average reduction in seed yield among combination treatments ranged between 17 (at 34 and 334 mg kg⁻¹ of Cr and Cu) and 60% (at 24 and 1338 mg kg⁻¹ of Cd and Cu), relative to the control. While comparing the sum of mean values of each metal treatment, the order of toxicity to seed mass decreased in the following order: Cd < Cu < Cr.

Indeed, the metals added to the soil in the present study had an adverse effect on the growth of greengram plants, that subsequently decreased the seed yield. Among the metals used, cadmium either alone or in combination with chromium and copper was found to be the most toxic metal for seed production. Similar evidence of cadmium toxicity in lentil crops has been reported (Wani et al., 2006). Furthermore, this study clearly showed a concentration-dependent decrease in seed yield of greengram compared with the control plants. The reduction in seed yield following heavy metal application has been attributed to the effects of metals on the proliferation of roots and shoots (Ibekwe et al., 1996). The reduction in roots and shoots then led to the suppressive effect on dry matter production, and consequently the seed yield (Bisessar et al., 1983).

3.6. Grain protein

The effect of heavy metals on grain protein was variable (Tab. I). Chromium, in general, consistently increased the grain protein with increasing concentrations. The average maximum grain protein (283 mg g⁻¹) was observed with

Cadmium uptake ($\mu\text{g/g}$)

Figure 1. Cadmium concentration in roots and shoots at 50 days and grains at 80 days after seeding the greengram in cadmium-amended soil. A maximum accumulation of 2, 0.72 and $0.5 \mu\text{g g}^{-1}$, of cadmium was recorded in roots, shoots and grains, respectively, at 24 mg Cd kg^{-1} of soil.

chromium at 136 mg kg^{-1} and was greater by 11% compared with control. In comparison, other metals used either alone or in combination decreased the grain protein consistently with an increase in concentration relative to control. Cadmium with copper decreased the grain protein by 7 (at 6 and 334.5), 8 (12 and 669) and 10% (at 24 and 1338 mg kg^{-1} of soil), respectively, relative to the control. The combinations of metals in general had the greatest adverse effect on grain protein compared with single metal treatments. The protein content in greengram seeds was below the normal range of 253 mg g^{-1} under the majority of the metal treatments except when chromium was used separately. The reduction in grain protein could be due to the high affinity of these metals for ligands of proteins, suggesting that the enzymes and functional proteins are the main targets of the cadmium and copper tested in this study. Moreover, the indirect effect of the tested metals on the active metabolism of the plants and perhaps their symbiotic partner, and decreased availability of N to the seed in turn might have accounted for decreased grain protein. Similarly, the reduction in grain proteins of other legumes has been reported (Wani et al., 2006). However, chromium in general did not affect the plant growth and symbiosis adversely, which could be the reason why seed production or grain protein increased with chromium application. Additionally, since the soil used in this study was non-sterilized, there is every possibility of the presence of chromium-reducing bacteria that might have alleviated the phytotoxic effect of chromium in this study (Faisal and Hasnain, 2005). The concentration of cadmium, chromium and copper in plant tissues and seeds of greengram under metal stress is discussed in the following section.

3.7. Concentration of cadmium, chromium and copper in plant tissues and grains

The concentration of cadmium, chromium and copper in plant tissues, e.g. roots and shoots, at 50 days after seeding

Chromium uptake ($\mu\text{g/g}$)

Figure 2. Chromium concentration in roots and shoots at 50 days and grains at 80 days after seeding the greengram in chromium-amended soil. A maximum accumulation of 29.9, 10.5 and $4.5 \mu\text{g g}^{-1}$ of chromium was recorded in roots, shoots and grains, respectively, at $136 \text{ mg Cr kg}^{-1}$ of soil.

Copper uptake ($\mu\text{g/g}$)

Figure 3. Copper concentration in roots and shoots at 50 days and grains at 80 days after seeding the greengram in copper-amended soil. A higher concentration of copper was recorded in roots, shoots and grains at $1338 \text{ mg Cu kg}^{-1}$ of soil.

and grains at 80 DAS differed among treatments. The metals in general in roots, shoots and grains were influenced greatly by the concentration of each metal tested. A higher amount of cadmium (Fig. 1), chromium (Fig. 2) and copper (Fig. 3) in roots, shoots and grains was observed when these metals were used individually compared with dual metal application. The greengram plants showed a maximum accumulation of cadmium in roots ($2 \mu\text{g g}^{-1}$), shoots ($0.72 \mu\text{g g}^{-1}$) and grains ($0.5 \mu\text{g g}^{-1}$) at 24 mg kg^{-1} of soil (Fig. 1). In comparison, the concentration of chromium was higher in roots ($29.9 \mu\text{g g}^{-1}$), shoots ($10.5 \mu\text{g g}^{-1}$) and grains ($4.5 \mu\text{g g}^{-1}$) at 136 mg kg^{-1} of soil (Fig. 2). The concentration of copper was higher in roots ($60.1 \mu\text{g g}^{-1}$), shoots ($26.2 \mu\text{g g}^{-1}$) and grains (15.7) at 1338 mg kg^{-1} of soil (Fig. 3). Following the dual metal treatments, the concentrations of cadmium, chromium and copper in plant tissues and grains were in general reduced marginally at 24 and 136 mg kg^{-1} of cadmium with chromium (Fig. 4),

Figure 4. Cadmium and chromium concentration in roots and shoots at 50 days and grains at 80 days after seeding the greengram in cadmium- and chromium-amended soil. A maximum accumulation of 1.8, 0.65 and 0.33 $\mu\text{g g}^{-1}$ of cadmium and 29, 10 and 4.4 $\mu\text{g g}^{-1}$ of chromium was recorded in roots, shoots and grains, respectively, at 24 mg Cd kg^{-1} with 136 mg Cr kg^{-1} of soil.

Figure 5. Cadmium and copper concentration in roots and shoots at 50 days and grains at 80 days after seeding the greengram in cadmium- and copper-amended soil. A maximum accumulation of 1.8, 0.65 and 0.34 $\mu\text{g g}^{-1}$ of cadmium and 19, 15 and 7.3 $\mu\text{g g}^{-1}$ of copper was recorded in roots, shoots and grains, respectively, at 24 mg Cd kg^{-1} with 1338 mg Cu kg^{-1} of soil.

24 and 1338 mg kg^{-1} of cadmium with copper (Fig. 5) and 136 and 1338 mg kg^{-1} of chromium with copper (Fig. 6). The concentration of chromium was maximum both at 24 and 136 mg kg^{-1} of Cd with Cr and 136 and 1338 mg kg^{-1} of Cr with Cu in roots (29 $\mu\text{g g}^{-1}$), shoots (10 $\mu\text{g g}^{-1}$) and grains (4.3 $\mu\text{g g}^{-1}$) compared with the other treatments. (Figs. 4 and 6). The phyto-accumulation of heavy metals was higher in roots compared with the shoots or grains at all rates of metals, applied singly or in dual treatments. It is also clear from this study that accumulation of metals by greengram plants was altered in the presence of additional metals. The variation in the uptake of metals by the greengram plants could be due to several reasons. For instance, the smaller uptake of metals by plant tissues in amended soil could be due to the antagonistic effect of one metal on another. A second possibility could be the existence of interaction at the root surface between metals for plant uptake. Lastly, there was probably competition between metals for adsorption onto soil. A similar variation

in the accumulation of metals in different plants has been reported (Charlier et al., 2005).

4. CONCLUSION

This study suggests that the increasing metal concentrations reduced plant growth and nodulation, leading eventually to decreased seed yield. It was also found that the cadmium, chromium and copper could enter the food chain through their accumulation in grains, which when consumed could lead to human health problems. The respective effect of each metal used either separately or in combination thus seems to depend on the concentration of metals and the greengram – metal – *Bradyrhizobium* symbiosis interaction after seeding. Understanding the mechanistic basis of metals with respect to toxicity to greengram plants and the extent of their accumulation will be important in modeling better the full impact of

Figure 6. Chromium and copper concentration in roots and shoots at 50 days and grains at 80 days after seeding the greengram in chromium- and copper-amended soil. A maximum accumulation of 29, 10 and 4.5 $\mu\text{g g}^{-1}$ of chromium and 19, 15 and 7.3 $\mu\text{g g}^{-1}$ of copper was recorded in roots, shoots and grains, respectively, at 136 mg Cr kg^{-1} with 1338 mg Cu kg^{-1} of soil.

metal contamination on legume crops. Furthermore, coal-fired power plants, oil refineries, smelters and other polluting industries are becoming more frequently placed in or around agricultural areas worldwide and are contaminating the agronomic soils. The metals released from these sources are thus making soil unsuitable for sustainable agriculture. Therefore, based on the present findings of the two-year trial, we suggest that growers who often use sewage water containing toxic metals, for greengram cultivation, should avoid the use of such contaminated water or should not allow the metals showing toxicity in this study to accumulate to toxic levels in the soil.

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