Summary

Metals are discharged into the environment from industrial operations such as smelting, mining, metal forging, manufacturing of alkaline storage batteries, and combustion of fossil fuel and sewage sludge. After discharge from these sources, metals accumulate in soil and, at higher concentrations, adversely affect the soil microbial activity and soil fertility. Moreover, the elevated concentrations of metals in soil, when taken up by plants also causes the disintegration of cell organelles and disruption of membrane, acts as a genotoxic substance and adversely affect the physiological processes, such as photosynthesis, protein synthesis, respiration and carbohydrate metabolism, and concomitantly results in losses in the yields of various crops including legumes. However, agronomically important rhizospheric microorganisms capable of alleviating the toxicity of metals and can promote the growth and yields of plants even in the metal contaminated soils. Among these microbes, the plant growth promoting rhizobacteria (PGPR) including phosphate solubilizing bacteria (PSB) and symbiotic nitrogen fixing organisms can provide protection to the plants against the toxic effects of metals through adsorption/desorption mechanisms, besides providing the essential nutrients (P by PSB and N by N₂-fixers) and plant growth promoting substances (phytohormones) including siderophores to the plants. With these consideration and lack of sufficient data on growth promoting potentials of plant growth promoting rhizobacteria, toxicity of metals to both plant growth promoting rhizobacteria and their metabolic activities and on the over all performance of legumes, cultivated in conventional and derelict soils, the present study was, therefore, deigned with the following specific aims and objectives:

- quantitative assay of heavy metals and soil microflora in the metal polluted and non-polluted soils of Aligarh and adjoining industrial area
- isolation of nitrogen fixing bacteria from the nodules of legumes grown in metal contaminated/conventional Indian soils and phosphate solubilizing bacteria from the rhizospheric soils of mustard and tomato
- assessment of the tolerance level of plant growth promoting rhizobacteria to cadmium, chromium, nickel, lead, zinc and copper
- to investigate the antibiotic resistant profile of heavy metal tolerant strains of plant growth promoting rhizobacteria including N₂ fixers
to assess the plant growth promoting potentials of plant growth promoting rhizobacteria, both in the absence and presence of metal ions

to assess the chromium (VI) reducing and lead and zinc solubilizing activity of selected bacterial strains under *in vitro* conditions

to evaluate the performance of inoculated chickpea, greengram, lentil and pea, when grown in metal treated sandy clay loam soils. Also, to assess the antioxidant enzyme activity and uptake of metals and nitrogen by plant organs and

to assess the bioremediation potentials of metal tolerant strains of nitrogen fixers and phosphate solubilizing bacteria, using chickpea, greengram, lentil and pea, as test legumes in pot house conditions. Quantitative assay of antioxidant enzyme activity, uptake of nitrogen and heavy metals by legumes grown both in conventional and metal stressed soils was also studied.

In this study, the concentration of heavy metals in polluted and non-polluted soils was determined by flame atomic absorption spectrophotometry. The heavy metal concentration in polluted soils of Mathura Road (S1) was (mg/kg soil): 11.5 (Cd); 67.5 (Cr); 290.1 (Ni); 4890 (Zn); 669.1 (Cu) and 195 (Pb) while in polluted soils of Exhibition ground (S2), were 9.8 (Cd); 64.2 (Cr); 334 (Ni); 3550 (Zn); 535 (Cu) and 191 (Pb). In comparison, the heavy metal concentration of the conventional agricultural soils of Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh (S3) were 10.8 mg Ni/kg, 8.1 mg Pb/kg, 19.2 mg Zn/kg, 6.3 mg Cr/kg, 12.2 mg Cu/kg and 0.2 mg Cd/kg. The rhizospheric soils of chickpea, greengram and brinjal (S1); chickpea, greengram, lentil and pea (S2) and mustard and tomato (S3) were subjected further for microbiological analysis. The viable counts of bacteria, fungi and phosphate solubilizing microorganisms (PSM) differed among rhizospheric soils. Generally, the microbial populations were less in polluted soils (S1 and S2) compared to conventional soils (S3). Phosphate solubilizing bacteria were recorded more than the phosphate solubilizing fungi in both polluted and conventional soils of Aligarh. Among all the rhizosphere soils, the population of both phosphate solubilizing bacteria and fungi was greater in the rhizosphere of mustard. Based on the morphological and biochemical characteristics, the phosphate solubilizing bacteria were presumptively identified as *Pseudomonas* sp. (PSB5) while others were identified as *Bacillus* spp.. Similarly, 50 rhizobial species were isolated from nodules produced on the root systems of
each of chickpea (*Mesorhizobium* spp.), greengram (*Bradyrhizobium* spp.), lentil (*Rhizobium* spp.) and pea (*Rhizobium* spp.), grown at the metal contaminated/non contaminated Indian soils and were characterized on the basis of physiological, morphological and biochemical properties. Due to lack of facilities for molecular characterization, the isolated cultures were not characterized at genetic level.

The nitrogen fixers and phosphate solubilizers were further screened for their multiple plant growth promoting (PGP) activities under *in vitro* conditions. The mesorhizobial strains were grouped into four PGP groups where group I included 30% of strains which showed four PGP traits, followed by PGP group II that included 40% of strains. Of the total 10 bacterial strains in PGP group III, 20% of the strains showed a positive reaction to ammonia and IAA, while PGP group IV contained only one strain (*Mesorhizobium* RC10) which possessed the property of synthesizing indole acetic acid. In a similar manner, the *Rhizobium* strains isolated from pea nodules were categorized into four PGP groups where PGP group I included two isolates (*Rhizobium* RP5 and RP7) and displayed four PGP traits (i.e. synthesis of ammonia, HCN, siderophore and IAA). This was followed by group II, which had only one strain (RP3) and was positive for ammonia, siderophore and indole acetic acid; group III contained 22% of the strains which were found to be positive for ammonia, hydrogen cyanide and indole acetic acid while group IV contained 56% of the strains and were found to be positive for ammonia and indole acetic acid. *Rhizobium* strains isolated from lentil nodules were grouped into four PGP groups. The PGP group I contained four (26.7%) isolates and showed four PGP traits (ammonia, HCN, siderophore and IAA); group II included only one strain (*Rhizobium* RL3) and was positive for ammonia, siderophore and indole acetic acid; group III contained 6.7% of the strains and was positive for ammonia, hydrogen cyanide and indole acetic acid while group IV contained 60% of the strains which were positive for ammonia and indole acetic acid only. Similarly, *Bradyrhizobium* strains isolated from greengram nodules were categorized into two PGP groups: the PGP group I contained 21% of isolates and showed four PGP traits (ammonia, HCN, siderophore and IAA). This was followed by group II which included 79% of strains and were positive for ammonia and indole acetic acid. Likewise, bacterial strains possessing phosphate solubilizing activity were grouped into four PGP groups where group I contained three (30%) isolates and showed five PGP traits (ammonia, HCN, siderophore,
IAA and phosphate solubilization) which was followed by group II (having only five strains) which was positive for ammonia, siderophore, indole acetic acid and phosphate solubilization; group III had 10% of the strains and were found to be positive for ammonia, hydrogen cyanide, IAA and phosphate solubilization while group IV included only one strain (Bacillus PSB9) and was positive for ammonia, IAA and phosphate solubilization.

The selected phosphate solubilizing and rhizobial strains were tested further for their ability to tolerate various concentrations of heavy metals, like, cadmium, chromium, nickel, lead, zinc and copper using agar plate dilution method. The phosphate solubilizers (Bacillus and Pseudomonas) and rhizobial strains differed considerably in terms of their ability to tolerate metals and were influenced by the type and concentration of metals. Among the phosphate solubilizers, Bacillus PSB1, PSB7 and PSB 10 tolerated multiple metals. Among Bacillus species strain PSB1 showed a higher tolerance to cadmium, nickel and copper (400 μg/ml for each metal), chromium (500 μg/ml) and 1400 μg/ml each to lead and zinc, while strain PSB7 showed a higher tolerance to cadmium and nickel (300 μg/ml for each metal), chromium and copper (400 μg/ml for each metal) and 1600 μg/ml to lead and 1400 μg/ml to zinc. Bacillus sp. PSB10 displayed a higher level of tolerance to cadmium and copper (300 μg/ml), 550 μg/ml to chromium, 400 μg/ml to nickel, 1600 μg/ml to lead and 1400 μg/ml to zinc. The order of tolerance of phosphate solubilizers to metals decreased in the following order- Zn < Pb < Cr < Ni < Cu < Cd. Similarly, the rhizobial strains were tolerant to one or more metal ions. Among these strains, Mesorhizobium sp. RC3 showed tolerance to multiple metals and tolerated a concentration of 400, 500, 500, 1500, 1500 and 400 μg/ml of cadmium, chromium, nickel, lead, zinc and copper, respectively, amended in agar plates. In contrast, the lentil and greengram rhizobia were highly resistant to zinc, followed by lead while pea rhizobial strains in general, were most resistant to lead, which was followed by zinc. Among the pea, lentil and greengram rhizobia, Rhizobium strain RP5, Rhizobium RL9 and Bradyrhizobium sp. RM8 exhibited highest tolerance to most of the metals. Among these, strain RP5 showed a higher tolerance to cadmium (250 μg/ml), chromium (350 μg/ml), nickel (350 μg/ml), lead (1200 μg/ml), zinc (1500 μg/ml) and copper (200 μg/ml). Of the 15 strains of Rhizobium isolated from lentil nodules, strain RL9 tolerated cadmium, chromium, nickel, lead, zinc and copper to a level of 300, 400, 500, 1400, 1000 and 300 μg/ml, respectively, while strain Bradyrhizobium RM8 showed a higher tolerance of 75
μg/ml to cadmium, 200 μg/ml to chromium, 300 μg/ml to nickel, 1300 μg/ml to lead, 1500 μg/ml to zinc and 100 μg/ml to copper. These bacterial isolates further varied considerably in their response to the sensitivity/resistance towards different antibacterial drugs (antibiotics). Among *Mesorhizobium* spp., 33% strains were resistant to both nitrofurantoin and methicillin while 33% *Rhizobium* spp. isolated from lentil nodules were resistant to nalidixic acid and ampicillin. Among the bradyrhizobial isolates, only one isolate (RM8) was resistant to ampicillin. In comparison, none of the strains of *Rhizobium*, isolated from pea nodules were resistant to any antibiotics tested. Generally, the growth of the bacterial isolates declined progressively with increasing concentrations of the metals under *in vitro* experiments.

The plant growth promoting rhizobacteria exhibited a substantial production of IAA after 24 h of incubation and showed concentration dependent increase in IAA. Among the phosphate solubilizing bacteria, *Bacillus* strains PSB1, PSB7 and PSB10 produced maximum amounts of IAA (19.3, 17.7 and 17.4 μg/ml, respectively) in Luria Bertani (LB) broth supplemented with 100 μg/ml tryptophan, which decreased consistently with increase in the concentration of the tested metals. Similarly, among greengram rhizobia, *Bradyrhizobium* strain RM8 produced 13.3 μg/ml of IAA in LB broth at 100 μg/ml tryptophan, which increased to 13.6 μg/ml with 50 μg Ni/ml, 13.5 μg/ml with 300 μg/Zn ml, 13.9 μg/ml with 50 μg/ml Cr and 13.5 μg/ml with 300 μg/Pb ml. Similarly, other rhizobial strains produced a maximum amount of IAA in LB broth supplemented with 100 μg/ml tryptophan, both in the absence and presence of heavy metals. Interestingly, the production of phytohormones did not differ significantly among metal amended or metal free medium. Furthermore, production of siderophores by the PGPR strains was also determined on CAS agar plates supplemented with or without hexavalent chromium, nickel, lead and zinc. The selected PGPR strains including nitrogen fixing organisms showed siderophore activity as indicated by the development of orange coloured zone on CAS agar plates amended with or without metal ions. A maximum reduction in zone size with increase in metal concentration varied between 8 (by *Rhizobium* sp. RP3 at 150 μg/ml of Cr and Ni and 300 μg/ml of Pb and Zn) to 13% (by *Rhizobium* sp. RP7 at 150 μg/ml of Cr and Ni) after four days of incubation, in comparison to control. The reduction in zone size by bradyrhizobial strains on CAS agar
plates varied between 11% (by *Bradyrhizobium* RM8 at 150 µg/ml of Cr, 100 and 150 µg/ml of Ni, 900 µg/ml of Pb and 600 and 900 µg/ml of Zn) to 25% (by *Bradyrhizobium* spp. RM1 at 150 µg/ml of Cr and Ni) after four days of incubation. Similarly, a maximum reduction in siderophore zone on metal amended CAS agar plates for *Bacillus* strains varied between 7 (by PSB10 at 600 and 900 µg/ml of Zn) to 9% (by PSB7 at 150 µg Cr/ml, 100 and 150 µg Ni/ml and 600 and 900 µg/ml of Pb and Zn, respectively). Further, the ethyl acetate extraction from culture supernatant of *Mesorhizobium* strains yielded a maximum amount of 17 and 24.5 mg/l of salicylic acid (SA) and dihydroxy benzoic acid (DHBA) by RC3, grown in the Modi medium. When, 50 µg/ml of chromium (VI) and nickel and 300 µg/ml of lead and zinc were also added to medium, the *Mesorhizobium* strain RC3 slightly increased the SA and DHBA compared to control. The amount of SA and DHBA in the supernatant of mesorhizobial strains decreased consistently with increase in each metal concentration. The ethyl acetate extraction from culture supernatant of strains grown in absence of each metal, yielded 24.2 and 20 mg/l of SA and DHBA by *Rhizobium* strain RP3, 24.2 and 21.2 mg/l of SA and DHBA by strain RP5 and 14.2 and 15.2 mg/l of SA and DHBA by strain RP7, respectively. Chromium and nickel at 50 µg/ml and lead and zinc at 300 µg/ml slightly increased the SA and DHBA, in comparison to control. Moreover, Cr and Ni at 150 µg/ml and Pb and Zn at 900 µg/ml did not affect the siderophore activity adversely. The *Rhizobium* (lentil) RL9 yielded a maximum amount of 15 and 18.3 mg/l of SA and DHBA, grown in the Modi medium devoid of each metal. Chromium (VI), Ni, Pb and Zn at 50 µg/ml however, marginally increased the SA and DHBA by RL9 compared to control. The amount of SA and DHBA in the supernatant of rhizobial strains specific to lentil decreased consistently with increase in each metal concentration. *Bradyrhizobium* strain RM8 yielded 17.4 and 16.3 mg/l of SA and DHBA, respectively. Chromium and Ni at 50 and Zn and Pb at 300 µg/ml either did not affect or slightly increased SA and DHBA. The ethyl acetate extraction from culture supernatant of phosphate solubilizing strains yielded 13 and 16.5 mg/l of SA and DHBA by *Bacillus* PSB 1, 12.6 and 10 mg/l of SA and DHBA by *Bacillus* PSB 7 and 13.5 and 14.5 mg/l of SA and DHBA by *Bacillus* PSB 10, respectively. In contrast, Cr and Ni at 50 and Pb and Zn at 300 µg/ml (except Zn at 300 µg/ml in case of PSB7) marginally increased the SA and
DHBA by *Bacillus* PSB 1, PSB 7 and PSB 10 compared to control. Further, strains were also found positive for HCN and ammonia, both in the presence and absence of metals.

A total of 20% of the phosphate solubilizing strains showed the phosphate solubilizing activity on Pikovskaya medium. Of these, P solubilizing stains, *Bacillus* PSB1, PSB7 and PSB10 showed largest zone of P solubilization on solid Pikovskaya medium amended with or without heavy metals. Further, the phosphate solubilizers and selected group of nitrogen fixers were tested for their ability to reduce chromium using nutrient broth (for PSB) and YEM broth (for rhizobia). The chromium reduction by two groups of organisms was affected by concentration of metals, pH and incubation periods. For example, *Bacillus* sp. PSB 10 reduced Cr (VI) by 87% which was followed by PSB 1 (83%) and PSB7 (74%) at pH 7 in nutrient broth after 120 h of incubation. A concentration of 50 μg ml⁻¹ of Cr (VI) was completely reduced by *Bacillus* sp. PSB 1 (after 100 h), PSB 10 (after 100 h) and PSB7 (after 120 h). Among the *Mesorhizobium* strains, strain RC3 reduced Cr (VI) by 90% which was followed by RC1 (84%) and RC4 (83%) at pH 7 in nutrient broth after 120 h of incubation. Strains RC1, RC3 and RC4 completely reduced 50 μg/ml of Cr (VI) at 120 h of incubation. Generally, the maximum reduction of Cr occurred at pH 7 by the test isolates that progressively increased with increase in incubation. Furthermore, *Bacillus* PSB1, PSB7 and PSB10 also solubilized lead and zinc under *in vitro* conditions. The higher concentrations of the metals in general, reduced considerably the P solubilization and lead and zinc solubilization activity.

Soils contaminated with heavy metals present a major concern for sustainable agriculture. In addition, legumes are used as a rich source of protein in Indian dietary systems, and hence, understanding the effects of these metals on the legume productivity will be useful. Therefore, the phytotoxic effects of three concentrations of cadmium, chromium, copper, nickel, zinc and lead (for chickpea) and cadmium, chromium and copper used either separately or as mixtures (for greengram, lentil and pea) on the biological and chemical characteristics of these legumes, in pot trials was studied. Also, the metal uptake by the legume organs (roots, shoots and grains) was determined at different stages of plant growth. Generally, cadmium, when used alone or in combination with other metals was found to be the most toxic metal for chickpea, greengram and lentil while copper had the most toxic effect on pea plants and substantially decreased the biological and chemical
properties. Chromium and lead (for chickpea), chromium (for greengram and lentil) and cadmium and chromium (for pea) enhanced the measured biological and chemical parameters, compared to control.

A maximum reduction of 43, 14 and 36% in total dry matter production of chickpea at 60, 90 and 135 DAS, respectively was observed with cadmium at 24 mg/kg soil, which was followed by the application of zinc (9780 mg/kg soil) to soils that substantially reduced the measured parameters. Chromium at 68 mg/kg increased the biomass by 48% (90 DAS) and at 136 mg/kg increased the total dry weight of chickpea by 22% (at 135 DAS), compared to control. In comparison, lead at 97.5 mg/kg increased the dry matter accumulation by 42 (90 DAS) and 23% (135 DAS) while 2445 mg/kg of zinc and 669 mg/kg of copper added to soil, increased the dry biomass by 23% at 90 DAS, compared to control. Among the dual metal combinations, chromium with nickel (34+145 mg/kg soil) had the largest stimulatory effect on chickpea plants which increased the total dry matter accumulation by 27% at 90 DAS, compared to control. Combination treatment of cadmium with nickel (24 mg Cd and 580 mg Ni/kg) decreased the total dry biomass production significantly (P ≤ 0.05) by 54% at 60 DAS, 11% at 90 DAS and 43% at 135 DAS, respectively, compared with the control plant. When cadmium (24 mg/kg) was applied along with Cr (136 mg/kg) and Ni (580 mg/kg), declined the dry matter by 58, 53 and 59% at 60, 90 and 135 DAS, over control. The multiple metal application of Pb + Zn + Zn (390 + 9780 + 1338 mg/kg soil), showed an increase of 5 and 2% at 60 DAS and 10% each at 135 DAS, respectively, in dry matter production over combination of Cd + Cr (24 + 136 mg/kg soil) and Cd + Ni (24 + 580 mg/kg), respectively.

Comparison between the metal free control and each metal treatment, revealed an increase of 23 (at 34 mg Cr/kg and 136 mg Cr /kg) to 54% (at 68 mg Cr/kg) in the number of nodules per plant at 60 DAS and 22 (34 mg Cr/kg) to 44% (at 136 mg Cr/kg) at 90 DAS, compared to control. Similarly, lead at 97.5 mg/kg soil, significantly increased the number of nodules per plant at 90 DAS by 18 and 70% over chromium (136 mg/kg) and control (27 nodules/plant), respectively. Among the single metal treatments, cadmium showed a profound toxic effect on symbiosis and reduced the number of nodules per plant by 69% (at 24 mg/kg) at 60 DAS, while at 90 DAS, it reduced the number of nodules per plant by 22% at the same rate of application. Similarly, the dual metal treatments, cadmium with
chromium (at 24 + 136 mg/kg soil) and cadmium with nickel (136 + 580 mg/kg soil), resulted in the largest adverse effect as did the mixtures of Cd + Cr + Ni (24 + 136 + 580 mg/kg soil) and reduced the number of nodules by 77% at 60 DAS and by 52%, at 90 DAS, respectively, compared to 13 and 27 nodules/plant observed at 60 and 90 DAS in control treatment. In contrast, chromium with lead (at 34 + 97.5 mg/kg) enhanced the number of nodules by 19% at 90 DAS while 136 and 390 mg/kg of Cr and Pb respectively, increased the number of nodules per plant by 7% only at 90 DAS, compared to control. In comparison, the triple metal treatment showed greatest adverse effect on nodulation compared with either the control plants or dual metal treatments. The reduction in nodulation was accompanied by a significant decrease in dry mass of nodules.

Cadmium at 24 mg/kg reduced the root N content in chickpea by 33, 22 and 29%, at 60, 90 and 135 DAS, respectively, compared with the control. Generally, the maximum reduction in N content was observed with dual or multiple metal application treatments relative to the control. For instance, cadmium with nickel (at 24 + 580 mg/kg soil) decreased the root N content by 39% at 60 DAS while cadmium with lead (at 24 + 390 mg/kg soil) decreased the root N content by 43% and 40% at 90 and 135 DAS, respectively, compared to control. In comparison, the triple metal combination of cadmium, chromium and nickel (at 24 + 136 + 580 mg/kg soil) reduced the root N content by 41, 58 and 46% at 60, 90 and 135 DAS, respectively, relative to the control plants. In general, maximum reduction in N content in shoots occurred at double the normal concentration of all metal treatments. The toxicity of the metals on shoot N content increased with increasing rates of all metals, except lead and the mixtures of chromium + lead, which consistently increased the N contents at 60, 90 and 135 DAS, compared to control. The N content of roots was more severely affected than the N content of shoots, at all the concentrations of the metals used. In comparison, lead at 390 mg/kg soil significantly increased the root N content by 10% at 60 DAS and chromium at 136 mg/kg soil increased the root N content by 9% at 90 DAS. The N content in roots increased consistently with increasing rates of combination of Cr + Pb, Cr + Zn and Ni + Pb at 60 DAS only, compared to those observed for control plants (28.7 mg/g at 60 DAS). A maximum increase of 10% in N content at 60 DAS was observed with 136 mg Cr/kg and 16 and 12% at 90 and 135 DAS, respectively, for 195 mg Pb/kg, respectively, compared with control.
Seed yield in chickpea decreased consistently for each metals, used either singly or in combination but was only significantly (P ≤ 0.05) reduced at double the normal concentration of all metals (except chromium and lead) and half (0.5 x) and normal (1 x) concentration of cadmium, zinc and copper. Among the dual metal treatments, cadmium with nickel had the highest adverse effect on grain yield and decreased it significantly by 28% at 24 + 580 mg/kg. The reduction in grain yield following multiple metals ranged between 19 (6 + 34 + 145 mg/kg of cadmium with chromium and nickel) to 33% (24 + 136 + 580 of cadmium with chromium and nickel) and 11 (97.5 + 2445 + 334.5 mg/kg of lead, zinc and copper) to 26% (390 + 9780 + 1338 mg/kg of lead, zinc and copper), compared to control. The order of toxicity on seed mass increased in the following order: lead > chromium > nickel > copper > zinc > cadmium. In contrast, chromium and lead consistently and significantly increased the grain yield, relative to the control plants. The average maximum increase of 12.9% and 11% was observed with lead at 97.5 and chromium at 34 mg/kg respectively, compared with those obtained for metal free but inoculated control (5.4 g/plant). In chickpea plants, double the normal concentration of all metal treatments significantly decreased the grain protein. Among the double metal treatments, the mixture of cadmium + nickel declined the grain protein by 10% at 6 + 145 mg/kg Cd + Ni and 14% at 12 + 290 mg/kg of cadmium + nickel, respectively, relative to the control. Among all metal treatments, the mixtures of Cd + Cr + Ni and Pb + Zn + Cu resulted in the highest decrease in grain protein at double the normal concentrations, compared with the control. In comparison, the average maximum protein (256 mg/g) in chickpea grain was obtained at 390 mg/kg Pb and was significantly (P ≤ 0.05) greater than those obtained for inoculated but metal free control (242 mg/g).

The most phytotoxic metal for greengram plants was cadmium that reduced the total dry matter accumulation significantly (P ≤ 0.05) by 27% (at 50 DAS) and 21% (at 80 DAS) at 24 mg/kg soil, compared to control (273 and 290 mg/plant at 50 and 80 DAS). This was followed by copper which decreased the total dry matter by 18% at 50 DAS and 20% at 80 DAS at 1338 mg/kg soil, compared to control. In contrast, chromium at 136 mg/kg soil increased the total dry matter production 1.3 fold (at 50 DAS) and 1.4 times (at 80 DAS), relative to the control. The reduction in dry biomass of greengram plants following mixtures of metals ranged between 24 (Cd with Cr at 6 and 34 mg/kg soil) to 41% (Cd with Cu at 24
and 1338 mg/kg), above the control at 50 and 80 DAS, respectively. In contrast, the combination of chromium and copper increased the dry matter by 31 and 26% at 136 and 1338 mg/kg soil, at 50 and 80 DAS respectively, relative to the control. Cadmium and copper at 24 and 1338 mg kg\(^{-1}\) soil declined the number of nodules per plant by 38 and 23% at pod fill stage and 36 and 27% at harvest, respectively, compared to control. In contrast, chromium at 136 mg Cr/kg soil significantly (P < 0.05) increased the number of nodules by 100%, each at pod fill and at harvest stage, in comparison to control. Similarly, the mixture of metals at all concentrations except chromium applied with Cu (at 34 and 334.5 mg/kg soil) decreased the number of nodules per plant at pod fill stage, compared to control. Among the metal combinations, when Cd was used with Cu at 24 and 1338 mg/kg soil showed a largest adverse effect and significantly (P < 0.05) reduced the number of nodules per plant at pod fill stage and at harvest by 62 and 64%, respectively, above control. The reduction in nodulation was accompanied by significant decrease in dry matter accumulation in nodules as well.

The average maximum decline in root N in greengram occurred at 50 (35 mg) and 80 DAS (30 mg) following 24 mg Cd/kg and decreased significantly (P ≤ 0.05) by 22 and 25% respectively, above the control. Cadmium with copper (at 24 and 1338 mg/kg soil) profoundly reduced the N content by 29 and 30% at 50 and 80 DAS, respectively, compared to the control. A trend similar to root N was observed for shoot N with three metals and their combinations. The N content of the roots was more severely affected than the shoot N at all the concentrations of tested metals, but the N concentration in roots and shoots in general, were less at 80 DAS compared to 50 DAS. In comparison, chromium progressively enhanced the root N by 29, 33 and 42% (at 50 DAS) and 33, 38 and 48% (at 80 DAS) at 34, 68 and 136 mg/kg soil, compared to control. The average maximum increase in shoot N with chromium occurred at 136 mg Cr/kg soil (31%) at 50 DAS and at 136 mg Cr/kg soil (18%) at 80 DAS, compared to control. Seed yield in greengram declined progressively for each metal with increasing concentration, used either separately (except the three concentrations of Cr) or in combination. Cadmium at 24 mg/kg soil significantly (P ≤ 0.05) decreased the seed yield by 40%, compared to control, which was followed by a significant decrease of 26% when 1338 mg Cu/kg soil was applied to soils, compared to control. The average reduction in seed yield among combination treatments ranged between 17 (at 34 and 334
mg/kg Cr and Cu) to 60 % (at 24 and 1338 mg/kg Cd and Cu), relative to the control. While comparing the sum of mean values of each metal treatment, the order of toxicity on seed mass decreased in the following order: Cd < Cu < Cr. The average maximum increase of 62 and 74% in seed yield of greengram was observed with 136 mg Cr/kg soil, in comparison to 34 mg Cr/kg soil and control. Cadmium at 24 and copper at 1338 mg/kg soil, decreased the grain protein of greengram plants by 8 and 6%, respectively, compared to control. Among the dual metal combination treatments, cadmium with copper declined the grain protein by 10% (at 24 and 1338 mg/kg of cadmium and copper respectively), relative to the control. Generally, the combination of metals showed greatest toxic effect on grain protein compared to single metal treatments. In contrast, chromium in general, consistently increased the grain protein with increasing concentrations; the average maximum increase in grain protein being 283 mg/g observed with 136 mg Cr/kg which was greater by 11 % than observed for control.

The total dry matter production by lentil plants increased with plant age but decreased substantially with increasing rates of each single or combined metal treatment. Cadmium at 24 mg/kg soil displayed the highest phytotoxic effect and reduced the dry biomass of plants by 12% at 120 DAS, relative to the metal free control. Chromium or copper when applied with cadmium also had a toxic effect on the dry mass production of lentil plants. A maximum decrease of 16% in dry matter was observed for 24 and 1338 mg/kg of Cd-Cu at 120 DAS, which was followed by the combination of Cd-Cr (24 and 136 mg/kg soil) that reduced the total biomass by 13%, compared to control. Generally the three concentrations of each metal (except 34 mg/kg of Cr) used either alone or as mixture decreased the number of nodules per plant, compared to untreated control. Cadmium at 24 mg /kg soil decreased the number of nodules by 46 (at 90 DAS) and 60% (at 120 DAS), respectively, compared to control. In contrast, the number of nodules produced on the root system of lentil plants increased significantly (P ≤ 0.05) by 12% at 90 DAS with 34 mg Cr/kg. Similarly, mixtures of metals at all levels decreased the number of nodules per plant compared to control plants. For example, Cd (24 mg/kg) with Cu (1338 mg/kg) showed the largest adverse effect and significantly (P ≤ 0.05) reduced the number of nodules per plant by 62 and 70%, at 90 and 120 DAS respectively, above the control. The reduction in nodulation was also accompanied by significant decrease in dry mass of nodules.
The average maximum decline in root N in lentil occurred at 24 mg Cd/kg that reduced the root N by 6% (at 90 DAS) and 8% (at 120 DAS), compared to control. Among the dual metal treatments, Cd (24 mg/kg soil) when applied with copper (1338 mg/kg soil) reduced the N content by 11 and 14% at 90 and 120 DAS, respectively, compared to the control. The dual combinations of cadmium (24 mg kg\(^{-1}\) soil) and Cu (1338 mg/kg soil) reduced the shoot N by 11 and 6% at 90 and 120 DAS, respectively, compared to the control. Though, chromium enhanced the root N marginally at 90 and 120 DAS at 34 mg/kg soil, but decreased consistently with increase in concentration of metals and plant age. Seed yield in lentil decreased progressively with increase in concentrations of metals. Cadmium at 24 mg/kg and Cd (24 mg/kg) and Cu (1338 mg/kg) decreased the seed yield by 17 and 29%, respectively, compared to control plants (100 mg/plant). In contrast, chromium at 34 mg/kg had the greatest stimulatory effect and increased the seed yield by 4% compared to control. Cadmium at 24 mg/kg and chromium with copper at 24 and 1338 mg/kg decreased the grain protein by 5% and 9%, respectively control (240 mg/g). The effect of three concentrations of cadmium, chromium and copper on dry matter accumulation in whole pea plants was variable. Among the single metal treatments, copper at 1338 mg/kg soil was the most toxic and reduced the total dry matter significantly (\(P \leq 0.05\)) by 18% (at 90 DAS) and 17% (at 120 DAS) respectively, compared to control. In contrast, the three concentrations of Cd and Cr increased the dry matter, above the control, the maximum being 60 and 40% at 90 DAS and 59 and 36% at 120 DAS at 12 mg Cd/kg and 68 mg Cr/kg soil, respectively, compared to control. The dry matter accumulation was reduced even further when copper was used in combination with Cd and Cr. The reduction in dry biomass of pea following mixtures of metals ranged between 6 and 7 (Cr with Cu at 34 and 334.5 mg/kg soil) to 16 and 18% (Cr with Cu at 136 and 1338 mg/kg), at 90 and 120 DAS, respectively, above the control. In contrast, the mixture of Cd (24 mg/kg soil) and Cr (136 mg/kg soil) increased the dry matter by 25 and 13% at 90 and 120 DAS, respectively, relative to the control. Copper at 1338 mg/kg soil decreased the number of nodules by 16% (at 90 DAS) and 22 % (at 120 DAS) respectively, compared to control. Interestingly, the number of nodules increased significantly (\(P \leq 0.05\)) by 53% (at 90 DAS) and 72% (at 120 DAS) with 24 mg Cd/kg, compared to control and by 31% (at 90 DAS) and 50% (at 120 DAS) with 136 mg Cr/kg soil respectively, compared to control. Among the metal combinations, Cd (24 mg/kg) with
Cu (1338 mg/kg) showed largest adverse effect and significantly reduced the number of nodules by 33 and 30% at 90 and 120 DAS respectively, above the control.

The average maximum decline in root N in pea plants occurred at 1338 mg Cu/kg that significantly reduced the root N by 20% (at 90 DAS) and 17% (at 120 DAS), in comparison to control. Among the dual metal treatments, cadmium (24 mg/kg soil) when used with copper (1338 mg/kg soil) reduced the root N content by 26 and 20% after 90 and 120 DAS, respectively, compared to the control. Generally, the accumulation of N was more in roots at 90 DAS which progressively decreased with increase in plant age for all the treatments; the maximum being 16 (Cd alone at 6 mg/kg soil) to 13% (Cd-Cu at 6 and 334.5 mg/kg soil) at 120 DAS compared to those observed for 90 DAS. A trend similar to root N was observed for shoot N with three metals and their combinations. Cadmium (24 mg/kg soil) when used with copper (1338 mg/kg soil) reduced the shoot N content by 13 and 21% at 90 and 120 DAS, respectively, compared to the control. Like other legumes, the N content of pea shoots also decreased with plant age and suffered severe metal toxicity. In comparison, Cd at 12 mg/kg soil enhanced the root N by 14 % (at 90 DAS) and 17% (at 120 DAS) respectively, compared to control. A trend similar to root N was observed for shoot N and the average maximum increase in shoot N content at 12 mg Cd/ kg was 28% (at 90 DAS) and 29% (at 120 DAS), respectively, compared to control. Seed yield in pea plants also decreased progressively with increasing concentration of copper added to soil either separately or as mixture. Copper at 1338 mg Cu/kg soil, significantly decreased the seed yield by 12 and 15 %, relative to 334.5 mg Cu/kg soil and control. The average maximum reduction in seed yield among combination treatments was 20% when 24 and 1338 mg/kg of Cd-Cu was applied together, relative to the control. In comparison, the average maximum increase of 13 and 8% in seed yield was observed with cadmium at 24 mg/kg soil and chromium at 136 mg/kg soil respectively, compared to control. The combination of Cd-Cr (6 + 34 mg/kg) increased the seed yield by 7%, compared to control. Copper used either alone or as mixture decreased the grain protein (GP) of pea plants consistently with increasing levels, relative to control. Cadmium (24 mg/kg) with Cu (1338 mg/g) 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. In comparison, Cd and Cr in general,
progressively increased the GP with increasing concentrations. The average maximum GP was observed with 24 mg Cd/kg (232 mg/g) and 136 mg Cr/kg (230 mg/g).

The glutathione reductase (GR), an antioxidant enzyme, synthesized within roots and nodules of lentil and pea plants under heavy metal stress, increased considerably with increase in the concentration of cadmium, chromium and copper. Among these metals, cadmium induced the maximum production of glutathione reductase in both roots and nodules of lentil and pea plants, compared to other metals. Roots of both lentil and pea plants, in general, had the highest glutathione reductase activity, compared to nodules under all metal regimes. The maximum increase in GR activity of roots and nodules of lentil plants was observed for cadmium at 24 mg kg\(^{-1}\) which increased the GR activity of roots by 282 and 280% after 90 and 120 DAS, respectively, compared to those observed for control at 90 (17 nmol/mg protein) and 120 DAS (15 nmol/mg protein), respectively. In comparison, the same concentration of cadmium increased the GR activity in nodules by 300 and 308% after 90 and 120 DAS, respectively compared to control. In combination treatments, the maximum increase in GR activity in roots was observed with cadmium and copper (24 and 1338 mg/kg) which increased the GR activity by 335 and 336% after 90 and 120 DAS respectively, compared to control. Similarly, the GR activity in nodules increased by 327 (at 90 DAS) and 338% (at 120 DAS) at 24 mg Cd/kg and 1338 mg Cu/kg soil compared to control plants. Conversely, the maximum increase in GR activity of pea plants in this study was observed for cadmium at 24 mg/kg which increased the GR activity of roots by 260 and 306% after 90 and 120 DAS respectively, compared to those observed for control at 90 (20 nmol/mg protein) and 120 DAS (16 nmol/mg protein), respectively. In comparison, the same concentration of cadmium increased the GR activity in nodules by 319 and 307% after 90 and 120 DAS respectively, compared to control. For dual metal treatments, the maximum increase in GR activity in roots was observed with cadmium and copper (24 and 1338 mg/kg) which increased the GR activity by 280 and 319% after 90 and 120 DAS, respectively, relative to the control. Similarly, the GR activity in nodules increased by 338 (at 90 DAS) and 329% (at 120 DAS) at 24 mg Cd/kg and 1338 mg Cu/kg soil above the control plants. The dual metal application exhibited the greatest GR activity in both roots and nodules, compared to sole metal application.
The uptake of metals by the roots and shoots at 60, 90 and 135 DAS and grains at 135 DAS (for chickpea); roots and shoots at 50 and 80 DAS and grains at 80 DAS (for greengram); roots and shoots at 90 and 120 DAS and grains at 120 DAS (for lentil and pea) increased substantially with increase in the concentration of heavy metals. The accumulation of metals in roots, shoots and grains were influenced greatly by the concentration of each metal tested. A higher amount of metal in plant organs was observed when these metals were applied individually compared with the levels obtained for multiple metal ions. A greater uptake of zinc in chickpea was observed in both roots, shoots and grains compared to other metals. The greengram plants showed a maximum accumulation of cadmium at 50 and 80 days after seeding in roots (2 and 3.1 μg/g), shoots (0.72 and 0.84 μg/g) and grains (0.35 μg/g) at 24 mg kg⁻¹ soil. In comparison, the concentration of chromium at 50 and 80 DAS was higher in roots (29.9 and 32.2 μg/g), shoots (10.5 and 15.5 μg/g) and grains (4.5 μg/g) at 136 mg/kg soil. The concentration of copper was higher in roots (60.1 and 64.5 μg/g), shoots (26.2 and 28.2 μg/g) and grains (15.7) at 1338 mg/kg soil. The lentil plants showed a maximum accumulation of cadmium in roots (1.9 and 2.8 μg/g) and shoots (0.5 and 0.8 μg/g) after 90 and 120 DAS, respectively, and grains (0.3 μg/g) at 120 DAS with 24 mg/kg soil. In comparison, the higher concentration of chromium in roots (23.7 and 30.9 μg/g) and shoots (14.5 and 20.6 μg/g) at 90 and 120 DAS respectively, and grains (5.8 μg/g) after 120 DAS, at 136 mg/kg soil. The concentration of copper was higher in roots (72.1 and 82 μg/g) and shoots (38.3 and 42.2 μg/g) at 90 and 120 DAS, respectively, and grains (10.5) after 120 DAS at 1338 mg/kg soil. The pea plants showed a maximum accumulation of cadmium in roots (1.5 and 2.1 μg/g) and shoots (0.62 and 1.1 μg/g) after 90 and 120 DAS respectively, and grains (0.32 μg/g) after 120 DAS with 24 mg/kg soil. In comparison, the higher concentration of chromium was observed at 90 and 120 DAS in roots (24.4 and 28.4 μg/g, respectively) and shoots (15.5 and 17.9 μg/g, respectively) and at 120 DAS for grains (2.7 μg/g). The application of 1338 mg/kg soil of copper showed the higher accumulation of copper in roots at 90 and 120 DAS (14.4 and 17.7 μg/g) and shoots (8.5 and 11.7 μg/g) and at 120 DAS for grains (3.7).

The plant growth promoting activities and the bioremediation potential of the selected strains were further evaluated with increasing concentrations of the tested metals using chickpea, greengram, lentil and pea plants inoculated with their respective metal
tolerant rhizobia or phosphate solubilizers. Chromium tolerant *Mesorhizobium* strain RC3 increased the biological and chemical characteristics of chickpea in chromium amended soil, compared to non-inoculated plants but chromium amended soil. A maximum increase of 86, 55, 71, 129, 46 and 40% at 90 DAS, in nodule numbers, dry nodule mass, total dry mass, chlorophyll, leghaemoglobin, root N and shoot N respectively and 31,45, 26, 27 and 8% at 135 DAS in total dry mass, root N, shoot N, seed yield and grain protein, respectively, was observed at 136 mg Cr/kg soil compared to non-inoculated plants but having the same concentration of chromium. The bio-inoculant decreased the uptake of chromium in roots, shoots and grains, respectively compared to un-inoculated plants. Similarly, the bio-inoculant *Bacillus* species PSB10 when added with 136 mg Cr/kg increased the nodule numbers, nodule dry weight, total dry weight, root N and shoot N by, 115, 59, 71, 4 and 3% at 90 DAS, respectively, while these parameters increased marginally at 135 DAS but seed yield and grain protein increased by 4 and 1%, respectively at 135 DAS, compared to control.

The bio-inoculant strain *Bradyrhizobium* RM8 tolerant to nickel and zinc, substantially enhanced the plant growth, nodule numbers, chlorophyll content, leghaemoglobin, seed yield, grain protein, root N and shoot N of greengram plants compared to uninoculated but metal treated soil. The bio-inoculant strain RM8 significantly (*P* ≤ 0.05) increased the nodule numbers, nodule dry mass, total dry mass, chlorophyll, leghaemoglobin, root N and shoot N by 54, 56 and 18, 19, 120, 41 and 37%, respectively, at 50 DAS and the nodule numbers, nodule dry mass, total dry mass, root N, shoot N, seed yield and grain protein by 22, 33, 21, 38, 38 and 13% respectively, at 80 DAS, when plants were grown in soil treated with 290 mg Ni/kg, compared to inoculated but without metal soil. Similarly, plants inoculated with strain RM 8 significantly (*P* ≤ 0.05) increased root nodule numbers, dry nodule mass, total dry mass, chlorophyll, leghaemoglobin, root N and shoot N by 50, 71, 28, 9, 100, 47 and 42%, respectively, at 50 DAS and nodule numbers, nodule dry mass, total dry mass, root N, shoot N, seed yield and grain protein by 73, 67, 26, 15, 39, 36 and 13% at 80 DAS, respectively, when plants were grown in soil amended with 4890 mg Zn/kg, compared to plants grown in the absence of bio-inoculant, but with the same concentration of metal. Furthermore, strain RM8 reduced the uptake of nickel and zinc by plant organs compared to plants grown in the absence of bio-inoculant. In
a similar manner, the bio-inoculant *Rhizobium* sp. RP5 displayed a substantial increase of 23, 32, 19, 19, 112, 26 and 47% at 90 DAS in nodule numbers, nodule dry mass, total dry matter, chlorophyll content, leghaemoglobin, root N and shoot N and 23, 28, 18, 40, 55, 26 and 8% at 120 DAS in nodule numbers, nodule dry mass, total dry matter, root N, shoot N, seed yield and grain protein respectively, at 290 mg Ni/kg soil, compared to non-inoculated but amended with same rate of nickel. Similarly, when strain RP5 was also added with 4890 mg Zn/kg soil, increased the nodule numbers, nodule dry mass, total dry matter, chlorophyll, leghaemoglobin, root N and shoot N by 23, 28, 16, 16, 78, 25 and 42% at 90 DAS and nodule numbers, nodule dry mass, total dry matter, root N, shoots N, seed yield and grain protein by 21, 22, 15, 25, 45, 26 and 6% at 120 DAS respectively, compared to plants grown in the absence of bio-inoculant but treated with the same dose of zinc. The bio-inoculant decreased the uptake of nickel and zinc in roots, shoots and grains, respectively, compared to un-inoculated plants. Similar increase in the biological and chemical parameters of lentil plants was observed when nickel, zinc and lead tolerant *Rhizobium* RL9 was also used in heavy metal treated soils. Rhizobial strain RL9 when used with 290 mg Ni/kg had the highest stimulatory effect and increased the nodule numbers, nodule dry weight and total dry weight by 50, 157 and 160% at 90 DAS and 82, 109 and 147% at 120 DAS, respectively, compared to un-inoculated but 290 mg Ni/kg treated soil. Likewise, the bio-inoculant increased the N content, seed yield and grain protein even in the presence of different concentration of nickel, the maximum being 14 and 7% at 90 DAS and 19 and 8% in root N and shoot N respectively, 97% in seed yield and 15% in grain protein at 290 mg/kg compared to non-inoculated but 290 mg Ni/kg amended soil. The bio-inoculant *Rhizobium* RP5 capable of forming symbiosis specifically with pea plants and *Rhizobium* RL9 with lentil plants increased the glutathione reductase activity of roots and nodules at all the concentrations of nickel and zinc (pea) and nickel, lead and zinc (lentil), compared to un-inoculated but plants grown in metal amended soils. Generally, when rhizobial or *Bacillus* strains applied as seed inoculant (biofertilizers) were used along with the metals, the inoculated strains prevented the uptake of metals by the legume organs. The study thus suggested that the rhizobia or *Bacillus* due to their intrinsic abilities of growth promotion and attenuation of the toxic effects of metals could be developed as inoculant and be exploited for remediation or restoration of metal derelict lands.