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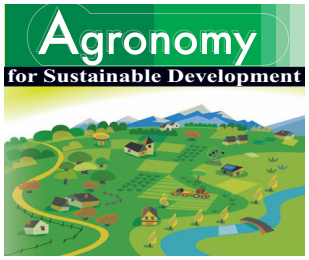

Research article

Impact of zinc-tolerant plant growth-promoting rhizobacteria on lentil grown in zinc-amended soil

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Abstract – Though zinc is a plant nutrient at low levels, Zn ions can be highly phytotoxic at higher concentrations found in contaminated soils. Plant growth-promoting rhizobacteria can be used to decrease this toxicity. Indeed, in addition to their role in plant-growth promotion, rhizobacteria also reduce the toxicity of heavy metals. In turn, they can be effective for crops grown in metal-contaminated soils. Here, we isolated a zinc-tolerant plant growth-promoting rhizobacterium, *Rhizobium* species RL9, from a zinc-contaminated soil and assayed its plant growth-promoting activities in vitro. We found that the rhizobacterium strain RL9 tolerated zinc up to a concentration of 400 μg mL^{-1} on yeast extract mannitol agar medium. It produced 33 μg $mL^{-1}$of indole acetic acid in Luria Bertani broth at 100 μg mL^{-1} of tryptophan and was positive for siderophore, hydrogen cyanide and ammonia. Such phytohormones released by this strain could help in promoting the growth of legumes. We further tested the effect of rhizobacterium strain RL9 on lentils grown in zinc-amended soil. We found that when the rhizobacterium strain RL9 was added to soil contaminated with Zn at 4890 mg/kg, lentil dry matter increased by 150%, nodule numbers by 15%, nodule dry mass by 27%, leghaemogloblin by 30%, seed yield by 10% and grain protein by 8%, compared with uninoculated plants. We also found that the concentration of zinc was higher in uninoculated plant organs than in the inoculated counterpart. Our findings thus suggest that rhizobacterium strain RL9 could be exploited for bacteria-assisted reduction of zinc toxicity in zinc-contaminated soils due to its intrinsic abilities of expressing growth-promoting substances and reduction of the toxic effects of zinc.

lentil / zinc tolerance / zinc uptake / plant-growth promotion

1. INTRODUCTION

Heavy metal pollution is a significant problem worldwide, due to its effects on 12% of the world's agricultural land (Moffat, 1999). Industrial operations such as smelting, mining, metal forging, manufacturing of alkaline storage batteries, combustion of fossil fuel and sewage sludge used as landfill material cause a phenomenal increase in the extent of heavy metal pollution (Mcllveen and Negusanti, 1994). Moreover, the introduction of pollutants to different environments such as groundwater and soil can often overwhelm the self-cleaning capacity of the recipient ecosystem and thus result in their accumulation to harmful levels, and consequently makes the soil unsuitable for sustainable agriculture. The increasing exposure to toxic heavy metal is a serious threat for both human and plant health (Aziz et al., 1996). Metal toxicity can cause multiple direct and indirect effects on plants. For instance, metals can induce interactions with sulfhydryl groups, resulting in the inactivation of plant proteins (Assche and Clijsters, 1990). Chaudri et al. (2000) reported that the application of 90-250 mg Zn kg^{-1}, 10 years after addition to sludge-treated soils, diminished the rhizobial populations. Furthermore, a pronounced metal toxicity in white clover (*Trifolium repens* L.) was confirmed in a sludge-treated soil where N_2 fixation was halved by increasing metal concentrations in the soil (Broos et al., 2005).

Microorganisms can be exploited for bioremediation under field conditions. It is important to use indigenous microorganisms as they become well adapted to the polluted soil. To prevent stress from heavy metal contamination, the rhizosphere microorganisms have developed a number of mechanisms that include the formation and sequestration of heavy metals, reduction of toxic metal to less toxic forms and direct efflux of metal out of the cell (Nies, 1999; Outten et al., 2000). Among the rhizosphere microorganisms, plant growth-promoting bacteria are known to reduce the heavy metal toxicity (Gupta et al., 2004; Tripathi et al., 2005). Furthermore, when these bacteria are applied to seeds or are incorporated into soil, the growth of plants is promoted through their ability to synthesize plant growth-promoting substances. Among the plant growth-promoting activities, the production of siderophore, indole acetic acid, hydrogen cyanide and ammonia (Ahmad et al., 2006; Ayanaba et al., 1983), which indirectly increase the plant growth, are well documented. Furthermore, a promising approach recently employed to enhance plant growth is phytoremediation, i.e the ability of plants to remove, destroy or sequester hazardous substances from the environment

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(Cunningham et al., 1995). However, successful phytoremediation may not depend solely on the plant itself, but also on the interaction of plant roots with the bacteria and the concentration of heavy metals available in soil (Wang et al., 1989).

Lentil (*Lens esculenta Moench*) is a grain legume and is grown over an area of 3.29 million ha with a total production of around 2.89 million tonnes worldwide (Rathore, 2000). In India, lentil is grown over an area of one million ha with a production of half a million tonnes. Lentil is grown on a variety of soils ranging from light loam to black vertisols. The nodule bacteria are well known for their N_2-fixing ability, consequently enhancing legume growth. Furthermore, these bacteria can also protect the plants against the toxic effects of zinc. However, to the best of our knowledge, no reports are available on the role of zinc-tolerant plant growth-promoting rhizobacteria on lentil growth. The present study was therefore designed to (1) isolate zinc-tolerant rhizobial strains; (2) evaluate the production of plant growth-promoting substances such as indole acetic acid, siderophore, ammonia and hydrogen cyanide by zinc-tolerant *Rhizobium*; (3) evaluate the effects of zinc-tolerant strains of *Rhizobium* species on the growth of lentil plants grown in zinc-treated soil, and (4) assess the uptake of zinc by lentil plants under clay pot conditions.

2. MATERIALS AND METHODS

2.1. Rhizobial strains and zinc tolerance

The rhizobial strains were isolated from nodules produced on the root systems of lentil, grown in zinc-contaminated soils of Aligarh (27° 29' latitude and 72° 29' longitude), India, using standard methods. For the isolation of *Rhizobium* strains, one mL of nodule suspensions was diluted and plated on solid yeast extract mannitol medium (10 g L^{-1} mannitol, 0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 1 g L^{-1} NaCl, 1 g L^{-1} yeast extract, 2 g L^{-1} $CaCO_3$, pH 7). The rhizobial strains were maintained on yeast extract mannitol medium until use. The selected rhizobial strains were re-streaked four times on the same medium to check the purity of the cultures. The isolated strains were further characterized using colony morphology and cultural and biochemical characteristics (Holt et al., 1994) to confirm the identity of cultures. The rhizobial strain was also checked for its nodulating efficiency, using lentil as a test crop under pothouse conditions. Rhizobial strains were tested further for their ability to tolerate zinc by the agar plate dilution method (Holt et al., 1994) using yeast extract mannitol agar (pH 7) medium. In the plate dilution method, freshly prepared agar plates amended with 0 to 400 μg Zn mL^{-1} of zinc chloride were spot-inoculated with 2×10^8 cells mL^{-1} of rhizobial strains. Plates were incubated at 28 ± 2 °C for 72 h and the lowest concentration of zinc inhibiting rhizobial growth on yeast extract mannitol agar plates was defined as the minimum inhibitory concentration. The rhizobial strain showing the greatest tolerance to zinc was used for further studies.

2.2. Bioassay of plant growth-promoting substances

The rhizobial strains tolerant to zinc were further evaluated for their ability to express the production of phytohormones. The indole-3-acetic acid was quantitatively assayed by the method of Brick et al. (1991). The rhizobial isolate was grown in Luria Bertani broth (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, pH 7.5). One hundred mL of Luria Bertani broth amended with 20, 60 and 100 μg mL^{-1} of tryptophan was inoculated with one mL of culture (2×10^8 cells mL^{-1}) and incubated at 28 ± 2 °C for 24 h with proper shaking. After 24 h, five mL of each culture were centrifuged (1 100×g) for 15 min. and two mL of Salkowsky reagent (2% 0.5M $FeCl_3$ in 35% perchloric acid) were added to two mL of supernatant and incubated at 28 ± 2 °C in darkness for 1 h. The absorbance of the pink color developed was read at 530 nm. The indole acetic acid concentration in the supernatant was determined using a calibration curve of pure indole acetic acid as a standard. The experiments were conducted three times at different time intervals.

The production of siderophore by the zinc-tolerant rhizobial isolate was detected using the Chrome Azurol S method of Alexander and Zuberer (1991). The Chrome Azurol S agar was prepared and 30 mL were poured onto Petri plates (8.5 cm diameter). One hundred μL of 2×10^8 cells mL^{-1} rhizobial inoculum were placed in the center of the Chrome Azurol S agar plates. Plates were incubated at 28 ± 2 °C for five days and were observed daily. Positive reactions were qualitatively estimated by changes in color of the assay reagent from blue to orange. The assay was considered negative when no change in blue color was observed within five days. The experiment was repeated three times at different sub-culture intervals. Further, the rhizobial culture (2×10^8 cells mL^{-1}) was grown in Modi medium (0.05% K_2 HPO_4, 0.04% $MgSO_4$, 0.01% NaCl, 1% mannitol, 0.1% glutamine, 0.1% NH_4NO_3) for five days and catechol-type phenolates were quantitatively measured by the method of Reeves et al. (1983). Hydrogen cyanide production by the zinc-tolerant rhizobial isolate was assayed by the method of Bakker and Schipper (1987). Rhizobial isolates were grown on a hydrogen cyanide induction medium (30 g L^{-1} tryptic soy broth, 4.4 g L^{-1} glycine, 15 g L^{-1} agar) at 28 ± 2 °C for five days to determine hydrogen cyanide. For each rhizobial isolate, 100 μL of inoculum (2×10^8 cells mL^{-1}) were placed in the center of the plates. A disk of Whatman filter paper No.1 soaked in 0.5% picric acid and 2% Na_2CO_3 was placed in the lid of the Petri plates, which were sealed with Para film. After five days of incubation at 28 ± 2 °C, a brown to orange color of the paper indicating hydrogen cyanide production was observed. In the case of ammonia production, rhizobial isolates were grown in peptone water (10 g L^{-1} peptone, 5 g L^{-1} NaCl, pH 7) and were incubated at 28 ± 2 °C for five days. One mL of Nessler's reagent was added to each tube and the development of yellow color, indicating a positive test for ammonia (Dye, 1962) production, was recorded.

2.3. Plant growth and zinc uptake

The experimental soil was sandy clay loam: 0.4%, organic carbon, 0.75 g kg^{-1} Kjeldahl N, 16 mg kg^{-1} Olsen P, pH 7.2, 0.44 mL g^{-1} water-holding capacity and 19.2 mg kg^{-1} zinc.

Seeds of lentil var. Malka were surface-sterilized using 70% ethanol, 3 min; 3% sodium hypochlorite, 3 min, to prevent the growth of seed-borne pathogens, rinsed six times with sterile water and dried. Seeds were coated with *Rhizobium* species RL 9 in liquid culture medium for two hours using 10% gum arabic as an adhesive to deliver approximately 10^8 cells per seed. The non-coated sterilized seeds used as control were soaked in sterile water only. The uninoculated and inoculated seeds (10 seeds per pot) were sown in clay pots (25 cm high, 22 cm internal diameter) using three kg unsterilized soil with control (without zinc) and three treatments each with 2445, 4890 and 9780 mg Zn kg^{-1} soil. The zinc was applied in the form of zinc sulfate and the pH of the zinc-treated soil was 6.9. The zinc concentration (4890 mg kg^{-1} soil) applied in this study was comparable with those detected in sewage-treated soils used for lentil cultivation. The inoculated and uninoculated control pots received N:P:K fertilizers at rates of 20:50:50 mg kg^{-1} soil. Nitrogen was applied as urea, P as diammonium phosphate and K as muriate of potash. The pots were arranged in a complete randomized design with six replications of each treatment. Ten days after emergence, plants in each pot were thinned to three plants. The pots were kept in open field conditions and were watered with tap water when necessary. The experiment was conducted for two consecutive years, i.e. 2005 and 2006, to ensure the reproducibility of the results.

All plants in three pots for each treatment were removed at 90 d and were observed for nodulation on the root system. The roots were carefully washed and nodules were detached, counted, oven-dried (80 °C) and weighed. Plants removed at 90 d were oven-dried (80 °C) to measure the total phytomass. The total nitrogen content in roots and shoots of lentil plants was measured at 90 d by the micro-Kjeldahl method (Iswaran and Marwah, 1980). The leghaemoglobin content in fresh nodules recovered from the root system of lentil plants was quantified at 90 d (Sadasivam and Manikam, 1992). Briefly, 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. The zinc content in tissues of roots and shoots was measured at 90 d, while in grains the zinc content was determined at 120 d. The organ tissues and grains were digested in nitric acid and perchloric acid (4:1) following the method of Ouzounidou et al. (1992) and zinc concentration was determined using flame atomic absorption spectrophotometry (Model GBC 932B Plus flame atomic Absorption Spectrophotometer). The remaining three pots of each treatment with three plants per pot were maintained until harvest (120 d) and seed yield and grain protein were determined (Sadasivam and Manikam, 1992).

2.4. Statistical analysis

Since the experiment was conducted for two consecutive years under identical environmental conditions using the same treatments and the data obtained were homogenous, the data of the measured parameters were pooled together and subjected to analysis of variance (ANOVA). The difference among treatment means was compared by high-range statistical domain using Tukey's test. Data were also subjected to ANOVA for the two-factor pot culture experiment, i.e. inoculation and zinc concentration, and least significant difference was calculated at the 95% probability level.

3. RESULTS AND DISCUSSION

3.1. Zinc tolerance and plant growth-promoting potential

In this study, we isolated 50 rhizobial strains from nodules of lentil plants grown in metal-contaminated Indian soils. Of these, the *Rhizobium* species RL 9 was specially chosen due to its ability to tolerate a high concentration of zinc up to the concentration of 400 μg mL^{-1}. The zinc-tolerant strain RL9 was Gram-negative and produced circular and white gummy colonies on yeast extract mannitol agar plates. The freshly grown culture of strain RL9 was positive for catalase, triple sugar iron agar, nitrate, urease and citrate, and could hydrolyze gelatin. Strain RL9 gave negative results for starch hydrolysis, indole, methyl red and Voges Proskauer tests (Tab. I).

Generally, microorganisms affect metal concentrations in the environment and exhibit a strong ability to remove metals from solution. The removal of metals from the metal-contaminated environment by the rhizosphere microbes can be achieved either through enzymatic or non-enzymatic mechanisms. To circumvent heavy metal stress, plant growth-promoting rhizobacteria have developed a number of mechanisms to tolerate the uptake of heavy metal ions. The mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell and reduction of toxic metals to less toxic forms (Outten et al., 2000). In this context, Gram-negative bacteria exhibiting varying degrees of resistance to zinc salts have been reported. For instance, *Rhizobium. leguminosarum* showed tolerance to zinc up to a concentration of 92.9 μM (Delorme et al., 2003). Conflicting reports are, however, available in the literature on the tolerance level of bacteria, which could possibly be due to the differences in the types of media used and growth conditions employed (Rajkumar et al., 2005). The ability of nodule bacterium (*Rhizobium* species RL 9) to tolerate relatively high concentrations of zinc in addition to their intrinsic property of nitrogen fixation makes them one of the most potentially effective rhizospheric microorganisms for legume improvement in zinc-contaminated soil.

Furthermore, zinc-tolerant strain RL9 showed the production of substantial amounts of indole acetic acid during 24 h of growth in Luria Bertani medium supplemented with 20, 60 and 100 μg mL^{-1} of tryptophan (Tab. I). Generally, we observed here that the amounts of indole acetic acid produced by this strain increased consistently with the increase in tryptophan concentration in Luria Bertani medium. A maximum amount of 33 μg mL^{-1} of indole acetic acid was observed

Table II. Effect of inoculation with *Rhizobium leguminosarum* RL 9 on the biological and chemical characteristics of lentil plants subjected to different soil zinc concentrations.

Treatment	Dose of Zinc	Dry weight	Nodulation		Leghaemoglobin content	N content (mg g^{-1})		Seed yield	Grain protein
	(mg kg^{-1} soil)	(g $plant^{-1}$)	No. $plant^{-1}$	Dry mass (mg$plant^{-1}$)	[m mol (g f.m)$^{-1}$]	Root	Shoot	(g /1000seed)	(mg g^{-1})
Uninoculated	Control	0.18c	12cd	13de	0.09bc	13.7ab	41.0a	95cd	236cd
	2445	0.17c	11cd	12de	0.09bc	13.3bc	40.4a	91de	233cd
	4890	0.16c	10cd	10de	0.07cd	13.1bc	40.1a	87de	230cd
	9780	0.15c	9d	9e	0.05d	12.8c	39.0a	83e	227d
Inoculated	Control	0.20c	13bc	15bc	0.10bc	14.2a	42.0a	100bc	240bc
	2445	0.40b	14ab	17ab	0.12ab	14.3a	42.2a	105ab	246b
	4890	0.50a	15a	19a	0.13a	14.7a	42.5a	110a	260a
	9780	0.20c	12cd	14cd	0.09*	13.8ab	39.5a	95cd	237cd
LSD ($P \leq 0.05$)		0.04	0.8	2.07	0.006	0.31	2.3a	4.4	2.6
F value	Inoculation (df = 1)	364.4*	252.0*	93.4*	675.0*	194.6*	6.9*	1093.5*	537.2*
	Zinc (df = 3)	79.5*	28.0*	7.7*	99.0*	16.5*	6.8*	122.5*	75.6*
	Inoculation × zinc (df = 3)	81.9*	18.6*	6.6*	594.6*	9.8*	NS	82.5*	82.2*

Mean values followed by different letters in the same column are different at $P \leq 0.05$ according to Tukey's test; * = Significantly different from the control at $P \leq 0.05$; NS = Not significant at $P \leq 0.05$.

Table I. Morphological, biochemical and plant growth-promoting activities of *Rhizobium* species isolated from metal-contaminated soil

Characteristics	**Strain RL9**
Morphological	
Gram reaction	Gram-negative
Cell shape	Short rods
Colony morphology	Circular and white gummy
Biochemical reactions	
Catalase	+
Nitrate	+
Urease	+
Citrate	+
Indole	-
Methyl red	-
Voges Proskauer	-
Hydrolysis	
Starch	-
Gelatin	+
Tipple sugar iron agar	+
Production of Siderophores on/in	
CAS agar (mm)	12 ± 2
SA (mg L^{-1})	15 ± 2
2,3 DBA (mg L^{-1})	18.3 ± 3
Synthesis of IAA (µg mL^{-1}) at	
20 T	6.4 ± 0.9
60 T	15.2 ± 1.5
100 T	33 ± 3
HCN	+
Ammonia	+
Tolerance to zinc	400 µg/mL

+ Indicates a positive reaction; each value is a mean of three replicates ± standard deviation; T = Tryptophan concentrations in mg mL^{-1}; CAS = Chrome Azurol S; SA = Salicylic acid; DBA = 2,3 dihydroxybenzoic acid; HCN = Hydrogen cyanide; IAA = Indole acetic acid.

when Luria Bertani medium was supplemented with 100 µg mL^{-1} of tryptophan, which was followed by 15.2 and 6.4 µg mL^{-1} of indole acetic acid at 60 and 20 µg tryptophan mL^{-1}, respectively. Moreover, the zinc-tolerant *Rhizobium* showed a positive siderophore activity, as indicated by the development of a 12-mm orange-colored zone on Chrome Azurol S agar plates, after four days of growth. The ethylacetate extraction of the culture supernatant yielded 15 mg L^{-1} of salicylate and 18.3 mg L^{-1} of 2,3-dihydroxybenzoic acid. In addition to N_2 fixation, nodule bacteria also affect legume plants by other mechanisms. For instance, they increase plant growth through the production of plant growth-promoting substances and siderophores, as reported by Ahmad et al. (2006) and Faisal and Hasnain (2006). Siderophores released by the plant growth-promoting rhizobacteria in the rhizospheres, in turn, bind to the available form of iron Fe^{3+} and make iron unavailable to the phytopathogens, leading thereby to an increase in plant health (Arora et al., 2001). Furthermore, the strain was positive for both hydrogen cyanide and ammonia (Tab. I). The ammonia released by the test strain plays a signaling role in the interaction between plant growth-promoting bacteria and plants (Becker et al., 2002). Moreover, the ammonia released by the bacterial strain is known to increase the glutamine synthetase activity (Chitra et al., 2002). In addition, ammonium transporters found in several plant growth-promoting rhizobacteria are thought to be involved in the reabsorption of NH_4^+ released as a consequence of NH_3 diffusion through the bacterial membrane (Van Dommelen et al., 1997). Similar evidence on the production of phytohormones (Rajkumar et al., 2006), siderophore and hydrogen cyanide (Ahmad et al., 2006), and indole acetic acid production by rhizobia has been reported (Duhan et al., 1998; Antoun et al., 1998). Such a nodule bacterium with the ability to reduce metal toxicity, producing phytohormones and providing N to legume crops could thus be very effective for legumes grown in metal-contaminated soils. The production of such phytohormones by the zinc-tolerant strain RL9 prompted us to assess the effect of

this strain on lentil, grown in soil treated with varying concentrations of zinc.

3.2. Zinc toxicity and inoculation effects on lentil plants

3.2.1. Plant growth and symbiosis

Uninoculated lentil plants grown in soil treated with increasing concentrations of zinc showed a phytotoxic effect (Tab. II). Generally, the plant dry weight and nodulation decreased with increasing concentrations of zinc applied to soil. The highest dose of zinc decreased the dry weight, number of nodules and nodule dry mass by 17, 25 and 31%, respectively, with respect to the untreated control. Furthermore, the leghaemoglobin content in fresh nodules from lentil plants grown in the highest concentration of zinc decreased by 44% with respect to the untreated control. The reduction in growth of lentil plants is possibly due to the presence of the higher concentration of zinc in the soil, as reported by others (Singh and Tewari, 2003; Rout and Das, 2003). A relatively higher concentration of metals exerts severe adverse effects on roots' growth and their function, resulting in root damage, which consequently diminishes the uptake of water and nutrients by the growing plants (Terry, 1981). Moreover, as a result of increased metal uptake, the normal metabolic functions of plants can be disrupted, eventually causing cellular injuries and, in extreme cases, even senescence. In the present study, the highest concentration of zinc had the greatest phytotoxic effect on lentil plants, possibly due to the inhibition of proteins, enzymes or DNA,through the generation of reactive oxygen intermediates (Asada, 1994).

In contrast, plants inoculated with strain RL9 increased the dry weight, nodule numbers and nodule dry mass by 150, 15 and 27%, respectively, with increasing zinc concentration up to 4890 mg Zn kg^{-1} with respect to uninoculated plants grown in zinc-treated soils (Tab. II). Furthermore, the leghaemoglobin content in fresh nodules increased significantly ($P \leq 0.05$) by 30% at 4890 mg Zn kg^{-1} soil, compared with control. In general, the sole inoculation of zinc-tolerant strain RL9 significantly enhanced the measured parameters compared with uninoculated plants. Two-factor ANOVA revealed that the individual effects of inoculation and zinc and their interaction (inoculation × zinc) were significant ($P \leq 0.05$) for the measured parameters. Plant growth-promoting rhizobacteria can positively influence plant growth and development either indirectly by preventing some of the deleterious effects caused by some toxic substances (Genrich et al., 2000), or directly by producing some substances which are beneficial for the promotion of plant growth (Ahmad et al., 2006). In this study, inoculation of lentil seeds with the zinc-tolerant phytohormone-producing strain RL9 increased lentil growth in the presence of different concentrations of zinc with respect to plants grown in the absence of a bioinoculant. This seems to suggest that the strain RL9 might have protected lentil plants against the inhibitory effects of zinc. Furthermore, it is likely that the siderophore-producing ability of this strain might have helped lentil root proliferation, enhancing the uptake of soil minerals by the host plant (Genrich et al., 2000). An alternative mechanism in plant-growth promotion could involve the release of indole acetic acid by the tested strain in this study. The indole acetic acid produced by the rhizobial strain promotes root growth by directly stimulating plant cell elongation or cell division (Minamisawa and Fukai, 1991). The observed growth promotion in the presence of the inoculant RL9 can thus possibly be attributed to the production of growth-promoting substances including siderophores, in addition to their ability to detoxify zinc through adsorption/desorption (Mamaril et al., 1997) and fix atmospheric nitrogen, making N available to the lentil plants. Similar evidence was reported for tomato (*Lycopersicon esculantum*), canola (*Brassica rapa*) and Indian mustard (*Brassica compestris*) in nickel-, lead- or zinc-treated soil (Genrich et al., 2000; Zaidi et al., 2006). Furthermore, lentil plants grown in the zinc-treated soil showed a significant ($P \leq 0.05$) decrease in root N content. A substantial reduction in N content and nodulation in field-grown pea (*Pisum sativum*) and greengram (*Vigna radiata* L. *wilczek*) following heavy metal application to soil has been reported (Chaudri et al., 2000; Wani et al., 2007). In contrast, plants grown in the presence of a bioinoculant increased nodulation, which suggested that the strain RL9 might have reduced the toxicity of zinc in sandy clay loam soil. In addition, a significant increase in nodulation and consequently N availability to the crops under different concentrations of zinc has also been reported (Ibekwe et al., 1995). The effect of zinc on the seed yield, grain protein and N content of lentil plants grown in the presence and absence of a bioinoculant was also assessed.

3.2.2. Seed yield, grain protein and N content

Seed yield and grain protein at 120 d and N content at 90 d decreased consistently with increasing concentrations of zinc for uninoculated lentil plants (Tab. II). The highest reduction in seed yield (13%), grain protein (4%), root N (7%) and shoot N (5%) were observed for the 9780 mg Zn kg^{-1} concentration with respect to the control. On the other hand, the plants inoculated with strain RL9 showed an increase in seed yield and grain protein with increasing concentrations of zinc at 120 d (Tab. II). A maximum increase of 10 and 8% in seed yield and seed protein, respectively, for the inoculated plants was observed at 4890 mg Zn kg^{-1} soil compared with uninoculated plants. However, nitrogen content in plant organs (e.g. roots and shoots), increased marginally in inoculated lentil plants with respect to the uninoculated plants. Generally, there was considerably more N in shoots compared with roots of lentil plants. Seed yield, grain protein and N content, however, decreased marginally at 9780 mg Zn kg^{-1} soil even in the presence of a bioinoculant compared with uninoculated but zinc-treated soil. Seed yield significantly ($P \leq 0.05$) correlated with N contents of roots (r = 0.5), shoots (r = 0.71) and grain protein (r = 0.94). Moreover, the grain protein strongly correlated with N contents of roots and shoots (r = 0.65 for both roots and shoots). The two-way ANOVA showed that the individual effects of inoculation and zinc and their interaction (inoculation × zinc) were significant for all the measured parameters except the N content of shoots.

In this study, the seed yield declined following zinc application to soils, possibly due to the effects of zinc on the proliferation of roots and shoots (Ibekwe et al., 1996). The reduction in roots and shoots in turn might have a suppressive effect on dry matter production and consequently on seed yield (Bisessar et al., 1983). In contrast, the plants grown in the presence of a bioinoculant increased the seed yield and seed protein consistently. Although the seed yield and grain protein content for inoculated lentil plants decreased with the highest level of zinc applied in this study (9780 mg Zn kg^{-1}), they were nevertheless higher than those observed for the uninoculated plants at any concentration of zinc applied to soil. The increase in the seed yield and grain protein could be due to the effective symbiosis shown by a zinc-tolerant organism endowed with plant growth-promoting activities. A similar increase in seed yield of pea following zinc and copper was reported (Chaudri et al., 2000). The accumulation of zinc in the root and shoot tissues and grains of lentil plants is discussed in the following section.

3.3. Zinc uptake

The concentration of zinc in plant roots, shoots and grains increased with increasing concentrations of zinc both for uninoculated and inoculated plants. The distribution profile of zinc accumulated in the different plant parts is shown in Figure 1. The average maximum accumulation of zinc in roots, shoots and grains was 333, 238 and 59 μg g^{-1}, respectively, for plants grown in the absence of a bioinoculant, at 9780 mg Zn kg^{-1} of soil. In contrast, the maximum accumulation of zinc in roots, shoots and grains was 320, 210 and 52 μg g^{-1}, respectively, for lentil plants grown in the presence of a bioinoculant, at the same dose of zinc applied to soil. The data revealed a lower concentration of zinc in roots, shoots and grains of inoculated plants compared with uninoculated plants. The two-factor ANOVA revealed that the individual effects of inoculation were significant ($P \leq 0.05$) for the concentration of zinc in roots, shoots and grains, whereas the interaction (inoculation × zinc) was significant ($P \leq 0.05$) only for roots and shoots. The lower concentrations of zinc in plant organs could be due to the removal of zinc through the adsorption/desorption mechanism of strain RL9 (Mamaril et al., 1997). Similar accumulations and distribution of zinc and cadmium and copper in plants grown in metal-contaminated soil have been reported (Longneckner and Robson, 1993; Mediouni et al., 2006). Moreover, in this report, we observed that the root tissues showed considerably more accumulation of zinc compared with the shoot tissues of lentil plants under both inoculated and uninoculated treatments. Furthermore, the zinc concentration in grains was directly related to the concentration of zinc applied to soil. Our findings are in accordance with the findings of Chaudri et al. (2000), who also observed a considerable accumulation of zinc and copper in pea and white clover shoots.

4. CONCLUSION

We demonstrated that the inoculation of the zinc-tolerant *Rhizobium* species RL9 endowed with plant growth-promoting activities protected not only the lentil plants from zinc toxicity but also stimulated the overall growth of the test plants. Consequently, the bioinoculant strain, when used with different concentrations of zinc, enhanced the yield and quality of lentil grains. However, at the highest level of zinc application, the strain RL9 showed no significant reduction in zinc toxicity to lentil plants but had a positive effect compared with the plants grown in only zinc-treated soil. Furthermore, the strain RL9 decreased the concentration of zinc in plant tissue and in grains by appreciable levels. The data suggest that the ability of strain RL9 to protect plants against the inhibitory effects of high concentrations of zinc is related to the *Rhizobium* reducing the toxicity of zinc and providing the lentil plants with sufficient N and growth-promoting substances. Thus, due to the ability to (i) tolerate relatively high level of zinc, (ii) produce plant growth-promoting substances, and (iii) reduce the toxicity of zinc, as observed in this study, the strain RL9 could therefore be used for the growth improvement of lentil plants as well as for the bioremediation of zinc-contaminated soil.

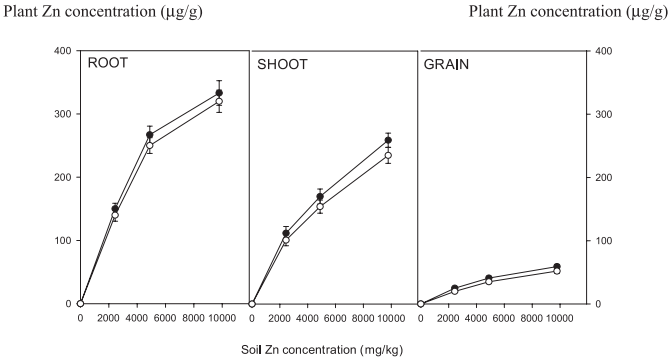

Figure 1. Concentration of zinc in roots, shoots and grains of lentil plants grown in the absence (•) and presence (○) of a bioinoculant with increasing soil zinc concentrations.

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