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Role of Ni-resistant rhizobacteria in the growth and Ni-uptake of maize in a calcareous soil

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ABSTRACT- A pot experiment was conducted to elucidate the effects of single and co-inoculation of maize plant with *Bacillus mycoides* and *Micrococcus roseus,* indigenous to HMs contaminated soils, on the plant growth and uptake of essential nutrients and Ni by maize in the soil polluted by 250 and 500 mg Ni kg^{-1}. At each level of Ni contamination, shoot dry weight and nutrients uptake significantly increased in *M. roseus* and *B. mycoides* treatments compared to non-inoculated plants. The highest plant growth promoting effect was found for *B. mycoides* at the level of 250 mg Ni kg^{-1} and for *M. roseus* at the level of 500 mg Ni kg^{-1} which enhanced plant biomass by 33.2% and 90%, respectively, compared to non-inoculated plants. At the levels of 250 and 500 mg Ni kg^{-1}, shoot Ni uptake of plants inoculated with *B. mycoides* or *M. roseus* significantly increased compared to non-inoculated plants. Root Ni uptake of plants inoculated with *B. mycoides* and *M. roseus* significantly decreased at the level of 250 mg Ni kg^{-1} and increased at the level of 500 mg Ni kg^{-1} compared to control plants. The lowest Ni transfer factor and maximum Ni translocation factor were in plants inoculated with *B. mycoides* at the level of 250 mg Ni kg^{-1} and in non-inoculated plants at the levels of 500 mg Ni kg^{-1}. Inoculation of plants with *B. mycoides* and *M. roseus* may be the effective treatments in Ni phytoextraction at the levels of 250 and 500 mg Ni kg^{-1}, respectively. Consortium of two bacteria had the lowest plant dry matter and shoots and roots Ni uptake and the maximum transfer factor compared to other treatments at the level of 500 mg Ni kg^{-1} that may be the effective treatment in Ni phytostabilization.

INTRODUCTION

The contamination of soils with heavy metals (HMS) especially Ni is a major environmental problem throughout the world. Soils polluted with Ni may threaten ecosystems and human health (Ma et al., 2009). Because of the increasing environmental concern regarding HM contamination, there has been an abundance of interest in the removal of HM ions from contaminated soils (Godtfredsen and Stone, 1994). The use of plants to remediate or clean-up contaminated soils can be used as a promising method to remove and/or stabilize soils contaminated with HM (Gaur and Adholeya, 2004). Plant growth-promoting rhizobacteria (PGPR) include a diverse group of soil bacteria that can improve host plant growth and development in HMs contaminated soils by mitigating toxic effects of HMs on the plants (Belimov et al., 2004). These metal resistant bacteria have an exceptional ability to promote the growth of the host plant by various mechanisms; namely, fixation of atmospheric nitrogen, production of siderophores, solubilization of phosphate, or production of plant growth regulators (hormones) (Glick et al., 1998; Abou-Shanab et al., 2005). PGP bacteria can be used to facilitate the process of phytoremediation and the growth of plants in metal-contaminated soils (Gamalero et al., 2009). Abou-Shanab et al. (2008) demonstrated an important role of four bacterial isolates in increasing Zn, Cr, Pb, and Cu accumulation in maize plants by 3.9, 2.7, 1.9, and 16 times, respectively, compared to non-inoculated plants. Therefore, isolating resistant PGPR for HM-contaminated soil and understanding mechanisms for tracing metal mobility and availability to plants through PGP characteristics should be studied. Therefore, the objective of this study was to determine the effects of inoculation of two resistant bacteria (*Bacillus mycoides* and *Micrococcus roseus*), indigenous to HMs-contaminated soils around a mine in Haft Emarat-Arak Markazi Province, on the growth of maize and accumulation of Ni in their parts. The results of this study may be applied as an advice for appropriate selection of microbial inoculants during phytoremediation of such contaminated areas.

MATERIALS AND METHODS

In the previous studies (Malekzadeh, 2010), 52 isolates were screened on supplemented HEPES-MES medium (Angle et al., 1992) which were treated by 250, 500, 750 and 1000 mg l^{-1} of Ni^{+2}. In order to determine the PGP

characteristics of resistant-bacteria, 10 Ni-resistant isolates were tested for some plant growth promoting characteristics. From 10 Ni-resistant isolates, two bacteria, namely, *Bacillus mycoides* and *Micrococcus roseus*, indigenous to polluted soils (previously identified by Motesharezadeh, 2008) had the ability to dissolve the insoluble organic and inorganic phosphate compounds, production of siderophore, indole acetic acid (IAA) and 1- aminocyclopropane-1-carboxylate (ACC)-deaminase enzyme (data not shown) (Malekzadeh, 2010).

The greenhouse experiment as factorial in completely randomized block design with two factors and three replications was conducted (36 pots): 1) PGPR with four levels, B_0 (without inoculation of bacteria), B_1 (*Bacillus mycoides*), B_2 (*Micrococcus roseus*) and B_1B_2 (*B. mycoides* + *M. roseus*) and 2) Ni with three levels (0, 250 and 500 mg Ni kg^{-1}). For greenhouse cultivation, a compound soil sample was taken from the depth of 0-30 cm (from the Beheshte sakineh located in Karaj, Tehran with coordinates of latitude of northern 35° 52' 58" and longitude of eastern 50° 52' 59" and 1254 meters above sea level). Air-dried soil samples were passed through 2 mm sieve, mixed uniformly and their physical, chemical and biological properties were assessed. The soil was sandy loam with field capacity of 20%, pH of 8.2, electrical conductivity of 0.5 dS m^{-1}, carbonate calcium equivalent of 11.6%, organic carbon of 0.8%, total Kjeldahl nitrogen of 0.1%, Olsen-phosphorus of 3.4 mg $kg^{-1}$and 1M NH_4OAc-extractable potassium of 1093 mg kg^{-1} , Fe, Cu, Mn, and Zn DTPA-extractable of 3, 2, 11.3 and 1.7 mg kg^{-1}, respectively and total microbial population of 1.3×10^4 cfu g^{-1} soil (Lindsay and Norvell, 1978; Page et al., 1982). Three Ni addition levels (0, 250 and 500 mg kg^{-1}) were applied in an analytical grade nickel chloride ($NiCl_2.6H_2O$) solution mixed thoroughly with 4 kg soil of each pot. In bacterial treatments, each seed of *Zea mays* L. (var. single cross 704) was inoculated with 1 ml of bacterial inoculum (1×10^8 cfu ml^{-1}). Totally, five seeds of maize were planted at each pot after whose germination, seedlings were thinned to three plants at each pot. Pots were placed in greenhouse conditions for three months (15-28 ˚C, with a 16/8 h light/dark period). After three months period of cultivation, dry weight of roots and shoots after being washed and dried at 65 ˚C for 72 h was measured. Soil Ni concentration (DTPA-extractable), shoot and root Ni concentration, shoot Fe, Zn and Mn concentrations by dry ash method with HCl using atomic absorption model A670 (Shimadzu, Japan) and shoot P concentration by colorimetry (spectrophotometer model UV-3100 (Shimadzu, Japan)) using the vanado-molybdate method were measured (Cottenie, 1980; Page et al., 1982).

Transfer factor (TF) (Eq.1) and translocation factor (TLF) (Eq.2) were assessed (Chen et al., 2006; Jankong and Visoottiviseth, 2008).

The data were analyzed statistically with SPSS 13.0 software and comparison of means was performed with MSTAT-C software by the Duncan's multiple range test at the 5% levels of significance.

$$TF = \frac{\text{Root Ni concentration}}{\text{Soil Ni concentration}} \quad (1)$$

$$TLF = \frac{\text{Shoot Ni concentration}}{\text{Root Ni concentration}} \quad (2)$$

RESULTS AND DISCUSSION

In this investigation, the bacterial strains were isolated from the HMs- contaminated soils with an objective to assess their effects on the plant growth and accumulation potential of Ni by maize in Ni-contaminated soil. In Ni-amended soil condition, with increasing the levels of Ni, the shoot and root dry weights of maize decreased considerably. Shoot dry weights of plants inoculated with *B. mycoides* at the level of 250 mg Ni kg^{-1} (33.2%) and plants inoculated with *M. roseus* at the level of 500 mg Ni kg^{-1} (90%), and root dry weight of plant inoculated with *M. roseus* at the level of 500 mg Ni kg^{-1}(62.9%) significantly increased in comparison with non-inoculated ones (Table 1). Therefore, inoculation of plants by *M. roseus* is probably highly efficient at protecting maize plant from growth inhibition caused by toxic effect of Ni at the highest concentrations of Ni.

Ni is known to inhibit enzymatic activity (Alam et al., 2007), biosythesis of chlorophyll and proteins (Sen and Bhattacharyya, 2004), and henceforth, the overall growth of plants. Since the process of metal uptake and accumulation by different plants depends on various internal and external factors (Gupta and Sinha, 2006), we assessed whether plant inoculation with PGPR affected the Ni uptake of maize plants. At the level of 250 mg Ni kg^{-1}, inoculation of plants with PGP bacteria increased shoot Ni content, whereas root Ni uptake decreased. At the level of 500 mg Ni kg^{-1}, shoot and root Ni contents in plants inoculated by *B. mycoides* and *M. roseus* increased and in consortium of two bacteria decreased. In this study, it was found that the inoculated and non-inoculated root systems accumulated considerably more Ni as compared to shoot systems (Table 2). The accumulation of Ni in the roots was more than shoots showing less translocation of metals from the underground part to the aerial part of the plant. This can be attributed to poor translocation of Ni from root to shoot (Burd et al., 2000). The present observations indicated that the PGPR protects the plants against the inhibitory effects of Ni. In general, at the levels of 250 and 500 mg Ni kg^{-1}, plant inoculation with PGPR increased Ni plant (shoot and root) uptake by 84.8% and 89% compared to control plants (Table 2) but the plant yield did not decrease (Table 1). The role of ACC deaminase synthesized by PGPRs in decreasing ethylene levels by the enzymatic hydrolysis of ACC into α-ketobutyrate and ammonia has been presented as one of the major mechanisms of PGPR in promoting root and levels by the enzymatic hydrolysis of ACC into α-ketobutyrate and ammonia has been presented as one of the major mechanisms of PGPR in promoting root and plant growth under metal stress condition (Belimov et al., 2005; Madhaiyan et al., 2006; Safronova et al., 2006).

Table 1. Shoot and root dry weights (DW) and DTPA-extractable Ni concentration of soil in maize plants inoculated with PGPR at different levels of Ni.

	Ni level (mg kg^{-1})	Treatment				F- value
		Blank	*Bacillus mycoides*	*Micrococcus roseus*	*B. mycoides* & *M. roseus*	B×Ni
Shoot dry weight (g pot^{-1})	0	15.49 ^{A}	14.70 ^{B}	13.56 ^{D}	14.18 ^{C}	
	250	7.57 ^{G}	10.09 ^{E}	9.55 ^{F}	9.66 ^{F}	222.9***
	500	3.54 ^{J}	6.12 ^{I}	6.73 ^{H}	3.24 ^{K}	
Root dry weight (g pot^{-1})	0	4.50 ^{A}	4.34 ^{B}	4.04 ^{C}	3.70 ^{E}	
	250	3.94 ^{CD}	3.88 ^{D}	4 ^{CD}	3.25 ^{F}	66.5***
	500	1.89 ^{I}	2.76 ^{H}	3.08 ^{G}	1.45 ^{J}	
Soil DTPA-extractable Ni concentration (mg kg^{-1})	0	0.35 ^{I}	0.35 ^{I}	0.37 ^{I}	0.22 ^{I}	
	250	28.44 ^{D}	26.56 ^{E}	16.08 ^{G}	25.09 ^{F}	420.5***
	500	32.39 ^{C}	34.70 ^{B}	36.59 ^{A}	7.17 ^{H}	

Significant at ***p<0.001; Within each reported plant response and soil Ni concentration, means with the same letter are not significantly different at the 0.05 level using Duncan's multiple range test.

Table 2. Shoot and root Ni contents, transfer and translocation factors in maize plants inoculated with PGPR at different levels of Ni.

	Ni level (mg kg^{-1})	Treatment				F- value
		Blank	*Bacillus mycoides*	*Micrococcus roseus*	*B. mycoides* & *M. roseus*	B×Ni
Shoot Ni uptake (µg pot^{-1})	0	0.77 ^{I}	3.92 ^{I}	58.76 ^{G}	75.63 ^{F}	
	250	113.1 ^{D}	158 ^{A}	126.3 ^{B}	120.1 ^{C}	487.8***
	500	86.22 ^{E}	108.6 ^{D}	112.4 ^{D}	49.41 ^{H}	
Root Ni uptake (µg pot^{-1})	0	45.45 ^{G}	63.77 ^{G}	104.5 ^{G}	58.20 ^{G}	
	250	3251 ^{A}	2684 ^{C}	2436 ^{D}	3015 ^{B}	538.8***
	500	1610 ^{E}	3027 ^{B}	3096 ^{B}	1147 ^{F}	
Transfer factor, TF	0	28.70 ^{EF}	42.50 ^{C}	68.71 ^{B}	71.94 ^{B}	
	250	29 ^{EF}	26.03 ^{F}	37.88 ^{CD}	37.21 ^{D}	168.7***
	500	26.33 ^{EF}	^{E} 31.62	27.48 ^{EF}	110.7 ^{A}	
Translocation factor, TLF	0	0.005 ^{F}	0.018 ^{DE}	0.168 ^{B}	0.339 ^{A}	
	250	0.018 ^{DE}	0.023 ^{CD}	0.022 ^{CD}	0.013 ^{E}	1233.2***
	500	0.029 ^{C}	0.016 ^{DE}	0.017 ^{DE}	0.019 ^{DE}	

Significant at ***p<0.001; Within each reported Ni uptake, TF, and TLF means with the same letter are not significantly different at the 0.05 level using Duncan's multiple range test.

The sidrophore is another important metabolite released by the PGPR that indirectly alleviate HMs toxicity by increasing the supply of iron to the plant (Burd et al., 1998, 2000) Shoot Fe uptake of plants inoculated with *B. mycoides* and *M. roseus* significantly increased compared to non-inoculated ones (Table 3). Both used bacteria displayed a positive sidrophore activity as indicated by the development of orange-colored zone on CAS agar plates (Malekzadeh, 2010). In general, the reduction of plant growth in Ni contaminated soil is often associated with iron deficiency and reduced uptake of some other essential elements (Mishra and Kar, 1974). At the level of 500 mg Ni kg^{-1}, siderophore produced by *M. roseus* (Malekzadeh, 2010) might have helped plant root proliferation and enhanced the uptake of soil minerals such as Fe, Zn and Mn (676.7, 76.69 and 940.3 µg pot^{-1}, respectively) (Table 3). IAA production by PGPR (Malekzadeh, 2010) might have promoted root growth by directly stimulating plant cell elongation or cell division (Glick et al., 1998). A low level of IAA produced by rhizosphere bacteria promotes primary root elongation whereas a high level of IAA stimulates lateral and adventitious root formation but inhibit primary root growth (Xie et al., 1996). In addition to IAA production, the phosphate solubilization by PGPR (Malekzadeh, 2010) is believed to play an important role in plant-bacterial interactions and plant growth in metal contaminated soils (Zaidi et al., 2006). The results showed at the level of 250 mg Ni kg^{-1}, phosphorus (P) uptake by shoot of plants inoculated with PGPR increased compared to control plants (Table 3). However, at the level of 500 mg Ni kg^{-1}, only single inoculation of plants with *B. mycoides* and *M. roseus* increased shoot P uptake compared to control plants (Table 3). At the level of 250 mg Ni kg^{-1}, inoculation of plants with PGP bacteria decreased root Ni uptake compared to control plants (Table 2). At this level of Ni, plants inoculated with *B. mycoides* with the highest shoot Ni uptake and compared to control plants (Table 3). At the level of 250 mg Ni kg^{-1}, inoculation of plants with PGP bacteria decreased root Ni uptake compared to control plants (Table 2).

Table 3. Shoot P, Fe, Zn and Mn contents in maize plants inoculated with PGPR at different levels of Ni.

	Ni level (mg kg^{-1})	Treatment				F- value
		Blank	*Bacillus mycoides*	*Micrococcus roseus*	*B. mycoides* & *M. roseus*	B×Ni
Shoot P uptake (mg pot^{-1})	0	71.50 ^{a}	69.31 ^{ab}	67.19 ^{b}	67.84 ^{b}	20.5***
	250	26.52 ^{E}	36.32 ^{C}	35.42 ^{C}	32.78 ^{D}	
	500	11.62 ^{G}	19.23 ^{F}	19.26 ^{D}	9.61 ^{H}	
Shoot Fe uptake (µg pot^{-1})	0	1738 ^{a}	1667 ^{b}	1636 ^{b}	1743 ^{a}	147.4***
	250	752.4 ^{D}	1073 ^{C}	1046 ^{C}	1079 ^{C}	
	500	394.5 ^{G}	620 ^{F}	676.7 ^{E}	318.5 ^{H}	
Shoot Ze uptake (µg pot^{-1})	0	1099 ^{A}	921.6 ^{B}	713.8 ^{C}	577.6 ^{D}	466.8***
	250	76.74 ^{H}	117.6 ^{G}	184.5 ^{E}	156.5 ^{F}	
	500	31.62 ^{J}	56.55 ^{HI}	76.69 ^{H}	52.80 ^{I}	
Shoot Mn uptake (µg pot^{-1})	0	1400 ^{A}	1251 ^{C}	1188 ^{D}	1309 ^{B}	74.5***
	250	798.2 ^{F}	1250^{C}	1402 ^{A}	1416 ^{A}	
	500	411.3^{G}	790.9 ^{F}	940.3 ^{E}	386.9 ^{G}	

Significant at ***P<0.001; Within each reported element uptake, means with the same letter are not significantly different at the 0.05 level using Duncan's multiple range test.

At this level of Ni, plants inoculated with *B. mycoides* with the highest shoot Ni uptake and Ni translocation factor may be the effective treatment in Ni phytoextraction (Table 2). At the level of 500 mg Ni kg^{-1} soil, plants inoculated with *M. roseus* compared to other treatments had the maximum plant dry matter (Table 1), Ni plant (shoot or root) uptake and postharvest DTPA-extractable Ni concentration of soil (Table 2) that may be an effective treatment in Ni phytoextraction. A possible explanation for the mechanisms used by *B. mycoides* and *M. roseus* might be :1) increased bioavailability of essential nutrients of P, Fe, Zn and Mn via production of_IAA phytohormone, siderophore, ACC deaminase and solubilization of phosphate and henceforth alleviation of toxicity of Ni due to the dilution effect caused by the increasing in plant dry weight; 2) immobilizing of Ni by binding it to the amino acids, proteins (metallothioneins and phytochelatins), or peptides and vacuoles accumulating and storing (tolerance strategies) (Pal et al., 2006; Denton, 2007); 3) siderophore production can also be stimulated by the presence of HMs (van der Lelie et al., 1999) and released by the PGPR which may indirectly alleviate HMs toxicity by increasing the supply of iron to the plant. Most siderophores showed affinity to bivalent metal ions (Neilands, 1981), and possibly affected their bioavailability as well.

The minimum postharvest DTPA-extractable Ni concentration of soil was measured in treatments inoculated with consortium of two bacteria (Table 1). Consortium of two bacteria had the lowest plant dry matter (Table 1) and shoots and roots Ni uptake and the maximum transfer factor (Table 2) compared to other treatments at the level of 500 mg Ni kg^{-1} that may be the effective treatment in Ni phytostabilization**.** At the levels of 250 and 500 mg Ni kg^{-1}, the minimum postharvest DTPA-extractable Ni concentration of soil (16 and 7.17 mg Ni kg^{-1} soil, respectively) was recorded in plants inoculated by *M. roseus* and consortium of two bacteria, respectively. The possible mechanisms might be the modification of rhizosphere pH, the exudation of metal-binding organic acids and the development of a thick mucous barrier on the root tip, which limit the uptake of Ni (avoidance strategies), thereby excluding them from plant tissues (Pal et al., 2006) .

Madhaiyan et al. (2007) studied the inoculation effects of PGPR *Methylobacterium oryzae* strain CMBM20 and *Burkholderia* sp. strain CMBM40, isolated from rice (*Oryza sativa*) tissues, on tomato (*Solanum tuberosum*), grown in nickel and cadmium-treated soil, demonstrated that bacterial strains significantly reduced the toxicity of both metals in tomato and promoted the plant growth under gnotobiotic and pot culture conditions. It was concluded that the bacterial strains reduced the uptake and consequent translocation of these metals to shoots and also synthesized phytohormones and ACC deaminase, which together accounted for increased growth of the test plant (Madhaiyan et al., 2007). In contrast, effects of inoculation were reported by Rajkumar and Freitas (2008), who showed that the addition of *Pseudomonas jessenii* to the root of *Ricinus communis* increased Ni, Cu and Zn concentrations in root tissues compared to non-inoculated controls.

However, more studies are necessary to examine the influence of PGPR inoculation on changes of HMs speciation in the rhizospheric soil and to determine whether such changes can alter the accumulation and distribution of HMs in various plants. It is recommended that future studies examine the co-inoculation of HMs-resistant PGPR under field conditions and their impact on other metals accumulation and in high yielding crop plants.

CONCLUSIONS

At the levels of 250 and 500 mg Ni kg^{-1}, inoculation of maize plants with Ni-resistant PGPR (*B. mycoides* or *M. roseus)* significantly increased Ni accumulation in plants without diminishing their biomass as compared to non-inoculated plants. Also, inoculation of plant with PGPR significantly increased nutrients uptake compared to that of control at medium and high applied levels of Ni. These results may be useful for Ni phytoextraction strategy (Blaylock et al., 1997). Treatments encompassing plants inoculated by consortium of two bacteria at high concentration of Ni may be effective for the purpose of Ni phytostabilization strategy.

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نقش ریزوباکتریهای مقاوم به نیکل در رشد و جذب نیکل گیاه ذرت در یک خاک آهکی

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چکیده- اثر مایه‌زنی جداگانه و توام دو جدایه *Bacillus mycoides* و *Micrococcus roseus* بومی خاک‌های آلوده به فلزات سنگین بر رشد گیاه و جذب عناصر غذایی ضروری و نیکل توسط گیاه ذرت در خاک آلوده به سطوح ۲۵۰ و ۵۰۰ میلی‌گرم نیکل در کیلوگرم خاک طی آزمایش گلدانی بررسی گردید. در هر یک از سطوح نیکل، وزن خشک اندام‌هوایی و جذب عناصر غذایی در تیمارهای *M. roseus* و *B. mycoides* در مقایسه با شاهد افزایش معنی‌داری داشتند. بیشترین اثر محرک رشد گیاه در سطح ۲۵۰ میلی‌گرم نیکل در کیلوگرم در تیمار *B. mycoides* و در سطح ۵۰۰ میلی‌گرم نیکل در کیلوگرم در تیمار *M. roseus* مشاهده گردید که به ترتیب نسبت به گیاهان شاهد مایه‌زنی نشده افزایش ۳۳/۲ و ۹۰ درصدی داشتند. در سطوح ۲۵۰ و ۵۰۰ میلی‌گرم نیکل در کیلوگرم، جذب نیکل در اندام هوایی گیاهان مایه‌زنی شده با *B. mycoides* و *M. roseus* به‌طور معنی‌داری در مقایسه با گیاهان مایه‌زنی نشده افزایش داشت. جذب نیکل در ریشه گیاهان مایه‌زنی شده با *B. mycoides* و *M. roseus* به‌طور معنی‌داری در سطوح ۲۵۰ و ۵۰۰ میلی‌گرم نیکل در کیلوگرم در مقایسه با گیاهان شاهد به ترتیب کاهش و افزایش یافت. کمترین فاکتور انتقال از خاک به ریشه و بیشترین فاکتور انتقال از ریشه به اندام‌هوایی در سطح ۲۵۰ میلی‌گرم نیکل در کیلوگرم در گیاهان مایه‌زنی شده با *B. mycoides* و در سطح ۵۰۰ میلی‌گرم نیکل در کیلوگرم در گیاهان مایه‌زنی نشده بود. احتمالا مایه‌زنی گیاهان با *B. mycoides* و *M. roseus* به ترتیب در سطوح ۲۵۰ و ۵۰۰ میلی‌گرم نیکل در کیلوگرم تیمارهای موثری در استخراج گیاهی نیکل باشند. در سطح ۵۰۰ میلی‌گرم نیکل در کیلوگرم، تیمار مایه‌زنی مشترک با دو باکتری کمترین وزن خشک گیاهی و جذب نیکل به اندام‌هوایی و ریشه، و بیشترین فاکتور انتقال از خاک به ریشه را در مقایسه با دیگر تیمارها داشت و ممکن است تیمار موثری در تثبیت گیاهی نیکل باشد.

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