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Full Length Research Paper

Growth response and heavy metals tolerance of *Axonopus affinis,* inoculated with plant growth-promoting rhizobacteria

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Different microorganisms have been used for bioremediation based on their resistance and ability to sequester heavy metals. The use of plant growth-promoting rhizobacteria (PGPR) for bioremediation of these contaminants has been successful. A PGPR isolated from hydrocarbons-contaminated soil identified as *Bacillus* sp., by microbiological and molecular tools and characterized as heavy metal tolerant by minimal inhibitory concentration (MIC) assay was inoculated into *Axonopus affinis* plants. Both of them were exposed to cadmium, nickel, and zinc and the effect of their relationship was analyzed by multivariate analysis. The results did not show a significant growth promotion and development of this Poaceae with rhizobacteria alone, but the presence of heavy metals plus the PGPR assured the survival of plants. This suggests that the plant's response is related with the metal concentration and the exposure time to the contaminants, as well as with its intrinsic tolerance. The *Bacillus* sp strain allowed the growth maintenance of *A. affinis* and enhanced its tolerance to the assayed heavy metals, suggesting a synergistic effect between this species and the rhizobacterium in response to contaminating agents.

Key words: Bioremediation, heavy metals, microorganisms, plants.

INTRODUCTION

Rhizosphere microorganisms that are closely associated with roots have been termed plant growth promoting rhizobacteria (PGPR) (Glick, 1995). These bacteria are capable of promoting plant growth by colonizing the plant root (Kloepper and Schroth, 1978). PGPR can be divided into two groups according to their relationship with the plants: symbiotic bacteria and free-living rhizobacteria.

Generally, PGPR function in three different ways: synthesizing particular compounds for the plants, facilitating the uptake of certain nutrients from the environment, and protecting the plants against diseases (Glick, 2003).

An extension of PGPR technology is the emerging use of bacteria with plants for environmental applications. Recent studies in this area include many different applications, such as growth promotion of soil stabilizing plants, to counteract flooding stress of plants, to aid plant growth in acidic conditions, to counteract high temperature stress, as well as for phytoremediation technologies (Burd et al., 2000; Zhuang et al., 2007).

Release of heavy metals from various industrial sources,

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agrochemicals and sewage sludge poses a major threat to the soil environment. The accumulation of heavy metals into soil leads to changes in microbial activity and soil fertility. Consequently, the enhanced concentration of metals in soil and its translocation to plant organs can have undesirable effects on growth of plants (Ahmed et al., 2008). Generally, heavy metals are not degraded biologically and persist in the environment indefinitely (Khan et al., 2009).

A diverse group of free-living soil bacteria can improve host plant growth and mitigate toxic effects of heavy metals on the plants (Belimov et al., 2004). Even more, metal bioavailability and uptake can be changed due to the microbial activity in the soil (Amezcua-Allieri and Rodríguez-Vázquez, 2008). It is also well known that heavy metals can even be toxic for metal-accumulating and metal-tolerant plants, if the metal concentration in the environment is high (Jing et al., 2007).

Accumulation of heavy metals in the soil environment and their uptake by rhizobacteria and plants is a matter of growing concern. Some metals such as zinc, copper, nickel and chromium are essential or beneficial micronutrients for plants, animals and microorganisms (Olson et al., 2001), whereas others (cadmium, mercury, and lead) have no known biological and/or physiological functions (Gadd, 1992). At higher concentrations, metal ions can either completely inhibit the microbial populations by inhibiting their metabolic activities or organisms can develop resistance or tolerance to the elevated metal levels. The ability to grow even at high metal concentration is found in many rhizospheric microorganisms (Lakzian et al., 2002) and may be the result of intrinsic or induced mechanisms (Giller et al., 1998).

In order to survive and proliferate in metal contaminated soils, tolerance has to be present in both microbes and their associated hosts. For survival in metal-stressed environments, the plant growth promoting rhizobacteria have evolved several mechanisms by which they can immobilize, mobilize, or transform metals, rendering them inactive, thereby allowing the plant to tolerate the uptake of heavy metal ions (Nies, 1999).

Different defense mechanisms allow these microorganisms to function metabolically in metal polluted environments. The exploitation of these bacterial properties for the remediation of heavy metal-contaminated sites has been shown to be a promising bioremediation alternative (Lovley and Coates, 1997; Lloyd and Lovley, 2001). Therefore, managing the microbial populations in the rhizosphere, by using microbial inocula, consisting of a consortium of plant growth promoting rhizobacteria and symbiotic nitrogen fixers as allied colonizers and biofertilizers, could provide plants with benefits crucial for ecosystem restoration (Khan, 2004). These microorganisms can be indigenous to a contaminated area (intrinsic bioremediation) or can be isolated from elsewhere and then introduced into the contaminated sites (bioaugmentation) (Whiting et al., 2001; Abou-Shanab et al., 2003).

The carpet grass (*Axonopus affinis*) has been employed for another experiments related with the response to heavy metals (Kuo et al., 2005) and with contaminated soils, mainly in regions of copper and gold with great importance for the development of rehabilitation of clean technologies in areas degraded by mining (phytoremediation) and in the mineral bioprospecting (Ernst-Frizzo and Porto, 2004).

This study evaluated the effects of the inoculation of *A. affinis* with plant growth promoting rhizobacterium and its tolerance to heavy metals as promising bioremediation alternative.

MATERIALS AND METHODS

Maintenance and identification of the rhizobacteria isolated from a soil contaminated with petroleum hydrocarbons

Twenty-three rhizobacteria strains were isolated from a rhizospheric contaminated soil with petroleum hydrocarbons with a Light Hydrocarbon Fraction Concentration (LHFC) of 90 ppm, Medium Hydrocarbon Fraction Concentration (MHFC) of 100 ppm and Heavy Hydrocarbon Fraction Concentration (HHFC) of 45 ppm. A pheno-typic phosphate solubilizing rhizobacterial strain was isolated from this soil sample using the method of Wu et al. (2006) with phos-phoric rock, this strain was maintained and preserved on nutrient broth agar medium plates for conventional bacterial analyses. The isolated rhizobacterial strain was identified by determining its 16S rRNA sequences. Colony PCR was performed from live cells cultured on solid nutrient broth agar medium. Cells were harvested after 24 h and processed for DNA isolation using the Allers and Lichten procedure (2000). Using the purified genomic DNA, the molecular target gene 16S rRNA was amplified using the universal primers set, fD1 and rD1, designed by Weisburg et al. (1991). The PCR mixture contained: 50 ng of genomic DNA, 3 mM $MgCl_2$, 2.5 units of *Taq* DNA polymerase (Invitrogene, CA), 200 mM of each dNTP and 20 pmol of each primer. The amplification reaction was cycled as follows: an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 2 min; 42°C for 40 sec, 72°C for 4 min, and finally a polymerizing cycle at 72°C for 20 min. Aliquots of PCR reaction products were electrophoresed in 1% agarose gel and then stained with ethidium bromide. These PCR products were purified and sequenced by the Unidad de Biotecnología y Prototipos at FES-Iztacala (UNAM). The sequences were then compared to similar sequences in the databases using BLAST analysis (Basic Logical Alignment Search Tool, BLAST at NCBI).

Determination of minimal inhibitory concentration (MIC) of the isolated rhizobacterial strain

The minimum inhibitory concentration for the isolated rhizobacterial strain was determined using the Sabry et al. (1997) method, by inoculating nutrient broth agar plates with a range of concentrations of each heavy metal, separately: 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 5.0, 10, and 15 mM for Cd and 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 10, and 15 mM for Ni and Zn, the analytical salts employed were: $3CdSO_4 \cdot 8H_2O$, $NiSO_4 \cdot 2H_2O$, and $ZnCl_2$. Results of the inoculated plates were read after 72 h of incubation at 28°C and recorded as positive

by colony appearance on the plate surface (CFU/ml). The lowest concentration that inhibited growth completely was considered the MIC, and the metal resistant strains were those in which their growth was not inhibited by 1 mM concentration of Cd, Ni, and Zn.

Assessment of *A. affinis* growth promotion by inoculated rhizobacteria and tolerance to heavy metals

Commercially obtained certified seeds of *A. affinis* were surface-sterilized with 10% sodium hypochlorite and then thoroughly rinsed with sterile distilled water. These sterilized seeds were incubated for 30 min at room temperature in either sterile distilled water as a blank control or a bacterial suspension in distilled water of 5 × 107 CFU/ml. Twenty seeds of each species, treated and non-treated with bacteria, were placed separately in baby food flasks with Magenta SIGMA caps with 25 ml of mineral medium containing: 0.20 M $NH_4H_2PO_4$, 0.50 M NH_4NO_3, 1.15 M $Ca(NO_3)_2$, 0.26 M $CaCl_2$, 0.2 M $MgCl_2 \cdot 6H_2O$, 0.20 M $Mg(NO_3)_2 \cdot 6H_2O$, 0.40 M $MgSO_4 \cdot 7H_2O$, 0.20 M KH_2PO_4, 1.2 M KNO_3, 0.5 M K_2SO_4, 0.04 M $FeCl_3 \cdot 6H_2O$, 1.2×10^{-2} M H_3BO_3, 1.2×10^{-4} M $CuCl_2 \cdot H_2O$, 2.3×10^{-3} M $ZnCl_2$, 4.4×10^{-4} M $MnCl_2 \cdot 4H_2O$, 6×10^{-6} M $Na_2MoO_4 \cdot H_2O$, EDTA and $FeSO_4 \cdot 7H_2O$, pH = ± 6.0 and 6% bacteriological agar; supplemented in each case with the corresponding Cd (0.01, 0.05 and 0.1 mM), Ni (0.1. 0.5 and 1 mM) and Zn (3, 4 and 5 mM) concentrations. The analytical salts employed were: $3CdSO_4 \cdot 8H_2O$, $NiSO_4 \cdot 2H_2O$ and $ZnCl_2$; the control experiments were performed without the metals. All the experi-ments were developed in sterile conditions and performed in triplicate. Experimental units were kept at ±36°C in a growth chamber with a 12:12 photoperiod for 8 days. The growth of plants was evaluated by measuring their root and shoot length. The Tolerance Index (TI), expressed as the ratio of the shoot and root lengths of plants grown in the presence and absence of a specific added metal, was obtained (Wilkins, 1978; Burd et al., 1998); TI = R or S L_m / R or S L_c where R or S L_m is the root or shoot length of plants grown in the presence of a specific added metal and R or Shoot L_c is the root or shoot length of plants grown in absence of metals (control).

Statistical analysis

All the results were analyzed with ANOVA and Tukey-Kramer's tests using the software Graph Pad Instat Ver. 2.03 (Aceves, 1993) and the experimental designs program FAUANL Ver. 1.4 (Olivares, 1989).

Finally, a multivariate analysis considering the plant growth results of *A. affinis* inoculated with the rhizobacterial strain in the presence or absence of heavy metals was performed through surface response analysis (Cleveland, 1994), using the software STATISTICA Ver. 8 (StatSoft Inc. 2007, USA).

RESULTS

Molecular characterization of the rhizobacterial strain isolated from the rhizosphere of contaminated soil

The rhizobacterial strain isolated was named rhizobacterium XIII strain, its microscopic morphology revealed characteristic short Gram positive and sporulated bacilli and was identified as *Bacillus* sp. strain based on its 16S rDNA sequence homology analysis.

Response to heavy metals of *Bacillus* sp. XIII strain by the MIC

The effect of the heavy metal on the rhizobacterial *Bacillus* sp. XIII strain's susceptibility was revealed by its bacterial count in the colony; the results showed that Cd was the most toxic metal at its lowest concentration of 0.1 mM, followed by Ni with a relatively toxic effect at concentrations between 0.1 and 1.0 mM. Zn was the heavy metal to which the rhizobacterium were tolerant at a high concentration of up to 5 mM.

Plant growth promoting activity of *Bacillus* sp. XIII strain on *A. affinis* seedlings

This rhizobacteria inoculated to *A. affinis* seeds showed that this strain not only has the ability to bind to seeds, but it can also stimulate growth of *A. affinis* seedlings in the hydroponic culture (Figure 1).

Growth response of *A. affinis* inoculated with rhizobacterial *Bacillus* sp. XIII strain and exposed to cadmium, nickel and zinc

The response of *A. affinis* exposed to Cd, Ni, and Zn showed that this species was susceptible to all the assayed concentrations, as heavy metal concentrations increased.

The results of the root and shoot lengths of *A. affinis* plants inoculated with *Bacillus* sp. XIII strain exposed to cadmium (Figure 2) showed that the rhizobacterium did not favor the growth of the plants at the highest concentrations of this heavy metal in the hydroponic cultures. This response was the same for the plants exposed to Ni, without an increase in the growth of root or shoots of the *A. affinis* plants at higher concentrations of the metal (Figure 3).

For zinc, the plant growth response of *A. affnis* inoculated with the rhizobacterium showed not only growth at 3 and 4 mM, but also a plant growth promoting effect at the 5 mM concentration of this heavy metal (Figure 4).

Determination of the effect of rhizobacteria inoculation on plants of *A. affinis* and the presence of heavy metals by the Tolerance Index

The effect of adding the rhizobacterial *Bacillus* sp. XIII strain to *A. affnins* before seeds germination, in presence of different concentrations of the assayed heavy metals was examined (Table 1). The results of these experiments are presented as TI (Tolerance index), to ease comparison of the effects of the different experimental conditions. A TI of 1.0 indicates that the treatment was

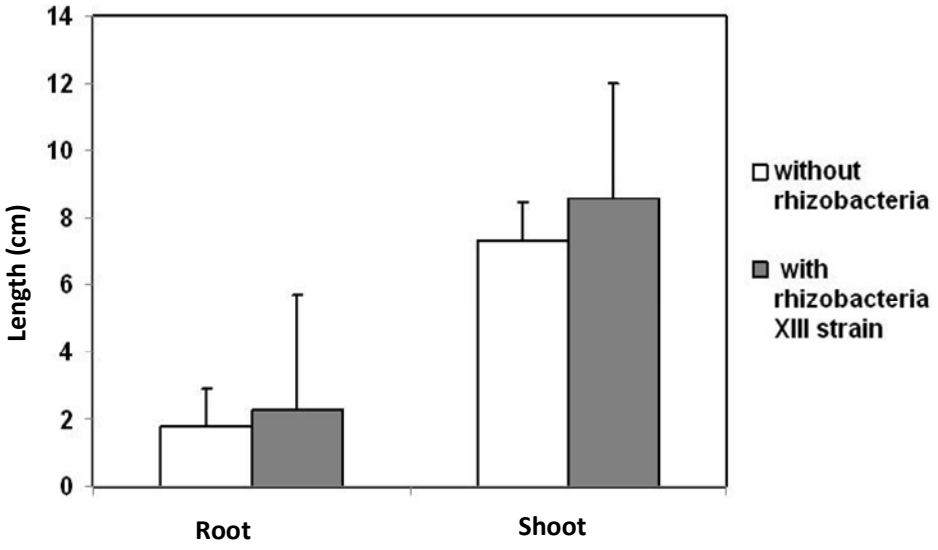

Figure 1. *A. affinis* shoot and root length not inoculated (control) and inoculated with rhizobacterial *Bacillus* sp. XIII strain. Mean values ± S.D. from three replicates are given. No significant differences were found between experiments ($P < 0.05$).

not inhibitory, whereas a TI of 0.1 indicates that the growth of treated plants was only 10% of the growth of the control plants (Burd et al., 1998).

These results show that for all the zinc concentrations tested in the inoculated plants of *A. affinis*, the addition of rhizobacteria increased the growth and tolerance of plants as revealed by their root and shoot lengths.

Multivariate analysis of the response of *Axonopus affinis* plants inoculated with the rhizobacterial *Bacillus* sp. XIII strain and exposed to heavy metals

Surface response graphs were obtained for the analysis of the root and shoot growth of *A. affinis* plants as related with the concentration of the heavy metals in absence (Figure 5a) and presence (Figure 5b) of rhizobacteria. There were no significant differences ($P < 0.005$) between the plant growth and the presence or absence of rhizobacteria in the heavy metal experiments.

DISCUSSION

Plants and bacteria can form specific associations in which the plant provides bacteria with a specific carbon source; this induces bacteria to reduce the phytotoxicity of contaminated soils. Alternatively, plants and bacteria can form nonspecific associations in which normal plant processes stimulate the microbial community, which in the course of its normal metabolic activity degrades contaminants in the soil. Plant roots can provide root exudates, as well as increase ion solubility. These biochemical mechanisms increase the remediation activity of bacteria associated with plant roots (Jing et al., 2007).

Multiple metal-resistance in bacteria seems to be the rule rather than the exception. Abou-Shanab et al. (2005) tested the patterns of tolerance to heavy metals in 107 rhizobacterial isolates at 1 mM concentrations and found that all the rhizobacterial strains were tolerant to multiple metal ions. Strains with hexa-, penta-, tetra- and tri-metal ions tolerance, respectively, were found more frequently than those with hepta-, double- and mono-tolerance. Notably, cadmium, copper, lead, and nickel resistance seemed to be restricted to those strains that were resistant to six metals or more. Sabry et al. (1997) have previously reported similar observations for rhizobacteria.

Abou -Shanab et al. (2005) investigated the correlation between metal resistance and metal mobilization abilities of rhizobacteria under heavy metal stress. The highest incidence of the biochemical activity of isolates and metal resistance was recorded for phosphate solubilizers with Cr, Zn and Pb (92.5, 82.2 and 68.2%, respectively); this implies that phosphate solubilization is not the only mechanism adopted by bacteria towards metals in soil but that siderophores and acid production are also involved in mobilizing metals.

It seems reasonable to assume that plant growth promoting rhizobacteria could be developed as inoculants to increase plant biomass and thereby, to stabilize and remediate metal polluted soils. Such soils can be made nutrient-rich by applying metal-tolerant microorganisms, especially plant growth promoting rhizobacteria, which would provide not only the essential nutrients to the

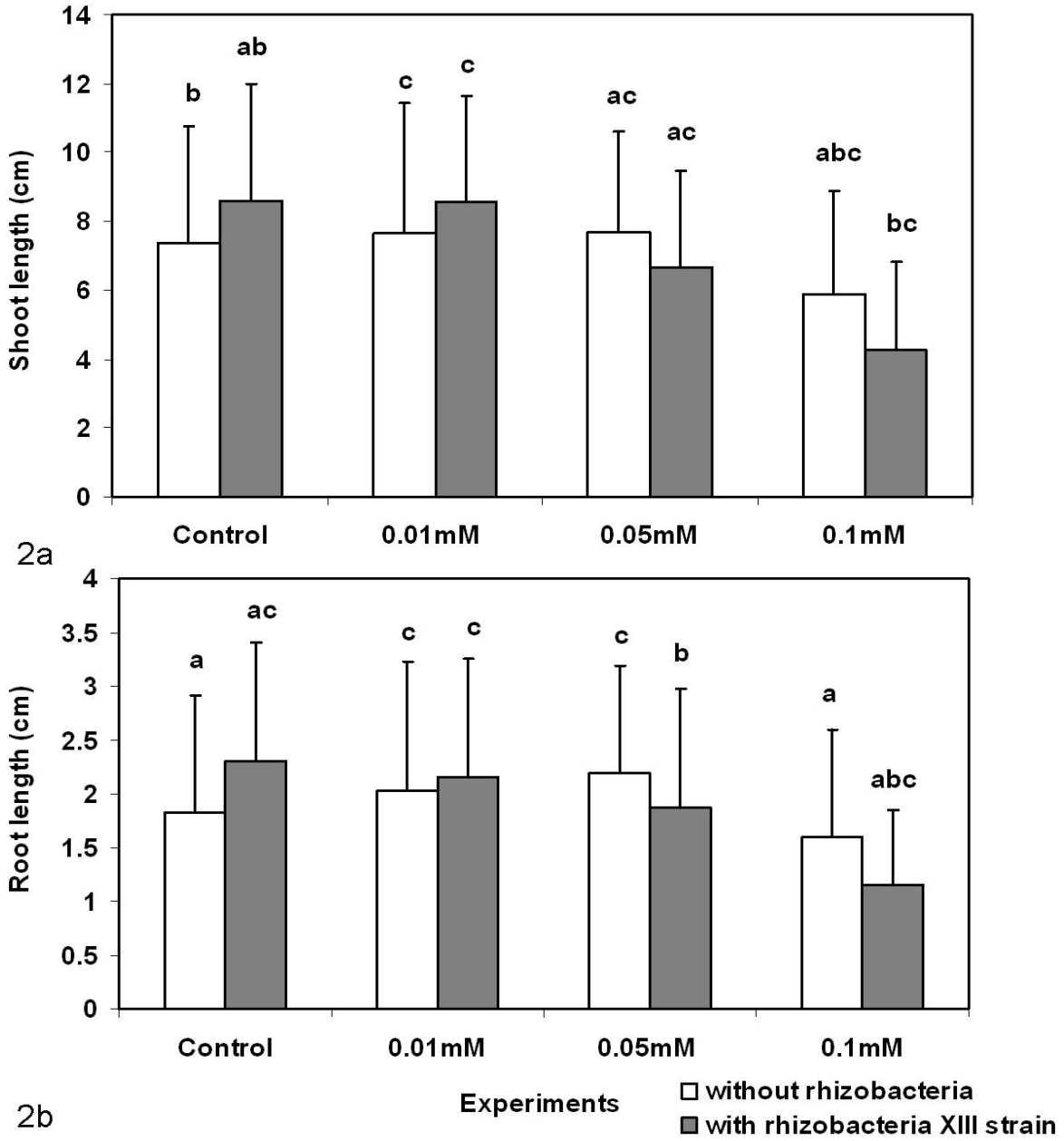

Figure 2. Effect of Cd on the shoot length (2a) and root length (2b) of *A. affinis* plants, without and with rhizobacterial *Bacillus* sp. XIII strain. Mean values ± S.D. from three replicates are given. The different lower-case letters indicate significant differences between experiments ($P < 0.05$).

plants growing in the contaminant sites but would also play a major role in detoxifying heavy metals (Mayak et al., 2004) and thus help plants capable of remediating heavy metals-contaminated soils (Glick, 2003).

Finally the current perspective about this research can resume in Karami and Shamsuddin (2010) comments, where they mentioned that the indirect impact of PGPR is usually achieved by increasing the plant tolerance to diseases (Guo et al., 2004); is important to extend the efficient use of PGPR and no limiting it to slight and moderately contaminated sites (Wu et al., 2006), They noted that the most important limiting factor for the application of PGPR is their tolerance to the concentration of heavy metals and the PGPR population between plants could be different among the same species in the contaminated soils, or even between the different growing stages of an individual plant. These authors mentioned too that several numbers of new researches

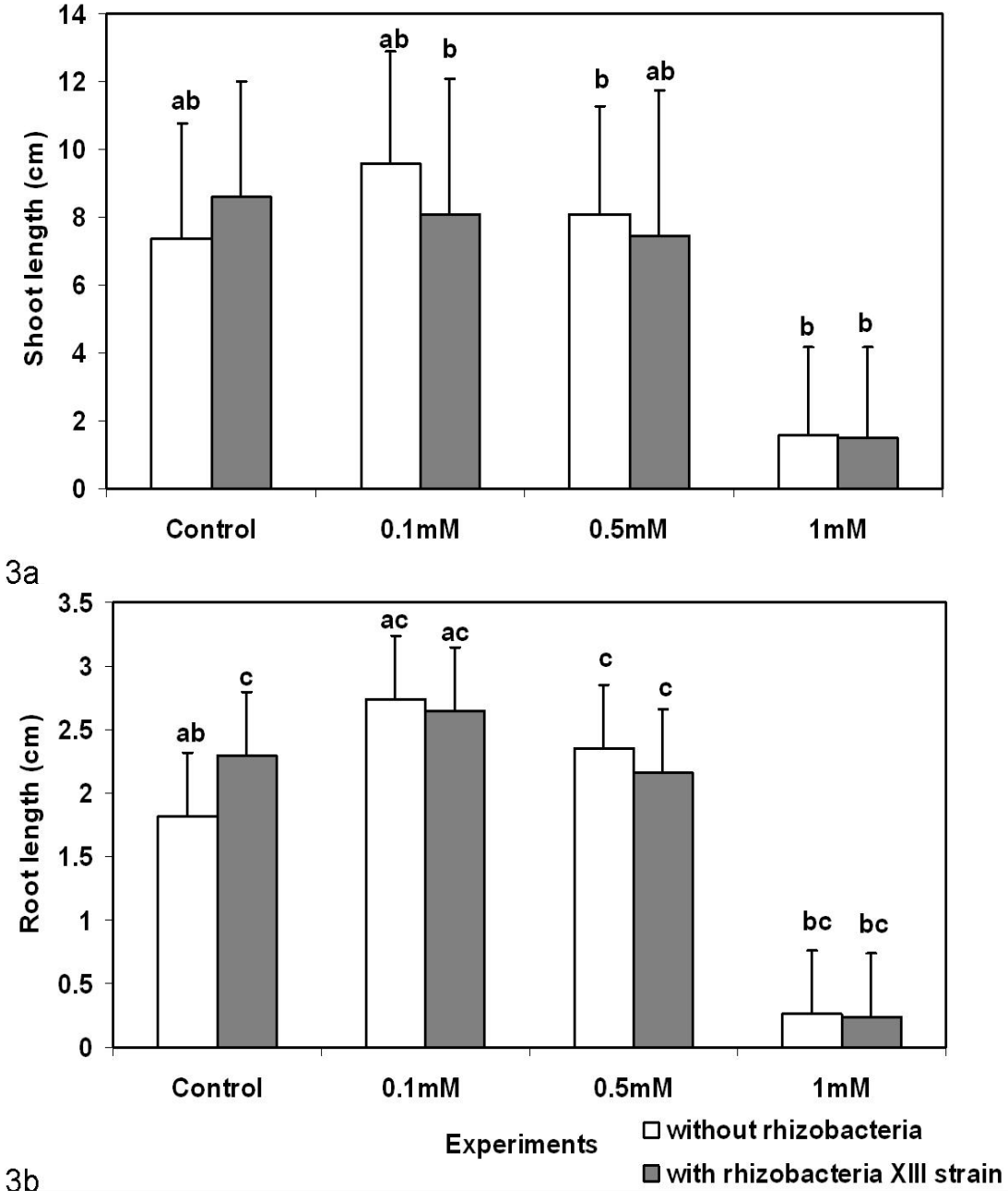

Figure 3. Effect of Ni on the shoot length (3a) and root length (3b) of *A. affinis* plants, without and with rhizobacterial *Bacillus* sp. XIII strain. Mean values ± S.D. from three replicates are given. The different lower-case letters indicate significant.

carried out in relation to the effects of PGPRs on the growth of plants and/or heavy metal concentrations in contaminated soils are reported today (Karami and Shamsuddin, 2010).

Some rhizobacteria can reduce the toxicity of heavy metals, resulting in the stimulation of plant growth. Several established studies indicate that PGPR can promote the growth of plants under the toxicity of Ni, Pb, or Zn. In addition, a variety of bacteria (mainly PGPR) have been reported as phytoextraction assistants: *Pseudomonas* spp., *Bacillus* spp., *Mesorhizobium* sp., *Microbacterium* spp., *Rhizobium* spp., *Variovorax* sp., *Rhodococcus* sp., *Psychrobacter* spp., *Flabobacterium* sp., *Sinorhizobium* sp., and *Achromobacter* sp. The exploration of new microbial resources, including PGPR, is still necessary for the development of *in situ* remediation

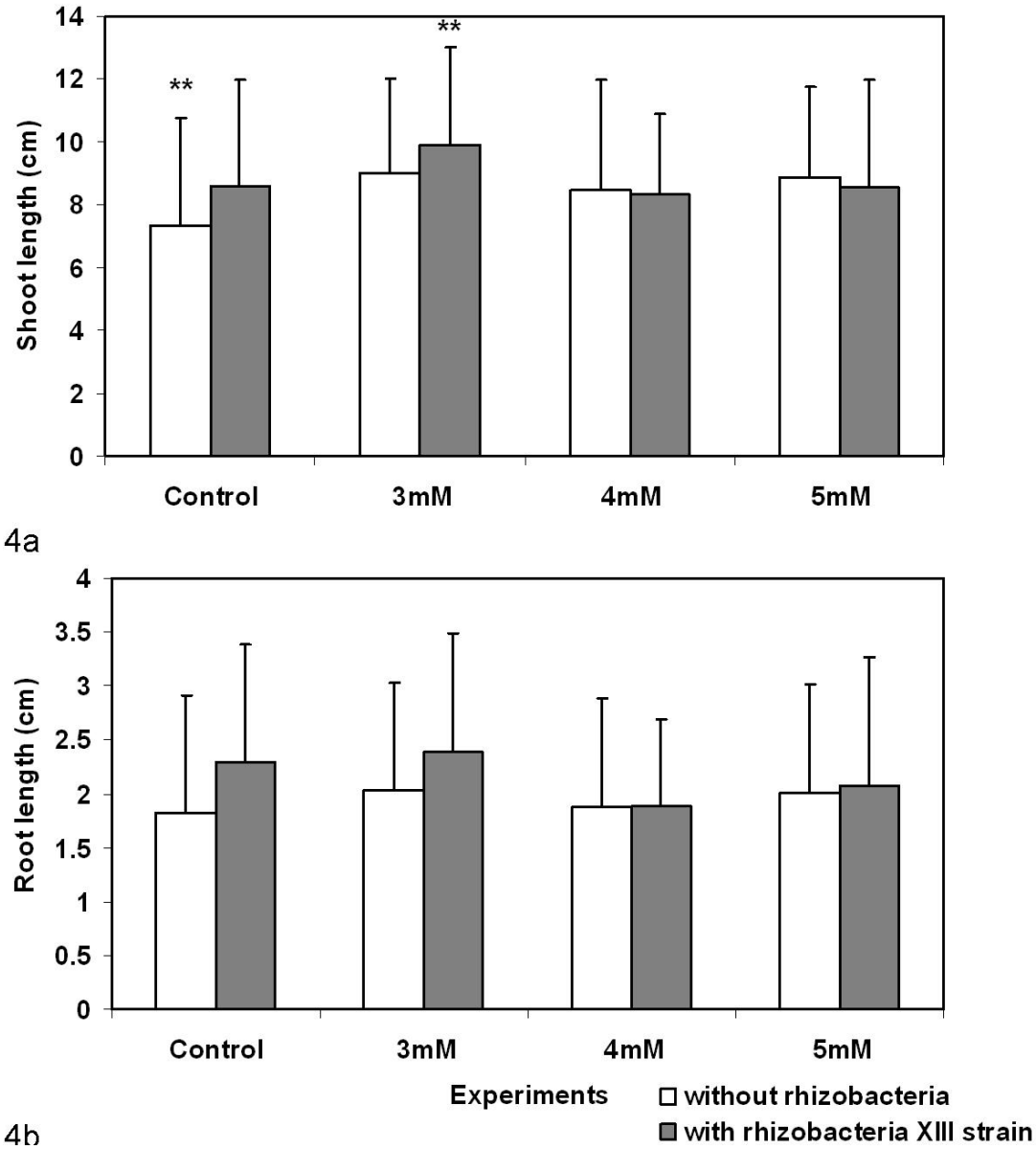

Figure 4. Effect of Zn on the shoot length (4a) and root length (4b) of *A. affinis* plants, without and with rhizobacterial *Bacillus* sp. XIII strain. Mean values ± S.D. from three replicates are given. The asterisks indicate significant differences between experiments ($P < 0.05$).

strategies under multifarious conditions and a better understanding of the interaction between PGPR and their host plants is important for enhancing the efficiency of microbe-assisted phytoremediation (Koo and Cho, 2009). Examples of the state of the art of the PGPR's research and their association with plants for the phytoremediation technologies as rhizoremediation, are the works of Koo and Cho, (2009) with a PGPR, *Serratia* sp. isolated from heavy-metal-contaminated soils and characterized by its plant growth-promoting abilities and the effects of its inoculation on the growth of *Zea mays* determined under both heavy metal-contamination and non contamination conditions, the Belimov et al. (2005) studies about the isolated and characterized Cd-tolerant bacteria associated with the roots of the metal accumulating plant *Brassica juncea* L. Czern. grown in heavy metal contaminated soils, making the selection of PGPR strains which might be useful to increase plant biomass production under unfavourable environmental conditions. Ma et al. (2009) isolated and characterized Ni-resistant bacteria associated with the rhizosphere of *Alyssum serpyllifolium* and *Phleum phleoides* grown in serpentine

Table 1. Tolerance index (TI) of the *A. affinis* plants inoculated with rhizobacterial *Bacillus* sp. XIII strain and exposed to heavy metals.

Cd (mM)				
	Organ	**0.01**	**0.05**	**0.1**
Without rhizobacteria	Shoot	1.03	1.04	0.79
	Root	1.11	1.20	0.87
With rhizobacteria	Shoot	1.16	0.90	0.57
	Root	1.18	1.02	0.63
Ni (mM)				
	Organ	**0.1**	**0.5**	**1.0**
Without rhizobacteria	Shoot	1.30	1.09	0.21
	Root	1.5	1.29	0.14
With rhizobacteria	Shoot	1.09	1.0	0.20
	Root	1.45	1.18	0.13
Zn (mM)				
	Organ	**3**	**4**	**5**
Without rhizobacteria	Shoot	1.22	1.15	1.20
	Root	1.11	1.03	1.10
With rhizobacteria	Shoot	1.34	1.12	1.16
	Root	1.31	1.03	1.13

soils, and selected plant growth-promoting bacteria which been useful to increase the plant growth and Ni uptake by *Brassica* species (*B. juncea* and *B. oxyrrhina*) in soil. He et al. (2009) characterized the Cd-resistant bacteria and evaluated the enhancement of plant growth promotion and observed that Cd and Pb uptakes in Cd-hyper-accumulator tomato plants grown in heavy metal-contaminated soil, improved the efficiency of phytore-mediation of Cd-contaminated soils.

Grandlic et al. (2009) mentioned that plant growth promoting rhizobacteria are introduced to seeds prior to planting to enhance one or more aspect of plant growth through a number of potential mechanisms and recently, they have demonstrate that these rhizobacteria can enhance plant biomass production in mine tailings at lower than optimal compost rates which represents a potential resource and cost savings (Grandlic et al., 2008). They mentioned too that the vast majority of previous studies have prepared seeds for inoculation by surface sterilization prior to introducing desired strains of PGPR, this event leads to a competitive advantage in colonizing the surface of the seed; as the first step of their influence to the development of plants making contribution of activities such as siderophore or plant hormone production.

Xiong et al. (2008) mentioned that there is little infor-mation available about the effects of terrestrial metal hyperaccumulating plants rhizospheric or rhizoplanic bacteria on multi- metals uptake by plants rooted in aquatic systems, so this research is needed to under-stand the role of microorganisms in the uptake of metals under both soil and hydroponics conditions. The knowledge from previous studies on *Sedum alfredii* focused on the physiological mechanism of heavy metal uptake and the application of phytoextraction of this species from contaminated soils were obtained, while the role of the rhizospheric bacteria in the plant tolerance against heavy metal toxicity and the removal of heavy metal from aqueous medium were ignored. Their research were to examine the possible effects of rhizospheric bacteria on plant growth and metal removal from wastewater by *S. alfredii* and the other possible factors involved in heavy metal accumulation.

In this work, the effect of the rhizobacterial *Bacillus* sp. XIII strain on the growth and development of *A. affinis* did not show a significant difference on root and shoot growth between the control and the Cd-exposed plants. This response is comparable with the results of Sheng and Xia (2006) and Belimov et al. (2005) with *Brassica juncea* L. and *Brassica napus*, where the addition of some plant growth promoting rhizobacteria favored the development of these species. Other examples are *Variovorax paradoxus*, *Rhodococcus* sp., and *Flavobacterium* sp., which stimulated root elongation of *Brasica campestris* seedlings either in the presence or absence of toxic cadmium (Belimov et al., 2005), sug-gesting that these bacterial strains could be developed as inoculants to improve growth of the metal-accumulating *B. campestris* in the presence of toxic cadmium concen-tration.

Cadmium, as a non-essential toxic heavy metal to plants, can inhibit root and shoot growth, affect nutrient

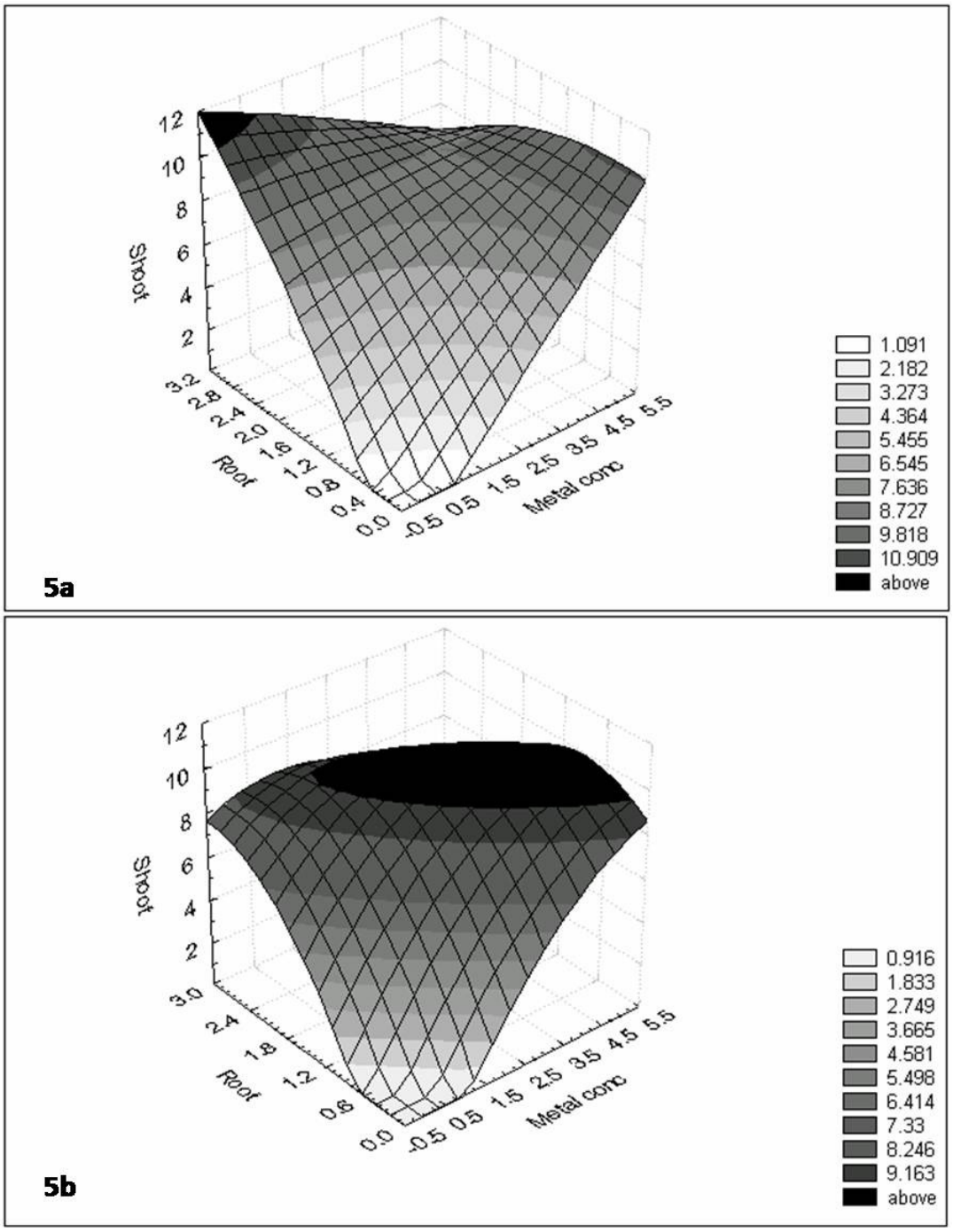

Figure 5. Surface response to evaluate the growth of *A. affinis* without (5a) and with (5b) rhizobacterial *Bacillus* sp. XIII strain and the effect of metals.

uptake and homeostasis and it is frequently accumulated by agriculturally important crops (Sanita di Toppi and Gabrielli, 1999; Jing et al., 2007).

The nickel concentrations tested for *A. affinis* and the presence of the rhizobacterial strain (Figure 3), just as the response to the other two metals, maintained the development of the plants but not the increment in their growth response, agreeing with the results reported by Burd et al. (1998) with the plant growth promoting rhizobacterium *Kluyvera ascorbata* SUD165 isolated from metal contaminated wetland, which, when applied to soil amended with nickel, zinc, lead and chromate, induced

an increase in the growth of *Brasica rapa*, while protecting the plants from nickel toxicity (Burd et al., 1998). Similarly, nickel resistant *K. ascorbata* protected *Lycopersicon esculentum* L., *Brasica campestris*, and *Brasica rapa* plants when grown in soils supplemented with nickel, lead, and zinc (Burd et al., 2000); Zaidi et al. (2006) obtained similar results with *Brassica juncea* and the bacterium *Bacillus subtilis* strain SJ-101.

In this study, all concentrations of nickel were toxic to plant development, even when the *A. affinis* plants were inoculated with the rhizobacterial *Bacillus* sp. XIII strain.

In the case of Zn, for all the experiments, root and shoot lengths of *A. affinis* were promoted. The presence of Zn favored also the growth of the plants and in the experiments with bacteria and the metal, the difference between the roots and shoot growth was not significant ($P < 0.05$). Only a slight decrease of plants growth was observed with the 4 mM zinc concentration for the inoculated and non-inoculated seeds.

Some plant growth-promoting rhizobacteria can significantly increase the growth of plants in the presence of heavy metals, including nickel, lead and zinc (Burd et al., 1998, 2000), thus allowing plants to develop longer roots and get better established during early stages of growth (Glick et al., 1998). Once the seedling is established, the bacterium can also help the plant acquire sufficient iron for optimal growth.

When plant growth promoting rhizobacteria, used as seed inoculants, were applied to soil, either treated/ amended intentionally with metals or already contaminated, a substantial reduction was observed in the toxicity of metals, as well as a concomitant improvement of the overall growth and yield of species, such as *Cicer arietinum* (Gupta et al., 2004), *Vignia radiata* L. *wilczek* (Wani et al., 2007), and *Pisum sativum* (Wani et al., 2008a). Besides their role in protecting the plants from metal toxicity, plant growth promoting rhizobacteria are also well known for their role in enhancing soil fertility and promoting crop productivity by providing essential nutrients (Zaidi et al., 2003; Zaidi and Khan, 2006).The use of such microorganisms possessing multiple properties of metal resistance/reduction and ability to promote plant growth through different mechanisms in metal-contaminated soils make them one of the most suitable choices for bioremediation studies (Khan et al., 2009).

Metal tolerant growth promoting rhizobacteria have also shown a substantial protection to plants against metal toxicity and consequently, improved the growth, symbiosis and seed yield of plants (Chaudri et al., 2000; Wani et al., 2008a, 2008b). The increase in the growth of agronomically important crops grown in metal-stressed soils by applying metal tolerant rhizobacteria has been attributed to the ability of rhizobacterial strains to mitigate the toxic effects of metals using mechanisms such as production of siderophores besides providing plants with the sufficient amounts of growth promoting substances (Khan et al., 2009).

This work demonstrates that the presence of heavy metals plus the PGPR assure the survival of plants. This suggests that the plant response is related with the metal concentration and the exposure time to the contaminants, as well as with its intrinsic tolerance. The presence of the *Bacillus* sp. strain induces growth maintenance of *A. affinis* and its tolerance to the heavy metals: cadmium, nickel and zinc, suggesting a synergistic effect between this species and the rhizobacteria on the response to the contaminants. Plant growth-promoting rhizobacteria, may eventually find a use in the development of phyto-remediation strategies to treat plants to increase their biomass and stabilize and remediate metal-contaminated soils.

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REFERENCES

Abou-Shanab RA, Angle JS, Delorme TA, Chaney RL, Verkum P van, Moawad H, Ghanem K, Ghozlan HA (2003). Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale*. New Phytol. 158: 219-22.

Abou-Shanab RA, Ghozlan H, Ghanem K, Noawad H (2005). Behavior of bacterial populations isolated from rhizosphere of *Diplachne fusca* dominant in industrial sites. World J. Microb. Biot. 21: 1095-1101.

Aceves J (1993). GraphPad Software. GraphPad InStat, V2.03.

Allers T, Linchen M (2000). A method for preparing genomic DNA that restrains branch migration of Holiday junctions. Nucleic Acids Res. 15: 28-36.

Ahmad P, Wani AE, Saghir MD, Khan AE, Zaidi A (2008). Effect of metal-tolerant plant growth-promoting *Rhizobium* on the performance of pea grown in metal-amended soil. Arch. Environ. Contam. Toxicol. 55:33-42.

Amezcua-Allieri MA, Rodríguez-Vázquez R (2008). Impact on metal Bioavailability and plant Uptake during the bioremediation of a phenanthrene-contaminated soil. Revista Terra Latinoamericana. 26: 351-359.

Belimov AA, Kunakova AM, Safronova VI, Stepanok VV, Yudkin LY, Alekseev YV, Kozhemyakov AP (2004). Employment of rhizobacteria for the inoculation of barley plants cultivated in soil contaminated with lead and cadmium. Microbiology (Moscow). 73: 99-106.

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR (2005). Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea L.* Czern.). Soil Biol. Biochem. 37: 241-250.

Burd GI, Dixon DG, Glick BR (1998). A plant growth-promoting bacterium that decreases nickel toxicity in seedlings. Appl. Environ. Microbiol. 64: 3663-3668.

Burd GI, Dixon DG, Glick BR (2000). Plant growth promoting bacteria that decrease heavy metal toxicity in plants. Can. J. Microbiol. 46: 237-

245.
Cleveland WS (1994). The elements of graphing data. AT&T Bell Laboratories, New Jersey, p. 297.
Chaudri AM, Allain CM, Barbosa-Jefferson VL, Nicholson FA, Chambers BJ, McGrath SP (2000). A study of the impacts of Zn and Cu on two rhizobial species in soils of a long term field experiment. Plant Soil. 22: 167-179.
Ernst-Frizzo TC, Porto ML (2004). Zoneamento da vegetação e sua relação com a ocorrência de estruturas mineralizadas na mina Volta Grande, Lavras do Sul, RS, Brasil. IHERINGIA, Sér. Bot. Porto Alegre. 59: 5-12.
Gadd GM (1992). Metals and microorganisms: a problem of definition. FEMS Microbiol. Lett. 100: 197-204.
Giller KE, Witter E, McGrath SP (1998). Toxicity of heavy metals to microorganisms and microbial process in agricultural soils: a review. Soil Biol. Biochem. 30: 1389-1414.
Glick BR, Penrose DM, Li JP (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. 190: 63-68.
Glick BR (1995). The enhancement of plant growth by free living bacteria. Can. J. Microbiol. 41: 109-117.
Glick BR (2003). Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotech. Adv. 21:383-393.
Grandlic CJ, Mendez MO, Chorover J, Machado B, Maier RM (2008). Plant growth-promoting bacteria for phytostabilization of mine tailings. Environ. Sci. Technol. 42: 2079-2084.
Grandlic CG, Palmer MW, Maier RM (2009). Optimization of plant growth-promoting bacteria-assisted phytostabilization of mine tailings. Soil Biol. Biochem. 41: 1734-1740.
Guo J, Qi H, Guo Y, Ge H, Gong L, Zhang L (2004). Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. Biol. Control, 29: 66-72.
Gupta DK, Rai UN, Sinha S, Tripathi RD, Nautiyal BD, Rai P, Inouhe M (2004). Role of *Rhizobium* (CA-1) inoculation in increasing growth and metal accumulation in *Cicer arietinum* L. growing under fly-ash stress condition. Bull. Environ. Contamin. Toxicol. 73: 424-431.
He LY, Chen ZL, Ren GD, Zhang YF, Qian M, Sheng XF (2009). Increased cadmium and lead uptake of a cadmium hyperaccumulator tomato by cadmium-resistant bacteria. Ecotox. Environ. Saf. 72: 1343-1348.
Jing Yan-de, Zhen-li HE, Xiao-e Y (2007). Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils. J. Zhejiang Univ. Sci. B. 8: 192-207.
Karami A, Shamsuddin ZHj (2010). Phytoremediation of heavy metals with several efficiency enhancer methods. Afr. J. Biotechnol. 25: 3689-3698.
Khan AG (2004). Mycotrophy and its significance in wetland ecology and wetland management. In: Wong MH (ed) Developments in ecosystems, vol 1. Elsevier, Northhampton, pp. 97-114.
Khan MS, Zaidi A, Wani PA, Oves M (2009). Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. Environ. Chem. Lett. 7: 1-19.
Kloepper JW, Schroth MN (1978). Plant growth promoting rhizobacteria on radishes, fourth international conference on plant pathogen bacteria, vol 2, Angers, France, pp. 879-882.
Koo SY, Cho KS (2009). Isolation and Characterization of a Plant Growth-Promoting Rhizobacterium *Serratia* sp. SY5. J. Microbiol. Biotechnol. 19: 1431-1438.
Kuo YJ, Chang YS, Lila MA, Chiu HY (2005). Screening growth and root formation in cadmium-treated turfgrass using a whole-plant microculture system. J. Plant Nutr. 8: 1041-1048.
Lakzian A, Murphy P, Turner A, Beynon JL, Giller KE (2002). *Rhizobium leguminosarum* bv. *viciae* populations in soils with increasing heavy metal contamination: abundance, plasmid profiles, diversity and metal tolerance. Soil Biol. Biochem. 34: 519-529.
Lloyd JR, Lovley DR (2001). Microbial detoxification of metals and radionuclides. Curr. Opin. Biotechnol. 12: 248-253.
Lovley DR, Coates JD (1997). Bioremediation of metal contamination. Curr. Opin. Biotechnol. 8: 285-289.
Ma Y, Rajkumar M, Freitas H (2009). Improvement of plant growth and nickel uptake by nickel resistant-plant-growth promoting bacteria. J. Hazard. Mater. 166: 1154-1161.
Mayak S, Tirosh S, Glick BR (2004). Plant growth promoting bacteria that confer resistance to water stress in tomatoes and peppers. Plant Physiol. 166: 525-530.
Nies DH (1999). Microbial heavy metal resistance. Appl. Microbiol. Biotechnol. 51: 730-750.
Olivares SE (1989). Paquete de diseños experimentales FAUANL. Versión 1.4. Facultad de Agronomía UANL. Marín, N.L. Mexico.
Olson JW, Mehta NS, Maier RJ (2001). Requirement of nickel metabolism protein HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. Mol. Microbiol. 39: 176-182.
Sabry SA, Ghozlan HA, Abou-Zeid DM (1997). Metal tolerance and antibiotic resistant patterns of a bacterial population isolated from sea water. J. Appl. Microbiol. 82: 245-252.
Sanita di Toppi L, Gabrielli R (1999). Response to cadmium in higher plants. Environ. Exp. Bot. 41: 105-130.
Sheng XF, Xia JJ (2006). Imrpovement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. Chemosphere, 64: 1036-1042.
Wani PA, Khan MS, Zaidi A (2007). Cadmium, chromium and copper in greengram plants. Agron. Sustain. Dev. 27: 145-153.
Wani PA, Khan MS, Zaidi A (2008a). Effect of metal tolerant plant growth promoting *Rhizobium* on the performance of pea grown in metal amended soil. Arch. Environ. Contam. Toxicol. 55: 33-42.
Wani PA, Khan MS, Zaidi A (2008b). Chromium reducing and plant growth promoting *Mesorhizobium* improves chickpea growth in chromium amended soil. Biotechnol. Lett. 30: 159-163.
Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991). 16Ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 131: 697-703.
Whiting SN, De Souza M, Terry N (2001). Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thalaspi caerulescens*. Environ. Sci. Technol. 35: 3144-3150.
Wilkins DA (1978). The measurement of tolerance to edaphic factors by means of root growth. New. Phytol. 80: 623-633.
Wu SC, Cheung KC, Luo YM, Wong MH (2006). Effects of inoculation of plant growth-promoting rhizobacteria on metal uptake by *Brassica juncea*. Environ. Pollut. 140: 124-35.
Xiong J, He Z, Liu D, Mahmood Q, Yang X (2008). The role of bacteria in the heavy metals removal and growth of *Sedum alfredii* Hance in an aqueous medium. Chemosphere, 70: 489-494.
Zaidi A, Khan MS (2006). Co-inoculation effects of phosphate solubilizing microorganisms and *Glomus fasciculatum* on greengram-*Bradyrhizobium* symbiosis. Turk. J. Agric. For. 30: 223-230.
Zaidi A, Khan MS, Amil M (2003). Interactive effect of rhizotrophic microorganisms on yield and nutrient uptake of chickpea (*Cicer arietinum* L.). Eur. J. Agron. 19: 15-21.
Zaidi S, Usmani S, Singh BR, Musarrat J (2006). Significance of *Bacillus subtilis* strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. Chemosphere, 64: 991-997.
Zhuang X, Chen J, Shim H, Bai Z (2007). New advances in plant growth-promoting rhizobacteria for bioremediation. Environ. Int. 33: 406-413.