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Study of the increase in phytoremediation efficiency in a nickel polluted soil by the usage of native bacteria: *Bacillus safensis* FO.036b and *Micrococcus roseus* M_2

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ABSTRACT
Nickel (Ni) is a heavy metal and soil pollutant but existence of small amount of it as a metallic part of urease enzyme in the plants is necessary. Remediation of spots contaminated with heavy metals is particularly challenging. Phytoremediation, the use of plants for environmental restoration, is a novel clean up technology. In this study, five levels of nickel [control (Ni_0), Ni_{125}, Ni_{250}, Ni_{500} and Ni_{1000} (mg kg^{-1})] as nickel chloride ($NiCl_2.6H_2O$) and three levels of bacterial inoculants [control (B_0), *Bacillus safensis* FO.036b (B_1) and *Micrococcus roseus* M_2 (B_2)] were used in sunflower (*Helianthus annus*), amaranthus (*Amaranthus retroflexus*) and alfalfa (*Medicago sativa*) for phytoextraction of nickel. A factorial experiment with a randomized complete block design (RCBD) with three replications was used. Results demonstrated that by increasing the nickel concentration in soil, its absorption by the plants has increased significantly. The highest concentration of nickel was found in shoot of amaranthus (176.83 mg kg^{-1}) and in the root of plants, in alfalfa (462.73 mg kg^{-1}) by usage of inoculant ($P<0.05$). The highest absorption of nickel occurred with B_1 inoculant in amaranthus, which was 459.41 µgPot^{-1}. Applying this inoculant may also cause an increase in concentration of iron and zinc in the root and shoot of the plants.

Keywords: *Bioaugmentation, Heavy metal, Nickel, Phytoremediation, Soil pollution.*

INTRODUCTION
Soil pollution, is a very important environmental problem and it has been attracting considerable attention in recent years (Garbisu and Alkorta, 2001; Marques *et al*., 2009). Industrial operation such as smelting, mining, metal forging, manufacturing of alkaline storage batteries, combustion of fossil fuel, utilization of fertilizers and pesticides and disposal of waste cause phenomenal increase in the extent of heavy metals in the environment (Alloway, 1995, McIlveen and negusanti, 1994). Nickel toxicity may be the cause of a number of biological and physiological processes in plants. Wilting and leaf necrosis have been described as typical visible symptoms of Ni^{2+} toxicity (Llamas *et al.,* 2008). Nickel concentration in non polluted soils is between 5-50 mg kg^{-1}, and in the plants is between 0.4-3 mg kg $^{-1}$ (Prasad, 2004). Phytoremediation is the technology of using plants to purify the environment (Yan-de *et al*., 2007).

Plant growth-promoting rhizobacteria (PGPR) are bacteria capable of promoting plant growth by colonizing the plant root (Abou-Shanab *et al.,* 2006; Shing and Xia, 2006). Recently, the application of PGPR has been extended to remediate contaminated soils in association with plants. First findings of using the plants to purify the soils relates to removal of nickel from soil by mustard and canola (Glick, 2003). According to the researchers' reports, inoculation of canola seeds with plant growth promoting rhizobacteria (PGPR) and resistance to nickel with the capability of producing 1-aminocyclopropane- 1-carboxylate (ACC) deaminase enzyme, caused decrease in the production of tension ethylene and stimulation of germination and growth in plants (Burd *et al.,* 1998). Synthesis of different kinds of phytohormones such as auxin and cytokinin, is a way to influence and stimulate growth of the plants by plant growth promoting rhizobacteria. Masalha *et al*. (2000) reported that growing of plants in non-sterile soils is better than sterile soils for iron nutrition. These results reveal the role of bacterial communities in iron nutrition. So, in addition to providing iron, siderophores decrease nickel poisoning (Yan-de *et al*., 2007). In a field study, using siderophores-producing bacteria in the soils infected with nickel compounds, it was observed that mustard seeds germinated in these soils and the size of the plant increased by 50% to 100% by adding a soil inoculant (Burd *et al*., 2000). Madhaiyan *et al* (2007) reported that the plant growth promoting bacteria *Methylobacterium oryzae* and *Burkholderia sp*. reduce the toxicity of Ni and Cd in tomato and promote plant growth. Examining the potential of metal absorption in domestic and exotic plants, biotic study and separation of native bacteria, resistant to heavy metals and with plant growth promoting properties has played a very important role in increasing efficiency of phytoremediation. Thus, with regard to the aforesaid issues and the importance of the subject as well as the need to identify approaches to increase efficiency of phytoremediation, this study was conducted with the following objectives:

1- Separation and identification of native bacteria, resistant to nickel and examination of their plant growth promoting properties
2- Examination of possibility of increasing phytoremediation in the Ni-polluted soil by inoculants of resistant native bacteria

MATERIALS AND METHODS

Nickel (Ni)-tolerant *Bacillus safensis* FO.036b and *Micrococcus roseus* M_2 were isolated from the lead and zinc mine of Shazand-Arak, Markazi province, located at longitude 35º 48' 35'' and latitude 50º 58' 18''. In the next step, colonies of bacteria were inoculated to Hepes and Mes culture and over different nickel concentration treatments of 0, 5, 10, 20, 30, 40, 50, 60, 100, 200, 500, 1000 mgl^{-1} Ni in the form of $NiCl_2.6H_2O$, and then 2 strains of bacteria resistant to nickel were separated. Nickel (Ni)-tolerant *Bacillus safensis* FO.036b and *Micrococcus roseus* M_2 were characterized based on 16S rRNA gene sequence analysis in the Institute of Biochemistry and Biophysics (***IBB***), ***University of Tehran*** following Berge's manual respectively (Holt *et al*., 1994). Two resistant strains had some of PGPR characteristics like siderophore production, IAA and ACC-deaminase enzyme production (Motesharezadeh and Savaghebi, 2010).

The soil used in the greenhouse experiment was chosen from the Campus of Agriculture and Natural Resources in the University of Tehran located in Karaj. Chemical and physical properties of the soil were determined (Table 1). Measurements of the soil N was done by Kjeldal method, (Bremner, 1996), available phosphorus by Olsen Method (Kuo, 1996), and available potassium by normal acetate ammonium method (Hemke and Sparks, 1996). Measurements of the soil pH was done on saturated extract (Thomas, 1996) and electrical conductivity was determined by Rhoades method (1996). Similarly equal calcium carbonate was measured by gravimetric ***method (Raad, 1976),*** organic carbon percentage by Walkly Black (Nelson and Sommers, 1982), texture of the soil was determined by hydrometric method (Bouyoucos, 1962) and cation exchange capacity was measured by Bower method (Sumner and Miller, 1996). Concentrations of available zinc, lead and cadmium, were measured by atomic absorption, (Shimadzu 670), and by DTPA method. The soil taken from Campus farm was passed through 4-mm sieve after air drying and threshing. Nickel concentration in soil treatments included: control(Ni_0), (Ni_{125})125, (Ni_{250})250, (Ni_{500})500 and (Ni_{1000})1000 mgkg^{-1}, nickel from nickel chloride ($NiCl_2.6H_2O$) and three inoculants including B_0 (control), B_1 (*Bacillus safensis* FO.036b) and B_2 (*Micrococcus reseus* M_2) – 1 ml in each seed- and plants using sunflower, amaranthus and alfalfa for greenhouse experiments. To pollute the soil, the considered amount of metal was dissolved in 200 ml distilled water and sprayed over each pot layer by layer as evenly as possible. During the cultivation period, irrigation was done based on pot at weighting 70%±10 of the FC with distilled water. After germination, seedlings were thinned in the pots so that in each pot, 6 alfalfas, 2 sunflowers and 2 amaranthus were grown. After 70 days, at the beginning of reproductive period, shoot and root of sunflower and amaranthus and three cuts of alfalfa during 140 days of cultivation were taken and after washing with distillated water and measuring fresh weight, they were placed in paper bags and dried to 70 ºC. Then the samples, were milled and concentration of nickel, iron and zinc were measured in nitric acid digestion extracts with ICP-OES, model ICAP-6500 (Madejon *et al*., 2003). To calculate translocation factor, or in other words to show the success in overloading and phytoextraction of heavy metals in shoot parts of the plant, the ratio of metal concentration in shoot to its concentration in the root was used (Marchiol *et al*., 2004). Finally, Statistical analysis of data in the form of factorial design with random basic design in three replications was done with SAS software and comparison of means was done with LSD test at 5% level. Gaphs were plotted with Excel software.

RESULTS AND DISCUSSION

Physical and chemical characteristics of the soil, before the addition of nickel, are included in Table 1. Results of soil analysis show that the soil had the required physical and chemical characteristics and texture to use in greenhouse cropping; and the basic concentration of heavy metals, was not limiting. According to the results of the soil test, required fertilizers were added to the pots to optimize plant growth and produce more biomass.

Table 1. Physical and chemical characteristics of the soil used in greenhouse experiment

Characteristics	Value	Characteristics	Value
Soil texture	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	SO_4($meql^{-1}$)	40.60
pH	7.90	Fe($mgkg^{-1}$)*	4.28
EC(dSm^{-1})	4.31	Cu($mgkg^{-1}$)*	4.06
CEC($Cmolkg^{-1}$)	26.00	Cd($mgkg^{-1}$)*	0.10
%OC	0.84	Zn($mgkg^{-1}$)*	0.81
FC%	17.80	Pb($mgkg^{-1}$)*	2.02
%$CaCO_3$	8.90	Ni($mgkg^{-1}$)*	0.10

* DTPA-Extractable

Table 2. Data on characteristics of Plant, Bacteria and Nickel in 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)	TF
Plant (P)	2	**15633	**83940	23.6*	**4921	**19193	**19330	**408/5	**143548	0.45**
Bacteria (B)	2	**398	435.5^{ns}	**131	*158	**690	**892	13.1**	**13090	0.003^{ns}
Nickel (Ni)	4	**51755	**7917	**139	*109	**2527	**12149	**284	**220854	0.28**
P*B	4	**327	**12122	21.6*	58.2^{ns}	**659	**777	**26	**15787	0.01^{ns}
P*Ni	8	3610.7**	**6614	**54	**322	**348	**2533	46.7**	**27208	*0.09
B*Ni	8	**225.8	**5813.3	**89.6	**118	**206.5	**262	**8.2	**22153	0.09^{ns}
P*B*Ni	16	**358.5	**9767	**21.7	**80.2	**296	**185.5	**7.1	**4407.5	*0.08
Error Experimental	90	71.7	1144.6	6.6	32.6	72.4	56	2.45	2286.5	0.044

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

Table 3. Data on characteristics of Plant, Bacteria and Nickel in Roots

		MS (Mean Square)						
S.O.V	df	Ni (Root)	Fe (Root)	Cu (Root)	Mn (Root)	Zn (Root)	Fresh Weight (Root)	Dry Weight (Root)
Plant (P)	2	**16761	**1005681	**1460	485.7^{ns}	**2280	**390	**31.5
Bacteria (B)	2	**6031	**1549913	**701	**11864	**5798	*7.8	**1.08
Nickel (Ni)	4	**340791	**2011539	**914	**2893	**1920	**67	**4.9
P*B	4	**7724	**1301279	**200	**1737	308^{ns}	**7.4	**1.5
P*Ni	8	**15435	**1205331	66.7^{ns}	**2169	**1314	**9.9	**0.88
B*Ni	8	**4949	**1133287	103^{ns}	**913	299^{ns}	**4.9	*0.21
P*B*Ni	16	**9824	**517739	83^{ns}	**1679	**826	**5.9	**0.35
Error Experimental	90	518.3	29153.7	75.3	253.2	194.9	1.6	0.09

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

In figures 1 to 10, triplicate effects of nickel, bacteria and plant were compared. According to the results in figure 1, the highest concentration of nickel in shoot, between the three mentioned plants, is for amaranthus with Ni_{1000} concentration ($p<0.05$). According to the results in figure 2, the highest concentration of nickel was seen in the roots of treatment $B1Ni_{1000}$ (Alfalfa), B_0Ni_{1000} and B_1Ni_{1000} (Amaranthus). And also the highest translocation factor was seen in treatments B_0Ni_{125} (Amaranthus), B_2Ni_0 (Sunflower) and B_2Ni_0 (Alfalfa), (Figure 9) and the highest absorption of nickel was in amaranthus and in treatments B_1Ni_{250}, B_0Ni_{250} and B_2Ni_{250} respectively (Figure 10).

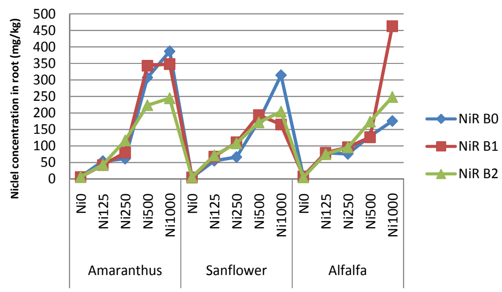

Fig. 1. Ni- concentration in the shoots

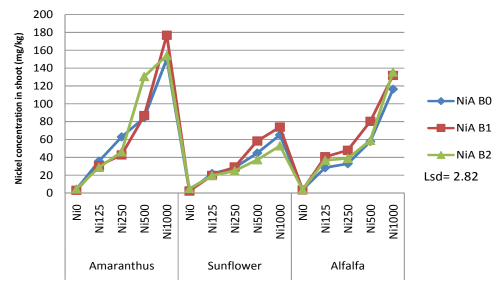

Fig. 2. Ni- concentration in the roots

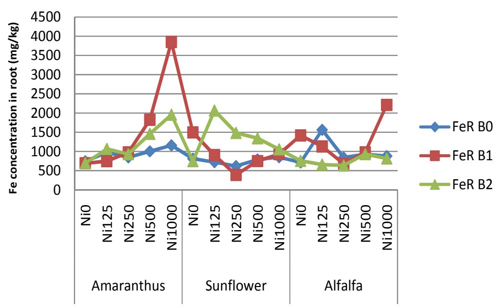

Fig. 3. Fe- Concentration in shoots

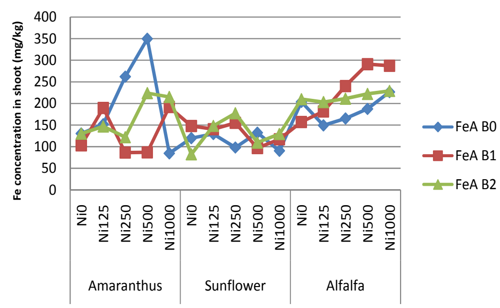

Fig.4. Fe- Concentration in roots

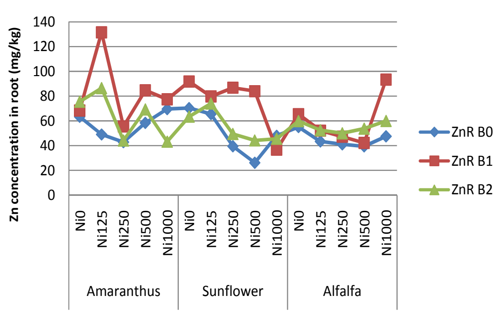

Fig. 5. Zn- Concentration in shoots

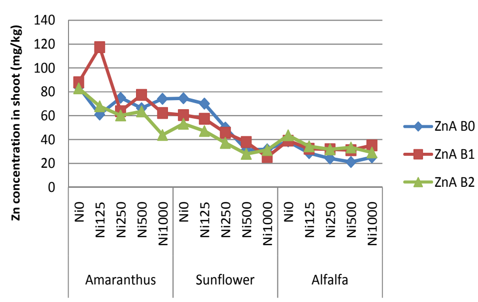

Fig. 6. Zn- Concentration in roots

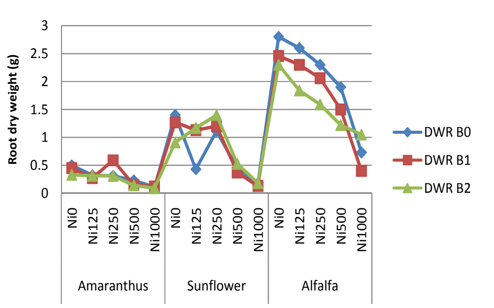

Fig. 7. Shoot Dry weight in three plants

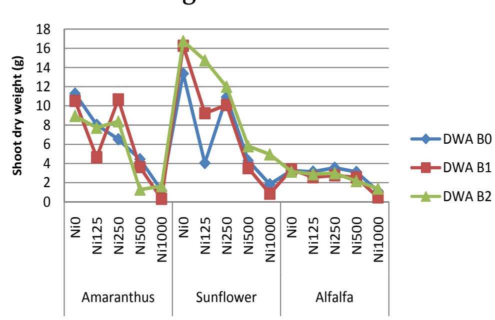

Fig. 8. Root Dry weight in three plants

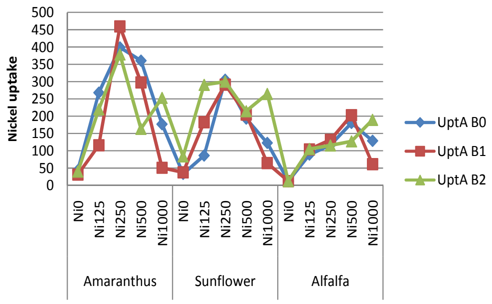

Fig. 9. Translocation Factor (TF) in three plants

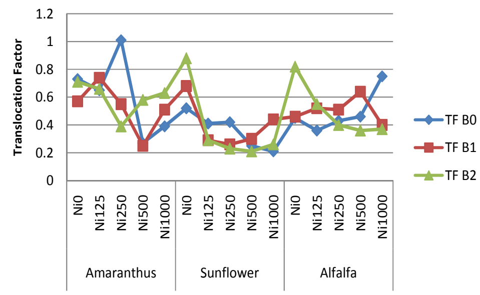

Fig. 10. Ni-uptake (μ g/Pot) in three plants

According to Figure 1, the highest concentration of nickel was found in amaranthus and in treatment Ni_{1000} by application of inoculant B_1. On the other hand, the highest concentration of nickel in roots was in alfalfa (Figure 2). These results show at first the success of amaranthus in phytoremediation of nickel and its transmission from roots to shoots, and secondly indicate the fixation of the metals by alfalfa. The highest absorption of nickel is seen in treatment with the usage of inoculants B_1 and in amaranthus (Figure 10). But it is not so easy to conclude about the selection of the appropriate plant for use in green remediation. In addition to these positive characteristics of amaranthus and alfalfa, the amount of biomass produced by sunflower is more than the others (Figure 7). Treatments with the usage of inoculants demonstrate better results. These results should be considered more according to the effects of inoculants in increasing sunflower and alfalfa's shoot iron and increasing of iron concentration in the roots of amaranthus (Figures 3 and 4). The increase in zinc in roots demonstrates the positive effects of the inoculant B_1 (Figure 6). But here, according to the type of the plant, results may not be positive; and there is no necessity to observe an increase in the concentration of these elements in shoots proportionate to the plant's root (Figure 5). In these conditions, severance of plants, metals or bacteria's effect is very difficult and results are under the influence of these factors and their antagonistic effects. So, these ideas and conclusions should be associated with more accuracy, caution and iteration in the investigations about organism (plant), and effects and interactions of another organism (bacteria) on the plant. According to research, exposure of plants to Ni, reduces Fe contents and results in Fe deficiency symptoms (Bollard, 1983; Ouzounidou *et al*., 2006). In general, observation of positive and stimulating effects of useful and plant growth promoting rhizobacteria (PGPR) on the plants, and on the other hand, capability of producing herbaceous biomass, show that amaranthus is appropriate for phytoremediation of soils polluted with nickel by the usage of phytoextraction mechanism (with potential for transmission of metal from the roots to harvestable parts of the plant). Moreover, after amaranthus, the sunflower absorbs high amount of nickel and also has high rate of biomass production (Figure 7). Its high potential for use in phytoremediation by plant fixation and the potential of alfalfa for phytoremediation by root fixation (Figure 8) was observed. The results on the useful effects of plant-growth-promoting rhizobacteria and the potential to use each of these plants in different methods of phytoremediation, are similar to those from other studies carried out in this field. Lombi *et al*. (2001) compared phytoremediation of metals by maize and Thlaspi and declared that metal concentration in the maize root is meaningfully more than in the shoot and the plant was appropriate for use in phytoremediation by plant fixation method. The seven capabilities of amaranthus in phytoremediation of soils polluted with heavy metals including zinc, cadmium, copper and nickel were reported by Bigaliev *et al*. (2000). They also examined the ability of two species of amaranthus in the absorption of these metals by soil. By increasing metals' concentration, in addition to delay in the seed germination, the wet weight of the shoots and roots decreased. Erakhrumen and Agbontalor (2007) emphasized on the advantages and disadvantages of using phytoremediation and the appropriate usage of crops like sunflower, alfalfa and other grasses in phytoextraction, rhizofiltration, phytodegradation and phytovolotalization; they also discussed about usage of these plants in phytoremediation of soils and polluted waters in different parts of the world such as America and Ukraine. Madejon *et al*. (2003) examined the potential of sunflower in phytoremediation of heavy metal polluted soils (Arsenic, Cadmium, Copper and Thallium) near one of mines in Spain, and declared that the amount of elements absorbed by the plant is meaningfully more in polluted soil than in the unpolluted one; and packing of several metals such as arsenic was higher in roots than in shoots, while cadmium was more in the shoots than in the roots. In this study, the results obtained on the usage of sunflower for phytoremediation demonstrate that the plant is appropriate for remediation of soils polluted with zinc, and in areas near the mines and can also be used to prevent the spread of pollution. These investigators suggest that with regard to the fast growth of sunflower, it can be used for amending soils polluted with metals and for confining pollution in one place; it is an appropriate choice in phytoremediation. Moreover, its leaves and stem are rarely eaten by animals, concentration of toxic metals in the seeds is low and the oil produced from the seeds is appropriate for industrial use. Moreover metal concentration of this plant has little danger for the food chain. Abou-Shanab *et al*. (2007) examined the potential of the crops and domestic plants for remediation of polluted soils with some metals and reported that by increasing metal concentration in the soil, increased absorption of the plant. In this research, sunflower, maize, sorghum and two domestic plants (Bermuda grass and alpine fleabane) were selected because of high production of biomass, fast growth and capability of removal of metals from polluted places; amount of produced biomass decreased by increasing concentration of metals. With regard to the high absorption of metals by roots of sunflower, it is suggested that this plant should be used in phytoremediation by rhizostabilization mechanism; the efficiency of the plant in the transmission of metal from roots to shoots was also low. Researchers have shown that the efficiency of phytoremediation is under the influence of availability of heavy metals in the soil and bacteria can provide heavy metals similar to the way that is available from plants (Yan-de *et al*., 2007). With regard to the low production of biomass in hyperacumulators and sensitivity of other plants' roots to high concentration of the metals, a lot of research was done on the possibility of using micro-organisms to develop phytoremediation technology and making this method economical (Glick, 2003; Ansari and Malik, 2007). According to Yan-de *et al*. (2007), Multiple Metal Resistance (MMR) in bacteria has more effect than resistance to one metal (inoculants B_1). Ansari and Malik (2007) examined resistance towards heavy metals in bacteria, separated from farming soils in India, which were irrigated with industrial wastewaters of the factories for two decades. According to this investigation, maximum inhibitory concentration of heavy metals for activity of bacteria is 200 for cadmium, 400 for zinc, and 800 for nickel and 1600 (micrograms in milligrams) for copper. Usage of resistant bacteria for remediation of pollution and metals has been reported in various researches (Cai *et al*., 1990; Hughes and Poole, 1989). Kuffner *et al*. (2008) studied the effects of rhizobacteria on absorption and accumulation of the metals in willow and identified and reported 10 bacteria species in polluted soils of lead mines. In the identified separators, six genena of bacteria were defined: Agromyces, Streptomyces, Flavobacterium, Serratia, Janthinobacterium and Pseudomonas. Of these, four strains two of which are Pseudomonas type and two of which belong to Streptomyces re capable of producing siderophore; and three species (two from Janthinobacterium type and one from Serratia) are capable of producing exine. Resistance of these bacteria towards zinc, cadmium and lead was evaluated. Zaidi *et al.* (2006) reported a nickel (Ni)-tolerant *Bacillus subtilis* strain SJ-101 for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. The role of this strain was ascertained in facilitating Ni accumulation in the *Indian mustard* plant. The data revealed that the plants exposed to $NiCl_2$ (1750 mg kg^{-1}) in soil bioaugmented with strain SJ-101 have accumulated 0.147% Ni vis-a`-vis 0.094% accumulation in dry biomass of the plants grown in uninoculated soil. The strain SJ-101 has also exhibited the capability of producing indole acetic acid (IAA) (55 μg ml^{-1}), and solubilizing inorganic phosphate (90 μg ml^{-1}) in specific culture media. Emphasizing on the results of using inoculants, we can also refer to Lasat (2002), Glick (2003), Aleem *et al*. (2003), Yan-de *et al*. (2007), Ansari and Malik (2007) and Kuffner *et al*. (2008).

CONCLUSION

Phytoremediation is a new and promising approach to remove contaminants in the environment. But using plants alone for remediation is confronted with many limitations. Recently, the application of PGPR has been extended to remediate contaminated soils in association with plants. In general, plant growth promoting rhizobacteria (PGPR) with direct or indirect effect on the root growth, stimulates growth of the root by production of siderophore that provide iron, phosphorus and other nutritional elements to fight against stress of heavy metals by producing ACC-deaminase enzyme which inhibits synthesis of stress ethylene, or by producing auxin hormone as indole-3-aceticacid (IAA). The mechanisms through which PGPRs can be effective in green remediation include: root growth stimulation, salinity and drought stress tolerance, plant absorption and translocation of heavy metals and decreasing effects of plant pathogenic factors.

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بررسی افزایش کارایی گیاه پالایی خاک آلوده به نیکل با استفاده از باکتری های بومی: *Bacillus safensis* FO.036b and *Micrococcus roseus* M_2

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چکیده

نیکل یکی از فلزات سنگین و آلاینده خاک است اما وجود مقادیر جزئی آن به عنوان جزء فلزی آنزیم آوره- آز در گیاه ضروری است. پالایش محل های آلوده به فلزات سنگین، یک چالش جدی است. گیاه پالایی، فناوری استفاده از گیاهان برای پالایش آلودگی از محیط زیست یک فناوری جدید پالایش است. در پژوهش حاضر، تاثیر پنج سطح نیکل شامل تیمار شاهد (Ni0) ، Ni125، Ni250، Ni500 و Ni1000 میلی گرم در کیلوگرم بصورت کلرید نیکل ($NiCl_2.6H_2O$) و سه سطح زاد مایه باکتریایی شاهد (B0) ، *Bacillus safensis FO.036b* (B1)، *Micrococcus roseus M2* (B2) در آفتابگردان، تاج خروس و یونجه بر گیاه جذبی نیکل توسط آفتابگردان، تاج خروس و یونجه استفاده شد. یک طرح آزمایش فاکتوریل با طرح پایه بلوک‌های کامل تصادفی با سه تکرار اجرا گردید. نتایج حاصله نشان داد که با افزایش غلظت نیکل در خاک، جذب آن توسط گیاهان نیز افزایش معنی داری داشت. بیشترین غلظت نیکل در اندام هوایی تاج خروس ($176.83\ mg.kg^{-1}$) و در ریشه سه گیاه، در یونجه ($462.73\ mg.kg^{-1}$) در نتیجه استفاده از زاد مایه مشاهده گردید($P<0.01$). بیشترین جذب نیکل نیز در تاج خروس با مصرف زاد مایه B1 و به میزان $459.41\ \mu gPot^{-1}$ مشاهده گردید. همچنین کاربرد زاد مایه مذکور، سبب افزایش غلظت آهن و روی در ریشه و اندام هوایی گیاهان گردید.