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Bioaccumulation and phyto-translocation of Nickel by *Medicago sativa* in a calcareous soil of Iran

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Abstract

Nickel is a heavy metal distributed ubiquitously in nature. It accumulates in soil as a result of human activities, including mining and industries development. It may be poisonous to plants, humans, animals and microorganisms. The present study was implemented as a factorial experiment with a Randomized Complete Block Design (RCBD), of three replications in calcareous soils of Karaj and in greenhouse conditions for detection of the effect of nickel polluted soil, Ni125, Ni250, Ni500, Ni1000 ($mgkg^{-1}$) in comparison with control (Ni0). The inoculant of resistant native bacteria to nickel in three levels, of: control (B_0), *Bacillus mycoides* $M_1(B_1)$, *Micrococcus roseus* M_2 (B_2) were examine on nickel phytoremediation in alfalfa (*Medicago sativa L.*). Results demonstrated that by increasing the polluted nickel concentration in soil, its absorption by the alfalfa have increased significantly ($P<0.05$). The plant growth and biomass accumulation severely decreased by increasing nickel concentration in soil. Application of native inoculant (B_1 and B_2) resistant to nickel significant increased the nickel concentration in plant shoot compared to control, and also increased the concentration of iron, zinc, copper and manganese in plant shoots. The highest nickel uptake occurred with B_2 inoculant and during the second cutting of the plant growth, which was 350 $\mu gPot^{-1}$.

Keywords: Medicago sativa; Nickel; Bacteria inoculant; Soil pollution; Phytoremediation

1. Introduction

Nickel (Ni) is one of the heavy metals that is naturally released from bedrocks. Generally, Ni contamination of soils is the result of disposal of industrial effluents, sewage sludge and of fertilizers (Alloway, 1990; Panwar *et al.,* 2002). Phytoremediation is the direct use of living plants for *in situ* remediation of contaminated soil, sludges, sediments, and ground water through contaminant removal, degradation, or containment (EPA, 1999). In phytoremediation, structure and natural texture of the soil are preserved while the plant's roots can transfer chemical elements and compounds. Also in this method, a more economical use of the plant material can be made as compared with the chemical and physical remediation methods (Ghosh and Singh, 2005). Phytoremediation potential depends on the soil interactions, heavy metals, bacteria and plant. It is under the influence of such various factors as soil characteristics, plant and bacteria's activity, climate and so on (Yan-de *et al.*, 2007). Soil bacteria play an important role in the recovery of nutrients, development of the soil structure, removal of toxicity resulting from soil chemical activities, control of plant’s pests and also in stimulation of the plant's growth. For developing phytoremediation application, crops with such high biomass production as alfalfa, sunflower,

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maize, canola, pea, wheat, cabbage, oat, barley, indian mustard have been used as substitution samples in different researches (Abou-Shanab *et al*, 2007; Erakhrumen and Agbontalor, 2007; Marchiol *et al.,* 2004; Jankite and Vasarevicius, 2007; Madejon *et al*., 2003). On the other hand, production of low biomass in hyperaccumulator and sensitivity of some other plants' roots to high concentrations of metals requires lots of researches to be performed about the possibility of using microorganisms for development of phytoremediation technology and making this method economical (Ansari and Malik, 2007; Glick, 2003). Research shows that some of Plant Growth Promoting Rhizobacteria (PGPR) could increase absorption of metal by the plants through use of some mechanisms. Metal absorption may be increased by bacteria with production of siderophore, which releases iron and makes other metals movement possible in the soil (Yan-de *et al*., 2007). Also some bacteria would be prevented from production of stress ethylene by producing ACC-deaminase enzyme and decreasing the heavy metals influences in plant's tissues. Cooperation of the plant and bacteria could increase efficiency of using phytoremediation technology (Glick, 2003). The present research was implemented to study the ways of increasing phytoextraction of nickel in *Medicago sativa* (a plant that produces high biomass) and facilitating its translocation from roots to shoots by resistant native bacteria to nickel in Iran's calcareous soils and in greenhouse conditions.

2. Materials and methods

2.1. Characteristics of Resistant Native Bacteria

Bacteria were isolated from polluted soil, then applied to Hepes and Mes culture. Two native strains of bacteria, previously identified resistant to heavy metals in an experiment namely: *Bacillus mycoides* $M_1(B_1)$ and *Micrococcus roseus* M_2 (B_2) were isolated and purified from the soils around lead and zinc mines of Haft Emarat in Arak-Markazi province, at longitude 35º 48' 35'' and latitude 50º 58' 18'' (Holt *et al*., 1994). B_1 isolate showed resistance to Cd, Pb, Ni and Zn, and B2 isolate demonstrated resistance to Cd and Ni. Characteristics of these bacteria and plant growth promoting rhizobacteria (PGPR) are listed in Table 1.

2.2. Selection of soil samples

The soil used in greenhouse experiment was chosen from Campus of Agriculture and Natural Resources, University of Tehran located at Karaj and classified as a fine loamy, super active thermic, xeric haplocambids and mixed. Evaluation of soil N was done through Kjeldal method, (Bremner, 1996), available phosphorus by Olsen Method (Kuo, 1996), available potassium through normal acetate ammonium method (Hemke and Sparks, 1996). Measurement of soil pH was done on saturated extract (Thomas, 1996) and electriced conductivity by Rhoades method (1996), Equal Calcium Carbonate by Bouyoucos method (Bouyoucos, 1962), organic carbon percentage by Walkly Black (Nelson and Sommers, 1982) and texture of the soil through hydrometric method (Bouyoucos, 1962) and cation exchange capacity by Bower method (Sumner and Miller, 1996). Available concentration of nickel, lead and zinc were extracted through DTPA method (Linsay and Norvell, 1978) and measured through Atomic Absorption Spectrometery (AAS). Chemical and physical properties, determined are presented in Table 2.

2.3. Alfalfa greenhouse experiment

The research was implemented as a factorial experiment with a Randomized Complete Block Design (RCBD) of three replications; with five Nickel treatments of (0, 125, 250, 500 and 1000 $mgkg^{-1}$) from Nickel Chloride source ($NiCl_2$. $6H_2O$) and three bacteria inoculants: control (B_0), B_1 and B_2 with equal bacterial population (5×10^8 $cfuml^{-1}$) through seed inoculation. To pollute the soil, the amount of metal was dissolved in 200 ml distilled water and sprayed over each pot layer by layer as evenly as possible. Twelve *Medicago sativa*, (Hamedani C.V.) seeds were panted in each pot after disinfection and germination. The pots were thinned at the first growth stage and six seedlings were kept in each pot. During the plant growth period, irrigation performed on a weight basis (%70±10 of the FC) with distilled water. The growth period of alfalfa was 140 days during which 3 cuts were collected at 46 days interval. After washing the roots by distilled water the fresh weight was measured and then placed in paper bags and dried at 70 ºC. The plant samples were then milled, and concentration of nickel, iron, zinc, copper and manganese measured in nitric acid digestion extracts through ICP-OES, model ICAP-6500 (Madejon *et al*., 2003). Finally, statistical analysis of data in the form of factorial design with random basic design in three replications was done using SAS software and a comparison of means was done with LSD test at 5% level and graphs drawn by use of Excel software.

Table 1. The PGPR characteristics and selection number of different strains used in greenhouse experiment

Bacteria	Positive or Negative Gram	Siderophore[3]	ACC-Deaminase[2]	IAA[1] (ppm)	Resistance to Cd ($1000 mgl^{-1}$)	Resistance to Zn ($1000 mgl^{-1}$)	Resistance to Pb ($1000 mgl^{-1}$)	Resistance to Ni ($1000 mgl^{-1}$)
B0 (distilled water)	-	-	-	-	-	-	-	-
*Bacillus mycoides M*1 (B1)	+	-	+	127	+	+	+	+
Micrococcus roseus M2 (B2)	+	+	+	10	+	-	-	+

1) Patten and Glick, 2002 2) Penrose and Glick, 2001 3) Alexander and Zuberer, 1991

Table 2. Selected properties of the soil used for this study

Characteristics	Value	Characteristics	Value
Soil texture Class	Loam	Total N (%)	0.08
Clay (%)	25.00	Available P ($mgkg^{-1}$)	17.10
Silt (%)	36.00	Available K ($mgkg^{-1}$)	247.00
Sand (%)	39.00	SO4 ($meql^{-1}$)	40.60
pH	7.90	Fe ($mgkg^{-1}$)*	4.28
EC(dSm^{-1})	4.31	Cu ($mgkg^{-1}$)*	4.061
%CaCO3	8.90	Mn ($mgkg^{-1}$)*	8.244
%OC	0.84	Zn ($mgkg^{-1}$)*	0.812
%SP	35.6	Pb ($mgkg^{-1}$)*	2.023
CEC ($cmolkg^{-1}$)	26.00	Ni ($mgkg^{-1}$)*	0.10

* DTPA-Extractable

3. Results and discussion

Table 1 show that the bacteria under this study possess some such appropriate growth promoting characteristics such as capability of producing phyto-hormone auxin, ACC-deaminase enzyme, siderophore. Bacteria, used in the study were distinguished as being resistant to nickel and cadmium. According to Hada and Sizemore (1981), although bacteria may adapt themselves to high concentrations of metals in non-polluted areas, observations show that resistant separators in polluted areas outnumber those in non-polluted areas. Moreover, gram positive and negative bacteria could resist heavy metals (Silver and Misra, 1998). Yan-de *et al*., (2007) declared that Multi Metal Resistance (MMR) trait in bacteria is more effective than mere resistance against a metal. In a similar study in India, Malik and Jaiswal (2000) isolated 45 pseudomonas strains from soils of industrial sewage polluted and non-polluted lands, and determined their morphological and biochemical characteristics. According to Malik and Jaiswal (2000), plant growth promoting rhizobacteria could increase absorption of metals by plants through several different mechanisms.

In figures 1 to 8, triplicate effects of nickel, bacteria and alfalfa cuttings were compared. The highest shoot Ni concentration was observed in B0Ni1000 (first cutting), B2Ni1000 (second cutting) and B1Ni1000 (first cutting) treatments respectively (Fig. 1). The highest shoot Fe concentration observed in B1Ni500, B1Ni1000 (first cutting) and B0Ni10 (third cutting) treatments (Fig. 2). The highest shoot Mn concentration observed in the second cutting of alfalfa and B1Ni1000, B2Ni1000 and B1Ni500 treatments (Fig. 3). The highest shoot Cu concentration observed in B1Ni125, B1Ni1000 (second cutting) and B0Ni0 (third cutting) treatments (Fig. 4). Also the highest shoot concentration of zinc observed in B2Ni0, B2Ni500 (first cutting), and B0Ni1000 (second cutting) treatments (Fig. 5); and the highest Ni-uptake among the three alfalfa cuttings was observed in B2Ni1000 (second cutting), B1Ni500 (first cutting), and B0Ni500 (third cutting) treatments (Fig. 6).

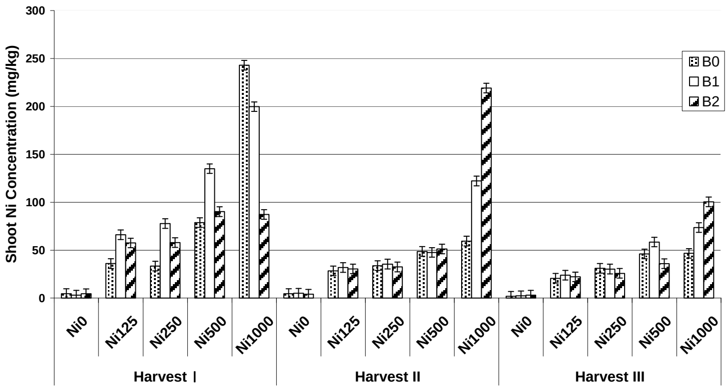

Fig. 1. The interaction effects of nickel, bacteria and alfalfa cutting on shoot Ni concentration

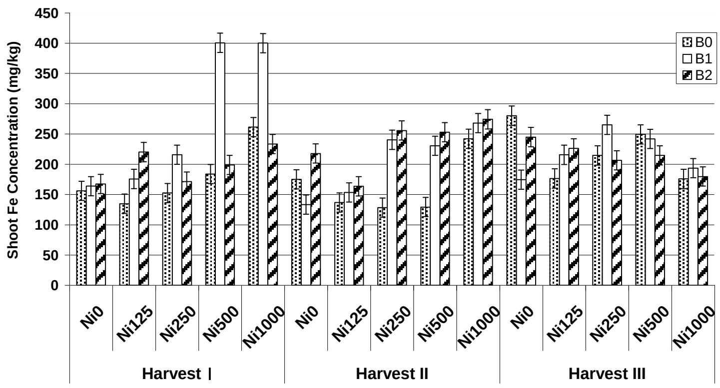

Fig. 2. The interaction effects of nickel, bacteria and alfalfa cutting on shoot Fe concentration

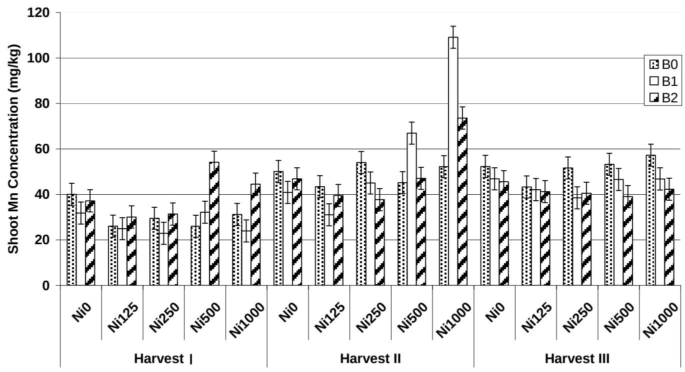

Fig. 3. The interaction effects of nickel, bacteria and alfalfa cutting on shoot Mn concentration

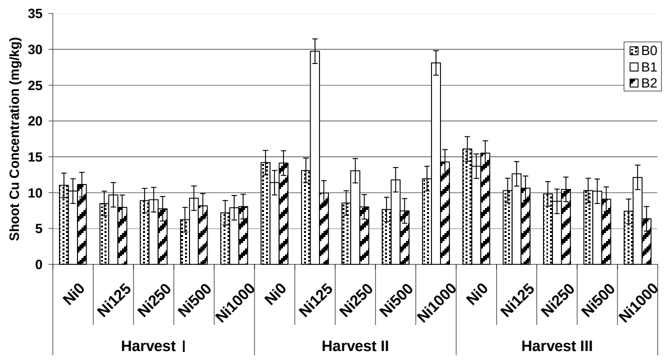

Fig. 4. The interaction effects of nickel, bacteria and alfalfa cutting on shoot Cu concentration

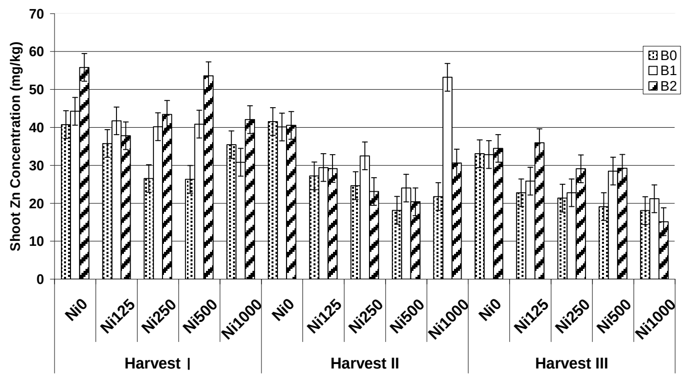

Fig 5. The interaction effects of nickel, bacteria and alfalfa cutting on shoot Zn concentration

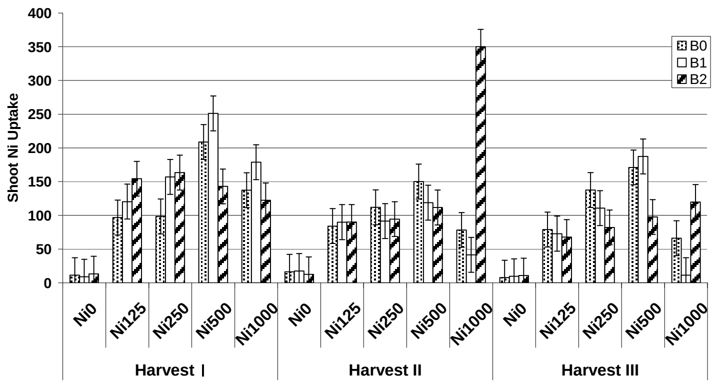

Fig. 6. The interaction effects of nickel, bacteria and alfalfa cutting on shoot Ni-Uptake (μg/Pot)

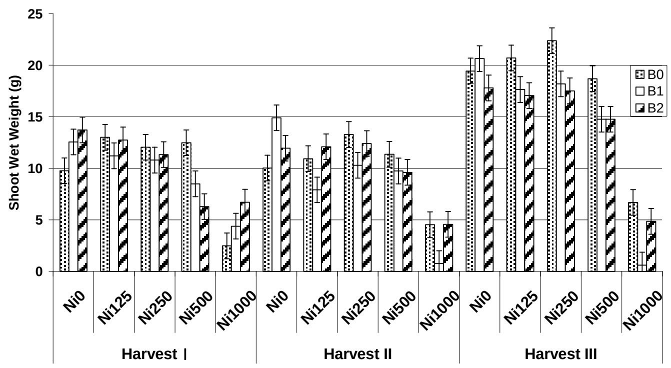

Fig. 7. The interaction effects of nickel, bacteria and alfalfa cutting on shoot fresh weight

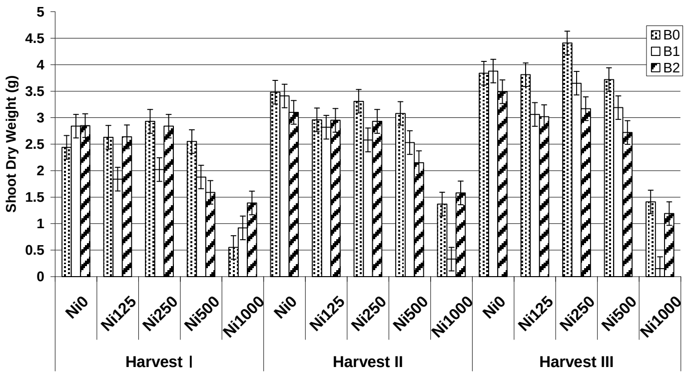

Fig. 8. The interaction effects of nickel, bacteria and alfalfa cutting on shoot dry weight

According to the results shown in Figures 7 and 8, the highest fresh and dry weight of the shoots was observed in the third cutting treated with inoculants. Analysis of variance shows that nickel and bacteria had significant effects on concentrations of nickel and iron in the shoots and as well on the absorption of nickel (Table 3). Also, the bacteria effects on the concentration of nickel, iron, copper, zinc, fresh and dry weight of the shoots were significant when the three cuttings compared.

Glick (2003) reported that plant growth promoting rhizobacteria could have direct and indirect positive effects on plants' growth. In the direct method, plant's growth is increased through an improvement of absorption and synthesis of the nutrients. Plant growth promoting rhizobacteria may increase nitrogen fixation and its availability to plants; and/or increase solubility of iron in the soil through production of siderophore facilitating the absorption of iron by plants. Synthesis of any kind of phyto hormones such as auxines, are among the ways that influence and stimulate plant's growth by plant growth promoting rhizobacteria. Stress ethylene was decreased through ACC-deaminase enzyme synthesis resulting in plant growth. In addition, in an indirect stimulation, available bacteria diminish or prevent the effects of phytopathogens (Glick, 2003). Glick declared the negative effects of high concentrations of heavy metals on the plants' growth due to two problems of: stress ethylene and limitation of iron's concentration in the plants.

As indicated in table 1 and graphs 1 to 8 application of inoculants (B_1 and B_2) improved plant growth and nutrient uptake as well as the metal uptake. Similar results were also reported by Glick (2003) for the effects of inoculants and siderophore on iron uptake by plants. Also the positive effects of plant growth promoting rhizobacteria (PGPR) such as auxin production resulting in root growth and ACC-deaminase production causing growth improvement of plants stress conditions have been shown through other research (Kuffner *et al.*, 2008; Ansari and Malik, 2007).

Table 3. Results of ANOVA characteristics of Bacteria, Nickel and Cut in Alfalfa shoots

MS (Mean Square)									
S.O.V	df	Ni (Shoot)	Fe (Shoot)	Cu (Shoot)	Mn (Shoot)	Zn (Shoot)	Fresh Weight (Shoot)	Dry Weight (Shoot)	Ni Uptake (Shoot)
Bacteria (B)	2	1907.7**	23401.2**	150.46**	2.5ns	676**	31.01*	2.8**	1962ns
Nickel (Ni)	4	58596.3**	22866.6**	92.3**	1253.8**	635.1**	529.9**	22.5**	81897**
Cut (C)	2	21872.8**	4047.6**	263.3**	4597.9**	2262.2**	487.4**	8.19**	20603**
B*Ni	8	335.3**	9819.7**	46.12**	232.4**	63.5ns	23.08**	0.91**	12348**
C*B	4	3763.2**	15928**	83.85**	739.3**	236**	15.2**	0.55**	8946**
C*Ni	8	3761.8**	14859.6**	69.96**	603.3**	177.5**	33.4**	0.65**	6122**
C*Ni*B	16	4434.4**	3926.9**	20.7**	319.9**	112.6**	5.5ns	0.22ns	6700**
Error Experimental	60	75.2	758.9	8.7	70.7	39.9	4.6	0.14	2004.8

*, ** and ns in order significant different at level 5% and 1% and non significant

Observance of the results in Figure 6, demonstrates that application of inoculants have the positive effects of absorbing and translocating nickel to the shoots, increasing of its absorption through use of inoculants. Considering the production of biomass in alfalfa and the possibility of obtaining several cuttings, alfalfa is appropriate for phytoremediation by being resistant to nickel in concentrations of 500 and 1000 $mgkg^{-1}$. Toxicity of nickel, becoming evident as dry and fresh weight of the shoots in alfalfa decreased significantly (Figures 7 and 8). Application of inoculants had the positive effects on increasing concentrations of copper (Figure 4), iron (Figure 2), manganese (Figure 3) and zinc (Figure 5), showing the facilitation of absorption and translocation of the plant's necessary metals in contrast with heavy metals' stress in alfalfa. These results confirmed other investigators' reports in this field. So, Erakhrumen and Agbontalor (2007) emphasized on the usage of alfalfa next to tall fescue for remediation of heavy metal polluted soils and waters. Usage of this plant for phytoremediation of heavy metals from soil by phytoextraction method has been reported in Utah State. Yan-de *et al* (2007), reported usage of resistant strains with plant growth promoting (PGP) abilities for confronting toxicity of nickel, cadmium, zinc, copper, cobalt, chrome and lead in the cereals; they declared that an ideal plant for phytoremediation should grow fast, have high production of biomass and resistance to high concentrations of shoot metals. Thus, high biomass producing plants (such as alfalfa), beside using PGPR, could be effective in phytoremediation technology. Growth of the plant's roots could increase as a result of production of auxin (IAA) by plant growth promoting rhizobacteria. Also, existence of ACC-deaminase enzyme with decreasing of stress ethylene stimulates the plant's growth, so assists in less toxicity of heavy metals in plant.

4. Conclusion

Altogether, from two aspect points of view, alfalfa can be regarded as a suitable plant for future phytoremediation research: On one hand, the considerable biomass production of alfalfa, and on the other, the effects of the applied resistant native bacteria’s inoculant. Results showed that the highest Ni concentration in the three cuttings of alfalfa was recorded for the first cutting of alfalfa grown in the soils polluted at a rate of 1000 $mgkg^{-1}$ Ni. Also usage of inoculant, increased Ni, Fe and Zn in alfalfa shoots. Finally, the highest uptake level of Ni was shown in the second cutting, with inoculant employed in the treatments of 1000 $mgkg^{-1}$ Ni pollution. This is in agreement with the reports of many studies (Aleem *et al.*, 2003; Ansari and Malik, 2007; Kuffner *et al.*, 2008). Finaly according to the results obtained the native and resistant bacteria, by their effective growth promoting abilities, especially providing necessary iron for the plants by production of microbial siderophores, influencing phytopathogens, could bring out the drought and salinity resistance and also producing indole acetic acid (IAA), and stimulating root, caused enhancement in translocation of metal from soil to the plant and as a result increased the efficiency of phytoremediation.

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