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Full Length Research Paper

Increased heavy metal tolerance of cowpea plants by dual inoculation of an arbuscular mycorrhizal fungi and nitrogen-fixer *Rhizobium* bacterium

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Through biological inoculation technology, the bacterial-mycorrhizal-legume tripartite symbiosis in artificially heavy metal polluted soil was documented and the effects of dual inoculation with arbuscular mycorrhizal (AM) fungus and *Rhizobium* (N-fixing bacteria, NFB) on the host plant cowpea (*Vigna sinensis*) in pot cultures were investigated at six concentrations of Zn (0.0 – 1000 mg/kg dry soil) and Cd (0.0 – 100 mg/kg dry soil). From a number of physiological indices measured in this study, microsymbionts significantly increased dry weight, root : shoot ratios, leaf number and area, plant length, leaf pigments, total carbohydrates, N and P content of infected plants as compared with non infected controls at all levels of heavy metal concentrations. Tolerance index of cowpea plants was increased in the presence of microsymbionts than in their absence in polluted soil. Microsymbionts dependencies of cowpea plants tended to be increased at higher levels of Zn and Cd in polluted soil. Metals accumulated by microsymbionts-infected cowpea plant were mostly distributed in root tissues, suggesting that an exclusion strategy for metal tolerance widely exists in them. This study provides evidence for benefits of NFB to AM fungi in the protection of host plants against the detrimental effects of heavy metals. If so, bacterial-AM-legume tripartite symbiosis could be a new approach to increase the heavy metal tolerance of legumes plants under heavy metal polluted soil.

Key word: Mycorrhiza, *Vigna sinesis*, heavy metals, microsymbiosis, *Rhizobium*.

INTRODUCTION

With the development of industries, mining activities, application of waste water and sewage sludge on land, heavy metal pollution of soils is increasingly becoming a serious environmental problem. Phytoremediation (such as phytoextraction, phytostabilization and rhizofiltration) of soils contaminated by heavy metals has been widely accepted as a cost-effective and environmental-friendly cleanup technolology. However, the progress in this field is hindered by lack of understanding of complex interact-tions in the rhizosphere and plant –based mechanisms which allow metal translocation and accumulation in plant (Yu et al., 2004).

Complex interactions between roots, microorganisms and fauna in the rhizosphere have a fundamental effect on metal uptake and plant growth. The arbuscular mycorrhiza (AM) fungi are important rhizospheric micro-organisms. They can increase plant uptake of nutrients especially relatively immobile elements such as P, Zn and Cu (Ryan and Angus, 2003), and consequently, they increase root and shoot biomass and improve plant growth. It has been indicated that AM fungi can colonize plant roots in metal contaminated soil (Vogel-Mikus et al., 2005), while their effects on metal uptake by plants are conflicting. In slightly metal contaminated soil, most studies show that AM fungi increased shoot uptake of metals (Weinstein et al., 1995), while in severely contaminated soil, AM fungi could reduce shoot metal concentration and protect plants against harmful effects of metals (Malcova et al., 2003). Thus the benefits of mycorrhizae may be associated with metal tolerance, and also with metal plant nutrition. Therefore, in degraded and contaminated soils, that are often poor in nutrients and with low water holding capacities, mycorrhizae formation would be of great importance.

A biotechnological goal is to use a combined inoculation of selected rhizosphere microorganisms to minimize toxic effects of pollutants and to maximize plant growth and nutrition. Selected combinations of microbial inocula enhanced the positive effect achieved by each microbial group, improving plant development and tolerance in polluted soils. The application of bioinoculants like AM fungi and one of the plant growth-promoting rhizobacteria such as *Azospirillum, Agrobacteria, Rhizobium, Pseudomonas,* several Gram positive *Bacillus* species is an environment-friendly, energy efficient and economically viable approach for reclaiming wastelands and increasing biomass production. The beneficial effects of these bacteria in combination with AM fungi have been reported by a number of workers (Tain et al., 2002; Domenech et al., 2004; Rabie and Almadini, 2005). It has been reported that these bacteria may affect AM fungi and their plant host through a variety of mechanisms that include (1) effects on the receptivity of the root; (2) effects on the root-fungus recognition; (3) effects on the fungal growth; (4) modification of the chemistry of the rhizospheric soil; and (5) effects on the germination of the fungal propagules. On the other hands, other reports stated that the presence of AM fungi is known to enhance nodulation and N fixation by legumes (Amora-Lazecano et al., 1998; Johansson et al., 2004). Moreover, AM fungi and N-fixing bacteria often act synergistically on infection rate, mineral nutrition and plant growth. Although the interaction between AM fungi and N-fixing bacteria was previously reported, less attention was paid to bacteria-AM-legume tripartite symbioses under heavy metal stress. Therefore, this article is considered a good attempt to increase the heavy metal tolerance of one of the most popular legume of the world, cowpea (*Vigna sinensis*), by using dual inoculation of the AM-fungi and nitrogen -fixing bacteria under heavy metal stress conditions.

MATERIALS AND METHODS

Soil

Sandy loam soil (1:1) was air dried, passed through 2 mm sieve, mixed thoroughly for homogencity and sterilized by autoclaving at 121°C for 20 min to kill soil microflora. The soil is non-saline, with pH 7.9, field capacity 653 ml/kg air dry soil and has 1.35 % organic matters. The total soluble salts were 1.17 %, with total nitrogen content of about 0.89 mg/kg and phosphorus content of 0.042 mg/kg. It contains (µg/g air dry soil) 13.4 Mn, 94 K, 138 Mg, 10.7 Zn, 0.83 Cd, 3.11 Cu and 0.49 Pb.

Seeds

Seeds of cowpea (*V. sinesis*) were kindly provided from Hada Al-Sham farm, Faculty of Metrology, King Abdulaziz University, Jeddah, Saudi Arabia.

Microsymbiont inoculations

Mycorrhizal spores: The spores of AM fungi were extracted from their cultivation and propagation pots, planted by *Zea mays*, using wet sieving and decanting method (Daniels and Skipper, 1982). The spore suspension was diluted with water, so that each ml has 55 spores. For soil inoculation, the surface soil crushed and mixed thoroughly with 15 ml spore suspension and return back to its pot.

***Rhizobium* strain (ARC 610):** It was kindly provided by Biofertilization Unit of Faculty of Agriculture, Ain-Shams University. Cairo, Egypt. The culture was cultivated, propagated and maintained in mannitol yeast extract medium (Allen, 1961), with regularly sub-culturing every two weeks. The inoculum (bacterial cells) was prepared from liquid mannitol yeast extract medium after 7 days of incubation at 37°C. The inoculum was 10 ml/pot (2 kg soil) having about 17.5 × 10^6 cells.

Growth conditions

The experiment was a 6 x 2 x 5 complete factorial combination for each heavy metal used. It was comprised of 6 metal concentrations and 2 inoculation treatments with 5 replicates for each treatment. The seeds sizes and weights were homogenous. They were surface sterilized (0.1% $HgCl_2$ + 0.2% HCl for 5 min), followed by repeated washes with sterile distilled water (Vincent, 1976). Seeds were planted in plastic pots (18 cm in diameter and 13 cm in depth) loaded with 2 kg air dried sterilized sandy loam soil. The pots were arranged in randomized block. It was carried out with followed treatments: non-inoculation control and inoculation with AM spores and *Rhizobium* cells in paired inoculum. Pots received 60 ml weekly of Hoagland's solution, minus phosphate, (Hoagland and Arnon, 1950; Downs and Hellmers, 1975). Ten seeds were planted in each pot at equal intervals. Field capacity of tap water was applied per pot for irrigation. The seeds were irrigated three times a week, until the plant seedlings emerge (about 5 cm height) and thinned to five per pot.

Cowpea (*V. sinesis*) seedlings were allowed to grow under greenhouse conditions (30°C and illumination period of 13 h/day) for seven weeks. At the end of the tested growth period, plant samples were carefully uprooted, washed thoroughly with tap water and rinsed twice with distilled water. After washing, the root and leaves were separated, thereafter the necessary analyses are carried out.

Analytical methods

Soil characteristics: The pH of sandy loam soil (1:1) was recorded using pH-meter (Richards, 1954) and the moisture content was calculated using an oven at 105°C (Kramer, 1983). The organic matter was estimated at 550°C using a muffle (Peach and Tracey, 1956) and the total soluble salts were determined by weight after evaporation of soil filtrate at 105°C. The field capacity was calculated by weight (Premachandra et al., 1992).

Growth parameters of cowpea: The plant height (cm), root system length (cm), leaf number and leaf area (cm^2) were measured. The fresh and dry weights of the root and shoot systems were also determined.

Total carbohydrates: Total carbohydrates were determined, after hydrolysis, colourimetrically using anthrone reagent (Fales, 1951).

Plant pigments: Chlorophyll a, b and carotenoids were estimated spectrophotometrically (Metzner et al., 1965), after acetone extraction of the pigments from fresh leaves.

Total nitrogen content: It was estimated colourimetrically using Nessler reagent (Delory, 1949; Humphries, 1956).

Table 1. Effect of different concentrations of Zn and Cd on some parameters of cowpea (*Vigna sinesis*) in the presence and absence of microsymbionts (of *Rhizobium* cells and AM spores) after 7 weeks of planting.

Heavy metal conc. (mg/kg dry soil)	Microsym b-iont	Dry weight (g/plant)		Leaf		Plant length (cm)	
		Total	Root: shoot ratio	No./plant	Area (cm^2)	Shoot	Root
Control	N	0.46	0.179	21.4	10.4	53.2	14.9
0.0	I	o.54	0.2	27.2	11.9	64.7	16.2
Zn	N	0.48	0.171	22.3	10.7	54.6	15.3
50	I	0.57	0.24	29.1	12.7	67.5	18.7
100	N	0.51	0.186	25.2	11.2	59.3	17.1
	I	0.64	0.25	31.7	13.6	74.7	19.6
200	N	0.46	0.179	24.3	11.0	61.2	19.1
	I	0.58	0.26	28.6	13.1	84.5	20.4
500	N	0.34	0.15	19.4	7.5	47.9	14.2
	I	0.46	0.26	25.1	9.2	74.1	16.4
1000	N	0.28	0.167	16.2	5.2	37.4	10.3
	I	0.44	0.179	20.8	7.3	52.3	14.2
L.S.D.at 5%		0.19	-	3.41	1.89	9.37	1.61
Cd	N	0.46	0.24	21.1	10.1	54.1	14.9
5	I	0.57	0.24	27.6	12.7	66.3	16.4
10	N	0.4	0.21	20.3	10.0	53.7	13.7
	I	0.54	0.26	26.9	11.9	65.4	16.1
20	N	0.30	0.24	19.5	8.2	53.5	13.7
	I	0.43	0.26	25.7	11.3	63.5	15.9
50	N	0.26	0.3	13.4	5.5	47.2	13.1
	I	0.39	0.26	18.6	7.5	59.6	15.3
100	N	0.15	0.36	9.6	3.6	30.9	11.1
	I	0.28	0.33	13.2	5.9	46.1	12.1
L.S.D.at 5%		0.13	-	2.49	1.63	6.28	2.35

I: Plants inoculated by microsymbiont of *Rhizobium* cells and AM spores.
N: Non-inoculated plants.

Determination of phosphorus, Zn and Cd: The plant material digested in nitric-perchloric acid mixture (5:3) and analyzed colourimetrically with malachite green reagent (Fernandez et al., 1985) to determine P concentration, and by atomic absorption spectrophotometry in Perkin – Elmer 500 instrument for Zn and Cd.

Mycorrhizal root infection: Mycorrhizal colonization was assessed using the grid – line intersect method (Giovannetti and Mosse, 1980) for examination of cleared and stained (Phillips and Hayman, 1970) root samples.

Rhizobial root nodules: The root nodules were estimated for each plant, separated and collected to determine their fresh and dry mass (Vejsadova et al., 1992).

Mycorrsymbiont dependency: The mycorrhizal dependency (M.D.) of the plants was calculated according to Gerdemann (1975) as:

$$\text{M.D.} = \frac{\text{d.w. infected plant at a particular level of metal}}{\text{d.w. non-infected plant at the same level of metal}} \times 100$$

Tolerance indices (Ti): Tolerance indices on infected and non-infected plants to heavy metals in soil were determined (Rabie, 2005) as:

$$\text{Ti} = \frac{\text{d.w. plant in polluted soil at a particular level of metal}}{\text{d.w. plant in non-polluted soil at a 0.0 level of metal}} \times 100$$

The translocation factor (TF): The translocation factor for metals within a plant was expressed by the ratio of metal (shoot) / metal (root) to show metal translocation properties from roots to shoots (Stoltz and Greger, 2002).

Statistical analysis

Each treatment was carried out in five replicates and the recorded results were the arithmetic mean. Data were statistically analyzed using one way analysis of variance ANOVA on the basis of which LSD values ($P \leq 0.05$ for $N = 5$) for any two compared means were calculated.

RESULTS

The results in Table 1, showed that the effect of dual inoculations of AM fungi and *Rhizobium* (a nitrogen fixing

Table 2. Effect of different concentrations of Zn and Cd on cowpea inoculated with or without microsymbiont of *Rhizobium* cells and AM spores after 7 weeks of planting.

Heavy metal conc. (mg/kg dry soil)	Microsymbionts	Leaf pigments (mg/g)	Total carbohydrate (mg/g dry wt.)	Total N_2 (mg/g dry wt.)	Total P (mg/g dry wt.)
Control	N	8.69	201.9	17.93	1.32
0.0	I	9.62	213.5	24.1	2.69
Zn	N	9.33	206.4	19.49	2.17
50	I	10.53	222.9	26.51	2.98
100	N	8.1	215.4	20.09	2.11
	I	10.11	246.6	29.75	3.05
200	N	7.45	208.3	20.39	2.04
	I	8.72	228.8	31.21	2.06
500	N	5.56	117.2	11.74	1.37
	I	6.95	189.6	17.72	2.96
1000	N	3.98	99.5	9.11	1.05
	I	6.05	145.8	14.53	1.65
L.S.D. at 5%		2.56	14.52	3.23	0.15
Cd	N	8.79	185.4	18.09	2.32
5	I	10.0	217.2	24.36	2.76
10	N	7.88	130.4	16.71	2.24
	I	9.67	204.6	22.06	2.69
20	N	7.51	115.3	15.18	2.04
	I	9.27	146.9	20.85	2.46
50	N	6.15	65.5	10.74	1.32
	I	7.88	119.3	13.38	1.84
100	N	5.6	37.5	6.63	0.93
	I	7.2	86.2	11.07	1.4
L.S.D. at 5%		1.74	22.15	3.01	0.21

I: Plants inoculated by microsymbiont of *Rhizobium* cells and AM spores
N: Non – inoculated plants

bacteria, NFB) on dry weight, root : shoot dry weight ratios, height and leaf number and area of cowpea plants under various levels of heavy metals, Zn and Cd. Zn level of 100 mg/kg soil was the best to attain higher values of tested growth parameters of cowpea (leaf number, leaf area and dry weight), either the soil has no microsymbiont of AM fungi and *Rhizobium* or it is inoculated with them. In the presence of microsymbionts, root shoot dry weight ratios were slightly increased with increasing Zn concentrations up to 500 mg/kg dry soil, while it decreased at 1 g/kg dry soil. However, Zn concentration of 200 mg/kg air dry soil was optimum for stem and root lengths either with microsymbiont inoculation or none, and lower or higher concentrations were concomitant with lower stem and root lengths. Generally, it is safe to conclude that Zn at 100 to 200 mg/kg soil was necessary to attain the best growth parameters, under the tested condition by cowpea plant. However, higher levels of Zn are conducive to the tested parameters. The results fairly indicated that the presence of AM fungi and *Rhizobium* bacterium noticeably improving the tolerance of cowpea plants against toxicity of Zn. Thus at the more toxic Zn concentration (1 g Zn/kg air dry soil) microsymbiont inoculation increases dry weights, leaf number and area as well as stem and root length by about 64, 28, 40, 40 and 38%, respectively, as compared to non-inoculated soils.

Inoculation of plants with AM fungi and NFB significantly increases Cd tolerance of cowpea plants at all concentrations used in this study. The maximum height of plants were obtained in plants inoculated with both AM fungi and NFB at corresponding levels of Cd concentrations (Table 1). The plant height increased from 47.7 to 66.5 cm in absence and presence of microsymbionts at 100 mg/kg dry soil, respectively. It was also revealed that the effect of dual inoculation of NFB and AM fungi on dry weight and leaf number and area of cowpea plants grown in Cd-polluted soil follow similar trends of these inoculants on the height of cowpea plants under the same soil conditions. Thus, at the most toxic Cd level (0.1 g/kg soil) microsymbiont inoculated soils showed increases in plant growth parameters of dry weights, leaf number and area, as well as stem and root lengths reached to about 87, 38, 64, 49 and 9%, respectively, as compared to non-inoculated soils. On the other hand, root shoot dry weight ratios exhibited constant values with increasing Cd concentrations in the presence of microsymbionts.

The data presented in Table 2 indicated that inoculation of microsymbiont to soil, in presence of different concentrations of Zn improves cowpea growth and nutrient assimilation in compared with microsymbiont-free soil. Zn concentrations of more than 50 mg/kg air dry soil were conducive to pigments formation by cowpea leaves either in absence or presence of microsymbionts in the soil. However, at a high Zn concentration (1000 mg/kg) pigments production was inhibited by about 55 and 37% in the absence and presence of microsymbionts in the soil, respectively. The results in Table 2 also indicated that carbohydrates biosynthesis by cowpea stimulated by microsymbionts inoculation more than non-microsymbionts and Zn concentration of 100 mg/kg was necessary for maximal carbohydrates formation. Higher soils. Cd level of 5 mg/kg air dry soil (inoculated or non-inoculated) induced better pigments formation, total carbohydrates, nitrogen and phosphorus content than control treatment (0.0 Cd), i.e., cowpea needed Cd within 5 mg/kg soil to attain improved plant growth. On the other hand, pigments formation, total carbohydrates, nitrogen and phosphorus content of non-inoculated and inoculated plants were inhibited at Cd concentration more than 5 mg/kg soil and the inoculated plants still showed higher pigments formation, total carbohydrates nitrogen and phosphorus contents than those of corresponding non-inoculated plant (Table 2) .

The translocation factors (TF), the ratio of the shoot to root metals, indicate internal metal translocation. The data presented in Table 3 showed that Zn and Cd metals accumulated by cowpea plant in roots as shown by general TF values < 1 either in the presence and absence of AM fungi and a nitrogen-fixing bacteria. However, the values of translocation factor were much lowered in plants infected with microsymbionts than that of non-infected especially at higher concentrations of Zn and Cd metals.

The effect of heavy metal pollutants on the plant studied as indicated by tolerance index was recorded in Table 3. The results showed that the tolerance index of cowpea plants markedly increased by the presence of microsymbionts. Therefore, it was proposed that rhizobial–mycorrhizal infection has potentially of increasing heavy metal tolerance for cowpea plants. Moreover, evidence from the data in Table 3 indicates that microsymbiont dependencies for plant dry mass increased by raising Zn and Cd concentrations in polluted soil. The increase varied from 118.8% at lower Zn level to 157.1% at higher level, while it increased from 123.9 to 186% at low and high Cd concentrations, respectively, in this experiment.

Zn levels of more than 200 mg/kg soil were of inhibitory action on carbohydrates biosynthesis, but inoculated plant still exhibit higher carbohydrates content than those of corresponding non-inoculated plant. As for the total nitrogen and phosphorus content, the inoculated plants accumulate nitrogen and phosphorus compounds more than do the non-inoculated one at all Zn tested levels (Table 2). The results also reveal that Zn level (100 mg/kg soil) was responsible for maximal total nitrogen and phosphorus accumulation in the cowpea plant, while more than 200 mg Zn/kg soil was conducive to total nitrogen and phosphorus accumulation in cowpea plant, either in absence or presence of microsymbiont but inoculated plant still exhibit higher nitrogen and phosphorus contents than those of corresponding non-inoculated plant.

When Cd is considered, the results (Table 2) revealed that microsymbiont inoculation in presence of different concentrations of Cd improves cowpea metabolites of leaf pigments, total carbohydrates, nitrogen and phosphorus content, as compared to non-inoculated

The results (Figure 1) showed concomitant increase of rhizobial nodulation parameters (number, fresh and dry weight/plant) as the concentration of Zn increased up to 200 mg/kg soil. Thus, under these conditions about 23% increase in both nodules number and their fresh weight, and about 10% increase in nodules dry weight. However, 0.5 g Zn/kg soil was drastic to rhizobial nodulation, and failed to be formed at 1 g Zn/kg soil. While AM successively colonize cowpea roots as Zn level increases up to 200 mg/kg (87% infection), it dropped suddenly to be 24% infection at 0.5 g Zn concentration and weak infection (8%) was recorded at 1 g Zn. On the other hand, Cd levels (5 to 100 mg/kg soil) appeared to be more toxic for rhizobial nodulation. However, AM fungi showed moderate root infection at the tested concentrations of Cd and with lower magnitude at higher Cd levels.

DISCUSSION

Associative and symbiotic nitrogen fixing bacteria and AM fungi are common beneficial microbes of leguminous plants. It is frequently suggested that AM may improve P nutrition, enhance N uptake, improve disease resistance in their host plants or adaptation to various environmental stresses. Other microbes, e.g., N-fixing bacteria, may synergistically interact with AM fungi and thereby benefit plant development and growth. The mycorrhizal symbiosis becomes even more important in sustainable agricultural systems such as in heavy metals polluted soil. The principal objective of this work was to attempt using dual inoculation of AM fungi and N-fixing bacteria to induce heavy metal tolerance of cowpea plants grown under heavy metals stress.

This study (Table 1) indicates that dual inoculation with AM fungi and N-fixing bacteria can increase the dry

Table 3. Metal concentration, translocation factor, tolerance index and dependency of cowpea plants inoculated with or without microsymbiont (of *Rhizobium* cells and AM spores) after 7 weeks of planting.

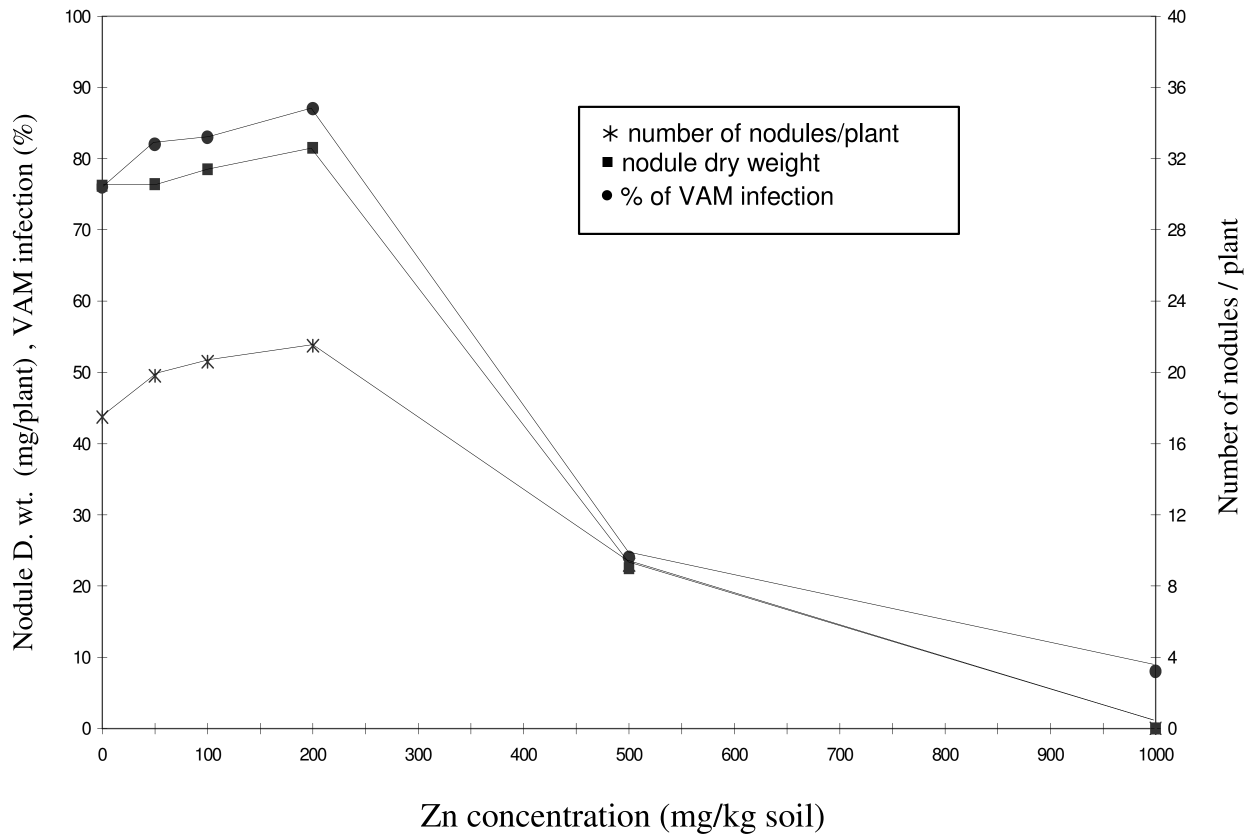
Heavy metal conc. (mg/kg dry soil)	Microsym bionts	Metal conc. (mg/g dry wt plant)		Translocation factor		Tolerance index (%)	Microsymbiont dependency
		Zn	Cd	Z	Cd		
Control	N	38.6	0.4	0.74	0.48	–	
0.0	I	61.2	0.3	0.68	0.29	–	117.4
Zn	N	58.5	DN	0.91	–	104.3	
50	I	74.0	ND	0.78	–	123.9	118.8
100	N	79.8	ND	0.76	–	110.8	
	I	92.7	ND	0.65	–	139.1	125.5
200	N	160.9	ND	0.80	–	100	
	I	171.1	ND	0.52	–	126.1	126.1
500	N	366.9	ND	0.75	–	73.0	
	I	422.2	ND	0.25	–	100	135.3
1000	N	623.1	ND	0.50	–	61.1	157.1
	I	779.4	ND	0.22	–	95.7	
Cd	N	–	0.7	–	0.71	100	
5	I	–	0.72	–	0.36	123.9	123.9
10	N	–	1.32	–	0.69	87.0	135
	I	–	1.32	–	0.35	117.4	
20	N	–	2.81	–	0.75	65.2	
	I	–	2.93	–	0.30	93.4	143.3
50	N	–	8.41	–	0.47	56.5	
	I	–	9.17	–	0.23	78.3	150
100	N	–	16.25	–	0.32	32.6	
	I	–	20.31	–	0.12	60.9	186

I: Plants inoculated by microsymbiont of *Rhizobium* cells and AM spores
N: Non-inoculated plants

weight, root shoot ratio, leaf number and area as well as length of cowpea plants more than non-inoculated one at all Zn and Cd levels. In this connection, Andrad et al. (2004) and Rabie (2005) proved that VA-mycorrhizal fungi and N-fixing bacteria can play an important role in the establishment of plants in soil contaminated with heavy metals.

The data in Table 2 showed that physiological indices, as expressed by some plant metabolites, accounted for plants infected with the two symbionts were significantly higher than that for non-infected one grown in heavy metal contaminated soil. This finding supported results from previous studies reporting that AM fungi and N-fixing bacteria has the ability to alleviate many anthropogenic stresses including effects of metals, polychlorinated aliphatic and phenolic compounds and polycyclic aromatic hydrocarbons (Entry et al., 2002; Yu et al., 2004; Johansson et al., 2004). These results indicated that the two microsymbionts effects on physiological growth of infected plants, probably improved plant development and indirectly minimized the stress caused by excess heavy metals in the soil.

The agriculturally important symbiotic microorganisms play a remarkable role in nutrient (nitrogen, phosphorus, potassium and microelements) acquisition for plants. In pursuit of that goal, various workers (Chezhiyan et al., 1999; Cairney, 2000; AlKaraki et al., 2001; Rabie et al., 2005) have used AM fungi and N-fixing bacteria, as single inoculants and in combination with each others in various plants, regardless of the presence or the absence of anthropogenic stresses. These symbiotic organisms have high ability to increase N, P and K as well as other nutrients in inoculated plants (Patreze and Cordeiro, 2004). The present study (Table 2) indicates that microsymbionts inoculation increased the concentration of N and P in cowpea plant tissue compared with non-inoculated one. These results that agree with previous works suggest that the combined or single effect of microbial symbiosis may play a great role in nutrient acquisition for plants. In this connection, Jha et al. (1993), Johansson et al. (2004) and Rabie and Almadini (2005)



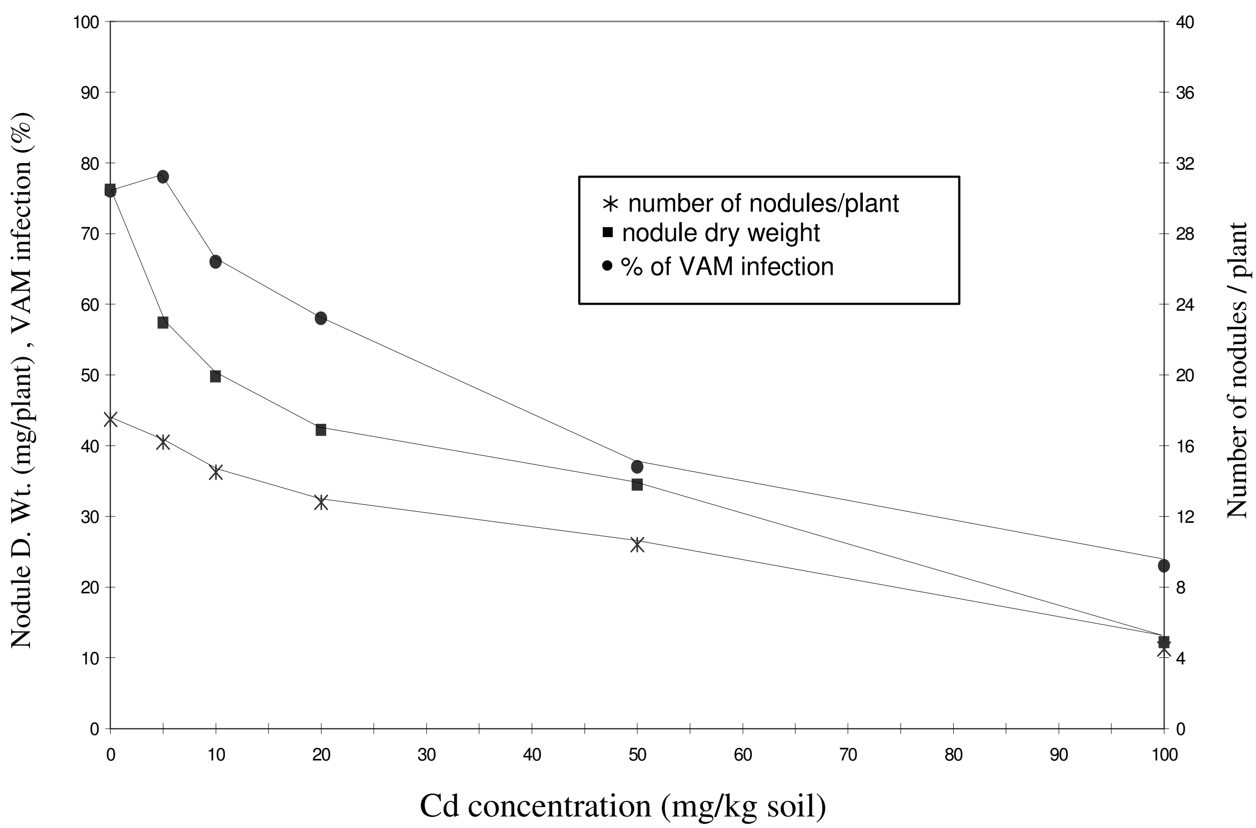


Figure 1. Effect of different concentrations of Zn and Cd on root nodulation.

showed that dual inoculation with AM fungi and N-fixing bacteria can support both needs for N and P and increase the growth of host plant.

Extrapolation the data in Figure 1 evinces that the nodule formation and percentage of mycorrhizal infection in cowpea were greatly reduced with raising Zn and Cd concentrations. Nevertheless, Cd is more toxic than Zn to inoculated plants. This finding is in line with results of Hildebrandt et al. (1999) and Vogel- Mikus et al. (2005) who reported that sensitivity of AM symbionts to heavy metal contaminated soil expressed as a reduction in spore germination, hyphal growth or root colonization, while sensitivity of N-fixing bacteria showed as decreasing in nodule formation and bacterial numbers (Andrad et al., 2004). Based on aforemention results (Tables 1, 2 and Figure 1) it is conceivable to relates the more inhibitory effects of Cd than Zn concentrations on growth of infected cowpea plants to the more toxicity of Cd to AM fungi and N-fixing bacteria.

The results in Table 3 revealed that cowpea plants species accumulate higher amount of Zn and Cd in their tissues in polluted soil than that in control (non-polluted one). Metal accumulation by plants in response to increased inputs is not consistent. Studies by Pichtel et al. (2000) have found no increase. Other work was not affected by soil metal concentrations (Oudeh et al., 2002). In contrast, increases in metal resulted in higher plant concentrations even at low soil concentrations (Rufyikiri et al., 2004).

In the present study microsymbionts acquisition may account for a high proportion of Zn and Cd in cowpea plant tissue that grew at heavy metal contaminated soil. The higher heavy metal concentration in microsymbionts–infected plants could be explained by the fact that AM infection increased plant uptake of metals by mechanisms such as enlargement of the absorbing area, volume of accessible soil, and efficient hyphal translocation (Yu et al., 2004). In addition, although heavy metal concentrations in infected-plants were much higher than that in non-infected one, the physiological indices (growth parameters) of infected-cowpea plants were significantly higher than that for non-infected plants grown in heavy metal polluted soil. This result suggests that bacterial-AM-legume tripartite symbiosis offers some protection against metal toxicity. Most reports note a positive effect of AM inoculation on the growth of plants in metal-contaminated soils. This protective benefit may be related to the adsorptive or binding capability for metals of the relatively large fungal biomass associated with the host plant roots, which may physically minimize or exclude the entry of metals into host plant (Cairney and Meharg, 2000).

The noteworthy result in Table 3 was that the presence of AM fungi and N-fixing bacteria inoculations would increase the metal tolerance index of cowpea plants compared with non-inoculated plants that grew in heavy metal polluted soil. This result emphasizes that bacterial-AM-legume tripartite symbiosis could be potentially effective in protecting plants exposed to high levels of heavy metal. The AM fungi and *Rhizobium* abilities to alleviate heavy metal stress of plants grown in heavy metal contaminated soil was previously proved by Burleigh et al. (2003), Andrad et al. (2004) and Rabie (2005). Another interesting result in Table 3 was that increasing Zn and Cd concentrations in the soil would increase the microsymbiont dependencies of cowpea plants exposed to high concentration of heavy metals. These finding that agree with others (Khan, 2001; Gonzalez-Chavez et al., 2002; Rabie, 2004) suggested that microsymbionts application to the soil may play an intelligible main role in the synergistic interactions. Moreover it is conceivable to conclude that uses of biopreparations of AM fungi and N-fixing bacteria as microsymbionts could reduce the inhibitory effects of heavy metals on plants grown in heavy metal polluted soil.

Accumulation and exclusion are two basic strategies by which plants respond to elevated concentrations of heavy metals. In metal accumulator species, shoot/root ratios greater than 1 are common. Whereas in metal excluder species the factors are typically lower than 1 (Stoltz and Greger, 2002). However, according to Stoltz and Greger (2002), exclusion of heavy metals was suggested as a tolerance strategy by AM cowpea plants on the basis of translocation factor as shown in Table 3. Shoot/root ratios of less than 1 were common for Zn and Cd, which is typical of excluders (Dahmani-Muller et al., 2000). These results suggested that the AM fungi acts in cowpea plants as a heavy metal filter to maintain low heavy metal concentrations in above ground plant tissues. If so, AM cowpea plants could be good candidates for the revegetation and phytostabilization of heavy metal polluted soil. In this connection, Dang et al. (2004) reported that species able to accumulate relatively high metal concentrations in above ground tissues could be good candidates for phytoextraction, while plant species which have strong ability to reduce metal translocation from roots to shoots are suitable as phytostabilizers for revegetation of metal contaminated lands.

Benefits of N-fixing bacteria to AM fungi were previously proved by Johansson et al. (2004) and Rabie et al. (2005). These authors reported that in bacterial-AM-legume tripartite symbiosis relationships nodulation of NFB and establishment of AM often occur simultaneously and synergically. Besides, NFB provide fixed nitrogen not only to the plant, but also to the fungus. Moreover NFB can also assist in mobilizing nutrients from the soil and improving the growth of infected plants. If so, the author suggested that bacterial-AM-legume tripartite symbiosis could be a new approach to increase the heavy metal tolerance of legume plants under heavy metal pollution conditions. Therefore additional researches are needed to explore the behavior of the two microsymbionts in various plant species and families for plant protection in heavy metal polluted soil. Nevertheless, the beneficial effects of the microsymbionts, observed in this study, arouse an interest in considering the role of bacterial-AM-plant tripartite symbiosis in plant-based strategies of remediation of highly heavy metal contaminated soils.

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